Published in final edited form as:
Curr Biol. 2012 April 24; 22(8): 689–694. doi:10.1016/j.cub.2012.02.051.

HESO1, a nucleotidyl transferase in *Arabidopsis*, uridylates unmethylated miRNAs and siRNAs to trigger their degradation

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SUMMARY

microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) impact numerous biological processes in eukaryotes. In addition to biogenesis, turnover contributes to the steady-state levels of small RNAs. One major factor that stabilizes miRNAs and siRNAs in plants as well as siRNAs and piRNAs in animals is 2′;-*O*-methylation on the 3′; terminal ribose by the methyltransferase HUA ENHANCER1 (HEN1) [1–6]. Genetic studies with *Arabidopsis*, *Drosophila* and zebrafish *hen1* mutants show that 2′-*O*-methylation protects small RNAs from 3′-to-5′ truncation and 3′ uridylation, the addition of non-templated nucleotides, predominantly uridine [2, 7, 8]. Uridylation is a widespread phenomenon that is not restricted to small RNAs in *hen1* mutants, and is often associated with their reduced accumulation ([7, 9, 10]; reviewed in [11]). The enzymes responsible for 3′ uridylation of small RNAs when they lack methylation in plants or animals have remained elusive. Here, we identify the *Arabidopsis HEN1 SUPPRESSOR1* (*HESO1*) gene as responsible for small RNA uridylation in *hen1* mutants. HESO1 exhibits terminal nucleotidyl transferase activity, prefers uridine as the substrate nucleotide, and is completely inhibited by 2′-*O*-methylation. We show that uridylation leads to miRNA degradation, and the degradation is most likely through an enzyme that is distinct from that causing the 3′ truncation in *hen1* mutants.

Keywords

miRNA; siRNA; HESO1; nucleotidyl transferase; methylation; uridylation

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RESULTS AND DISCUSSION

Loss of function in *HESO1* suppresses the morphological defects of a partial loss-of-function *hen1* mutant

We hypothesized that a terminal nucleotidyl transferase uridylates miRNAs in *Arabidopsis hen1* mutants and that uridylation serves as a signal to trigger miRNA degradation. Hence, we predicted that loss of function in this nucleotidyl transferase gene would lead to increased miRNA accumulation in *hen1* mutants and may rescue the morphological phenotypes associated with reduced miRNA accumulation in *hen1*. To identify the nucleotidyl transferase, we first searched for *Arabidopsis* proteins with sequence similarities to known nucleotidyl transferases with uridylation activity such as the human TUT4 [12], fission yeast cid-1 [13], and *Chlamydomonas* MUT-68 [9]. Ten putative *Arabidopsis* nucleotidyl transferases of unknown biological function were identified. Next, we obtained T-DNA mutants in each of the ten putative nucleotidyl transferase genes and crossed each mutant with *hen1-8*, a partial loss-of-function *hen1* mutant in the Col accession [14], the same accession in which the nucleotidyl transferase mutants were generated. We found that only the mutation in At2g39740 (Figures S1A and S1B) partially rescued the morphological phenotypes of *hen1-8* (Figures 1A and S1C). Therefore, we named this gene *HEN1 SUPPRESSOR1* (*HESO1*). The *hen1-8 heso1-1* double mutant was larger and had better fertility than the *hen1-8* single mutant (Figures 1A and S1C). To ensure that the *heso1-1* mutation was solely responsible for the phenotypic rescue of *hen1-8*, we introduced a *HESO1* genomic fragment fused in-frame to GFP (*pHESO1:HESO1-GFP*) into the *hen1-8 heso1-1* double mutant. Many independent transgenic lines showed phenotypes that resembled *hen1-8* mutants (Figure S1C), indicating that it was the *heso1-1* mutation that suppressed the defects of *hen1-8*. Some transgenic lines showed phenotypes even more severe than *hen1-8* mutants. It is possible that these lines had higher levels of *HESO1* expression, which enhanced *hen1-8* defects.

Loss of function in *HESO1* results in an increase in miRNA abundance

Suppression of the morphological defects of *hen1-8* by *heso1-1* suggested that miRNA activities were partially rescued by *heso1-1*. One activity of plant miRNAs is the cleavage of their target transcripts to lead to their degradation. We examined the levels of four miRNA target transcripts in wild type, *hen1-8*, and *hen1-8 heso1-1* by real-time RT-PCR. As expected, in *hen1-8*, these transcripts accumulated to higher levels as compared to wild type (Figure 1B), due to the reduced levels of miRNAs [14]. Indeed, the increase in the transcript levels in *hen1-8* was partially or fully rescued by *heso1-1* (Figure 1B).

