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siRNAs compete with miRNAs for methylation by HEN1 in *Arabidopsis*

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ABSTRACT

Plant microRNAs (miRNAs) and small interfering RNAs (siRNAs) bear a 2′-*O*-methyl group on the 3′-terminal nucleotide. This methyl group is post-synthetically added by the methyltransferase protein HEN1 and protects small RNAs from enzymatic activities that target the 3′-OH. A mutagenesis screen for suppressors of the partial loss-of-function *hen1-2* allele in *Arabidopsis* identified second-site mutations that restore miRNA methylation. These mutations affect two subunits of the DNA-dependent RNA polymerase IV (Pol IV), which is essential for the biogenesis of 24 nt endogenous siRNAs. A mutation in *RNA-dependent RNA polymerase 2*, another essential gene for the biogenesis of endogenous 24-nt siRNAs, also rescued the defects in miRNA methylation of *hen1-2*, revealing a previously unsuspected, negative influence of siRNAs on HEN1-mediated miRNA methylation. In addition, our findings imply the existence of a negative modifier of HEN1 activity in the Columbia genetic background.

INTRODUCTION

In *Arabidopsis*, microRNAs (miRNAs) and small interfering RNAs (siRNAs) represent an average of 15% and 85% of cellular small RNAs, respectively (1–4). A subset of endogenous siRNAs is 21-nt *trans*-acting siRNAs (ta-siRNAs) derived from non-coding RNAs (5,6). The largest class of endogenous siRNAs representing 84% of the cellular small RNA population is that of 24-nt siRNAs, which tend to be derived from repeat sequences and transposons (1,2,4).

The biogenesis of endogenous 24-nt siRNAs requires RNA-dependent RNA polymerase 2 (RDR2) (7), and also two DNA-dependent RNA polymerases, Pol IV and Pol V (8–12). In *Arabidopsis*, *NRPD1* and *NRPE1* encode the largest subunits of Pol IV and Pol V, respectively, while *NRPD2*/*NRPE2* (which we will hereafter refer to as *NRPD2*) encodes the shared, second largest subunit of the two polymerases. Pol IV is required for the biogenesis of almost all species of 24-nt siRNAs, while Pol V is only required for a subset of siRNAs, usually siRNAs from highly repeated sequences. It has been proposed that Pol IV transcribes all loci that give rise to siRNAs to generate precursors of siRNAs (8,11). Pol V generates non-coding transcripts at silenced loci (13) and is required for siRNA-mediated DNA methylation (9,12). It is thought that the role of Pol V in siRNA biogenesis is indirect such that Pol V-mediated DNA methylation at some loci leads to siRNA production in a feed-forward loop (12).

Plant miRNAs and siRNAs carry a 2′-*O*-methyl group on the 3′-terminal nucleotide, a modification introduced by the methyltransferase HEN1 (14,15). In plants carrying the severe *hen1-1* allele, small RNAs lack methylation, accumulate at a lower level, and become heterogeneous in size due to the presence of one to six additional nucleotides, usually uridines, at the 3′-end of the small RNAs (14,16). siRNAs and piRNAs from animals also carry a 2′-*O*-methyl group, which is introduced by animal HEN1 homologs (17,18).

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Here we report that loss-of-function *nrpd1*, *nrpd2* and *rdr2* alleles rescue miRNA methylation defects of *hen1-2*, a weak *hen1* allele, suggesting that siRNAs compete with miRNAs for methylation in *Arabidopsis* when HEN1 function is compromised. Furthermore, our results with partial loss-of-function *hen1* alleles from different ecotypes suggest the presence of a negative modulator of HEN1 activity in the Columbia (Col) genetic background.

MATERIALS AND METHODS

Plant strains

The mutants, *rdr2-1* (7), *nrpd1-4* (8), *nrpe1-11* [formerly *nrpd1b-1* (12) and later renamed *nrpd1b-11*] and *hen1-8* are in the Col genetic background. *hen1-1* and *hen1-2* (19) are in the L*er* genetic background.

