Methylation Protects miRNAs and siRNAs from a 3′-End Uridylation Activity in *Arabidopsis*

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Summary

Small RNAs of 21–25 nucleotides (nt), including small interfering RNAs (siRNAs) and microRNAs (miRNAs), act as guide RNAs to silence target-gene expression in a sequence-specific manner [1]. In addition to a Dicer homolog, DCL1, the biogenesis of miRNAs in *Arabidopsis* requires another protein, HEN1 [2, 3]. miRNAs are reduced in abundance and increased in size in *hen1* mutants [2, 4–7]. We found that HEN1 is a miRNA methyltransferase that adds a methyl group to the 3′-most nucleotide of miRNAs [8], but the role of miRNA methylation was unknown. Here, we show that siRNAs from sense transgenes, hairpin transgenes, and transposons or repeat sequences, as well as a new class of siRNAs known as *trans*-acting siRNAs, are also methylated in vivo by HEN1. In addition, we show that the size increase of small RNAs in the *hen1-1* mutant is due to the addition of one to five U residues to the 3′ ends of the small RNAs. Therefore, a novel uridylation activity targets the 3′ ends of unmethylated miRNAs and siRNAs in *hen1* mutants. We conclude that 3′-end methylation is a common step in miRNA and siRNA metabolism and likely protects the 3′ ends of the small RNAs from the uridylation activity.

Results and Discussion

miRNAs in *hen1* Mutants Are Heterogeneous in Size

Previous studies showed, with RNA filter hybridization, that miRNAs in *hen1* mutants are either reduced in abundance or totally absent [2, 4–7]. For some miRNAs, smearing bands larger than wild-type miRNAs were detected [2, 5]. When we resolved total RNAs from *hen1-1* on high-resolution acrylamide gels, heterogeneity in the size of miRNAs was dramatically revealed by the presence of a ladder of bands, and this ladder presumably represents molecules differing in length in 1 nt intervals (Figure 1A). Most of the miRNA species in *hen1-1* were larger than those in L*er*, ranging from 21 to 26 nt (Figure 1A). miRNA species apparently smaller than wild-type were also detected (Figure 1A, miR163, miR167, and miR173). A similar pattern of miRNA accumulation was observed in other

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hen1 alleles for miR171 (Figure 1A) and miR172 (Figure 1C). Therefore, HEN1 appears to maintain the correct size as well as the normal levels of miRNAs.

Heterogeneous miRNA Species in *hen1* Are Generated by DCL1

DCL1 is required for the generation of miRNAs [2, 3] and *trans*-acting siRNAs [9, 10]. The predominant size of these two types of small RNAs is 21–22 nt. *DCL3* is required for the accumulation of 24–25 nt endogenous siRNAs but not miRNAs [7]. Loss of DCL3 activity results in a shift in size of siRNA02, an endogenous siRNA, from 24–25 nt to 21–22 nt [7], presumably as a result of the activity of another DCL or other DCLs. To investigate whether the miRNA size heterogeneity in *hen1* mutants resulted from the mistargeting of miRNA precursors by DCL proteins other than DCL1, we analyzed the expression of miR172 in the *dcl1-9 hen1-1* double mutant (Figure 1C). The absence of miR172 in *dcl1-9 hen1-1* indicates that the heterogeneous miR172 species in *hen1-1* are all generated by DCL1.

Heterogeneous miRNAs in *hen1-1* Have the Same 5′ Ends as Wild-Type miRNAs

To begin to understand how the size heterogeneity of miRNAs in *hen1* mutants is generated, we first determined whether the heterogeneity resides in the 5′ ends of the miRNAs by primer extension. Seventeen- to eighteen-nucleotide DNA oligonucleotides complementary to the 3′ portions of miRNAs were used in primer-extension assays on L*er* and *hen1-1* RNAs. Extension products of the same sizes were obtained in L*er* and *hen1-1* for all the miRNAs examined (Figure 1B). The specificity of the primer extension was inferred from the reduced signals in the *dcl1-9* mutant (Figure 1B), which is known to have decreased or undetectable levels of the miRNAs ([2, 3]; Figure 1A). This indicates that the heterogeneous miRNA species in *hen1-1* have the same 5′ ends as the corresponding miRNA species in L*er*. Therefore, the size heterogeneity of miRNAs in *hen1-1* should reside in the 3′ ends.

miRNAs in *hen1-1* Lack 3′ Methylation and Become 3′ Uridylated

How does the 3′-end heterogeneity of miRNAs in *hen1* mutants arise? We envisioned that at least two mechanisms account for the 3′-end heterogeneity. First, DCL1 imprecisely processes the pre-miRNAs in the absence of functional HEN1 to generate miRNAs with differing 3′ ends. In this scenario, HEN1 specifies the 3′ ends of the miRNAs in the pre-miRNAs during DCL1 processing. This seems unlikely given HEN1's role as a miRNA methyltransferase [8]. Second, DCL1 is able to precisely process the pre-miRNAs in *hen1* mutants, but additional nucleotides are added to the 3′ ends after miRNAs are generated. This model predicts that the extra nucleotides at the 3′ ends of miRNA species in *hen1* are unlikely to correspond to those 3′ to the miRNAs in the pre-miRNAs.