Next, we tested whether the *heso1-1* mutation resulted in higher abundance of miRNAs. Northern blotting was performed to detect miR166, miR167, and miR173 in wild type, *heso1-1*, *hen1-8*, and *hen1-8 heso1-1*. Like other *hen1* alleles [8], the *hen1-8* mutation resulted in both a reduction in miRNA levels and the accumulation of heterogeneous species - bands corresponding to 3′ truncated as well as 3′ tailed species were present (Figure 1C). The *heso1-1* mutation did not affect miRNA accumulation in the wild-type background, but it resulted in an obvious increase in the abundance of the heterogeneous miRNA species in the *hen1-8* background (Figure 1C). The increase in miRNA levels in the double mutant was rescued by the *pHESO1::HESO1-GFP* transgene (Figure S1D). For miR167 and miR173, it was also apparent that the increase in abundance was restricted to normal-sized species, 3′ truncated species, or species with short tails (Figure 1C). This suggested that 3′ tailing was reduced in the *hen1-8 heso1-1* double mutant relative to *hen1-8*.

Our previous studies show that mutations in RNA polymerase IV (Pol IV) subunit genes, *NRPD1* and *NRPD2*, rescue the morphological, as well as miRNA accumulation, defects of

a weak *hen1* allele, *hen1-2*, which harbors the same molecular lesion as *hen1-8* but is in the Landsberg accession [14]. Pol IV is a key factor in the biogenesis of a predominant class of endogenous siRNAs, the 24 nucleotide (nt) siRNAs [15–18]. As natural substrates of HEN1 [8], the 24 nt siRNAs compete with miRNAs for methylation by the mutant HEN1-2 protein such that elimination of these siRNAs in *nrpd1* or *nrpd2* mutants allows miRNAs to gain methylation and thus increase in abundance in the *hen1-2* background. With β-elimination followed by northern blotting [6], miR165/166 and miR173 were found to be unmethylated in both *hen1-8* and *hen1-8 heso1-1* (Figure S1E). Therefore, unlike *nrpd1* or *nrpd2* mutations, the *heso1-1* mutation led to an increase in miRNA accumulation without affecting their methylation status.

Loss of function in *HESO1* results in reduced 3′ uridylation without affecting 3′ truncation

Although northern blotting suggested that the *heso1-1* mutation caused a reduction in 3′ tailing, the concomitant increase in miRNA accumulation made it difficult to determine the degree of reduction in 3′ tailing. To obtain a global and quantitative view of 3′ modifications of miRNAs, we constructed small RNA libraries from wild type, *heso1-1*, *hen1-8*, and *hen1-8 heso1-1* and subjected them to deep sequencing. Two biological replicates were performed for each genotype. An algorithm was developed to classify reads corresponding to a known miRNA into four categories: full-length, 3′ truncated only, 3′ tailed only, and 3′ truncated and tailed (see Experimental Procedures).

We first focused on the status of 3′ tailing of miRNAs. For each biological replicate, 44 miRNAs that were represented by at least 50 transcripts per million (TPM) in all four libraries were included in the analysis. 3′ tailing was quantified for each miRNA as a percentage of (3′ tailed only reads + 3′ truncated and tailed reads)/total reads. In this analysis, the lengths of the tails were not taken into account as long as the read had at least one non-templated nucleotide, it was considered a tailed species. For each of the miRNAs, representatives of which are shown in Figure 2A (replicate 2) and Figure S2A (replicate 1), the percentage of tailed species was reduced in *hen1-8 heso1-1* relative to *hen1-8*, indicating that *HESO1* was partially responsible for 3′ tailing in *hen1-8*. The *heso1-1* mutation did not affect the tailing status of most miRNAs in the wild-type background, with the exception of miR319a, which showed higher levels of tailing relative to other miRNAs in wild type (Figures 2A and S2A).