To obtain the *rdr2 hen1-2* double mutant in the L*er* genetic background, we first constructed RDR2/*rd2-1 HEN1*/*hen1-2* by crossing *hen1-2* (L*er* background) with *rdr2-1* (Col background). Plants of this genotype were subjected to two rounds of crosses to L*er*. In the F1 populations of each round of crosses, the double heterozygous mutants were determined through genotyping of *rdr2-1* and *hen1-2* (7,19). The three-time backcrossed *RDR2*/*rdr2-1 HEN1*/*hen1-2* was crossed to *hen1-2*. In the F1 population, *RDR2*/*rdr2-1 hen1-2*/*hen1-2* plants were identified through genotyping and allowed to self. In the F2 population, *rdr2-1 hen1-2* double mutants were identified.

To construct *hen1-1 nrpd1-8* or *hen1-1 nrpd2-16*, *hen1-1* was crossed to *hen1-2 nrpd1-8* or *hen1-2 nrpd2-16*. The desired double mutants were then identified through genotyping of *hen1-1* (19) and *nrpd1-8 or nrpd2-16* in the F2 populations. The *nrpd1-8* mutation was genotyped through digestion of the F16M19-9F (5′-ggcgtttaat gccacaaact-3′)/F16M19-9R (5′-cagacatgttttgtttcgcttt -3′) PCR product with AccI, which could cut the PCR product from *nrpd1-8* but not from wild type. For *nrpd2-16* genotyping, the NRPD2-mobF (5′-caagag acgctcatgcagatt-3′)/NRPD2-mobR (5′-agccagttgcagaca ggcag-3′) PCR product was digested with MoblII. *nrpd2-16* resulted in the generation of a MoblII site.

To construct the *nrpd1 hen1-8* or *nrpe1 hen1-8* double mutant, *hen1-8* was crossed to *nrpd1-4* or *nrpe1-11*. The desired double mutants were identified in the F2 populations through genotyping *nrpd1-4*, *nrpe1-11* and *hen1-8* (8,12,19).

MAP-based cloning of *NRPD1* and *NRPD2*

hen1-2 suppressors were crossed to *hen1-8*, which contains the same point mutation in *HEN1* as *hen1-2* but is in the Col genetic background. In the F2 population, plants with long siliques were collected as the mapping population. Initial mapping showed that the two suppressors were linked to the markers nga280 on chromosome 1 and nga162 on chromosome 3, respectively. New markers in these two regions were developed according to polymorphisms between L*er* and Col (https://www.arabidopsis.org/cgi-bin/cereon/cereon_login.pl).

Complementation assay

A ~10 kb genomic fragment containing the *NRPD1* coding and promoter regions was amplified by PCR using primers 5′-gaggtaccttgctgaaatggtgattgaga-3′ and 5′-gaggatcctgggtcatcaagttgtcaaa-3′, and cloned into the pPZP211 binary vector to generate pPZP-NRPD1. Similarly, ~7.7 kb genomic fragment containing the *NRPD2* coding and promoter regions was amplified by PCR using primers 5′-cgggatccgtgtcccattgttgtgcaag-3′ and 5′-cgggatccggagcaaccccaactttgta-3′ and cloned into pPZP211 to generate pPZP-NRPD2. The pPZP-NRPD1 and pPZP-NRPD2 plasmids were transformed into *nrpd1-8 hen1-2* and *nrpd2-16 hen1-2*, respectively. The T1 transgenic plants were selected on medium containing 50 μg ml^{-1} kanamycin.

RNA and protein analysis

RNA isolation and hybridization for miRNAs and endogenous siRNAs were carried out as described (16). Radioactive signals were detected with a phosphorimager. Sodium periodate treatment and β elimination were done as described (15). Western blotting to determine the levels of HEN1 in L*er* and Col was performed with polyclonal anti-HEN1 antibodies generated in the Chen lab. The anti-Hsp73 mouse monoclonal antibody (Stressgen cat# SPA-818) was used to detect Hsp70 proteins from *Arabidopsis* as a loading control.

Bioinformatic analysis of miRNAs

The *rdr2* and wild-type libraries were described previously (Nobuta *et al*., 2008). miRNAs annotated in miRBase were selected and their normalized abundance (TP2M, transcripts per 2 million) was determined in both libraries. The relative abundance of a miRNA in the total miRNA population was calculated as: individual miRNA count/total miRNA count.

