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A Concerted DNA Methylation/Histone Methylation Switch Regulates rRNA Gene Dosage Control and Nucleolar Dominance

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Summary

Eukaryotes regulate the effective dosage of their ribosomal RNA (rRNA) genes, expressing fewer than half of the genes at any one time. Likewise, genetic hybrids displaying nucleolar dominance transcribe rRNA genes inherited from one parent but silence the other parental set. We show that rRNA gene dosage control and nucleolar dominance utilize a common mechanism. Central to the mechanism is an epigenetic switch in which concerted changes in promoter cytosine methylation density and specific histone modifications dictate the on and off states of the rRNA genes. A key component of the off switch is *HDT1*, a plant-specific histone deacetylase that localizes to the nucleolus and is required for H3 lysine 9 deacetylation and subsequent H3 lysine 9 methylation. Collectively, the data support a model in which cytosine methylation and histone deacetylation are each upstream of one another in a self-reinforcing repression cycle.

Introduction

In eukaryotes, rRNA genes transcribed by RNA polymerase I (pol I) are tandemly arrayed in hundreds to thousands of copies within nucleolus organizer regions (NORs) (Grummt, 2003; Moss and Stefanovsky, 2002). The synthesis and processing of nascent rRNA transcripts leads to the formation of the nucleolus, the subnuclear domain in which ribosome assembly takes place (Hernandez-Verdun et al., 2002).

One of the earliest recognized epigenetic phenomena, nucleolar dominance describes the transcription of rRNA genes inherited from only one parent in a plant or animal genetic hybrid (Pikaard, 2000; Reeder, 1985; Viegas et al., 2002). Such hybrids are typically vigorous, indicating that one parent's set of rRNA genes is dispensable. In fact, eukaryotic genomes seem to contain excess rRNA genes, with dosage control mechanisms regulating the number of active genes (reviewed in Grummt and Pikaard, 2003). For instance, psoralen crosslinking studies show that only 30%–50% of the rRNA genes in proliferating yeast or murine cells are in an open, psoralen-accessible chromatin structure resulting from active transcription (Conconi et al., 1989; Dammann et al., 1995; Sandmeier et al., 2002). Corroborating electron microscopy evidence has shown that only half of the ~150 yeast rRNA genes are loaded with pol I elongation complexes (French et al., 2003). Furthermore, chicken cells with two, three, or four NOR-bearing chromosomes synthesize the same amount of rRNA (Muscarella et al., 1987) and maize inbred lines can vary 10-fold in rRNA gene content without displaying differences in growth rate or vigor (Flavell, 1986).

Ribosomal RNA gene transcription varies with the demand for ribosome production and protein synthesis, being highest in proliferating cells and lowest in nongrowing cells (Grummt, 2003; Moss and Stefanovsky, 2002). Available evidence suggests that rRNA genes are regulated at two levels, first by controlling the number of rRNA genes in the on or off states (dosage control) and subsequently by modulating pol I initiation frequency among the active subset (reviewed in Grummt and Pikaard, 2003). The latter fine-tuning is most likely accomplished by the transcription factor RRN3/TIF-IA (the homologs in yeast and mammals, respectively), whose polymerase-stimulating activity varies with the growth status of the cell (Milkereit and Tschochner, 1998; Peyroche et al., 2000). By contrast, the mechanisms responsible for switching rRNA genes on or off within an NOR are poorly understood. Here, we present evidence for an epigenetic switch in which concerted changes in promoter cytosine methylation density and specific histone modifications dictate the on and off states of rRNA genes.

Results

Variation in rRNA Gene Promoter Methylation

To test the hypothesis that subsets of rRNA gene promoters differ in methylcytosine content, possibly correlated with alternative transcriptional states, the positions of methylated cytosines were mapped in *A. thaliana* rRNA gene promoters using bisulfite-mediated DNA sequencing (Frommer et al., 1992). Bisulfite catalyzes the conversion of cytosine to uracil, a reaction that is blocked if the cytosine is methylated. Sequence analysis of 67 independent *A. thaliana* rRNA gene promoter clones, amplified by PCR following bisulfite treatment of purified genomic DNA, revealed that most promoters were extensively methylated (Figure 1A) in all cytosine contexts (CpG, CpNpG, CpNpN). A less abundant class of promoters was sparsely methylated overall and completely unmethylated in the region previously defined as the minimal promoter (Figure 1B).