We utilized a modified miRNA-cloning approach to determine the identity of the extra nucleotides in the 3′ ends of miRNAs. Small RNAs of 18–28 nt from L*er* and *hen1-1* were isolated and ligated to a 3′ adaptor. Two miRNAs (miR167 and miR173) were selectively amplified with miRNA-specific primers (corresponding to the 5′ portion of the miRNAs) and the 3′-adaptor primer. The resulting PCR products were cloned and sequenced as described [2]. In the great majority of wild-type clones (100% for miR173 and 98% for miR167), at least two nucleotides immediately adjacent to the miRNA-specific primer are the same as those in the miRNAs. In most wild-type clones (89% for miR173 and 91% for

miR167), at least three nucleotides immediately adjacent to the primer sequence are the same as those in the miRNAs. This indicates that we selectively amplified the miRNAs of interest. Approximately 60% of the clones from the wild-type represent the full-length miRNAs, and the remaining ones are shorter than full length. The shorter miRNA species are either minor species normally present in the wild-type, or they were truncated during the experiment.

As predicted, species longer than miR173 and miR167 at the 3′ ends were cloned in the *hen1-1* mutant but not in the wild-type (Table 1). The extra nucleotides did not correspond to the 3′ flanking sequences in their precursors. For example, among the cloned miR173 species that were full length or longer in the *hen1-1* mutant (H5 to H10), 38% (H6 to H10) had extra nucleotides. Most of the extra nucleotides were U residues instead of those 3′ to miR173 in the precursor. For miR167, one clone (H13) had U residues after the full-length miRNA sequence. Most cloned miR167 species from *hen1-1* (H1 to H11) were not full-length miR167. It is very unlikely that they represent degradation intermediates from the handling of the RNAs in the experiment because many had U tails. It is unlikely that these U residues were artificially incorporated during our cloning procedure or that miRNAs with 3′ U residues were preferentially cloned because our previous miRNA cloning with a similar RNA-ligase based approach did not reveal a preference for miRNAs with 3′ U residues [2]. The miR173 and miR167 species cloned from the wild-type also did not reveal a preference for 3′ U residues. Therefore, we believe that the 3′-truncated miR167 species with U tails represent miR167 species that are present in vivo. Occasionally, other nucleotides were also found in the tails of miRNAs from *hen1-1* (Table 1).

To further confirm the presence of U tails in these and other miRNAs, we carried out a primer-extension assay on the previously mentioned miRNA-specific RT-PCR products amplified from miRNAs ligated to adaptors. Taq DNA polymerase was used to extend the RT-PCR products with a primer complementary to the 3′ portion of the 3′ adaptor, in the presence of only α-^{32}P-dATP (Figure 2A). As a result of the presence of three T residues at the 5′ end of the adaptor, products terminating at each of the T residues were generated in both wild-type and *hen1-1* samples (bars in Figure 2B). Whereas further extension beyond the adaptor was absent or rare in wild-type, a ladder of products with A residues was obvious in *hen1-1* (brackets in Figure 2B). To rule out the remote possibility of other deoxynucleotides contaminating the RT-PCR products that were purified from polyacrylamide gels, we also performed the primer extension with a primer complementary to the entire 3′-adaptor sequence in the presence of any one of the four radioactive deoxynucleotides. A ladder of primer-extension products was only observed in the *hen1-1* mutant when dATP, but not any of the other deoxynucleotides, was present (see Figure S1 in the Supplemental Data available with this article online). This confirms that the 3′ ends of miRNAs have U tails in *hen1-1*. We also carried out the assay on two miRNAs, miR389a1 and miR399f, whose production is DCL1 independent but requires HEN1 [11], and we obtained similar results (Figure 2B). We amplified by RT-PCR the putative pre-miR167a [3] from *hen1-1* and confirmed by sequencing that extra U residues were not present in the precursor (data not shown). Therefore, we conclude that the U residues are added after DCL1-mediated cleavage of the pre-miRNAs and that a novel enzymatic activity,

presumably that of a terminal transferase or polymerase, adds the U residues to the 3′ ends of miRNAs in *hen1-1*.