RESULTS

nrpd1 and *nrpd2* mutations suppress the *hen1-2* fertility defects

The *hen1-2* mutation results in the substitution of an aspartic acid located close to the S-adenosyl methionine-binding site by an asparagine (19). Both in terms of morphological and molecular (i. e. miRNA accumulation) defects, *hen1-2* is weaker than *hen1-1* (14,19), suggesting that the hen1-2 protein is partially functional. *hen1-2* plants have reduced fertility, as reflected by short fruits (siliques, Figure 1A). We carried out an EMS mutagenesis screen in *hen1-2* and isolated two suppressors with longer siliques (Figure 1A). The average length of siliques in the two suppressors was increased by 50% compared with that of *hen1-2* (Figure 1B). Backcrosses to *hen1-2* showed that the two suppressors carry recessive, extragenic mutations. The two suppressors complemented each other, indicating that the two lines carry mutations in two genes. We mapped one suppressor mutation to an approximately 200 kb region of chromosome 1 that contains *NRPD1*. Sequencing *NRPD1* revealed a G-to-A

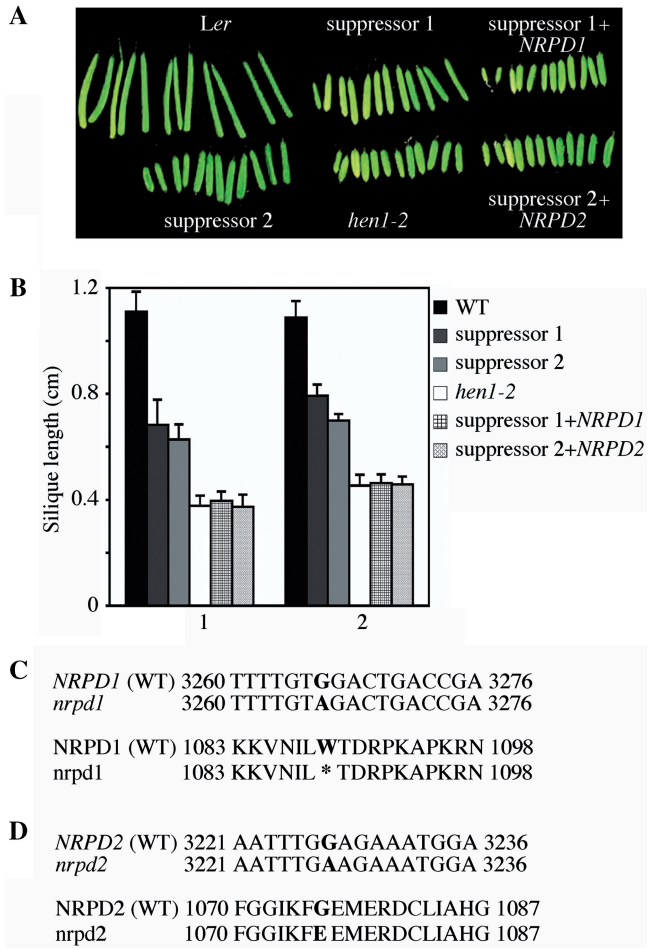

Figure 1. A mutation in *NRPD1* or *NRPD2* partially rescues the fertility defect of *hen1-2*. (**A**) The first 12 siliques from plants of the indicated genotypes. L*er*, wild type; suppressor 1 and 2 harbor extragenic mutations in the *hen1-2* background; suppressor 1+*NRPD1*, suppressor 1 harboring *NRDP1* genomic DNA. suppressor 2+*NRPD2*, suppressor 2 harboring *NRPD2* genomic DNA. (**B**) Quantification of silique length in various genotypes. 1, average length of the first five siliques; 2, average length of siliques 6–15. Eight plants from each genotype were included in the analysis. (**C**) Mutation in the *NRPD1* gene and protein in suppressor 1. A G-to-A mutation at nucleotide position 3266 of *NRPD1* results in a premature a stop codon at amino acid position 1089. (**D**) Mutation in the *NRPD2* gene and protein in suppressor 2. A G-to-A mutation at nucleotide position 3227 of *NRPD2* results in a glycine-to-glutamic acid conversion at amino acid position 1076. WT, wild type. The mutant gene and protein sequences are shown below the wild-type sequences.