Hypomethylated and Hypermethylated rRNA Genes Represent Alternative Chromatin States

To ascertain whether rRNA genes with hypermethylated or hypomethylated promoters are organized in functionally distinct chromatin environments, chromatin immu-

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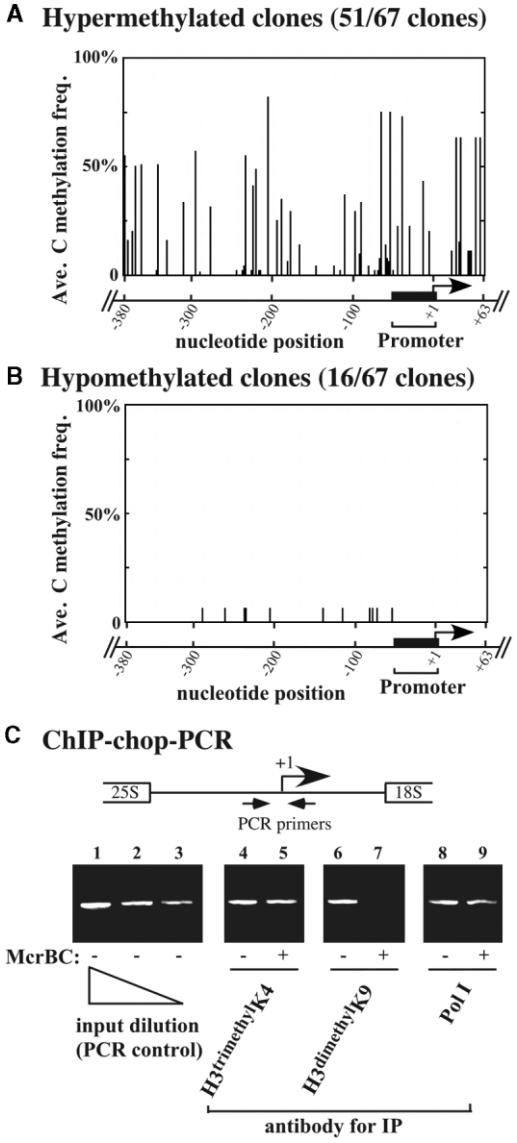

Figure 1. Hypermethylated and Hypomethylated rRNA Gene Promoters in *A. thaliana* Are Organized in Different Chromatin Environments

(A) Pooled data for 51 of 67 promoter clones recovered following sodium bisulfite treatment of genomic DNA. The horizontal axis shows nucleotide positions −380 to +63, numbered relative to the transcription start site, +1. The minimal promoter, −55 to +6 (Doelling and Pikaard, 1995), is denoted by a black rectangle. Each vertical bar represents a cytosine. The height of the bar reflects the frequency at which that cytosine was methylated, on average, among the 51 clones.

(B) Data displayed as (A) for 16 of the 67 clones that were unmethylated within the minimal promoter region.

(C) Chromatin immunoprecipitation followed by McrBC digestion and PCR (ChIP-chop-PCR) to evaluate the methylation density of immunoprecipitated rRNA gene promoters. Antibodies used were specific for $H3^{trimethyl}K4$, $H3^{dimethyl}K9$, or RNA polymerase I. Equal amounts of immunoprecipitated DNA were subjected to digestion with McrBC (+) or were mock-digested (−). PCR amplification of rRNA gene promoter regions used the same primers used for bisulfite-mediated methylcytosine mapping. Input controls in lanes 1–3 represent 0.08%, 0.04%, and 0.02%, respectively, of the chromatin subjected to immunoprecipitation. Note that the amount of PCR product is proportional to the amount of input chromatin template in lanes 1–3, suggesting that the results are semiquantitative.

noprecipitation (ChIP) using antibodies recognizing specific histone modifications or pol I was followed by treatment with McrBC. Only DNA that is methylated at two or more cytosines is digested by McrBC (Bourniquel and Bickle, 2002). PCR was then used to amplify the rRNA gene promoter region. A PCR product is obtained only if the promoter template DNA is not "chopped up" by McrBC. Our shorthand notation for this assay is thus ChIP-chop-PCR.