Recently, HEN1 was found to be a miRNA methyltransferase that adds a methyl group to the ribose of the last nucleotide of miRNAs [8]. Whereas miRNAs in the wild-type have a methyl group, the heterogeneous species in *hen1-1* lack methylation [8]. The methylation status of small RNAs can be evaluated by treatment of the RNAs with sodium periodate (oxidation) followed by β elimination [12]. RNAs with free 2′ and 3′ OH are sensitive to the chemical-modification reactions, which result in the elimination of the last nucleotide. The resulting RNAs would migrate faster in gel electrophoresis by approximately 2 nt as a result of the presence of a 3′ P [8, 12, 13]. We found that all miR171 species in *hen1-1* changed their apparent mobility after the chemical treatment, whereas miR171 from wild-type did not (Figure 1D). Similar analyses in different *hen1* alleles revealed that the state of 3′ methylation was inversely correlated with the accumulation of 3′-uridylated miRNAs. For example, the majority of miR171 in *hen1-3*, a weak allele, was still methylated (data not shown) and appeared wild-type in size (Figure 1A), whereas almost all miR171 species in the severe *hen1-1* allele lacked methylation (Figure 1D) and accumulated as 3′-uridylated species (Figure 1A). This suggests that methylation prevents or attenuates the uridylation activity.

The 3′ Ends of the Two Strands of the miRNA/miRNA* Duplex Are Asymmetrically Affected in *hen1*

Experimental evidence supports the existence of the predicted intermediate, miRNA/miRNA* duplex, in miRNA biogenesis in plants [3, 14, 15]. The sense strand is selectively assembled into an RNA-induced silencing complex (RISC), whereas the antisense strand (miRNA*) is presumably degraded [16–18]. We analyzed the 3′-end sequences of miR173* in the wild-type and *hen1-1* by a similar directional-cloning approach described above. Only 1 out of 18 clones in the miR173* population from *hen1-1* had extra Us at the 3′ end (Table 2), a proportion that is much lower than the 33.3% (13/39) in the miR173 population (Table 1). We further confirmed this observation by using the α-^{32}P-dATP incorporation assay on the RT-PCR products of miR173* ligated to the 3′ linker. This assay failed to detect 3′-uridylated miR173* in either the wild-type or *hen1-1* (Figure 2B). Therefore, there is a strand bias for 3′ uridylation similar to that for RISC assembly [17, 18]. Perhaps, the U tailing enzyme favors the miRNA over miRNA* in the miRNA/miRNA* duplex. It is also possible that this activity acts at a stage after RISC assembly. miR173* clones ending with a nonencoded A residue were also obtained in both wild-type and *hen1-1* samples (L2 and H1 in Table 2). It remains to be determined whether they represent in vivo miR173* species or merely nonspecific amplification products.

A 24 nt Endogenous siRNA Lacks 3′ Methylation and Becomes 3′ Uridylated in *hen1*

HEN1 is required for the accumulation of some 24–25 nt endogenous siRNAs [7, 19]. Whereas *AtSN1* siRNAs and siRNA1003 are barely detectable in *hen1-1*, the abundance of siRNA02 and cluster2 siRNAs is increased in *hen1-1* [7]. Intriguingly, the latter two siRNAs also appear larger in *hen1-1* than in the wild-type [7], suggesting that endogenous siRNAs may undergo 3′ uridylation similar to that of miRNAs.

We found that siRNA02 in all the *hen1* alleles except the weak *hen1-3* allele appeared as a ladder of molecules 23–26 nt in length, in contrast to the 24 nt siRNA02 species in the wild-type (Figure 3). Primer extension of siRNA02 in *hen1-4* and *hen1-6* showed that the 5′ ends of siRNA02 in these *hen1* alleles were the same as that in the wild-type (Figure 3C). Therefore, the heterogeneity in size of this endogenous siRNA resides in the 3′ ends. By using the α-^{32}P-dATP incorporation assay, we further demonstrated that these extra nucleotides are composed of several U residues (Figure 3D). We treated total RNAs with periodate followed by β elimination and detected siRNA02 by RNA filter hybridization to determine the methylation status of this siRNA. Indeed, the apparent mobility of siRNA02 after the reactions remained unchanged in the wild-type but appeared faster in *hen1-1* (Figure 3B). We conclude that siRNA02 is methylated by HEN1 in vivo.