We next examined the nature of the non-templated nucleotides in the tails and the lengths of the tails. For each miRNA, all tailed reads (both full-length species with tails and 3′ truncated species with tails) were categorized by tail length, and the frequencies of the four nucleotides in the tails were calculated. Although the tailing patterns differed to some extent among the miRNAs, two observations were obvious (Figures 2B and S2B). First, uridine was the predominant nucleotide of the tails in *hen1-8* as previously reported for other *hen1* alleles [8]. Second, there was a shift towards shorter tails in *hen1-8 heso1-1* as compared to *hen1-8*. For miR166a and many other miRNAs, tails of 1 to 7 nt were found in *hen1-8*, but 1 nt tails were the most predominant in *hen1-8 heso1-1* (Figures 2B and S2B). These results demonstrated that *HESO1* was responsible for 3′ uridylation of miRNAs in *hen1*.

We next examined the status of 3′ truncation in relationship to 3′ tailing by *HESO1* for all 44 miRNAs abundantly represented in the libraries. We found similar results for nearly all miRNAs; ten representative miRNAs from the two biological replicates are shown in Figures 3 and S3. In wild type and the *heso1-1* single mutant, in which miRNA methylation was unaffected, most miRNA reads belonged to the full-length category. In *hen1-8*, the lack of methylation led to an increase in truncated only, tailed only, and truncated and tailed species. In *hen1-8 heso1-1*, 3′ tailing was greatly reduced, such that species with 0 or 1 nt tails were most abundant. Intriguingly, reads representing 3′ truncation were largely

unaffected by the *heso1-1* mutation, which was inconsistent with the hypothesis that 3′ uridylation triggers 3′ truncation.

Endogenous siRNAs are also 3′ uridylated in *hen1* mutants [8]. To determine whether HESO1 was responsible for the uridylation of 24 nt siRNAs, we focused on three abundantly represented 24 nt siRNAs (Table S1). All three siRNAs were truncated and tailed in *hen1-8*; In *hen1-8 heso1-1*, an obvious reduction in 3′ tailing was observed but 3′ truncated species were still present (Figures 3 and S3). Therefore, siRNAs and miRNAs were similarly affected by loss of function in *HESO1*.

HESO1 exhibits terminal nucleotidyl transferase activity

The above genetic evidence suggested that *HESO1* is responsible for 3′ uridylation of unmethylated miRNAs and siRNAs in *hen1* mutants. We sought biochemical evidence that HESO1 uridylated small RNAs with a recombinant His-HESO1 protein produced in *E. coli*. His-HESO1 was incubated with an RNA oligonucleotide corresponding to unmethylated miR173 that was radiolabeled at its 5′ end. HESO1 was able to add a U tail to miR173 that lengthened with time and reached up to approximately 60 nt after 40 min of incubation (Figure 4, lanes 1–6). To rule out that the terminal nucleotidyl transferase activities were due to a contaminating protein from *E. coli*, we mutated two aspartate residues in His-HESO1 that are part of the metal binding pocket in the nucleotidyl transferase domain to alanine (Figure S4). The mutant His-HESO1m protein was similarly purified and assayed for activity. The His-HESO1m protein was unable to add a U tail to unmethylated miR173 (Figure 4, lanes 13–14).

Next, we examined the enzymatic properties of HESO1. HESO1 was able to use ATP, CTP, or GTP, but the tail lengths with these nucleotides were much shorter than U tails, indicating that HESO1 preferred uridine (Figure 4, lanes 7–9). When dATP was used in the reaction, only one nucleotide was added (Figure 4, lane 10), suggesting that the 3′ OH of the substrate RNA was the site of phosphodiester bond formation. More importantly, when 2′-*O*-methylated miR173 was incubated with HESO1 in the presence of UTP, no tailing was observed (Figure 4, lanes 15–18). This indicated that HESO1 activity was completely inhibited by 2′-*O*-methylation.

CONCLUSIONS

Genetic and biochemical evidence in this study supports HESO1 as the nucleotidyl transferase that uridylates miRNAs and siRNAs when they lack 2′-*O*-methylation. The large increase in miRNA abundance in *hen1-8 heso1-1* vs. *hen1-8* demonstrates that uridylation leads to degradation. The fact that the *heso1-1* mutation caused a large reduction in 3′ uridylation without strong effects on 3′ truncation suggests that 3′ truncation observed in *hen1* mutants is largely independent of 3′ uridylation. Two implications from these observations are: 1) uridylation triggers the degradation of small RNAs by a nuclease distinct from the one that causes 3′ truncation; and 2) if the nuclease that degrades uridylated small RNAs is a 3′-to-5′ exonuclease, then it should be highly processive such that few or no degradation intermediates (i.e. 3′ truncated species) would be present *in vivo*. The lack of an effect on miRNA accumulation by *heso1-1* in wild type suggests that *HESO1* may not be part of the normal small RNA degradation machinery. However, given the presence of other nucleotidyl transferase genes with potentially overlapping functions, a role of *HESO1* in miRNA degradation in wild-type plants cannot be excluded. Although HESO1 activity is completely inhibited by 2′-*O*-methylation, it is possible that HESO1 acts cooperatively with SDN1, which truncates small RNAs [19], or another nucleotidyl transferase that is not inhibited by 2′-*O*-methylation, to degrade miRNAs. The activities of these enzymes would generate unmethylated miRNAs on which HESO1 can uridylate.