mutation that results in a premature stop codon at amino acid 1089 (Figure 1C). We mapped the second suppressor mutation to an approximately 200 kb region of chromosome 3 that contains *NRPD2*. Sequencing *NRPD2* revealed a G-to-A mutation that results in the conversion of an invariant glycine among subunit II of RNA polymerase II and RNA polymerases IV and V to glutamic acid (Figure 1D). Introduction of *NRPD1* and *NRPD2* genomic sequences into the corresponding suppressor mutants reversed the fertility phenotype back to that of *hen1-2* (Figure 1A and B). Therefore, mutations in *NRPD1* and *NRPD2* are responsible for the partial rescue of *hen1-2* fertility. We named the new *nrpd* alleles

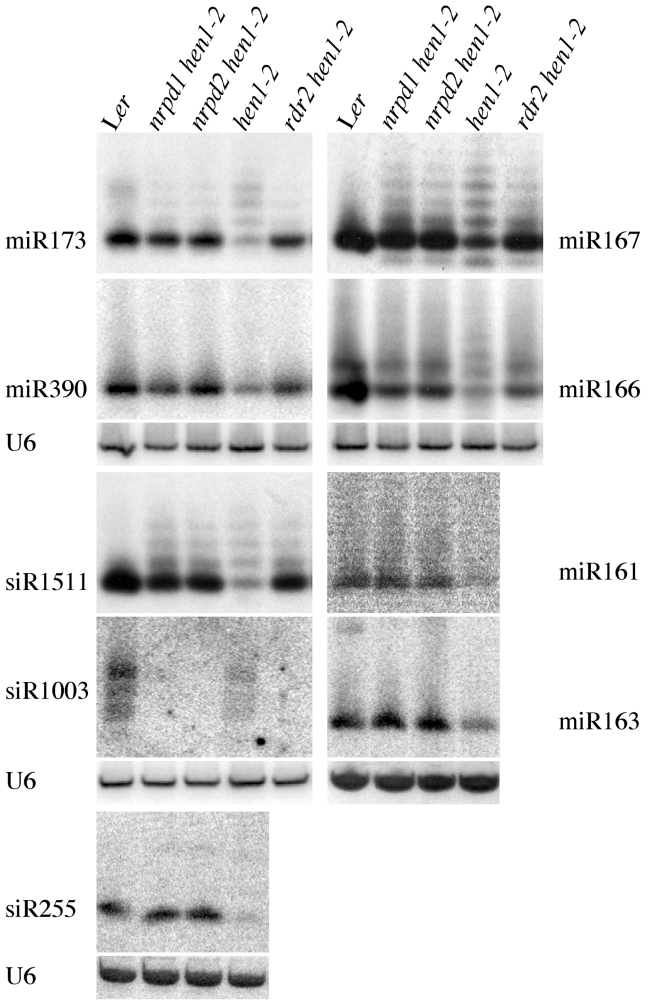

Figure 2. Loss-of-function *nrpd1*, *nrpd2* or *rdr2* mutations result in an increase in miRNA and ta-siRNA levels in *hen1-2*. Six miRNAs and three endogenous siRNAs in various genotypes were monitored by filter hybridization. U6 was used as a loading control. L*er*, wild type.

nrpd1-8 and *nrpd2-16*, respectively. Three Pol IV-dependent siRNAs, siRNA1003, cluster 2 and AtSN1 (8,9,11,12), were absent in the two suppressor lines (Figure 2 and Supplementary Figure S1B), indicating that *nrpd1-8* and *nrpd2-16* are potentially null alleles.

nrpd1 and *nrpd2* mutations increase the levels of miRNAs and ta-siRNAs in *hen1-2*

As the fertility defect of *hen1-2* is caused by the reduced accumulation of small RNAs, probably miRNAs, the *nrpd1* and *nrpd2* mutations may rescue the *hen1-2* fertility defect by increasing the accumulation of miRNAs. We first examined the abundance of six miRNAs in *nrpd1-8 hen1-2* and *nrpd2-16 hen1-2* by RNA filter hybridization. In the *hen1-2* mutant, the abundance of normal-sized miRNAs (i.e. miRNAs of the wild-type size) was greatly reduced as compared to wild type (Figure 2). In addition, uridylated (and therefore larger) species were detectable for miR173, miR167 and miR166 (Figure 2). In the two suppressor lines, the abundance of normal sized miRNAs