A. thaliana rRNA gene promoter DNA is immunoprecipitated using antibodies specific for histone H3 trimethylated on lysine 4 ($H3^{trimethyl}K4$), a known epigenetic mark of transcriptionally active protein-coding genes (Figure 1C, lane 4) (Grewal and Moazed, 2003; Richards and Elgin, 2002). Treatment of $H3^{trimethyl}K4$-immunoprecipitated DNA with McrBC prior to PCR amplification had no significant effect on the abundance of the PCR product (Figure 1C, lane 5), indicating that $H3^{trimethyl}K4$-associated promoters are hypomethylated. Ribosomal RNA gene promoter DNA is also immunoprecipitated by antibodies specific for $H3^{dimethyl}K9$ (Figure 1C, lane 6), a known epigenetic mark of transcriptionally inactive heterochromatin. McrBC treatment eliminated the ability to amplify rRNA gene promoters in chromatin precipitated by the $H3^{dimethyl}K9$-specific antibodies, indicating heavy DNA methylation (Figure 1C, lane 7). ChIP-chop-PCR was also carried out following immunoprecipitation with a pol I antibody (Figure 1C, lanes 8 and 9). Promoter DNA of rRNA genes associated with pol I was resistant to McrBC cleavage (lane 9), indicating that the active genes are hypomethylated. Collectively, the data of Figure 1 show that rRNA genes whose promoters are hypomethylated associate with $H3^{trimethyl}K4$ and pol I, indicating that these are the active subset of rRNA genes. By contrast, promoters that are hypermethylated associate with $H3^{dimethyl}K9$ and are presumably silenced.

Epigenetic Marks of Active and Silenced rRNA Genes in Nucleolar Dominance

To test the hypothesis that $H3^{dimethyl}K9$ is a mark of silenced rRNA genes, we exploited the fact that one parental set of rRNA genes is silenced in genetic hybrids that display nucleolar dominance. In *A. suecica*, the allotetraploid hybrid of *A. thaliana* and *A. arenosa* (Figure 2A), the rRNA genes inherited from *A. thaliana* are transcriptionally silenced whereas rRNA genes derived from *A. arenosa* are transcribed (Chen et al., 1998) and thus dominant (Figure 2B, column 2; compare results with the two probes). Importantly, the silencing of *A. thaliana*-derived rRNA genes in *A. suecica* is reversed by treatment with the DNA methylation inhibitor, 5′-aza-2′-deoxycytosine (aza-dC) or the histone deacetylase inhibitor, trichostatin A (TSA) (Figure 2B, columns 3–5). Transcription of the dominant *A. arenosa* rRNA genes is also upregulated 2- to 3-fold by aza-dC and TSA, suggesting that the mechanisms responsible for silencing the *A. thaliana*-derived rRNA genes are also responsible for dosage control among the dominant set.

Determination of the histone modifications associated with silenced and dominant rRNA genes in *A. suecica* was evaluated using ChIP followed by dot blotting and hybridization to species-specific probes corresponding to the ~3 kb intergenic spacers (Figure 2C). In mock-