EXPERIMENTAL PROCEDURES

See supplemental information on-line.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Elizabeth Luscher for comments on this manuscript and Dr. Detlef Weigel for sharing the pJL-blue plasmid. We thank Lei Gao, Tammy Luu and Renee Zamora for technical assistance. This work was supported by grants from National Science Foundation (NSF MCB-1021465) to X.C., Chinese National Science Foundation (30970265) to B.M., and NSF (0701745) to B.C.M. Thanh D. was supported by an NSF ChemGen IGERT fellowship (DGE0504249), and Jixian Z. was supported by a University of Delaware graduate fellowship.

References

1. Horwich MD, Li C, Matranga C, Vagin V, Farley G, Wang P, Zamore PD. The Drosophila RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. Curr Biol. 2007; 17:1265–1272. [PubMed: 17604629]
2. Kamminga LM, Luteijn MJ, den Broeder MJ, Redl S, Kaaij LJ, Roovers EF, Ladurner P, Berezikov E, Ketting RF. Hen1 is required for oocyte development and piRNA stability in zebrafish. The EMBO journal. 2010; 29:3688–3700. [PubMed: 20859253]
3. Kirino Y, Mourelatos Z. The mouse homolog of HEN1 is a potential methylase for Piwi-interacting RNAs. RNA. 2007; 13:1397–1401. [PubMed: 17652135]
4. Kurth HM, Mochizuki K. 2′-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in Tetrahymena. RNA. 2009; 15:675–685. [PubMed: 19240163]
5. Saito K, Sakaguchi Y, Suzuki T, Suzuki T, Siomi H, Siomi MC. Pimet, the Drosophila homolog of HEN1, mediates 2′-O-methylation of Piwi- interacting RNAs at their 3′ ends. Genes & development. 2007; 21:1603–1608. [PubMed: 17606638]
6. Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, Steward R, Chen X. Methylation as a crucial step in plant microRNA biogenesis. Science. 2005; 307:932–935. [PubMed: 15705854]
7. Ameres SL, Horwich MD, Hung JH, Xu J, Ghildiyal M, Weng Z, Zamore PD. Target RNA-directed trimming and tailing of small silencing RNAs. Science. 2010; 328:1534–1539. [PubMed: 20558712]
8. Li J, Yang Z, Yu B, Liu J, Chen X. Methylation protects miRNAs and siRNAs from a 3′-end uridylation activity in Arabidopsis. Curr Biol. 2005; 15:1501–1507. [PubMed: 16111943]
9. Ibrahim F, Rymarquis LA, Kim EJ, Becker J, Balassa E, Green PJ, Cerutti H. Uridylation of mature miRNAs and siRNAs by the MUT68 nucleotidyltransferase promotes their degradation in Chlamydomonas. Proc Natl Acad Sci U S A. 2010; 107:3906–3911. [PubMed: 20142471]
10. van Wolfswinkel JC, Claycomb JM, Batista PJ, Mello CC, Berezikov E, Ketting RF. CDE-1 affects chromosome segregation through uridylation of CSR-1-bound siRNAs. Cell. 2009; 139:135–148. [PubMed: 19804759]
11. Kim YK, Heo I, Kim VN. Modifications of small RNAs and their associated proteins. Cell. 2010; 143:703–709. [PubMed: 21111232]
12. Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, Yeom KH, Han J, Kim VN. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell. 2009; 138:696–708. [PubMed: 19703396]
13. Rissland OS, Mikulasova A, Norbury CJ. Efficient RNA polyuridylation by noncanonical poly(A) polymerases. Mol Cell Biol. 2007; 27:3612–3624. [PubMed: 17353264]
14. Yu B, Bi L, Zhai J, Agarwal M, Li S, Wu Q, Ding SW, Meyers BC, Vaucheret H, Chen X. siRNAs compete with miRNAs for methylation by HEN1 in Arabidopsis. Nucleic Acids Res. 2010; 38:5844–5850. [PubMed: 20448024]

