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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, NRPDF1 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 animal HEN1 homologs (17,18).
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 Ler genetic background.

To obtain the *rdr2 hen1-2* double mutant in the Ler genetic background, we first constructed RDR2/*rd2-1 HEN1/hen1-2* by crossing *hen1-2* (Ler background) with *rdr2-1* (Col background). Plants of this genotype were subjected to two rounds of crosses to Ler. 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/*rd2-1* HEN1/hen1-2 was crossed to *hen1-2*. In the F1 population, RDR2/*rd2-1 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'-gggtaggtcagtccagagta-3')/F16M19-9R (5'-cagacagttctgagttggca-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'-gaag acctgtagcttgccga-3')/NRPD2-mobR (5'-agcatgtagcagcagggggcc-3') PCR product was digested with MobIII. *nrpd2-16* resulted in the generation of a MobIII 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 Ler and Col (https://www.arabidopsis.org/cgi-bin/cereon/cereon_login.pl).

Complementation assay

A ~10kb genomic fragment containing the *NRPD1* coding and promoter regions was amplified by PCR using primers 5’-gaggtcagtagctagatgcttttatttctgaga-3’ and 5’-gaggtcagtagctagatgcttttatttctgaga-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’-cagacagttctgagttggca-3’ and 5’-gaggtcagtagctagatgcttttatttctgaga-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 Ler 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**

*nrdp1* 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

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 \textit{NRPD2}. Sequencing \textit{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 \textit{NRPD1} and \textit{NRPD2} genomic sequences into the corresponding suppressor mutants reversed the fertility phenotype back to that of \textit{hen1-2} (Figure 1A and B). Therefore, mutations in \textit{NRPD1} and \textit{NRPD2} are responsible for the partial rescue of \textit{hen1-2} fertility. We named the new \textit{nrpd} alleles \textit{nrpd1-8} and \textit{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 \textit{nrpd1-8} and \textit{nrpd2-16} are potentially null alleles.

\textit{nrpd1} and \textit{nrpd2} mutations increase the levels of miRNAs and ta-siRNAs in \textit{hen1-2}

As the fertility defect of \textit{hen1-2} is caused by the reduced accumulation of small RNAs, probably miRNAs, the \textit{nrpd1} and \textit{nrpd2} mutations may rescue the \textit{hen1-2} fertility defect by increasing the accumulation of miRNAs. We first examined the abundance of six miRNAs in \textit{nrpd1-8} and \textit{nrpd2-16} by RNA filter hybridization. U6 was used as a loading control. Ler, 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/b-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, nrpd2 hen1-2 but not in wild type. 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
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 uridyling 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 Ler 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 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*
to the presence of a negative modulator of chromosome 4, it is likely that another gene modulates two strong mutant alleles in the Arabidopsis 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 mopp1-1 mutant to wild type (mopp1 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 NRPDI 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 nrpdi alleles since they result in the absence of siRNAs in both Ler (Figure 1) and Col (8,12). The most likely reason that nrpdi rescues hen1-2 but not hen1-8 is that HEN1 has a stronger activity in Ler 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 Ler 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 Ler and Col inflorescences (Supplementary Figure S3). The HEN1 protein in Ler 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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**REFERENCES**