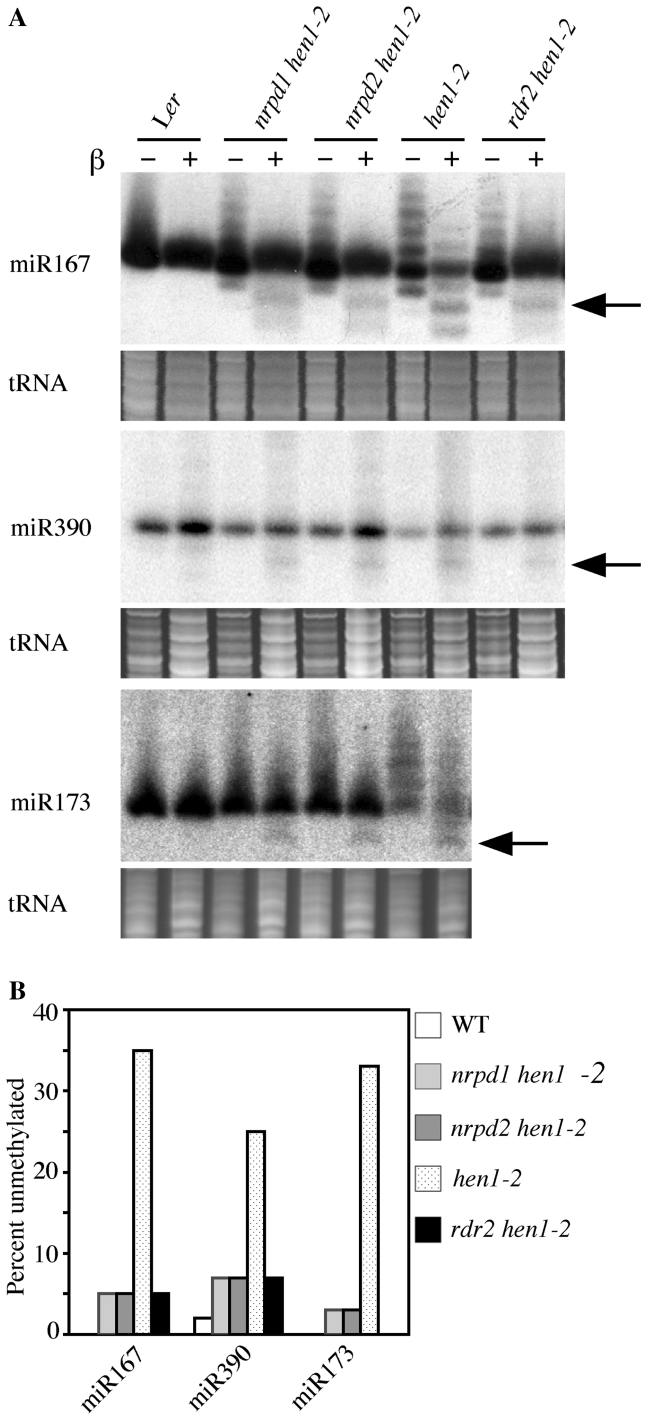

Figure 3. Increased methylation of miRNAs in *nrpd1-8 hen1-2, nrpd2-16 hen1-2* and *rdr2-1 hen1*-2. (**A**) Total RNAs treated (+) or not (–) with sodium periodate followed by β-elimination were separated on a 15% acrylamide gel and probed for various miRNAs by filter hybridization. Unmethylated miRNAs migrate ~2 nt faster after the chemical treatment, while methylated miRNAs do not change mobility. The arrow in each panel marks the expected position where a normal sized and unmethylated miRNA would migrate after the chemical treatments. The ethidium-bromide stained gel in the region of tRNAs was shown below the corresponding miRNA blot to indicate the amount of RNAs used. (**B**) The proportion of unmethylated miRNAs within the population of normal sized miRNA species in each genotype. The numbers were derived from quantification of the intensity of the marked bands (unmethylated miRNAs) and the intensity of the major species in the '–' lanes (total miRNAs of normal size) in Figure 3A and calculation of the ratio of the two intensities. L*er*, wild type.