S-PTGS, IR-PTGS, and *trans*-Acting siRNAs Are Methylated by HEN1

HEN1 was previously found to be required for S-PTGS and for the accumulation of siRNAs from sense transgenes [4]. *GUS* siRNAs from the silenced L1 locus in the wild-type were resistant to the oxidation and β elimination reactions, suggesting that they are methylated at the last nucleotide (Figure 4A). Although the greatly reduced accumulation of *GUS* siRNAs in *hen1-4* and *hen1-6* made it difficult to evaluate whether the siRNAs lack methylation in the *hen1* mutants, methylation is likely conferred by HEN1 given its role in S-PTGS.

The accumulation and function of IR-PTGS siRNAs are not affected by mutations in *HEN1* [4]. Our observation that both wild-type and *hen1-4* plants carrying an *AP1* IR-PTGS construct [20] show *ap1* loss-of-function phenotypes (data not shown) supports the notion that *HEN1* is not required for IR-PTGS. However, siRNAs from the *AP1* hairpin transgene were resistant to the oxidation and β elimination reactions in the wild-type but were sensitive to the reactions in the *hen1-4* mutant, suggesting that IR-PTGS siRNAs are also methylated by *HEN1* (Figure 4). Moreover, IR-PTGS *AP1* siRNAs appeared larger in *hen1-4* than in the wild-type (Figure 4B), suggesting that they may also be targeted by the uridylation activity in the absence of methylation. IR-PTGS and S-PTGS are thought to involve overlapping but also distinct pathways because a number of genes (including *HEN1*) required for S-PTGS are genetically dispensable for IR-PTGS [4, 21]. Our finding that IR-PTGS siRNAs are subjected to methylation by HEN1 as are S-PTGS siRNAs shows that differential genetic requirements do not preclude similar underlying biochemical pathways in the biogenesis and function of S-PTGS and IR-PTGS siRNAs. Perhaps the high levels of IR-PTGS siRNAs allow a bypass of some of the genetic requirements for S-PTGS.

HEN1 is required for the accumulation of *trans*-acting siRNAs (ta-siRNAs) [9, 10]. However, this requirement may be explained by the fact that the biogenesis of ta-siRNAs depends on certain miRNAs [22] whose biogenesis requires HEN1. However, a direct role of HEN1 in methylating the ta-siRNAs cannot be excluded. We found that ASRP1511, a ta-siRNA [22], was resistant to the chemical-modification reactions in wild-type by not in *hen1-1* plants (Figure 4C), indicating that this ta-siRNA is methylated in vivo by HEN1. In addition, the size of this ta-siRNA was heterogeneous in *hen1-1* (Figure 4C), suggesting that it is also U tailed in the absence of methylation.

We conclude that methylation on the ribose of the last nucleotide by HEN1 is a universal step in the biogenesis of miRNAs and siRNAs in plants. At least one function of the methylation is to prevent a novel uridylation activity from targeting the last nucleotide of the small RNAs. It was found that U residues are added to the $5'$ cleavage products of miRNA-guided cleavage of mRNAs and that the U tailing correlates with the exonucleolytic degradation of the $5'$ cleavage products [23]. Perhaps the same enzyme targets unmethylated small RNAs for uridylation, which may subsequently lead to the degradation of the small RNAs. In addition to the uridylation activity, a $3'$-to-$5'$ exonuclease activity is apparently counteracted by the $3'$ methylation as suggested by the presence of $3'$-truncated miRNAs and siRNAs in *hen1* (Figure 1; Table 1). The methylation of miRNAs and siRNAs by HEN1 may also prevent RNA-dependent RNA polymerases (RdRPs) from using the small RNAs as primers. However, the effect of the methyl group on the ability of RdRPs to use the small RNAs as primers needs to be experimentally evaluated.

Experimental Procedures

Directional Cloning and Sequencing of Specific miRNAs

microRNA cloning and sequencing were performed as described [2] with some modifications. In brief, 18–28 nt small RNAs were recovered from total RNAs after gel electrophoresis, dephosphorylated, and ligated to a $3'$ adaptor. The ligation products were reverse transcribed with a primer targeting the $3'$ adaptor. The RT products were PCR-amplified with a primer corresponding to the $3'$ adaptor and ones corresponding to the $5'$ portions of specific miRNAs. The PCR products were concatamerized, cloned, and sequenced.

α-^{32}P-dATP Incorporation Assay

Small-RNA purification, $3'$-adaptor ligation, and RT- PCR were first performed as described above. miRNA RT-PCR products were PAGE-purified to remove the free nucleotides. The incorporation reactions were run against the purified RT-PCR products with a primer complementary to the $3'$-adaptor primer at 94°C for 30 s, 55°C for 30 s, and 72°C for 10 s in the presence of α-^{32}P-dATP and Taq DNA polymerase. The final products were resolved in 15% denaturing PAGE gels and analyzed with a Storm PhosphorImager.

