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Mechanisms of HDA6-mediated rRNA gene silencing: suppression of intergenic Pol II transcription and differential effects on maintenance versus siRNA-directed cytosine methylation

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The *Arabidopsis* histone deacetylase HDA6 is required to silence transgenes, transposons, and ribosomal RNA (rRNA) genes subjected to nucleolar dominance in genetic hybrids. In nonhybrid *Arabidopsis thaliana*, we show that a class of 45S rRNA gene variants that is normally inactivated during development fails to be silenced in *hda6* mutants. In these mutants, symmetric cytosine methylation at CG and CHG motifs is reduced, and spurious RNA polymerase II (Pol II) transcription occurs throughout the intergenic spacers. The resulting sense and antisense spacer transcripts facilitate a massive overproduction of siRNAs that, in turn, direct de novo cytosine methylation of corresponding gene sequences. However, the resulting de novo DNA methylation fails to suppress Pol I or Pol II transcription in the absence of HDA6 activity; instead, euchromatic histone modifications typical of active genes accumulate. Collectively, the data reveal a futile cycle of unregulated transcription, siRNA production, and siRNA-directed DNA methylation in the absence of HDA6-mediated histone deacetylation. We propose that spurious Pol II transcription throughout the intergenic spacers in *hda6* mutants, combined with losses of histone deacetylase activity and/or maintenance DNA methylation, eliminates repressive chromatin modifications needed for developmental rRNA gene dosage control.

[**Keywords:** Chromatin; histone deacetylation; DNA methylation; epigenetics; RNA polymerase I; ribosomal RNA genes]

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In eukaryotes, ribosomal RNA (rRNA) genes are repeated head to tail at chromosomal loci known as nucleolar organizer regions (NORs) (see Fig. 1A). In the plant *Arabidopsis thaliana*, NORs composed of ~375 rRNA genes abut the telomeres on the northern ends of chromosomes 2 and 4 (Copenhaver et al. 1995; Copenhaver and Pikaard 1996a,b). Each rRNA gene repeat can be transcribed by RNA polymerase I (Pol I) to produce a 45S pre-rRNA primary transcript. The primary transcript is

then processed into the 18S, 5.8S, and 25S rRNAs that partner with 5S rRNA, transcribed by Pol III, and ~80 proteins, encoded by mRNAs transcribed by Pol II, to form the 40S and 60S ribosomal subunits (Korostelev and Noller 2007).

Each rRNA gene is separated from the adjacent rRNA gene by an intergenic spacer (IGS) that contains multiple regulatory elements (Reeder 1989). In *A. thaliana*, the IGS includes the gene promoter, spacer promoters, and so-called Sal repeats (Fig. 1A; Gruendler et al. 1991). Spacer promoters share >85% similarity with the gene promoter but are only ~10% as active in transient expression assays (Doelling et al. 1993). The Sal repeats, named for the presence of SalI restriction endonuclease sites, occupy the same positions as enhancer repeats in *Xenopus* and mice, and can functionally substitute for *Xenopus* enhancers in frog oocytes (Pikaard et al. 1990; Doelling

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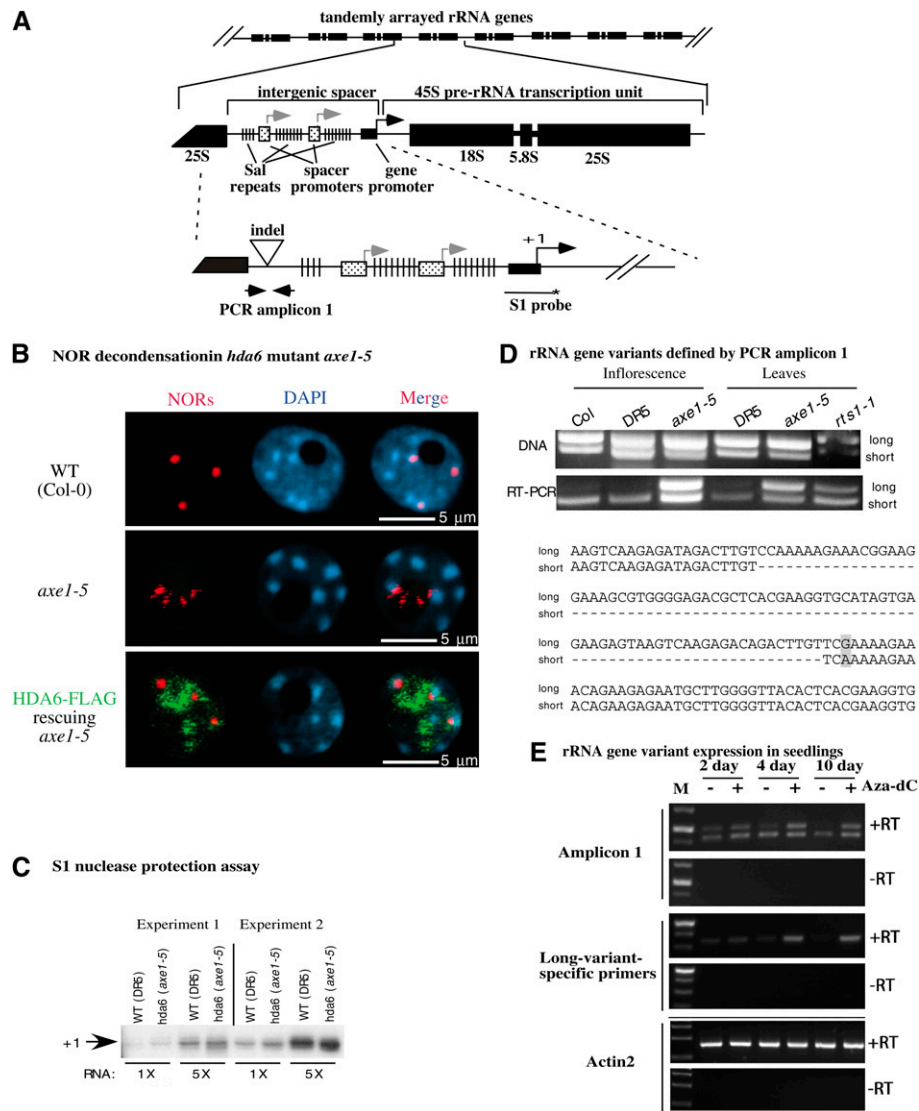


Figure 1. *HDA6* is required for developmentally regulated silencing of 45S rRNA gene variants. (A) rRNA gene organization showing the tandem arrangement of rRNA genes at NORs and the structure of individual rRNA gene repeats. In the *middle* drawing, the locations of the IGS and the 45S pre-rRNA transcription unit are highlighted, as well as the gene promoter (black arrow, +1), duplicated spacer promoters (gray arrows), and repetitive elements known as Sal repeats. The *bottom* drawing shows the locations of the promoter S1 probe and PCR amplicon 1, which includes an indel that allows long and short variants to be discriminated. (B) NORs are decondensed in *hda6* mutants. Nuclei isolated from wild-type (WT) plants, *axe1-5* mutants, or *axe1-5* transformed with an *HDA6-Flag* transgene were subjected to DNA-FISH using a 45S rRNA gene probe (red signals). *HDA6-Flag* protein was immunolocalized using anti-Flag antibody. Nuclei were counterstained with DAPI (blue signals). Bars, 5 μ m. (C) 45S pre-rRNA transcripts initiated at the RNA Pol I start site (+1), detected using S1 nuclease protection of RNA from wild-type (DR5) or *hda6* mutant (*axe1-5*) plants. RNA was isolated from two sets of plants grown independently (experiments 1 and 2) and tested at two input RNA concentrations, showing that the results are semiquantitative. The S1 probe location is shown in A. (D) Long and short rRNA gene variants defined by amplicon 1 are differentially expressed. The *top* panel shows ethidium bromide-stained amplicon 1 PCR products obtained using genomic DNA from inflorescence or leaves as the template. The *bottom* panel shows RT-PCR products amplified from cDNA generated by random priming of total RNA. Wild-type Col-0 and DR5 (a Col-0 transgenic line from which *axe1* mutants were isolated) controls are compared with the *hda6* mutants *axe1-5* and *rts1-1*. Sequences of long and short PCR products in the vicinity of the indel present within amplicon 1 are shown at the *bottom* of the panel. (E) Silencing of long rRNA gene variants is developmentally regulated. Wild-type Col-0 seedlings were germinated on media containing (+) or lacking (-) 10 mM aza-dC and then harvested 2, 4, or 10 d after germination. Ethidium bromide-stained RT-PCR reaction products, and products of controls in which reverse transcriptase was omitted (-RT), are shown using amplicon 1 primers (*top*) or primers specific for long rRNA gene variants (*middle*). The *ACTIN2* control shows that equal amounts of RNA were subjected to PCR amplification in all reactions. Size markers (M) are present in the first lane of each gel.

et al. 1993). Despite their enhancer activity in *Xenopus*, enhancer activity has not been demonstrated for Sal repeats in *Arabidopsis* (Wanzenböck et al. 1997).