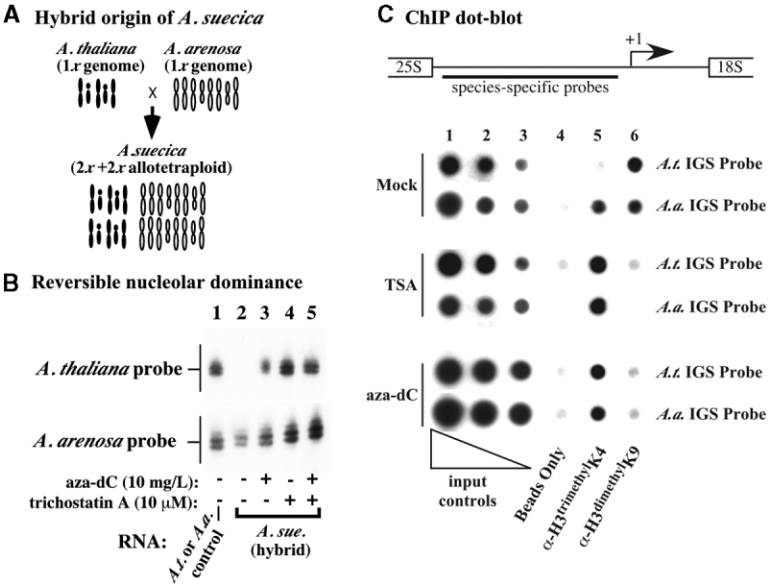

Figure 2. Active and Silenced rRNA Genes Subjected to Nucleolar Dominance in *Arabidopsis suecica* Associate Differently with $H3^{trimethyl}K4$ and $H3^{dimethyl}K9$

(A) *A. suecica* is an allotetraploid hybrid possessing a 2*x* chromosome complement from *A. thaliana* (1*x* = 5 chromosomes) and *A. arenosa* (1*x* = 8 chromosomes).

(B) *A. suecica* seedlings were grown on sterile media prepared with (+) or without (−) 5-aza-2′-deoxycytosine (aza-dC), trichostatin A, or both. Equal amounts of purified RNA were then subjected to S1 nuclease protection using probes specific for *A. thaliana*- or *A. arenosa*-derived rRNA gene transcripts. RNA isolated from *A. thaliana* and *A. arenosa* served as controls in column 1. In the absence of chemical treatment, *A. thaliana*-derived rRNA genes are silenced and *A. arenosa*-derived genes are active (dominant) in *A. suecica* (column 2). Aza-dc and/or TSA derepress *A. thaliana*-derived rRNA genes and upregulate the dominant *A. arenosa*-like genes (columns 3–5). Though aza-dC and TSA appear to exert additive effects only for *A. arenosa* rRNA genes, other repetitions of this experiment show that aza-dC and TSA together are no more effective than either treatment alone. Note that S1 nuclease digestion results in a cluster of probe fragments, each differing in size by one nucleotide. The most intense band is due to precise trimming of the probe DNA-RNA hybrid at +1. Removal of the DNA nucleotide corresponding to RNA position −1 is inefficient, resulting in a longer fragment. Shorter bands result from melting and subsequent nucleotide removal at the termini of DNA-RNA hybrids.

(C) Silenced *A. thaliana*-derived rRNA genes in *A. suecica* associate with histone H3 dimethylated on lysine 9 whereas active and derepressed genes associate with histone H3 trimethylated on lysine 4. *A. suecica* chromatin was immunoprecipitated using antibodies specific for $H3^{trimethyl}K4$ (column 5) or $H3^{dimethyl}K9$ (column 6). Equal aliquots of immunoprecipitated chromatin from control (mock-treated), trichostatin A (TSA)-treated, or aza-dC-treated seedlings were dot blotted in duplicate rows for each treatment. Each row was then hybridized to a radioactive probe specific for either the *A. thaliana*- or *A. arenosa*-derived rRNA gene intergenic spacers (these span ~3 kb in each species). Columns 1–3 are controls showing hybridization signals for 5%, 2.5%, and 1.25% of the input chromatin used for ChIP. Column 4 shows background signals captured on the protein A beads (no antibodies).

treated (control) *A. suecica*, only background amounts of the silenced *A. thaliana* rRNA genes were precipitated with the antibody specific for $H3^{trimethyl}K4$ (Figure 2C, see column 5). Instead, the *A. thaliana* rRNA genes associate with $H3^{dimethyl}K9$ (see column 6). By contrast, the dominant *A. arenosa rRNA* genes associate with both $H3^{trimethyl}K4$ and $H3^{dimethyl}K9$. These data confirm that $H3^{dimethyl}K9$ is an epigenetic mark of silenced rRNA genes. The data further suggest that only some of the dominant *A. arenosa* rRNA genes are active and associated with $H3^{trimethyl}K4$, whereas the remainder are presumably excess, silenced (dosage controlled), and associated with $H3^{dimethyl}K9$. Consistent with these interpretations, treatment of *A. suecica* with TSA or aza-dC, which derepress the underdominant *A. thaliana*-derived rRNA genes and upregulate the dominant *A. arenosa* genes (see Figure 2B), caused the loss of $H3^{dimethyl}K9$ association and a switch to $H3^{trimethyl}K4$ association both for the *A. thaliana*- and *A. arenosa*-derived rRNA genes.