15. Herr AJ, Jensen MB, Dalmay T, Baulcombe DC. RNA polymerase IV directs silencing of endogenous DNA. Science. 2005; 308:118–120. [PubMed: 15692015]

16. Kanno T, Huettel B, Mette MF, Aufsatz W, Jaligot E, Daxinger L, Kreil DP, Matzke M, Matzke AJ. Atypical RNA polymerase subunits required for RNA-directed DNA methylation. Nat Genet. 2005; 37:761–765. [PubMed: 15924141]

17. Onodera Y, Haag JR, Ream T, Costa Nunes P, Pontes O, Pikaard CS. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell. 2005; 120:613–622. [PubMed: 15766525]

18. Pontier D, Yahubyan G, Vega D, Bulski A, Saez-Vasquez J, Hakimi MA, Lerbs-Mache S, Colot V, Lagrange T. Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in Arabidopsis. Genes & development. 2005; 19:2030–2040. [PubMed: 16140984]

19. Ramachandran V, Chen X. Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. Science. 2008; 321:1490–1492. [PubMed: 18787168]

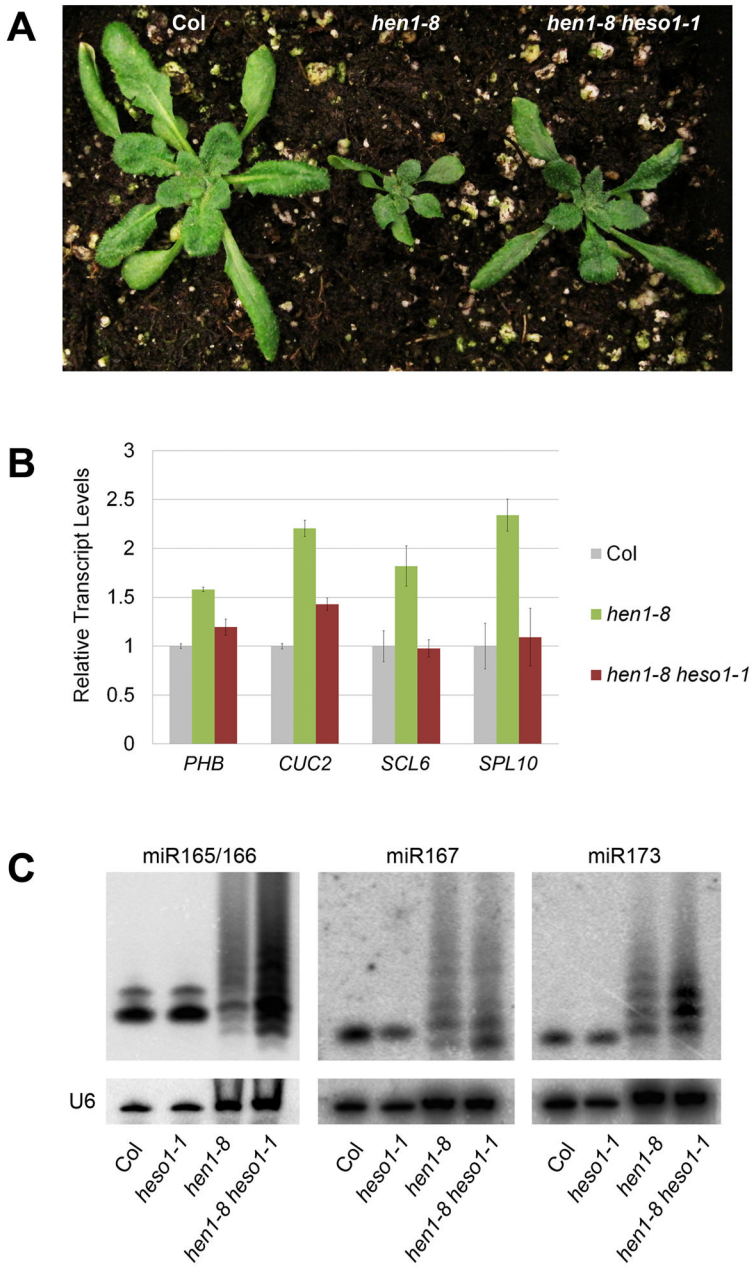


Figure 1.