Only a subset of rRNA genes is active in somatic cells, with distinct post-translational chromatin modifications reflecting the “on” or “off” transcriptional states (Grummt and Pikaard 2003; Preuss and Pikaard 2007; McStay and Grummt 2008). Enzymes that regulate histone acetylation, histone methylation, DNA methylation, and nucleosome positioning are involved in the on/off switch (Sandmeier et al. 2002; Santoro et al. 2002; Lawrence et al. 2004; Earley et al. 2006a; Espada et al. 2007; Preuss et al. 2008). Other mechanisms, including phosphorylation of Pol I transcription factors, regulate the transcriptional capacity of genes in the on state, thereby fine-tuning the dosage control system (Grummt and Pikaard 2003; Russell and Zomerdijk 2006; Moss et al. 2007; McStay and Grummt 2008). As a result of fine-tuning, changes in the number of active genes do not necessarily result in increased rRNA synthesis, due to compensatory changes in the number of transcribing RNA Pol I enzymes per gene (French et al. 2003).

Evidence that histone deacetylation is involved in rRNA gene silencing first came from the study of nucleolar dominance, an epigenetic phenomenon in genetic hybrids that describes the expression of rRNA genes inherited from only one progenitor (McStay 2006; Preuss and Pikaard 2007). Initially, the histone deacetylase (HDAC) inhibitors Trichostatin A (TSA) and sodium butyrate were shown to derepress rRNA genes subjected to nucleolar dominance (Lawrence et al. 2004; Chen 2007). Subsequently, RNAi-mediated knockdown of the 16 predicted HDACs in *Arabidopsis suecica*, the allotetraploid hybrid of *A. thaliana* and *Arabidopsis arenosa*, revealed that HDA6 is required for the preferential silencing of *A. thaliana*-derived rRNA genes (Earley et al. 2006a). In vitro, HDA6 is a TSA-sensitive enzyme that removes acetyl groups from multiple core histone lysines, suggesting that HDA6 facilitates rRNA gene silencing via regional erasure of histone acetylation (Earley et al. 2006a). However, the rRNA gene intervals where HDA6 exerts its control have not been identified.

In addition to affecting rRNA gene silencing, *HDA6* is required for transgene silencing, as revealed by multiple genetic screens, including screens targeting components of the RNA-directed de novo cytosine methylation pathway (Furner et al. 1998; Murfett et al. 2001; Aufsatz et al. 2002; Probst et al. 2004; Matzke and Birchler 2005; He et al. 2009; Matzke et al. 2009). *ATCOPIA*, *ATLINE1-4*, *ATLANTYS*, *ATGP1*, and *Sadhu* transposable elements are also suppressed by HDA6, revealing a broad influence of HDA6 throughout the genome (Lippman et al. 2003; May et al. 2005; Rangwala and Richards 2007).

On a chromosomal scale, disruption of nucleolar dominance in allotetraploid *A. suecica* *HDA6*-RNAi lines correlates with decondensation of the multimegabase NORs (Earley et al. 2006a). On a genic scale, histone hyperacetylation, increased histone H3 Lys 4 (H3K4) trimethylation, and decreased cytosine methylation occur at rRNA gene promoters in *HDA6*-RNAi lines (Earley

et al. 2006a). In the diploid species *A. thaliana*, similar chromatin modifications occur in *hda6* mutants; likewise, NORs are decondensed (Probst et al. 2004), suggesting an increase in the number of active rRNA genes. However, no change in the abundance of transcripts initiated at rRNA gene promoters could be detected in a previous study of *A. thaliana hda6* mutants (Probst et al. 2004), making the basis for NOR decondensation in *hda6* mutants unclear.

In this study, we explored the molecular basis for HDA6-mediated repression of rRNA genes in *A. thaliana*. We show that a major function of HDA6 is to suppress spurious RNA Pol II transcription throughout the IGS. Consequently, sense and antisense IGS transcripts are overproduced in *hda6* mutants, giving rise to abundant siRNAs that are diced by DCL3 and DCL4. Resulting siRNAs, in turn, direct de novo cytosine methylation throughout the IGS. Interestingly, losses in maintenance methylation at CG and CHG motifs in *hda6* mutants occur in parallel with the gain of de novo asymmetric CHH methylation. Although siRNA-directed cytosine hypermethylation is typically repressive, this is not the case in *hda6* mutants. Instead, histone modifications typical of active genes accumulate, and Pol I and Pol II association with rRNA genes increases. Importantly, an abundant class of rRNA gene variants, normally expressed only during early seedling development, fails to be silenced in *hda6* mutants, and therefore remains active in mature plants. We propose that, in *hda6* mutants, spurious transcription throughout the IGS, combined with losses of maintenance cytosine methylation and HDAC activity, disrupts the chromatin modifications needed to repress rRNA gene promoters during development.

Results

HDA6 suppresses IGS transcription

NORs are dramatically decondensed in *A. thaliana hda6* mutants (Fig. 1B; Probst et al. 2004). In diploid *A. thaliana*, there are four NORs: one pair present on chromosome 2, and the other pair located on chromosome 4. NORs tend to coalesce such that fewer than four DNA-FISH (DNA fluorescent in situ hybridization) signals are typical in interphase nuclei using rRNA gene probes. For example, three NOR signals (red) are detected by DNA-FISH in the wild-type (ecotype Col-0) nucleus shown in Figure 1B. These bright FISH signals correspond to the portions of the NORs where excess, inactive rRNA genes are highly condensed and sequestered in heterochromatin at the external periphery of the nucleolus. In contrast, active rRNA genes are transcribed within the nucleolus, which appears as a black hole in nuclei stained with the fluorescent DNA-binding dye DAPI (blue signals). The filaments of highly decondensed, active rRNA genes do not yield FISH signals bright enough to be detected in Figure 1B.

Unlike wild-type nuclei, whose prominent NOR signals are external to the nucleolus, nuclei of *hda6* mutants

such as *axe1-5* (Murfett et al. 2001) display decondensed NOR signals within the nucleolus (Fig. 1B, middle panel). Transformation of *axe1-5* with a transgene encoding HDA6 fused to a Flag epitope tag (HDA6-Flag) rescues the mutation, such that compact NOR signals external to the nucleolus are again observed, as in wild-type nuclei (Fig. 1B, bottom panel). Localization of recombinant HDA6 by virtue of its Flag tag shows that HDA6 localizes within the nucleolus, as well as the nucleoplasm, in agreement with a previous study (Earley et al. 2006a).

NOR decondensation typically correlates with increased rRNA gene transcription. However, using S1 nuclease protection to monitor levels of accurately initiated transcripts at rRNA gene promoters, we observe no change in *axe1-5* mutants relative to the nonmutant control DR5 (Fig. 1C), in agreement with prior results (Probst et al. 2004). However, RT-PCR analyses reveal an effect of HDA6 on the differential expression of rRNA gene variants that are polymorphic at their 3' ends (Fig. 1D). Long and short rRNA gene variants can be discriminated by an 81-base-pair (bp) insertion/deletion (indel) present within PCR amplicon 1 (Fig. 1A, bottom panel). This indel is located within a region of the primary transcript that is removed during pre-rRNA processing (S Tucker, F Pontvianne, and CS Pikaard, unpubl.). PCR amplification of genomic DNA reveals that long and short rRNA gene variants are similar in abundance (Fig. 1D, top panel). However, RT-PCR analysis of total RNA reveals that only short variants are expressed in inflorescence or leaves of mature wild-type (Col-0 and DR5) plants (Fig. 1D, bottom panel). In contrast, both long and short variant transcripts are detected in the *hda6* mutants *axe1-5* and *rts1-1*. Collectively, these data indicate that HDA6 is involved in repressing long rRNA gene variants, which represent a significant fraction of the total rRNA gene pool.

During early seedling development in *Arabidopsis*, condensation of chromocenters and NORs into heterochromatin and silencing of rRNA genes subjected to nucleolar dominance begin 2 to 4 d after germination (Mathieu et al. 2003; Pontes et al. 2007). Consistent with these observations, RT-PCR analysis using amplicon 1 primers, or a primer pair that amplifies only long variants, reveals that long variants are progressively inactivated during early seedling development (Fig. 1E). At 2 d after germination, transcripts of both long and short rRNA gene variants are detected in similar abundance, but, by 10 d, only short variant transcripts are expressed abundantly. If plants are grown on media containing 5-aza-2'-deoxycytidine (aza-dC), an inhibitor of cytosine methylation, suppression of long rRNA gene variants is precluded (Fig. 1E). Taken together, these results indicate that long variant expression in *A. thaliana* is regulated epigenetically, with the developmental silencing of variants involving HDA6 and cytosine methylation, as is also the case for uniparental rRNA gene silencing in nucleolar dominance in the hybrid *A. suecica* (Chen and Pikaard 1997; Earley et al. 2006a; Preuss et al. 2008).