Acknowledgments

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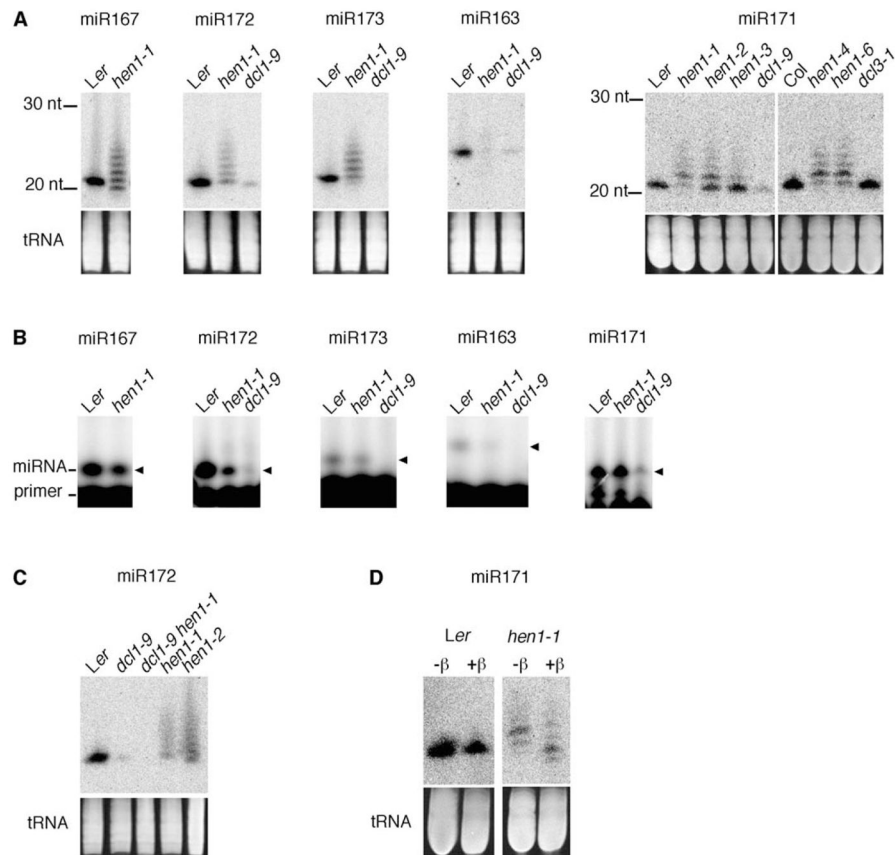
Figure 1. miRNAs in *hen1* Have 3′-End Defects

(A) RNA filter hybridization of miR167 (21 nt), miR172 (21 nt), miR173 (22 nt), miR163 (24 nt), and miR171 (21 nt) in various genetic backgrounds. L*er* is the wild-type control for *hen1-1*, *hen1-2*, *hen1-3*, and *dcl1-9*. Col is the wild-type control for *hen1-4*, *hen1-6* (SALK_090960), and *dcl3-1*.

(B) 5′ ends of miRNAs in *hen1-1* are the same as those in L*er* as detected by primer extension. Primer extension was performed as described [24], with 17–18 nt DNA primers spanning the 3′ portions of mature miRNAs. The extension products are indicated by the arrowheads.