The *heso1-1* mutation partially rescues the developmental and molecular defects of *hen1-8* plants. (A) 20-day-old plants of wild-type (Col), *hen1-8*, and *hen1-8 heso1-1* genotypes, as indicated. (B) Relative transcript levels from four miRNA target genes in the three genotypes as determined by real-time RT-PCR. The de-repression of the genes in *hen1-8*, due to reduced miRNA accumulation, was fully or partially rescued by *heso1-1. PHB*, *CUC2*, *SCL6*, and *SPL10* are targets of miR165/166, miR164, miR171, and miR156/157, respectively. (C) The accumulation of three miRNAs in the four genotypes as determined by northern blotting. Note that 50 μg of total RNA was used for *hen1-8* and *hen1-8 heso1-1* whereas 5 μg of total RNA was used for Col and *heso1-1*.

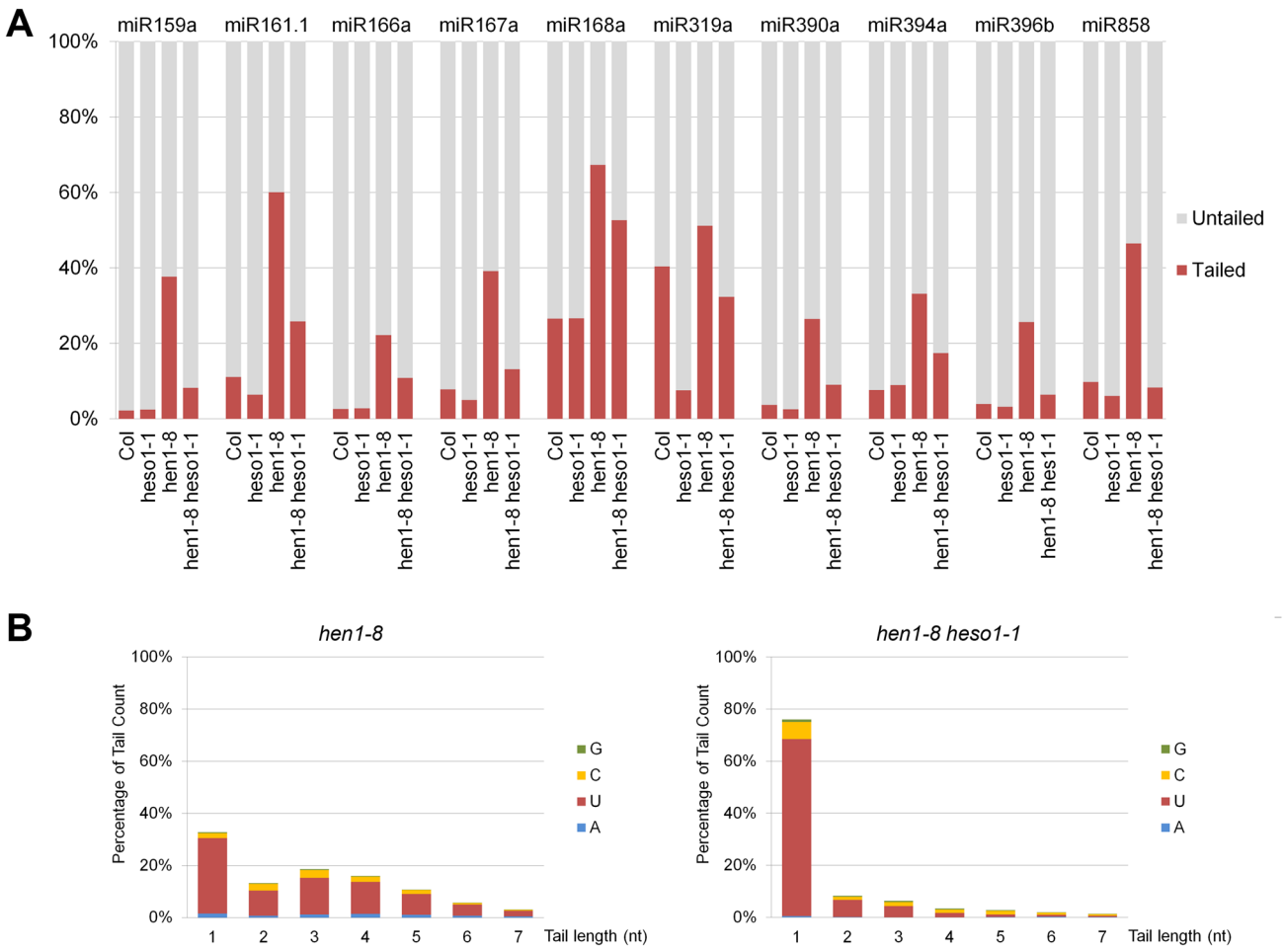


Figure 2.