To examine the genic regions where HDA6 exerts its repressive effects, we used RT-PCR to compare transcript

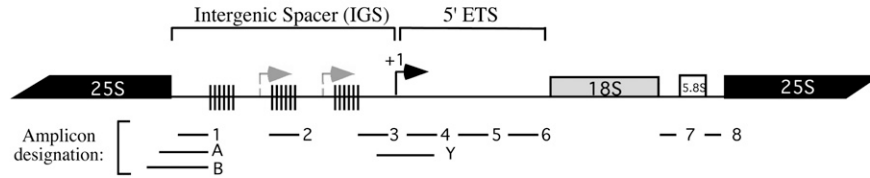
levels at various intervals throughout the ~10-kb rRNA gene repeat (Fig. 2A) in both wild-type (DR5) and *hda6* mutant plants (Fig. 2B,C). In *axe1-4*, *axe1-5* (Murfett et al. 2001), or *rts1-1* (Aufsatz et al. 2002) mutants, RNA transcript levels are elevated throughout the IGS (Fig. 2B; amplicons 1, 2, 3, A, B, and Y). RT-PCR reactions employing strand-specific primers revealed that these IGS transcripts correspond to both DNA strands, indicative of bidirectional transcription (Fig. 2C). However, downstream from the gene promoter, no significant difference in RNA transcript levels is detected in *hda6* mutants compared with the DR5 (wild-type) control (Fig. 2B, amplicons 4–8). These data suggest that HDA6-mediated transcriptional repression is focused on the IGS.

Enzymatic activity of HDA6 is required to suppress spurious IGS transcription

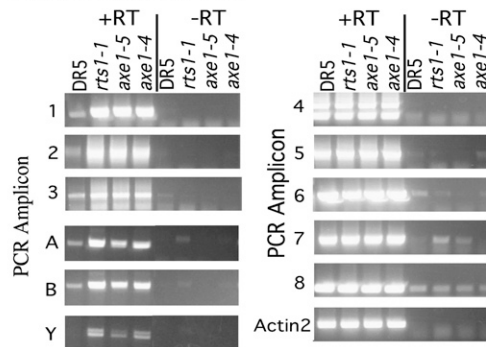
HDACs including human HDAC1, *Aquifex aeolicus* HDAC HDLP (Finnin et al. 1999), *Arabidopsis* HDA19, and *Arabidopsis* HDA6 are similar in sequence in the vicinity of their catalytic centers (Fig. 3A). The crystal structure of HDLP in complex with TSA suggests that conserved amino acids corresponding to HDA6 positions 186, 188, 190, 191, and 193 contribute to the active site (Fig. 3A; Finnin et al. 1999). Consistent with this prediction, HDAC1 aspartates circled in Figure 3A, corresponding to D186 and D188 of HDA6, are critical for HDAC activity (Hassig et al. 1998). Using site-directed mutagenesis, we changed each of five amino acids of HDA6 that are invariant among HDAC1, HDLP, HDA19, and HDA6. Mutant and wild-type versions of the *HDA6* gene were then transformed into *hda6* mutant plants (*axe1-5*), and resulting HDA6-Flag proteins expressed in the transgenic plants were affinity-purified. Immunoblotting confirmed that similar quantities of recombinant HDA6-Flag were recovered from each transgenic line (Fig. 3B, top panel). HDAC activity of the recovered proteins was tested using core histones acetylated in vitro using recombinant *A. thaliana* HAC1 and tritiated acetyl-CoA (Earley et al. 2006a, 2007). Whereas wild-type HDA6 deacetylated the histones, causing a loss of signal on the fluorogram (duplicate reactions are shown), HDA6 bearing any of the active site mutations lacks HDAC activity (Fig. 3B, middle panel). Mutant HDA6 proteins also lack HDAC activity on histone H4 N-terminal tail peptides acetylated using human pCAF2 (data not shown).

To determine if the HDAC activity of HDA6 is necessary for rRNA gene variant silencing, we assayed for long and short variant expression in wild-type (Col-0), *hda6* (*rts1-1* or *axe1-5*) mutants, *axe1-5* mutants rescued by a wild-type HDA6 transgene, or *axe1-5* plants expressing HDA6 active site mutant proteins. Only short rRNA gene variants are expressed in vegetative tissues of mature control plants, whereas in *hda6* mutants (*rts1-1* and *axe1-5*), both the long and short variants are expressed (Fig. 3C, amplicon 1 results). The wild-type HDA6 transgene rescues the *axe1-5* mutant, such that only short variants are expressed, and also restores IGS transcripts to control levels (Fig. 3C, amplicon 3 results). In contrast, none of

A. Positions of RT-PCR amplicons



B. Random-primed RT-PCR



C. Strand-specific RT-PCR

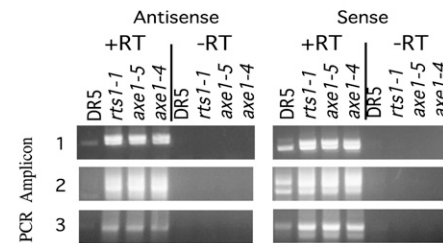


Figure 2. IGS transcripts are overproduced in *hda6* mutants. (A) Diagram showing the positions of RT-PCR amplicons examined in B and C. (B) RT-PCR analysis of RNAs corresponding to the IGS, 5' ETS, or rRNA coding regions. First-strand cDNA was synthesized from total RNA using random primers and then subjected to PCR using primers specific to the indicated amplicons. Ethidium bromide-stained reaction products derived from RNA of wild-type (DR5) or *hda6* mutants (*axe1-5*, *axe1-4*, or *rts1-1*) are compared. Control reactions from which reverse transcriptase was omitted (–RT) reveal background signals attributable to DNA contamination. Amplification of *ACTIN2* served as a control to show that equivalent amounts of RNA from each genotype were tested. (C) Strand-specific RT-PCR amplification of IGS transcripts. Sense or antisense strand-specific primers were used to synthesize first-strand cDNA, followed by PCR amplification using amplicon 1, 2, or 3 primers.

the *HDA6* transgenes bearing active site mutations rescues *axe1-5* (Fig. 3C). Collectively, the results indicate that the HDAC activity of HDA6 is required for rRNA gene variant silencing.

IGS siRNAs are overproduced in *hda6* mutants

We reasoned that bidirectional IGS transcription in *hda6* mutants might give rise to dsRNAs that could be diced into siRNAs. Indeed, RNA blot analyses using probes corresponding to several regions of the IGS reveal a massive accumulation of IGS siRNAs in *hda6* mutants compared with wild-type plants (Fig. 4A). Downstream from the transcription start site, within the external transcribed spacer (ETS), no hyperaccumulation of siRNAs is detected, consistent with the RT-PCR evidence that IGS, but not ETS, transcripts accumulate in *hda6* mutants (refer to Fig. 2B). Using the IGS2 probe, blot hybridization shows that siRNA overproduction in *hda6* is suppressed upon complementation of *axe1-5* with wild-type HDA6-Flag (Fig. 4B, two independent transgenic lines are shown). However, *HDA6* transgenes bearing D186A, H190A, or five clustered active site mutations fail to rescue the *axe1-5* mutation (Fig. 4B). These data indicate that the HDAC activity of HDA6 is required to suppress siRNA overproduction, presumably due to suppressing precursor transcription (see Fig. 2B,C).

IGS siRNAs overproduced in *hda6* mutants vary in size in different regions of the IGS. The IGS1 probe primarily

detects 23- and 24-nucleotide (nt) siRNAs, whereas the IGS2 and IGS3 probes predominantly detect 21-nt siRNAs. To determine which dicers generate IGS siRNAs in *hda6* mutants, we assayed siRNA levels in *hda6 dcl* double mutants. In the *rts1-1 dcl4-4* double mutant, 24-nt IGS2 siRNAs are unaffected, but 21-nt siRNAs are lost and are replaced, instead, by 22-nt siRNAs, presumably generated by DCL2 (Fig. 4C). In *rts1-1 dcl2-1* double mutants, no change in siRNA size classes occurs, consistent with DCL2 playing a negligible role in IGS dsRNA dicing (unless DCL4 is mutated). In the *rts1-1 dcl3-1* double mutant, the 24-nt siRNAs are lost, but 21-nt siRNAs are unaffected. Collectively, the data indicate that DCL4 and DCL3 are primarily responsible for the 21-nt and 24-nt siRNAs, respectively, that are overproduced in *hda6* mutants.