was increased to a level similar to that of wild type. Furthermore, the proportion of uridylated forms of miR173, miR167 and miR166 was reduced in the two suppressor lines as compared to *hen1-2* (Figure 2). The profiles of miR173 species were nearly identical between *hen1-2* and the two suppressor lines rescued with *NRPD1* or *NRPD2* (Supplementary Figure S1A), demonstrating that the loss of *NRPD1* and *NRPD2* in *hen1-2* caused the increased abundance of normal sized miRNAs and the reduced levels of uridylated miRNAs. In addition, we found that the levels of two ta-siRNAs (20), siRNA255, a ta-siRNA from the *TAS1* locus and siRNA1511, a ta-siRNA from the *TAS2* locus, were increased in the two suppressor lines (Figure 2). Uridylated siRNA1511 species were detected in *hen1-2* and the two suppressor lines, but the proportion of uridylated siR1511 was reduced in the two suppressor lines.

nrpd1 and *nrpd2* mutations enhance miRNA methylation in *hen1-2*

The increased accumulation and decreased uridylation of miRNAs in the two suppressor lines would be best explained by increased miRNA methylation because methylation protects miRNAs from degradation and uridylation (14,15). Therefore, we examined whether the *nrpd1* or *nrpd2* mutation in *hen1-2* enhanced miRNA methylation using the periodate/β-elimination assay (15; Figure 3). Loss of methylation would result in faster migration of the RNA in this assay. After the chemical treatment, a band that migrated ~2 nt faster than the normal sized miRNAs was detected (Figure 3A, arrow) in *hen1-2*, *nrpd1 hen1-2*, *nrpd2 hen1-2* but not in wild type. The band represents the portion of the normal sized miRNAs that was unmethylated. We quantified the amount of the unmethylated miRNAs and calculated the proportion of the unmethylated miRNAs among total miRNAs of normal size. There was a clear reduction in the proportion of unmethylated miRNAs in *hen1-2 nrpd1* and *hen1-2 nrpd2* as compared to *hen1-2* (Figure 3B), demonstrating that the *nrpd* mutations enhance miRNA methylation in *hen1-2*. Another formally possible explanation for the elevated miRNA accumulation in the two suppressor lines is increased transcription of the *MIR* genes. Real-time RT–PCR showed that the levels of pri-miR173 were similar in *hen1-2*, *nrpd1 hen1-2* and *nrpd2 hen1-2*. Hence, it is unlikely that the elevated miRNA levels in the two suppressor lines resulted from increased transcription of *MIR* genes.

nrpd1 and *nrpd2* mutations do not rescue miRNA defects of *hen1-1*

How do *nrpd1* and *nrpd2* mutations increase the methylation of miRNAs in *hen1-2*? One possibility is that Pol IV is required for the uridylation activity. If uridylation and methylation are competitive processes, reduced uridylation could lead to increased methylation. The second possibility is that Pol IV directly or indirectly inhibits HEN1-mediated miRNA methylation. Loss of

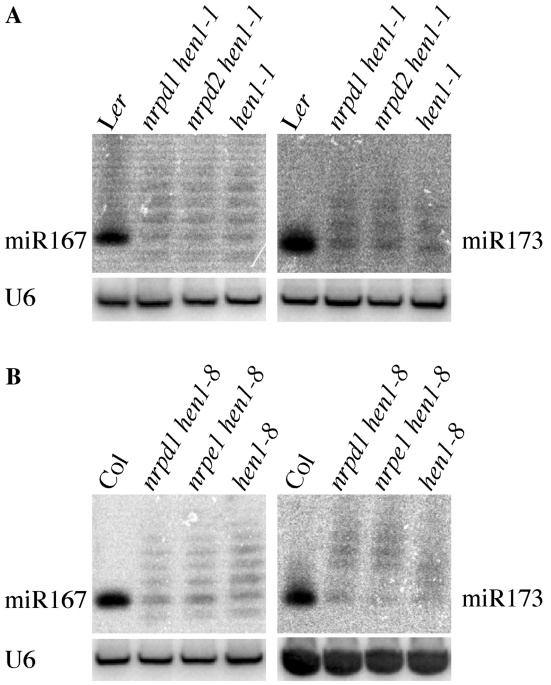

Figure 4. The accumulation of miRNAs in *hen1-1* or *hen1-8* is not changed by loss-of-function of Pol IV subunits. (**A**) The levels of miRNAs in *nrpd1-8 hen1-1* or *nrpd2-16 hen1-1*. L*er*, wild type. (**B**) The levels of miRNAs in *nrpd1-4 hen1-8* or *nrpe1-11 hen1-8*. U6 blots are loading controls.