To verify that silenced rRNA genes are marked by $H3^{dimethyl}K9$ whereas active rRNA genes associate with $H3^{trimethyl}K4$, the two *A. thaliana*-derived NORs and the six *A. arenosa*-derived NORs of *A. suecica* (Pontes et al., 2003) were examined in interphase cells by a combination of fluorescence in situ hybridization (FISH) and histone immunolocalization (Figure 3). As shown in Figure 3A, two of the major heterochromatic loci enriched for $H3^{dimethyl}K9$ (A) correspond to the two *A. thaliana* NORs (B and C). Other major $H3^{dimethyl}K9$-positive loci correspond to condensed pericentromeric heterochromatin, which stains more intensely with DAPI than does euchromatin (D and E). By contrast, the antibody specific for $H3^{trimethyl}K4$ interacts with decondensed euchromatin interspersed throughout interphase nuclei, with condensed heterochromatic domains appearing as dark holes (F). The silenced *A. thaliana* NORs (G) account for two of these dark holes (H). These data confirm and extend the ChIP-chop-PCR and ChIP dot blot results by showing that entire silenced NORs spanning millions of base pairs are enriched for the heterochromatic mark, $H3^{dimethyl}K9$, and are depleted for the euchromatic mark, $H3^{trimethyl}K4$. Assuming that transcribed coding regions are nucleosomal, we deduce that these rRNA coding sequences associate with histones displaying the same modifications as the intergenic regions that were assayed by ChIP.

Analysis of the *A. arenosa*-like NORs in *A. suecica* by FISH and immunolocalization (Figure 3B) revealed that the dominant class of rRNA genes colocalize with both $H3^{dimethyl}K9$ and $H3^{trimethyl}K4$, in agreement with the ChIP data of Figure 2C. The *A. arenosa*-derived NOR FISH signals were found to partially, but not completely, overlap $H3^{dimethyl}K9$-positive domains (A–C), suggesting that portions of the NORs are $H3^{dimethyl}K9$-positive heterochromatin. The *A. arenosa* NORs also overlap the euchromatic regions enriched for $H3^{trimethyl}K4$ (F–H). Collectively, these data are consistent with the interpretation that only a subset of the dominant *A. arenosa* rRNA genes is active, decondensed, and associated with $H3^{trimethyl}K4$ in *A. suecica* whereas the remaining excess,