Effects of dicer mutations on the abundance of IGS and ETS transcripts was tested by RT-PCR (Fig. 4D). Controls show that IGS transcripts corresponding to amplicons 1, 2, or 3 are not increased in abundance in either *dcl3* (Fig. 4D) or *dcl4* mutants (data not shown). However, in *rts1-1 dcl4* and *rts1-1 dcl3* double mutants, IGS transcript abundance is increased above the levels in *rts1-1* or *axe1-5* single mutants. One explanation of these results could be that IGS transcript stability is increased in the absence of either DCL3 or DCL4-mediated dicing. Alternatively, disrupted siRNA-mediated transcriptional silencing in the absence of dicer activity could potentially result in elevated IGS transcription.

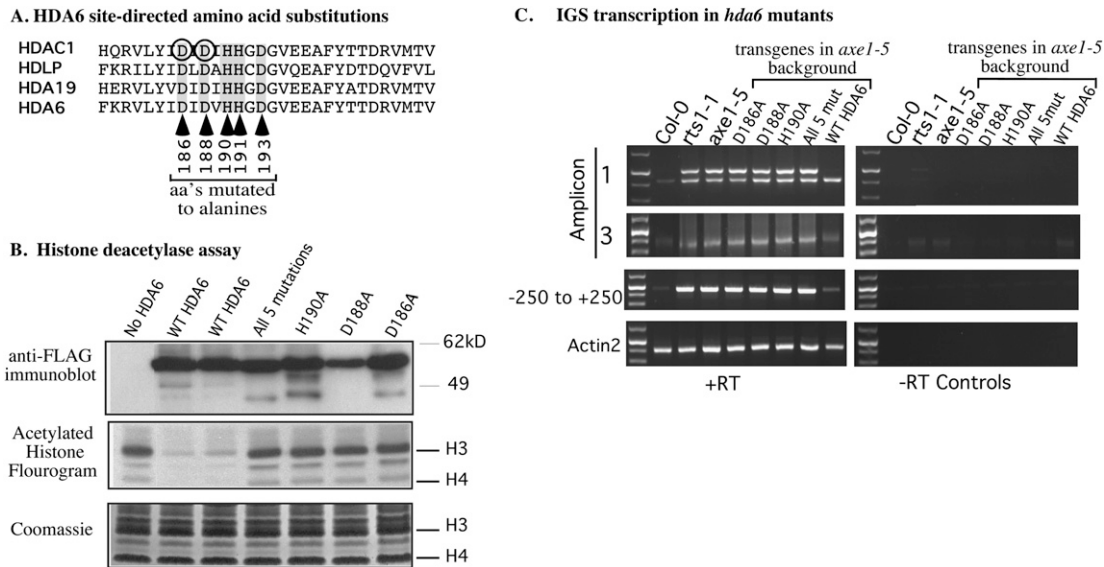


Figure 3. HDA6 enzymatic activity is required for rRNA gene variant silencing. (A) Multiple alignment of HDA6 and related HDACs in the vicinity of the active site. Circled amino acids are known to be required for human HDAC1 activity. Arrows indicate amino acids mutated to alanines in transgene-expressed recombinant HDA6 tested in B and C. (B) Alanine substitutions of conserved active site amino acids abolish HDA6 enzymatic activity. The *top* row shows an immunoblot of Flag-tagged HDA6 proteins expressed in the *A. thaliana axe1-5* mutant background, affinity-captured on anti-Flag beads, and resolved by SDS-PAGE. Single-alanine substitution mutants (D186A, D188A, or H190A) and an HDA6 mutant bearing alanine substitutions at five positions (186, 188, 190, 191, and 193) are shown alongside wild-type (unmutated) HDA6-Flag and the anti-Flag immunoprecipitate from *axe1-5* plants not bearing an HDA6 transgene (no HDA6 control). The *middle* panel shows a fluorogram of histones that were acetylated *in vitro* by recombinant *A. thaliana* HAC1 using radioactive (^3H) acetyl-CoA and then incubated with wild-type or mutated HDA6. (*Middle* panel) Reaction products were resolved by SDS-PAGE and gels were stained with Coomassie Blue, dried, and exposed to X-ray film using a ^3H -intensifying screen in order to generate the fluorogram shown. The Coomassie-stained gel revealing the histones is shown in the *bottom* panel. (C) The HDAC activity of HDA6 is required to suppress long variant expression and IGS transcription. Long and short variant expression (amplicon 1), IGS transcription upstream of the gene promoter (amplicon 3), and transcripts reading through the promoter (–250 to +250 amplicon) were detected using RT–PCR. Total RNA isolated from wild-type Col-0 and *hda6* mutants *axe1-5* or *rts1-1* were analyzed in parallel with RNA isolated from *axe1-5* plants transformed with transgenes expressing HDA6 bearing single amino acid mutations of the active site (D186A, D188A, or H190A), the five amino acid mutation of the active site (all 5 mut), or wild-type HDA6 (WT HDA6). Amplification of *ACTIN2* cDNA serves as a control to show that equivalent amounts of RNA were tested in all lanes. The *right* panel shows control reactions from which reverse transcriptase was omitted (–RT).

Cytosine methylation gains and losses in *hda6* mutants

To determine if siRNAs overproduced in *hda6* mutants program siRNA-directed DNA methylation of corresponding genomic sequences, we digested genomic DNA using methylation-sensitive restriction endonucleases and performed Southern blot hybridization using a promoter region (–250 to +250) or amplicon 1 probe (Fig. 5). The *hda6* mutants *axe1-5* and *rts1-1* and *axe1-5* rescued with the HDA6-Flag transgene were compared with wild-type (Col-0) and *ddm1* (*decrease in DNA methylation 1*) controls. The restriction endonucleases HpaII and MspI recognize the sequence CCGG, but differ in their methylation sensitivities such that MspI digestion provides insight into CHG methylation status (where H denotes A, T, or C), and HpaII provides insight into CG methylation. In *ddm1* mutants, methylation is decreased in multiple sequence contexts (Vongs et al. 1993), resulting in increased digestion of IGS sequences by both HpaII and MspI (Fig. 5A). In *hda6* mutants, IGS

sequences are somewhat more susceptible to HpaII digestion, but not as dramatically as in *ddm1*. However, using the promoter region probe, similar (but not identical) losses in CHG methylation are apparent in *ddm1* and *hda6* mutants. Unlike the promoter region, the amplicon 1 probe reveals a more pronounced loss of CHG methylation at MspI sites in *ddm1* mutants compared with *hda6* mutants. Importantly, CG methylation patterns are not restored to a wild-type pattern in *axe1-5* rescued by the HDA6 transgene (Fig. 5A), and CHG methylation is only partially restored, consistent with the fact that maintenance methylation patterns, once lost, are not easily regained (Vongs et al. 1993; Richards 1997).

Examination of cytosine methylation at AluI restriction sites, which reports on de novo methylation in a CHH sequence context, reveals a dramatic increase in de novo cytosine methylation in *hda6* mutants, and a modest increase in *ddm1* mutants, as shown using both the promoter region and amplicon 1 probes (Fig. 5A). The de novo cytosine methylation is consistent with the dramatic overproduction of siRNAs in *hda6* mutants (see