Pol IV results in increased miRNA methylation, which protects miRNAs from uridylation and degradation.

To distinguish these two possibilities, we evaluated the effect of the *nrpd1* and *nrpd2* mutations in *hen1-1*, a severe allele that leads to complete loss of miRNA methylation. The *nrpd1-8 hen1-1* and *nrpd2-16 hen1-1* double mutants appeared morphologically indistinguishable from *hen1-1* plants. At the molecular level, *nrpd1* and *nrpd2* mutations were unable to rescue the miRNA defects of *hen1-1* (Figure 4A). The levels of normal sized miRNAs as well as the uridylated miRNAs were similar in *nrpd1-8 hen1-1*, *nrpd2-16 hen1-1* and *hen1-1*.

The presence of uridylated miRNAs in *nrpd1* (or *2*) *hen1-1* plants argues against a role of Pol IV in uridylating unmethylated miRNAs. The fact that Pol IV mutations lead to increased miRNA accumulation in *hen1-2* but not *hen1-1* suggests that Pol IV mutations suppress *hen1-2* by allowing the hen1-2 protein to better methylate miRNAs.

The *rdr2-1* mutation acts similarly to *nrpd1* and *nrpd2* mutations in *hen1-2*

How does Pol IV negatively impact miRNA methylation? One possibility is that Pol IV inhibits HEN1-mediated miRNA methylation by promoting the production of 24-nt siRNAs, which represent 84% of the cellular small RNA population and compete with the remaining small RNAs (miRNAs and tasiRNAs) for methylation by HEN1. If this were true, mutations in other genes essential for endogenous siRNA biogenesis would also result in increased abundance and methylation of miRNAs in *hen1-2*. We examined the abundance and methylation of miRNAs in *rdr2-1 hen1-2*, in which the Col *rdr2-1* allele was introgressed into L*er* by four backcrosses. Indeed, introducing *rdr2-1* into *hen1-2* increased the abundance of normal-sized miRNAs and reduced the proportion of uridylated miRNAs (Figure 2). In addition, like *nrpd1* (or *2*) *hen1-2*, the methylation of miRNAs was enhanced in *rdr2-1 hen1-2* (Figure 3).

nrpd1 and *nrpe1* mutations do not suppress *hen1-8* defects

The fact that mutations in Pol IV suppress the *hen1-2* defects prompted us to test whether mutations in Pol V might also suppress *hen1-2*. Because *nrpe1* alleles are only available in the Col ecotype, we took advantage of the Col-derived *hen1-8* allele (isolated from an independent genetic screen) that carries an identical molecular lesion as *hen1-2* to address our question. Like *hen1-2* but in contrast to the strong *hen1-4* allele in Col (21), *hen1-8* behaves as a weak *hen1* allele in terms of both its ability to suppress sense transgene post-transcriptional gene silencing (PTGS) and its responses to viral infection (Supplementary Figure S2B and C).

We crossed *nrpe1-11* [in the Col ecotype; formerly known as *nrpd1b-1* (12)] to *hen1-8*. We also crossed *nrpd1-4* (8) [formerly known as *nrpd1a-4*, also known as *nrpd1a-1* (12)] to *hen1-8* as a positive control. *nrpe1-11* was unable to suppress the fertility defects of *hen1-8*. Surprisingly, *nrpd1-4* was also unable to suppress the fertility defects of *hen1-8*. In addition, filter hybridization showed that the levels of normal-sized and uridylated miRNAs were the same in *nrpd1-4 hen1-8*, *nrpe1-11 hen1-8* and *hen1-8* (Figure 4B), indicating that loss-of-function of Pol IV or Pol V did not rescue the defects of *hen1-8* in the Col background. This is in contrast to the fact that the loss of function of Pol IV suppresses *hen1-2* in the L*er* background. It is unknown whether loss of function of Pol V would suppress *hen1-2* in the L*er* background. The *nrpe1-11* mutation in the Col background needs to be introgressed into L*er* to test this.