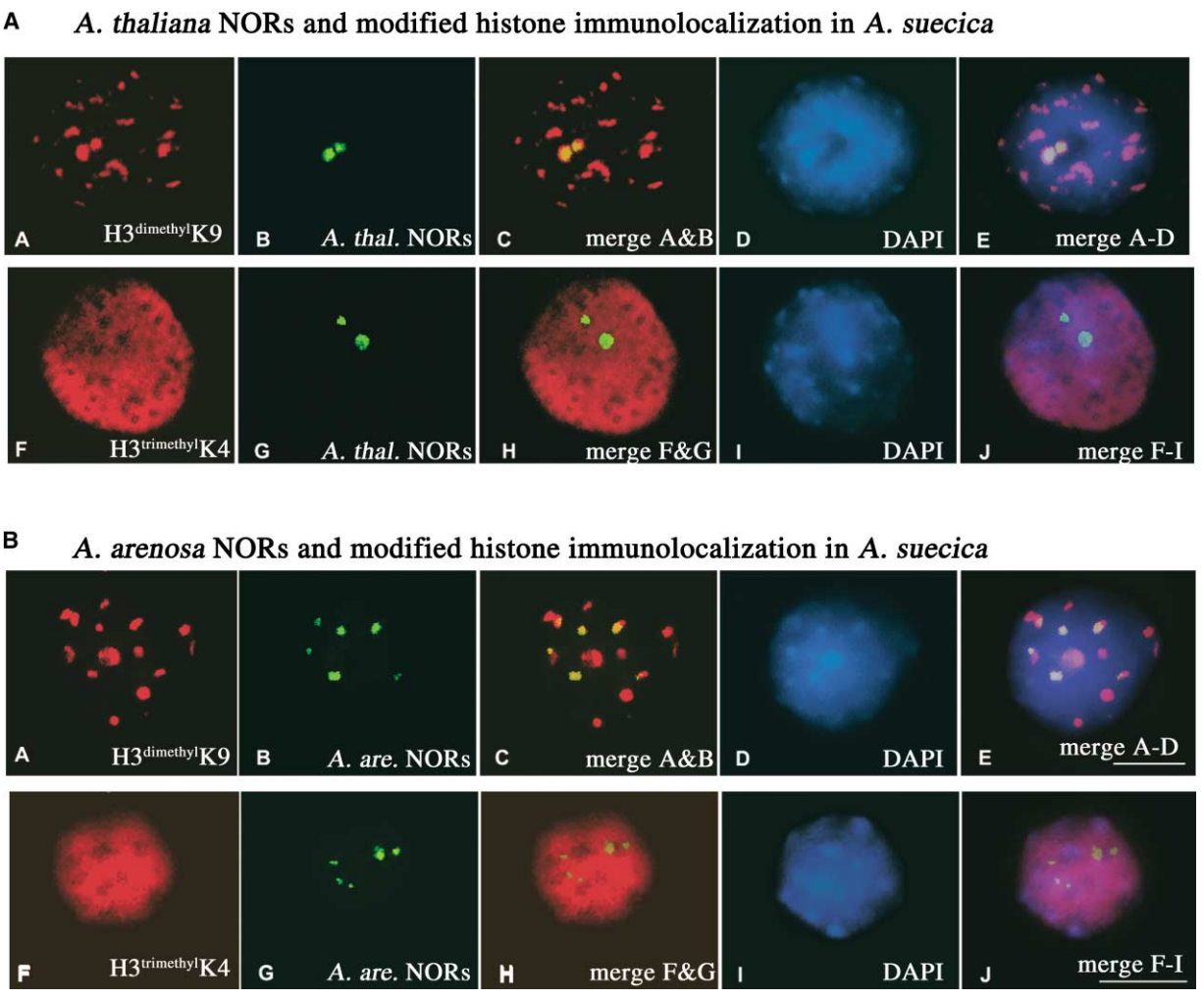

Figure 3. Fluorescence In Situ Hybridization and Histone Immunolocalization in *A. suecica* Meristematic Root-Tip Cell Nuclei

(A) Top row: (A) $H3^{dimethyl}K9$ immunolocalization (in red). (B) FISH using an *A. thaliana*-specific rRNA gene intergenic spacer probe (in green). (C) Superimposition of (A) and (B). (D) Chromatin counterstained with DAPI, which appears blue to bluish-white depending on signal intensity. (E) Superimposition of all images. Bottom row: (F) $H3^{trimethyl}K4$ immunolocalization (in red). (G) *A. thaliana* rDNA loci (green signals). (H) Superimposition of (F) and (G). (I) Chromatin counterstained with DAPI. (J) Superimposition of all images.

(B) Top row: (A) Immunodetection of $H3^{dimethyl}K9$ (in red). (B) *A. arenosa* rDNA loci revealed using FISH probe pCaIGS (green signals). (C) Superimposition of (A) and (B). (D) Chromatin counterstained with DAPI. (E) Superimposition of all images. Bottom row: (F) Immunodetection of $H3^{trimethyl}K4$ (in red). (G) FISH using probe pCaIGS (in green; the larger signal represents two adjacent NORs). (H) Superimposition of (F) and (G). (I) Chromatin counterstained with DAPI. (J) Superimposition of all images.

inactive fraction is condensed, heterochromatic, and associated with $H3^{dimethyl}K9$.