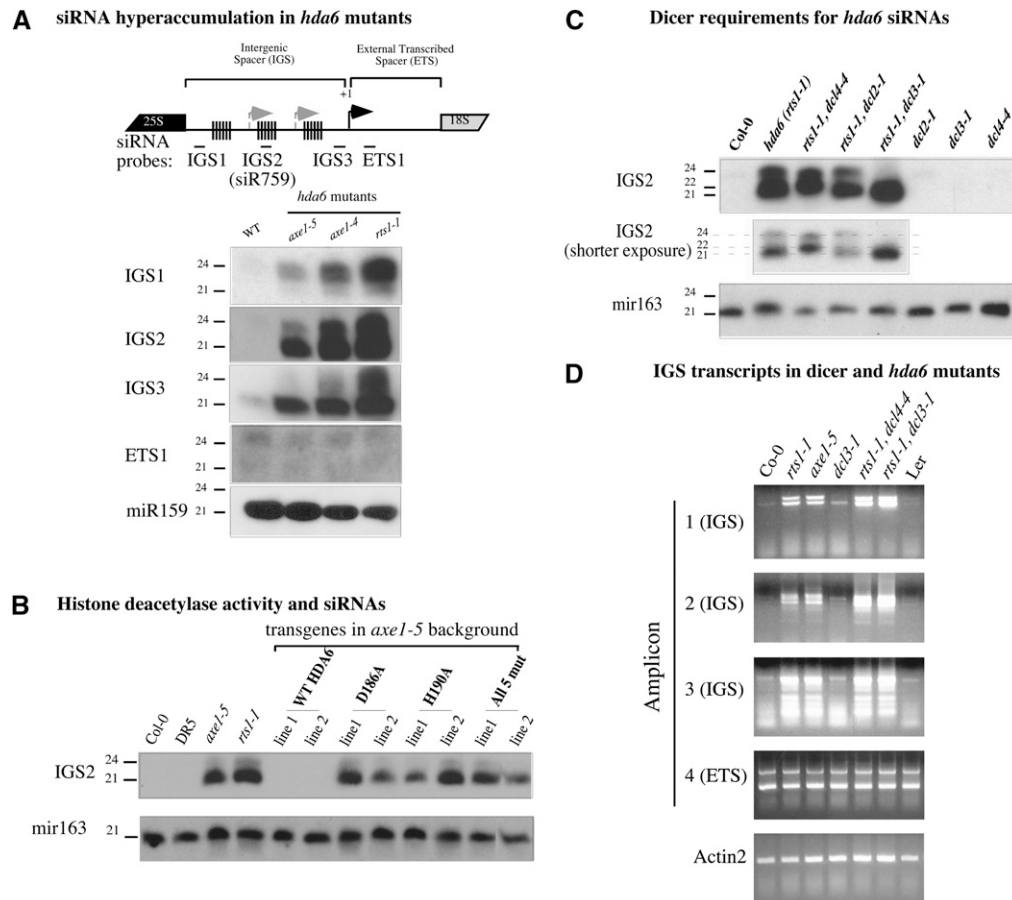


Figure 4. IGS siRNAs are overproduced in *hda6* mutants. (A) IGS and ETS probes were hybridized to Northern blots of small RNAs isolated from wild-type (Col-0) or *hda6* mutant plants (*axe1-5*, *axe1-4*, and *rts1-1*); see the diagram at the top for IGS and ETS probe locations. Detection of the microRNA miR159 served as an RNA loading control. (B) Small RNAs isolated from wild-type plants (Col-0 and DR5), *hda6* mutants (*axe1-5* and *rts1-1*), or *axe1-5* plants expressing transgenes encoding either wild-type HDA6 or an HDA6 mutant bearing the D186A, H190A, or five clustered (all 5 mut) active site mutations were examined by Northern blotting using the IGS2 hybridization probe. Two independent transgenic lines for each construct are shown. Hybridization to a miR163 probe controls for RNA loading. (C) Dicers DCL3 and DCL4 are primarily responsible for IGS siRNAs overproduced in *hda6* mutants. Small RNAs isolated from wild-type Col-0, *hda6* (*rts1-1*), *hda6* dicer double mutants (for dicers *dcl2*, *dcl3*, and *dcl4*), and dicer single mutants were examined by Northern blotting using the IGS2 probe or miR163 probe; the latter controls for RNA loading. (D) IGS transcripts hyperaccumulate in *hda6* *dcl4* and *hda6* *dcl3* double mutants. IGS transcripts were detected by RT-PCR using amplicon 1, 2, or 3 primers; ETS transcripts were detected using amplicon 4 primers (see Fig. 2 for amplicon positions). Total RNA tested was isolated from wild-type Col-0 or Ler plants, *hda6* mutants (*rts1-1* and *axe1-5*), *dcl3-1*, and the double mutants *rts1-1* *dcl4-4* and *rts1-1*/*dcl3-1*.

Fig. 4), and a modest increase in siRNA levels in *ddm1* mutants (data not shown). In *axe1-5* rescued by the HDA6-Flag transgene, CHH methylation is restored to wild-type levels, consistent with the return of siRNA abundance to wild-type levels (refer to Fig. 4B).

As an independent test of DNA methylation status, we used Chop-PCR, an assay in which genomic DNA is subjected to digestion with a methylation-sensitive restriction endonuclease (i.e., it is "chopped") and then tested as a template for PCR amplification using primers flanking the restriction sites. In wild-type plants, lack of DNA methylation at one or more AluI sites in the promoter region allows AluI to cut the DNA such that the template cannot be amplified by PCR (Fig. 5B; Supplemental Fig. S2). In contrast, the promoter region of wild-type plants is recalcitrant to digestion with MspI,

due to CHG methylation; as a result, the region can be PCR-amplified following MspI digestion. In agreement with the Southern blot data, increased de novo methylation at asymmetric sites occurs in the promoter region of *ddm1*, *rts1-1*, and *axe1-5* mutants, making the promoter region more resistant to AluI cleavage. At the same time, losses of CHG and CG methylation make promoter regions more susceptible to MspI and HpaII digestion, respectively (Fig. 5B; Supplemental Fig. S2). Importantly, wild-type methylation patterns at AluI sites are restored in *axe1-5* mutants transformed with the wild-type HDA6-Flag transgene (Fig. 5B). Taken together, the data show that symmetric CHG sites are hypomethylated, but asymmetric CHH sites are hypermethylated, in *hda6* mutants. We conclude that HDA6 is not required for de novo CHH methylation, but appears to be required for

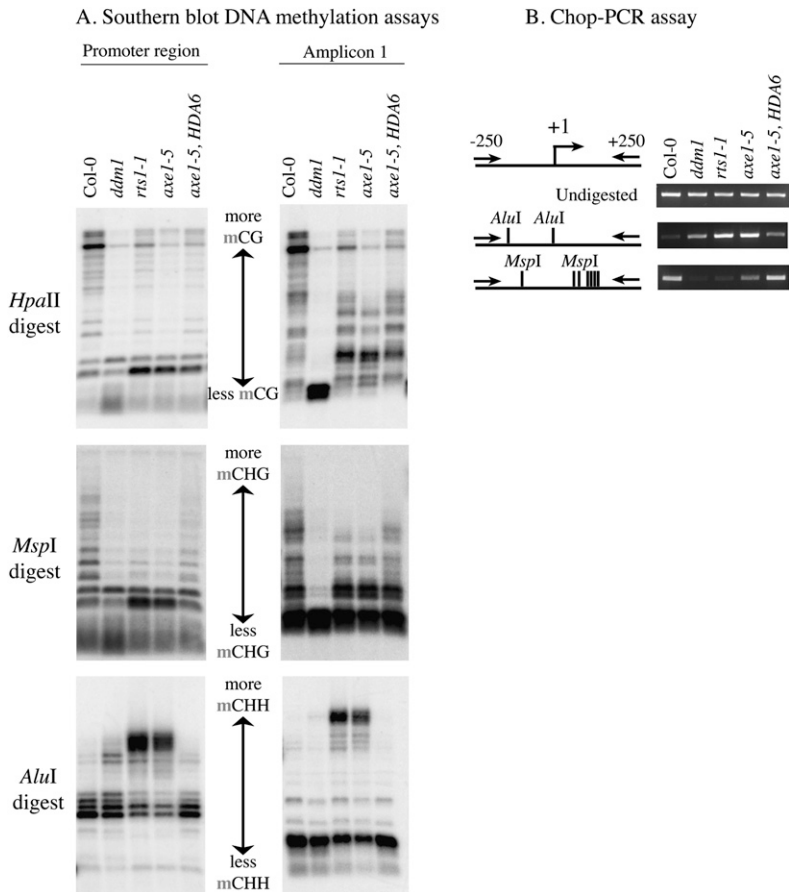


Figure 5. Symmetric cytosine methylation decreases, but asymmetric cytosine methylation is gained de novo, in *hda6* mutants. (A) DNA methylation assayed using methylation-sensitive restriction endonucleases and Southern blot hybridization. Total genomic DNA was isolated from wild-type Col-0, *ddm1*, *hda6* mutants (*rts1-1* and *axe1-5*), or *axe1-5* plants rescued with a wild-type HDA6 transgene. The DNA was digested using *HpaII*, which reports on methylation in a symmetric CG context; *MspI*, which reports on methylation in a symmetric CHG context; or *AluI*, which reports on CHH methylation. Following electrophoresis and blotting, nylon membranes were hybridized to promoter region (−250 to +250) or amplicon 1 large variant-specific probes. (B) Analysis of *AluI* and *MspI* site methylation using Chop-PCR. Undigested genomic DNA (top row) or genomic DNA digested with *MspI* (bottom row) or *AluI* (middle row) was subjected to PCR amplification using primers spanning the promoter region.

CG and CHG maintenance methylation. The possibility that HDA6 is also required for de novo methylation at CG and CHG motifs cannot be ruled out.