The *heso1-1* mutation reduces 3′ uridylation of miRNAs in *hen1-8* as revealed by small RNA high throughput sequencing. Small RNA reads corresponding to known miRNAs were categorized into four classes: full-length (class 1), tailed only (full-length reads plus tails) (class 2), truncated only (class 3), and truncated and tailed (class 4). (A) The proportion of tailed and untailed reads for ten miRNAs in *hen1-8* and *hen1-8 heso1-1*. The proportions of tailed and untailed species were calculated as %(sum of classes 2 and 4 read numbers/total read number) and %(sum of classes 1 and 3 read numbers/total read number), respectively. Data from the second biological replicate were used for the calculations here. Similar results were obtained from biological replicate one (Figure S2A). (B) Tail length distribution and nucleotide frequencies in the tails of miR166a. All tailed miR166a reads (classes 2 and 4) were analyzed for frequencies of tail length. The proportions of miR166a species with tails of 1-7 nt are shown. Nucleotide frequencies in the tails were calculated as % (number of a nucleotide in the tail/tail length).

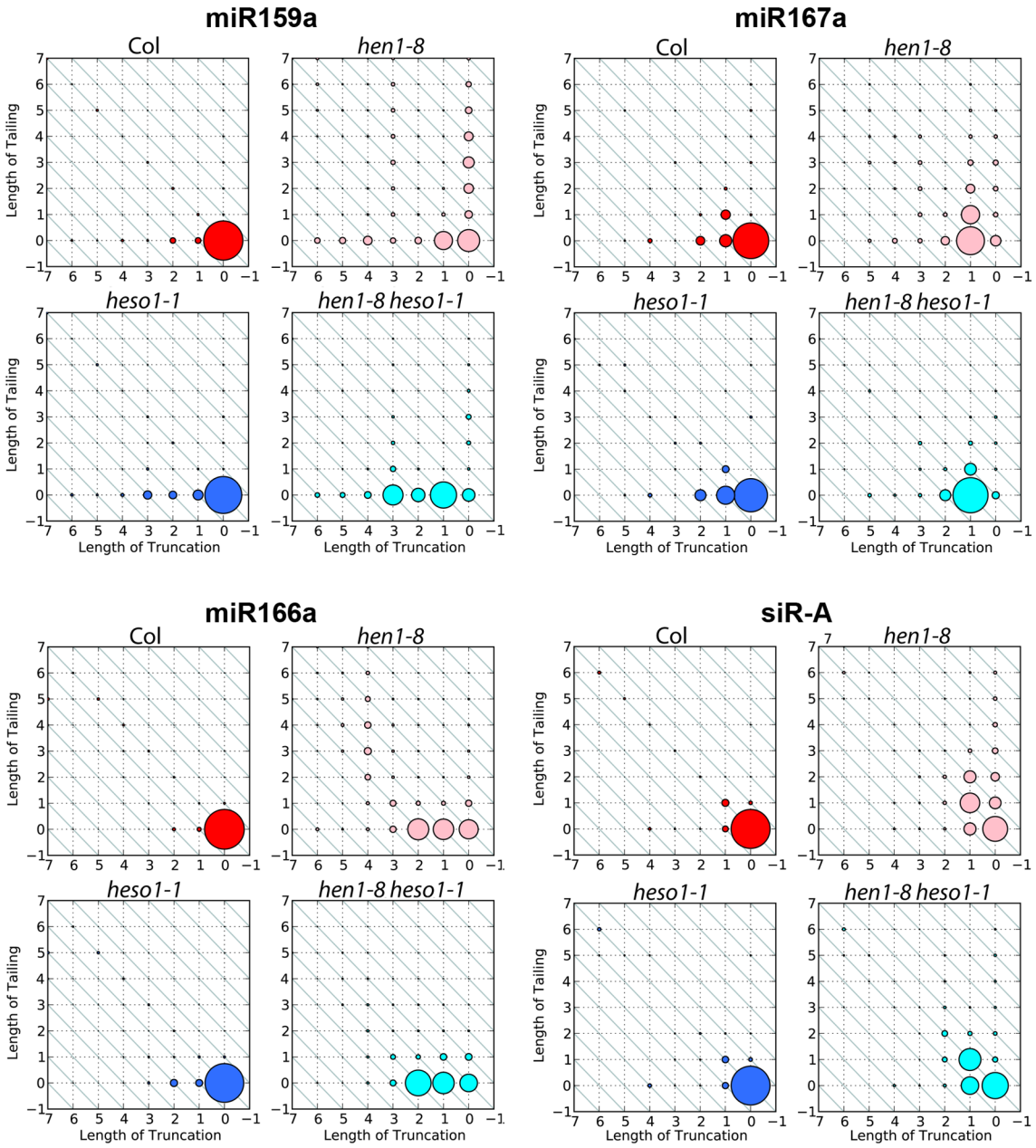
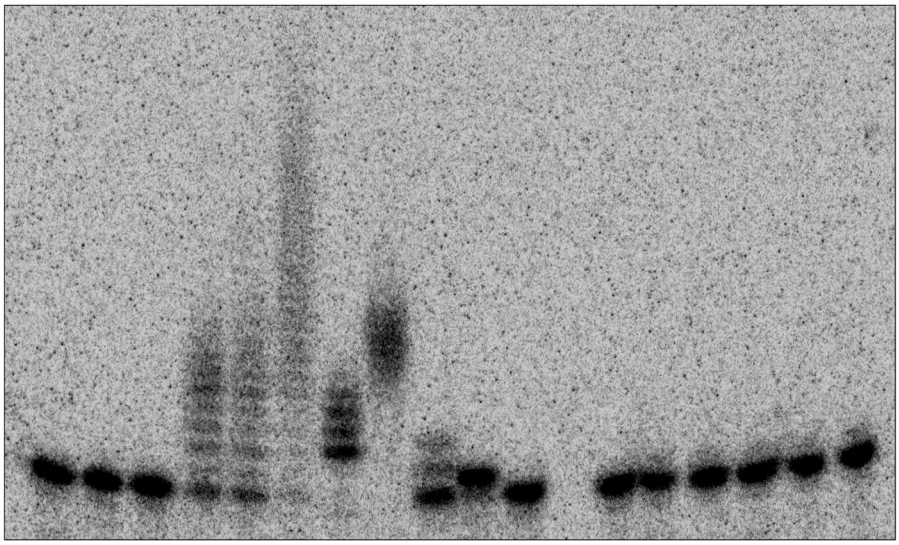