(C) Accumulation of miR172 in various genotypes as detected by RNA filter hybridization.
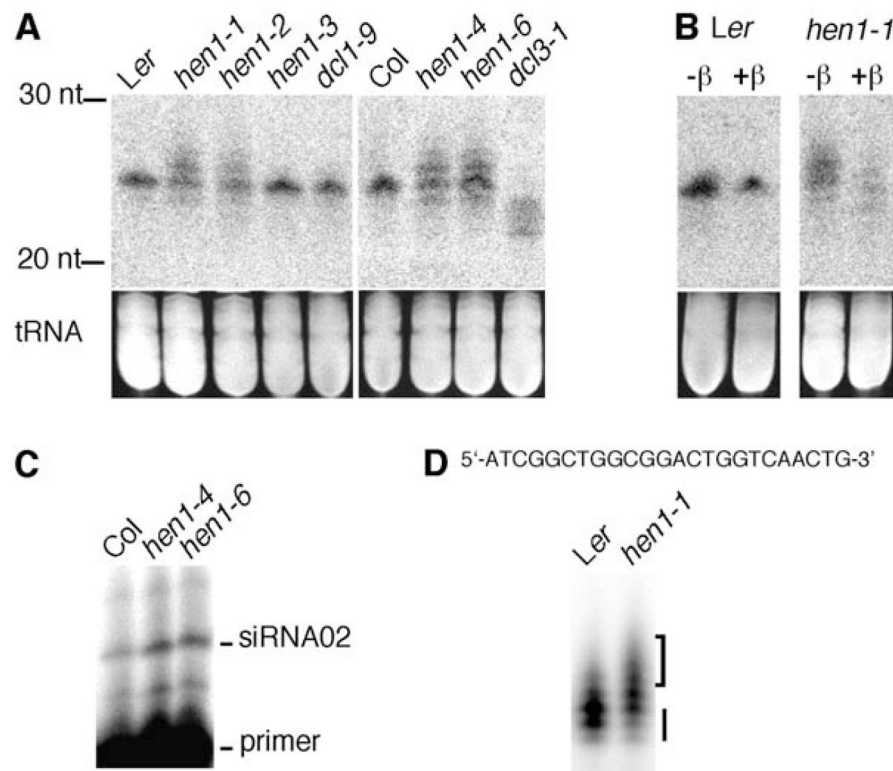
(D) Detection of 3′ methylation of miR171. Total RNAs were treated (+β) or not (−β) with the oxidation and β elimination reagents, and miR171 was detected by RNA filter hybridization. miR171 from the wild-type is resistant, whereas the heterogeneous miR171 species in *hen1-1* are sensitive to the chemical reactions. In (A), (C), and (D), images of ethidium-bromide-stained gels in the region of tRNAs are shown at the bottom to indicate near-equal loading.
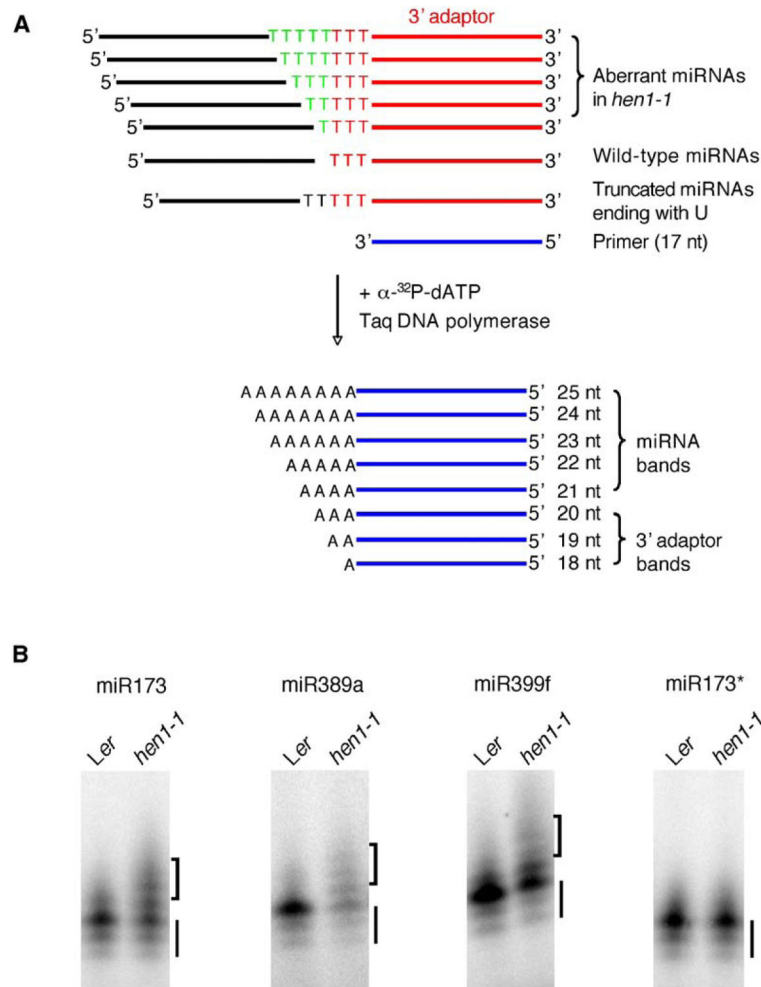
Figure 2. miRNAs in *hen1-1* Are Uridylated at Their 3′ Ends

(A) A schematic diagram of an α-³²P-dATP incorporation assay. The first three residues in the 3′ adaptor are T (red). T residues between the mature miRNAs (black line) and the 3′ adaptor (red line and letters) in the RT-PCR products are in green. The primer complementary to the 3′ adaptor (excluding the three T residues) is in blue.