Histone modifications and polymerase occupancy in *hda6* mutants

To assay chromatin modifications and polymerase occupancy in rRNA gene IGS and coding sequences (Fig. 6A), we used chromatin immunoprecipitation (ChIP) combined with quantitative PCR (see Fig. 6A for positions of amplicons tested). Mock ChIP reactions conducted in the absence of antibody revealed background signals (Fig. 6B). Antibodies recognizing histone modifications indicative of actively transcribed genes—namely, histone H3 acetylated on Lys 9 and Lys 14 (H3Ac); Histone H4 hyperacetylated on Lys 5, Lys 8, Lys 12, and Lys 16 (H4Ac); or histone H3 trimethylated on Lys 4 (H3K4me3)—yielded greater ChIP signals in *hda6* mutants compared with wild-type plants throughout rRNA gene coding and noncoding regions (Fig. 6C–E; Supplemental Fig. S1). Conversely, levels of histone H3 dimethylated on Lys 9 (H3K9me2), a modification characteristic of inactive heterochromatic sequences, are substantially decreased in *hda6* mutants relative to wild-type controls (Fig. 6F). At actin genes, which are constitutively transcribed by RNA Pol II, differences in H3K4me3 and H4Ac levels in wild-type

versus *hda6* mutant plants are not significant. However, at *AtSN1* family retrotransposons, which are silenced by HDA6 and RNA-directed DNA methylation, H3K4me3 and histone acetylation levels increase, and H3K9me2 levels decrease in *hda6* mutants compared with wild-type plants (Fig. 6). These chromatin changes are consistent with the derepression of *AtSN1* elements in *hda6* mutants (data not shown).

ChIP using antibodies specific for RNA Pol I revealed Pol I in association with the pre-rRNA transcription unit, including the gene promoter region (+1), the 5' ETS, the 25S coding sequences, and 3' ETS sequences that are located immediately adjacent to the 25S coding sequences (Fig. 6G). In each of these intervals, Pol I occupancy is increased nearly twofold in *hda6* mutants relative to wild-type controls, consistent with the derepression of long rRNA gene variants, which account for approximately one-half of the total rRNA gene pool. Pol I was not detected at substantial levels in the IGS adjacent to the Sal repeats in either wild-type or *hda6* mutant plants (Fig. 6G). At the *Actin 2* gene, transcribed by Pol II, and at *AtSN1* retrotransposons, thought to be transcribed by Pol III, ChIP signals were essentially undetectable using the anti-Pol I antibody.

ChIP performed using an antibody specific for Pol II revealed that, in wild-type plants, Pol II is not detected in association with rRNA genes at levels above background

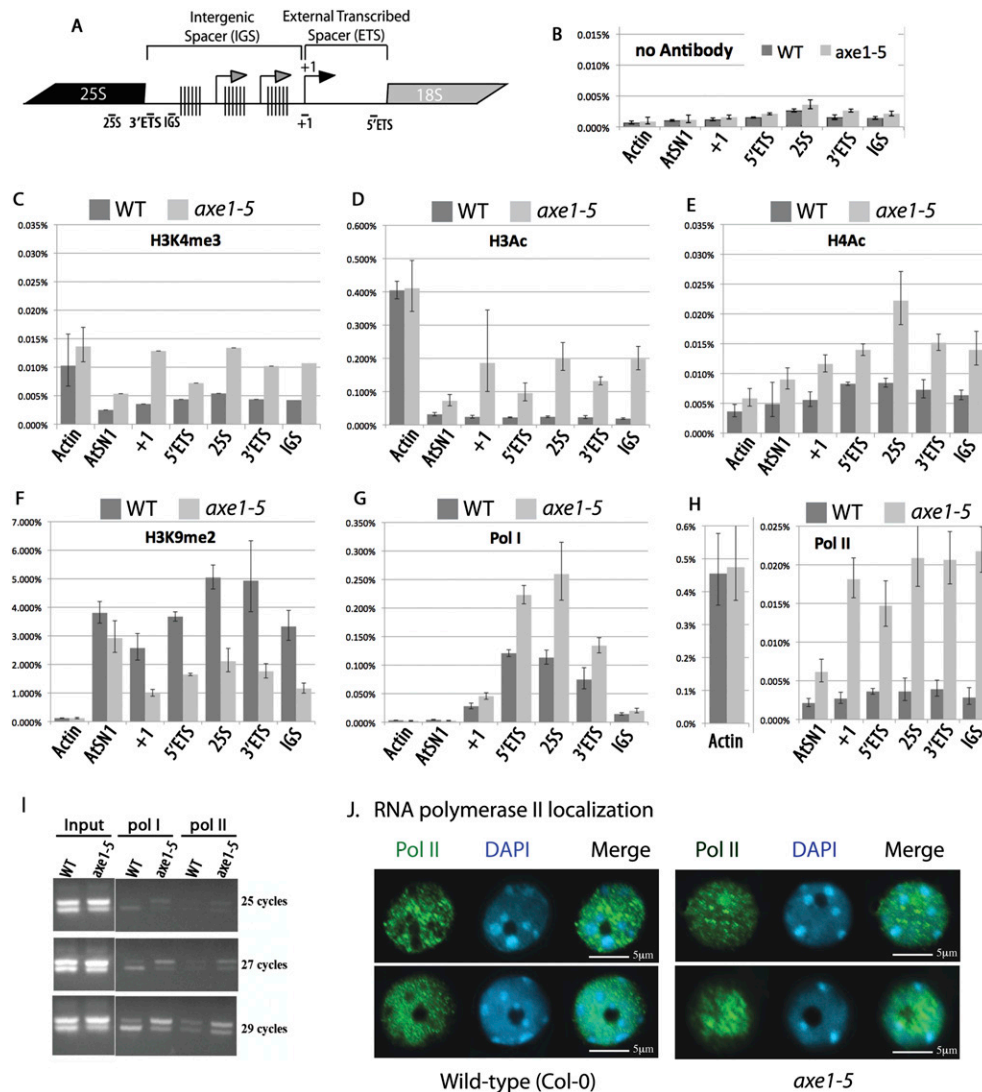


Figure 6. Chromatin marks and polymerase occupancy at rRNA genes in wild-type and *hda6* mutant plants. (A) Diagram showing the location of IGS, ETS, and coding region amplicons assayed by quantitative PCR following ChIP. (B–H) ChIP using chromatin isolated from shoots of 2-wk-old wild-type (Col-0) or *hda6* mutant (*axe1-5*) plants. Following chemical cross-linking, chromatin was isolated and mock-immunoprecipitated (B) or immunoprecipitated using antibodies recognizing different histone modifications (C–F), RNA Pol I (G), or RNA Pol II (H). *ACTIN2* and *AtSN1* served as controls for active or (normally) repressed loci, respectively. (I) Association of Pol I and Pol II with long or short rRNA gene variants. Chromatin of wild-type or *axe1-5* mutant plants was subjected to ChIP using anti-RNA Pol I or anti-RNA Pol II antibodies. Precipitated DNA was then subjected to PCR amplification using amplicon 1 primers. Reaction products following 25, 27, or 29 cycles of PCR amplification were resolved by agarose gel electrophoresis and stained with ethidium bromide. (J) Immunolocalization of RNA Pol II in interphase nuclei of wild-type or *hda6* (*axe1-5*) mutant plants (green signals). Nuclei were counterstained with DAPI (blue signals). Two representative wild-type and two representative *axe1-5* nuclei are shown. Bars, 5 μ m.

(Fig. 6, cf. H and B). However, in *hda6* mutants, Pol II associates with the rRNA genes throughout the coding and noncoding regions (Fig. 6H). Pol II association with *AtSN1* retrotransposons also increases in *hda6* mutants, unlike the actin gene control.

The ChIP data suggest that the high levels of IGS transcription occurring in *hda6* mutants is attributable to the derepression of cryptic Pol II transcription units. This raised the possibility that Pol II, rather than Pol I, might be solely responsible for transcripts that include

the indel defining the long and short rRNA gene variants. We tested this possibility using anti-Pol I and anti-Pol II antibodies for ChIP, followed by PCR amplification using amplicon 1 primers. In mature wild-type plants, Pol I is preferentially associated with the short variants (Fig. 6I), consistent with the preferential expression of this variant class (refer to Fig. 1). However, in *hda6* mutants (*axe1-5*), Pol I is associated with both long and short variants, in proportion to their relative abundance (see input chromatin controls). Pol II association with rRNA genes also

increases in *hda6* mutants (Fig. 6I), indicating that Pol II transcription contributes to the synthesis of RNA in the region that defines the long and short variants. Taken together, the data of Figure 6, G and I, suggest that Pol I transcribes both long and short variants, beginning at the gene promoter and including the polymorphic indel region in the 3' ETS. Superimposed onto this pattern of expression, spurious Pol II transcription occurs throughout the rRNA genes.