Figure 3.

The *heso1-1* mutation reduces 3′ tailing of three miRNAs and an siRNA without much effects on 3′ truncation in *hen1-8*. The status of 3′ truncation and/or 3′ tailing for each small RNA is represented by a two dimensional matrix in which the X-axis represents the extents of 3′ truncation, and the Y-axis represents the extents of 3′ tailing. The sizes of the circles indicate the relative abundance of the small RNA species. In *hen1-8*, both 3′ truncation and 3′ tailing occurred at a much higher frequency than in wild type. In *hen1-8 heso1-1*, 3′ tailing was drastically reduced but 3′ truncation was largely unaffected. Data from biological replicate two were used for the analysis here. Results from biological replicate one as well as results for more small RNAs from biological replicate two are shown in Figure S3.



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Time (min)	0	40	0	5	20	40	40	40	40	40	40	40	0	40	0	40	0	40
Nucleotide	U	U	U	U	U	U	A	C	G	dA	-	U	U	U	U	U	U	U
Protein	-	-	W	W	W	W	W	W	W	W	W	W	M	M	-	-	W	W
Oligo	miR173											-	miR173		miR173-2'-*O*-Me			

Figure 4.

HESO1 exhibited terminal nucleotidyl transferase activity. The nucleotidyl transferase assay was conducted with 5′ radiolabeled miR173 without or with 2′-*O*-methylation. Recombinant wild-type (W) or mutant His-HESO1, in which two catalytic residues were mutated (M; Figure S4), was incubated with the miRNA in the presence of various nucleotide triphosphates. Nucleotidyl transferase activity is represented by the presence of higher molecular weight bands relative to the input miRNA. The "-" signs indicate the absence of the nucleotide triphosphate, protein, or RNA oligonucleotide.