(B) α-³²P-dATP incorporation assay performed on miR173, miR389a1, miR399f, and miR173* from L*er* and *hen1-1*. The bottom three bands marked by the bars represent the extension products corresponding to the Ts in the 3′ adaptor. Extension products marked by the brackets indicate the T residues between the mature miRNAs and the 3′ adaptor.

Figure 3. A 24 nt Endogenous siRNA in *hen1* Is Unmethylated and 3′ Uridylated

(A) Accumulation of siRNA02 in various genotypes as detected by RNA filter hybridization.

(B) Detection of 3′ methylation of siRNA02. Total RNAs were treated (+β) or not (−β) with the oxidation and β elimination reagents, and siRNA02 was detected by filter hybridization. siRNA02 is methylated in L*er* and unmethylated in *hen1-1*. Images of ethidium-bromide-stained gels in the region of tRNAs in (A) and (B) are shown at the bottom to indicate near-equal loading.

(C) The 5′ ends of siRNA02 in *hen1-4* and *hen1-6* are the same as those in the wild-type as detected by primer extension. A 15 nt DNA primer complementary to the 3′ portion of siRNA02 was used.

(D) U tails at the 3′ ends of siRNA02 in *hen1-1* were detected by an α-^{32}P-dATP incorporation assay (diagrammed in Figure 2A). The bottom three bands marked by the bars represent the extension products corresponding to the three Ts in the 3′ adaptor. Extension products marked by the brackets indicate the T residues between the mature siRNA and the 3′ adaptor. The sequence of siRNA02 is shown. The presence of an extension product 1 nt beyond the 3′ adaptor in the wild-type is probably due to the presence of some siRNA02 species ending with the penultimate T nucleotide.
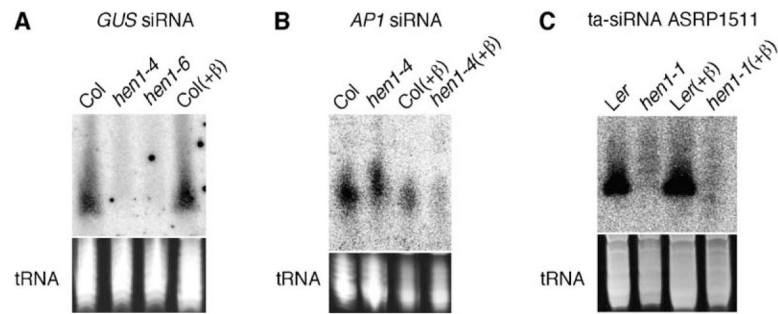
Figure 4. siRNAs Produced from Transgenes and a ta-siRNA Are Methylated in the Wild-Type but Not in *hen1*

(A) Accumulation of S-PTGS siRNAs from *GUS* transgene (L1 locus) in the wild-type (Col) before and after the oxidation and β elimination reactions (+β) and in *hen1-4* and *hen1-6* (SALK_090960).

(B) Accumulation of IR-PTGS siRNAs from an *AP1* hairpin transgene in the wild-type (Col) and *hen1-4* before and after (+β) the oxidation and β elimination reactions.

(C) Accumulation of a ta-siRNA in the wild-type (L*er*) and *hen1-1* before and after (+β) the oxidation and β elimination reactions. Images of ethidium-bromide-stained gels in the region of tRNAs are shown at the bottom.