RNA Pol II is generally not present in the nucleolus of wild-type *Arabidopsis* nuclei (Fig. 6J, two representative nuclei are shown). The ChIP data showing that Pol II associates with rRNA genes in *hda6* mutants prompted us to investigate whether Pol II localization is altered in the mutants. Indeed, Pol II can be observed throughout the nucleolus in many *hda6* nuclei (Fig. 6J, top right panel). In other *hda6* nuclei, Pol II is not distributed uniformly throughout nucleoli, but is nonetheless present within the internal periphery of the nucleolus (Fig. 6J, bottom right panel). Collectively, the ChIP and cytological evidence independently reveal the role of HDA6 in suppressing Pol II transcription of nucleolar rRNA genes.

Discussion

HDA6 is a key regulator of gene silencing that displays a complex interrelationship with DNA methylation. In the absence of HDA6 activity, symmetrical cytosine methylation is decreased at the same time that asymmetric, siRNA-directed de novo cytosine methylation accumulates. siRNA-directed DNA methylation is typically repressive, but this is not the case in *hda6* mutants, suggesting that HDA6 acts downstream from de novo DNA methylation. Conversely, the loss of maintenance methylation in *hda6* mutants suggests that HDA6 acts upstream of DNA methylation. These observations can be reduced to two simple models, which need not be mutually exclusive. One model is that HDA6-mediated histone modifications silence rRNA genes directly (Fig. 7A). An alternative model is that HDA6 acts indirectly through maintenance DNA methylation, which may be the critical determinant for gene silencing (Fig. 7B). Several observations support the direct action model. First, rescuing an *hda6* mutant with a wild-type *HDA6* transgene fully restores developmental silencing of long rRNA gene variants (Fig. 3C). However, CG and CHG maintenance methylation patterns are not fully restored in these lines (Fig. 5), arguing that restoration of HDA6 activity, and not maintenance methylation, is key. Second, the ability to clonally inherit maintenance methylation patterns suggests that loss of these patterns early in development in *hda6* mutants might yield a relatively constant molecular phenotype. However, IGS transcripts and siRNAs become more and more abundant in *hda6* mutants during early development, indicating that HDA6 activity is needed continuously to suppress their expression (Fig. 7C,D). Last, the fact that de novo methylation occurs in *hda6* mutants but cannot bring about silencing fits with the hypothesis that HDA6 is critical for silencing in its own right. Based on these considerations, we

favor a model in which HDA6 is critical for suppressing Pol II and Pol I transcription, but DNA methylation plays a reinforcing role, potentially helping recruit HDA6 (Fig. 7E).

In general, the regulation of RNA gene variant expression in nonhybrid *A. thaliana* shows an overall similarity to uniparental rRNA gene silencing (nucleolar dominance) in hybrid *A. suecica*. In both cases, silencing is developmentally regulated and requires HDA6 and cytosine methylation. These observations fit with our previous conclusion that nucleolar dominance is a manifestation of rRNA gene dosage control, which operates in all eukaryotes studied to date, including nonhybrids. Interestingly, in *A. suecica*, nucleolar dominance requires the de novo cytosine methyltransferase DRM2, whereas knocking down the maintenance methyltransferases MET1 or CMT3 does not abrogate rRNA gene silencing (Preuss et al. 2008). However, in the present study, de novo methylation increases in *hda6* mutants, yet rRNA genes are derepressed. Although these observations appear contradictory, the loss of CG and CHG methylation at the same time that CHH methylation is gained in *hda6* mutants is a complicating factor that precludes a simple interpretation. A combination of de novo methylation and HDA6-dependent maintenance methylation patterns may be important.

The fact that S1 nuclease protection assays do not detect a twofold increase in nascent transcript signals initiating at the rRNA gene promoter when both long and short variants are expressed, as opposed to when only short variants are expressed, is puzzling. This is especially true given that we do observe an approximately twofold increase in Pol I ChIP signals associated with pre-rRNA transcription units in *hda6* relative to wild-type plants. One possible explanation for this apparent paradox could be that the half-life of the ETS sequences immediately downstream from the transcription start site becomes shorter as more genes are expressed, perhaps because processing must become more efficient. Further studies will be needed to test this hypothesis.

A surprising aspect of our study is that Pol II transcription is not incompatible with Pol I transcription of rRNA genes in plants. Likewise, a recent study using mammalian cells defective for DNA methyltransferase activity showed that derepression of normally silent rRNA genes correlates with Pol II transcription (Gagnon-Kugler et al. 2009). We speculate that Pol II transcription causes the cotranscriptional replacement of repressive chromatin marks with histone modifications typical of active genes, thereby allowing the Pol I transcription machinery greater access to rRNA gene promoters. Importantly, there has long been evidence for a positive role of IGS transcription in rRNA gene expression in frogs and flies. *Xenopus laevis* and *Xenopus borealis* have IGS organizations very similar to the *A. thaliana* IGS organization depicted in Figure 1A. In *Xenopus*, full enhancer activity of spacer repeats requires the presence of at least one spacer promoter (DeWinter and Moss 1986). Likewise, at least one spacer promoter is needed in addition to enhancer repeats for *X. laevis* minigenes to outperform *X.*

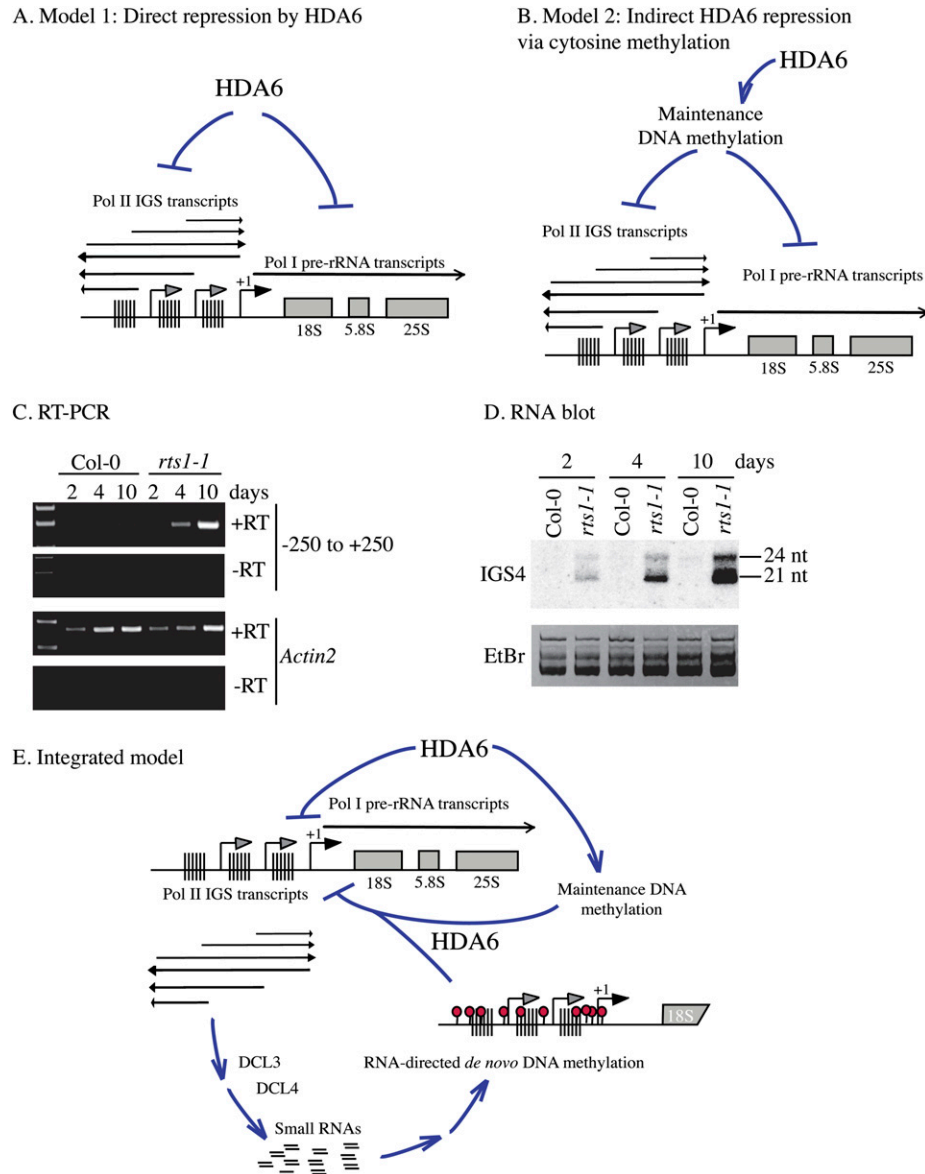


Figure 7. Models for the role of HDA6 in rRNA gene variant regulation. (A,B) Models for HDA6 acting directly or through cytosine maintenance methylation. (C) RT-PCR analysis of IGS transcripts in wild-type and *hda6* mutants 2, 4, and 10 d after germination. PCR products were subjected to gel electrophoresis and stained with ethidium bromide. (–RT) Controls lacking reverse transcriptase. (D) RNA blot analysis of IGS small RNAs present in total RNA isolated from wild-type and *hda6* mutants 2, 4, and 10 d after germination. Ethidium bromide staining shows that similar amounts of RNA were loaded in all lanes. (E) HDA6 suppresses sense and antisense IGS transcription by Pol II. Resulting siRNA production and siRNA-directed de novo cytosine methylation does not repress transcription in the absence of HDA6. Maintenance methylation at CG and CHG motifs is decreased in *hda6* mutants, indicating that HDA6 acts upstream of maintenance methylation. Collectively, losses of maintenance cytosine methylation or losses of repressive chromatin marks due to lack of HDA6 activity and rampant IGS transcription prevent the developmental silencing of rRNA gene variants.