DISCUSSION

In this study, we found that *nrpd1* and *nrpd2* mutations result in increased miRNA methylation in the *hen1-2* background and partially rescue the *hen1-2* fertility defects. The partial rescue of miRNA defects of *hen1-2* by a mutation in *RDR2*, which is also required for endogenous 24-nt siRNA biogenesis, strongly supports the conclusion that endogenous siRNAs compete with miRNAs for methylation by the partially defective hen1-2 protein.

Such a competition between siRNAs and miRNAs may also occur when HEN1 activity is not compromised, albeit at a smaller scale. The levels of a number of miRNAs are unchanged in *nrpd1* or *nrpd2* mutants (8,9,11,12), suggesting that HEN1 activity is not limiting for these miRNAs. However, northern blots showed that the levels of nine miRNAs, including miR771, miR772 and others, are increased in the *rdr2-1* mutant and a *dcl2*

dcl3 dcl4 triple mutant that lacks most endogenous siRNAs (2). Our analysis of high-throughput sequencing data on miRNAs from wild type and *rdr2-1* (22) confirms that rare miRNAs including those detected by Lu et al. are increased in relative abundance among total miRNAs in *rdr2-1* as compared to wild type (Supplementary Table S1). Variation among miRNA levels has also been demonstrated in sequence-based comparisons of the maize *mop1-1* mutant to wild type (*mop1* is the maize ortholog of the *Arabidopsis RDR2*; 22). One possible explanation is that without competition from siRNAs, these miRNAs can be more efficiently methylated and accumulate to higher levels. However, other causes for the increased accumulation of these miRNAs are also possible. Although not all miRNAs are affected (in terms of their abundance) by the competition from siRNAs under normal conditions, it is intriguing to speculate that such a competition for HEN1 activity between siRNAs and miRNAs could be augmented in certain cell types or under circumstances where a burst of small RNA synthesis occurs (such as under viral infection).

In the course of our studies, we unexpectedly discovered that loss-of-function mutations in *NRPD1* suppress the Landsberg *hen1-2* allele but not *hen1-8*, a Col allele that carries the same molecular lesion as *hen1-2*. This is not due to differences in the strength of the *nrpd1* alleles since they result in the absence of siRNAs in both L*er* (Figure 1) and Col (8,12). The most likely reason that *nrpd1* rescues *hen1-2* but not *hen1-8* is that HEN1 has a stronger activity in L*er* than in Col. In fact, *hen1-8* exhibits more severe fertility defects than *hen1-2*. At the molecular level, *hen1-8* exhibits similar levels of miRNA impairment as *hen1-4* and *hen1-5*, two strong *hen1* mutant alleles in the Col genetic background (21,23; Supplementary Figure S2A). While normal-sized miR167 accounts for the majority of miR167 species in *hen1-2* (Figures 2 and 3), no apparent enrichment for normal-sized miR167 is found in *hen1-8* (Figure 4B). The stronger methylation activity of *hen1-2* relative to *hen1-8* may be due to intrinsic differences between the L*er* and Col HEN1 proteins, or due to the presence of a negative modulator of *HEN1* expression or activity in Col. Western blotting showed that the levels of HEN1 were similar in L*er* and Col inflorescences (Supplementary Figure S3). The HEN1 protein in L*er* differs from that in Col by a single amino acid outside the methyltransferase domain (19). Although we cannot rule out that this single amino acid difference contributes to differences in HEN1 activity, genetic mapping pinpointed a locus on chromosome 1 that underlies the differences in phenotypic severity between *hen1-2* and *hen1-8* (Bin Yu, unpublished results). Since HEN1 resides on chromosome 4, it is likely that another gene modulates the activity of HEN1. *Arabidopsis* accessions exhibit natural variations in many processes, including flowering time, light response, lipid metabolism, and hormone responses (24). Our data indicate that natural genetic variation also modulates the biogenesis of small RNAs. Identification of this negative regulator of HEN1 will help elucidate the mechanisms controlling small RNA methylation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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