Table 1

miR167 and miR173 Cloned from L*er* and *hen1-1*

Name	Sequences[a]	Length (nt)	Number of Clones	Genotype
Sequences from the miR173 Cloning				
miR173	UUCGCUUGCAGAGAGAAAUCAC*agugguca*	22		L*er*
L1	UUCGCUUGCAGAGAGAAAUC	20	2	L*er*
L2	UUCGCUUGCAGAGAGAAAUCu	21	2	L*er*
L3	UUCGCUUGCAGAGAGAAAUCA	21	9	L*er*
L4	UUCGCUUGCAGAGAGAAAUCAa	22	2	L*er*
L5	UUCGCUUGCAGAGAGAAAUCAC	22	21	L*er*
H1	UUCGCUUGCAGAGAGAAAUau	21	1	*hen1-1*
H2	UUCGCUUGCAGAGAGAAAUCc	21	1	*hen1-1*
H3	UUCGCUUGCAGAGAGAAAUCA	21	2	*hen1-1*
H4	UUCGCUUGCAGAGAGAAAUCAu	22	1	*hen1-1*
H5	UUCGCUUGCAGAGAGAAAUCAC	22	21	*hen1-1*
H6	UUCGCUUGCAGAGAGAAAUCACa	23	1	*hen1-1*
H7	UUCGCUUGCAGAGAGAAAUCACu	23	7	*hen1-1*
H8	UUCGCUUGCAGAGAGAAAUCACuu	24	2	*hen1-1*
H9	UUCGCUUGCAGAGAGAAAUCACuuc	25	1	*hen1-1*
H10	UUCGCUUGCAGAGAGAAAUCACuuu	25	2	*hen1-1*
Sequences from the miR167 Cloning				
miR167a	UGAAGCUGCCAGCAUGAUCUA*auuagcuu*	21		L*er*
miR167b	UGAAGCUGCCAGCAUGAUCUA*ucuuuggu*	21		L*er*
miR167c	UGAAGCUGCCAGCAUGAUCUU*gucuuccu*	21		L*er*
miR167d	UGAAGCUGCCAGCAUGAUCUGG*uaaucg*	22		L*er*
L1	UGAAGCUGCCAGCAUGAUu	19	1	L*er*
L2	UGAAGCUGCCAGCAUGAUC	19	2	L*er*
L3	UGAAGCUGCCAGCAUGAUCaa	21	1	L*er*
L4	UGAAGCUGCCAGCAUGAUCU	20	13	L*er*
L5	UGAAGCUGCCAGCAUGAUCUA	21	26	L*er*
L6	UGAAGCUGCCAGCAUGAUCUGG	22	1	L*er*
H1	UGAAGCUGCCAGCAUGAUu	20	1	*hen1-1*
H2	UGAAGCUGCCAGCAUGAUuu	22	2	*hen1-1*
H3	UGAAGCUGCCAGCAUGAUuuuu	24	1	*hen1-1*
H4	UGAAGCUGCCAGCAUGAUuuuuu	25	2	*hen1-1*
H5	UGAAGCUGCCAGCAUGAUC	20	3	*hen1-1*
H6	UGAAGCUGCCAGCAUGAUCa	21	1	*hen1-1*
H7	UGAAGCUGCCAGCAUGAUCU	21	8	*hen1-1*
H8	UGAAGCUGCCAGCAUGAUCUu[b]	22	4	*hen1-1*
H9	UGAAGCUGCCAGCAUGAUCUuu[b]	23	4	*hen1-1*

Name	Sequences[a]	Length (nt)	Number of Clones	Genotype
H10	<u>UGAAGCUGCCAGCAUGA</u>UCUuuu[b]	24	1	*hen1-1*
H11	<u>UGAAGCUGCCAGCAUGA</u>UCUuuuu[b]	25	1	*hen1-1*
H12	<u>UGAAGCUGCCAGCAUGA</u>UCUA	22	2	*hen1-1*
H13	<u>UGAAGCUGCCAGCAUGA</u>UCUAuuu	25	1	*hen1-1*

[a] Sequences of the miR173 and miR167 genomic loci and the cloned sequences from L*er* (L) and *hen1-1* (H) are shown. The nucleotides in mature miRNAs are in uppercase letters. The 3′ flanking sequences in the genomic loci are represented by italic, lowercase letters. The sequence of the 5′ cloning primer used for PCR is underlined. Nucleotides in the cloned sequences that do not correspond to those in the mature miRNAs are in lowercase letters. For miR167, the sequences from four different loci (a, b, c, and d) in the genome (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml) are listed individually; these loci potentially encode three forms of miR167. The expression of each individual has not been examined previously.

[b] These sequences can theoretically be regarded as miR167c and miR167c with U tails. However, because miR167c was never cloned from L*er*, we believe they are truncated miR167a, b, or d with U tails.

Table 2

miR173* Cloned from L*er* and *hen1-1*

Name	Sequences[a]	Length (nt)	Number of Clones	Genotype
miR173*	GAUUCUCUGUGUAAGCGAAAG*agcuug*	21		L*er*
L1	GAUUCUCUGUGUAAGCGAAA	20	2	L*er*
L2	GAUUCUCUGUGUAAGCGAAAa	21	1	L*er*
L3	GAUUCUCUGUGUAAGCGAAAG	21	14	L*er*
H1	GAUUCUCUGUGUAAGCGAAAa	21	3	*hen1-1*
H2	GAUUCUCUGUGUAAGCGAAAG	21	14	*hen1-1*
H3	GAUUCUCUGUGUAAGCGAAAGu	22	1	*hen1-1*

[a]The sequences of the miR173* genomic locus and cloned miR173* sequences from L*er* (L1–L3) and *hen1-1* (H1–H3) are shown. The nucleotides in mature miR173* are in uppercase letters. The 3′ flanking sequence in the genomic locus is represented by italic, lowercase letters. The sequence of the 5′ cloning primer used for PCR is underlined. Nucleotides that are in the cloned sequences and do not correspond to those in the mature miRNAs are in lowercase letters.