borealis minigenes in nucleolar dominance-like competition assays (Caudy and Pikaard 2002). Much of the *Drosophila* IGS is composed of duplicated spacer promoters that stimulate transcription from the downstream promoter (Grimaldi et al. 1990). Collectively, these observations suggest that spacer transcripts, or possibly the act of spacer transcription, assist in rRNA gene promoter activation.

In parallel with evidence that spacer transcription can positively influence rRNA gene expression, there is evidence that IGS transcripts can repress transcription. In mice, structured RNAs that include promoter sequences recruit the NoRC repressor complex (Mayer et al. 2006, 2008). Moreover, our prior studies of nucleolar dominance in *A. suecica* implicate IGS siRNAs, generated by DCL3, in the RNA-directed repression of rRNA

genes. The evidence that spacer transcription can be activating or repressing suggests that not all spacer transcripts are equal, such that their influence on rRNA gene transcription is dependent on the precise transcription unit that is used (Santoro et al. 2010). Deciphering the origins and regulation of IGS transcripts in future studies should yield important new insights into the complex regulation of rRNA genes in both plants and mammals.

Materials and methods

Mutant and control plant lines

DR5, *axe1-5*, and *axe1-4* seeds were provided by J. Murfett and T. Guilfoyle; *rts1-1* was provided by W. Aufsatz and M. Matzke; and *ddm1* was provided by E. Richards. Plants were grown in growth chambers or a greenhouse.

HDA6 mutagenesis, purification, and assay

The *HDA6* gene was PCR-amplified from *A. thaliana* (ecotype Col-0) genomic DNA using Platinum Pfx (Invitrogen) polymerase and forward (5'-CACCCCTTTACTACTTTACTCTCAAGTCAACCG-3') and reverse (5'-AGACGATGGAGGATTCACGTCTGG-3') primers and then was cloned into pENTR-D TOPO (Invitrogen). Site-directed ligase-independent mutagenesis (SLIM) was performed on pENTR-D-*HDA6* (Chiu et al. 2004) using primer pairs listed in the Supplemental Material. *HDA6* genes in pENTR vectors were recombined into the pEarleyGate 302 destination vector (Earley et al. 2006b) using LR clonase and were transfected into *Arabidopsis tumefaciens* strain GV3101. Transformation of *A. thaliana* (Col-0) was by the floral dip method (Clough and Bent 1998). *HDA6* purification, histone acetylation, and histone deacetylation assays were performed as described (Earley et al. 2006a, 2007).

Small RNA analyses

Small RNAs were isolated using a mirVana kit (Ambion). Gel electrophoresis and blotting were performed as described (Onodera et al. 2005). Hybridization probes were generated using a mirVana probe construction kit (Ambion), and were purified using Performa DTR Gel Filtration Cartridges, as described previously (Onodera et al. 2005). Oligonucleotide probes are listed in the Supplemental Material.

RT-PCR

Total RNA was isolated using a RNeasy Plant mini-kit (Qiagen). S1 nuclease protection using a 5'-end-labeled promoter probe was performed as described previously (Lawrence et al. 2004). For RT-PCR assays, RNA was DNase-treated for 60 min using RQ1 DNase (Promega) or 45 min using Turbo DNase (Ambion) according to the manufacturers' instructions. Random-primed cDNA was amplified from 500 ng of total RNA using SuperScript III reverse transcriptase (Invitrogen). PCR amplification conditions were 25–26 cycles of 30 sec at 94°C, 30 sec at 53°C, and 60 sec at 72°C unless noted otherwise. PCR primers are listed in the Supplemental Material.

DNA methylation assays

Genomic DNA was purified from 2-wk-old shoots using a Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences). For Southern blots, 3 g of DNA was digested overnight

with 30 U of HpaII, MspI, or AluI. Following 0.8% agarose gel electrophoresis, DNA was depurinated and denatured within the gel and transferred to Hybond-N⁺ nylon membranes (GE Healthcare). Hybridization was performed overnight at 55°C in 0.25 M sodium phosphate (pH 7.2), 1 mM EDTA, 6.6% SDS, and 1% BSA. Amplicon 1 and promoter (–250 to +250) PCR products were made into probes by random priming (Feinberg and Vogelstein 1983) and labeling with α -[³²P]-dCTP. Membranes were washed twice for 5 min with 2× SSC and 0.1% SDS and twice for 20 min in 0.1× SSC and 0.1% SDS and were subjected to phosphorimaging. For Chop-PCR, 100 ng of DNA was digested 60 min with 5 U of HpaII, MspI, or AluI (New England Biolabs) at 37°C. An aliquot of the reaction was then subjected to 20 cycles of PCR amplification, followed by agarose gel electrophoresis and ethidium bromide staining.

ChIP

ChIP was performed as described previously (Wierzbicki et al. 2008). Anti-H3K9me2 (catalog no. ab7312), anti-H3K4me3 (catalog no. ab8580), and anti-H3Ac (catalog no. ab4441) antibodies were purchased from Abcam. Anti-H4Ac (catalog no. 06.598) was obtained from Upstate Biotechnologies. Anti-Pol I and anti-Pol II antibodies were raised against the N-terminal domains of the NRPA2 (At1g29940) or NRPB2 (At4g21710) subunits, respectively. Quantitative PCR was performed on purified immunoprecipitated DNA using 0.5 U of Platinum Taq (Invitrogen), SYBR Green I (Invitrogen), and Internal Reference dye (Sigma). Results were analyzed using the comparative C_T method (Livak and Schmittgen 2001) relative to input.

DNA-FISH and protein immunolocalization

DNA-FISH to detect 45S rRNA gene loci (NORs), and protein immunolocalization using anti-Flag antibodies, were performed using isolated nuclei, as described previously (Pontes et al. 2003, 2006). For dual *HDA6*-Flag/NOR localization, slides were subjected to immunofluorescence and post-fixed in 4% formaldehyde/PBS, followed by DNA-FISH. DNA was counterstained with DAPI (1 g/mL) in Vectashield (Vector Laboratories). Nuclei were examined using a Nikon Eclipse E800i epifluorescence microscope equipped with a Photometrics Coolsnap ES Mono digital camera and a Nikon A1 laser-scanning confocal microscope using 100× objectives. Lasers were 405 nm for DAPI, 488 nm for Alexa 488, and 561 nm for Alexa 594. Confocal and wide-field images were processed using Imaris (Bitplane) and Adobe Photoshop, respectively. For each experiment, 100–200 nuclei were analyzed. Representative nuclei are shown.

Acknowledgments

K.W.E. created all transgenic lines and produced Figures 1, A,C,D; 2, A–C; 3, A,B; 4, B–D; and 6I. F.P. produced Figures 1E, 3C, 5B, and 7 (except part D) and Supplemental Figure S1. A.T.W. produced Figure 6, B–H; S.T. produced Figure 4A; O.P. produced Figures 1B and 6J; and T.B. generated Figures 5A and 7D. P.C.N. first noted accumulation of IGS small RNAs in *hda6* mutants. K.W.E., F.P., and C.S.P. wrote the manuscript. We thank Jeremy Haag, Tom Ream, Alexa Vitins, Ek Han Tan, and Sasha Preuss for helpful discussions. This research was supported by NIH grant GM60380 (to C.S.P.). P.C.N. was supported by fellowships SFRH/BD/6520/2001 and SFRH/BPD/30386/2006 from the Fundação para a Ciência e Tecnologia, Portugal. T.B. was supported by a Ruth L. Kirschstein National Research Service Award. O.P. was supported by a grant from the Edward Mallinckrodt Foundation.

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