We next tested whether or not promoter DNA methylation was tightly correlated with H3K9 dimethylation in nucleolar dominance in *A. suecica*, as was the case in the pure species *A. thaliana* (refer to Figure 1C). To do so, chromatin isolated from *A. suecica* was tested using the ChIP-chop-PCR assay using primers that specifically amplify either the *A. thaliana*- or the *A. arenosa*-derived rRNA gene promoters (Figure 4). In mock-treated (control) *A. suecica*, *A. thaliana* rRNA gene promoter DNA was not amplified from the chromatin immunoprecipitated with antibodies against $H3^{trimethyl}K4$ (columns 4 and 5) but was enriched within the chromatin immunoprecipitated with antibodies against $H3^{dimethyl}K9$ (columns 6 and 7), consistent with all previous results. Importantly, the *A. thaliana* rRNA gene promoter region could not be amplified by PCR if the chromatin immunoprecipitated with antibodies against $H3^{dimethyl}K9$ was first treated with McrBC (column 7). These data indicate that the *A. thaliana* rRNA genes in *A. suecica* are hypermethylated and are exclusively associated with $H3^{dimethyl}K9$. By contrast, the dominant *A. arenosa* rRNA genes within mock-treated *A. suecica* were readily amplified using chromatin immunoprecipitated with antibodies recognizing either $H3^{trimethyl}K4$ or $H3^{dimethyl}K9$, also consistent with all previous results. Those promoters associated with $H3^{trimethyl}K4$ were resistant to McrBC, indicating that they are hypomethylated (column 5), but those associated with $H3^{dimethyl}K9$ were heavily methylated such that McrBC digestion precluded subsequent PCR amplification (column 7). Treatment of *A. suecica* with aza-dC or TSA to derepress the silenced *A. thaliana* rRNA genes and upregulate the dominant *A. arenosa* rRNA genes

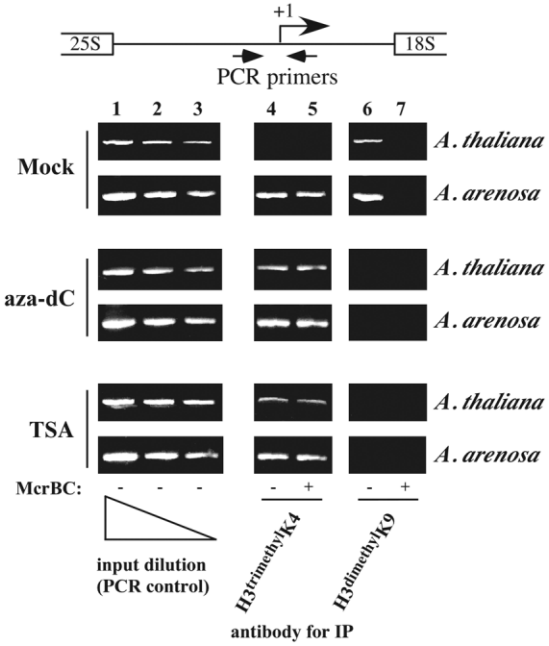

Figure 4. Dominant and Derepressed rRNA Genes in *A. suecica* Associate with $H3^{trimethyl}K4$ and Have Hypomethylated Promoters Whereas Silenced Genes Associate with $H3^{dimethyl}K9$ and Have Hypermethylated Promoters

The ChIP-chop-PCR assay (as in Figure 1C) was used to examine chromatin immunoprecipitated from control (mock-treated), aza-dC-treated, or trichostatin A (TSA)-treated *A. suecica* seedlings. *A. thaliana*- or *A. arenosa*-like promoter regions were amplified by PCR using species-specific primers. Chromatin immunoprecipitated with the $H3^{trimethyl}K4$ antibody was hypomethylated and thus resistant to McrBC cleavage. By contrast, chromatin immunoprecipitated with the $H3^{dimethyl}K9$ was readily digested by McrBC, preventing subsequent PCR amplification of the promoter region. Input controls in lanes 1–3 represent 0.08%, 0.04%, and 0.02%, respectively, of the chromatin subjected to immunoprecipitation.

(refer to Figure 2B) caused *A. thaliana* rRNA genes to switch from exclusive $H3^{dimethyl}K9$ association and heavy promoter DNA methylation to an association with $H3^{trimethyl}K4$ and loss of promoter methylation. Likewise, aza-dC or TSA treatment caused the dominant *A. arenosa* rRNA genes to be found exclusively associated with $H3^{trimethyl}K4$ and to be demethylated in their promoter regions. Loss of promoter methylation upon treatment with the cytosine methylation inhibitor aza-dC is expected, but promoter demethylation by a histone deacetylase inhibitor, TSA, is more surprising, though not unprecedented (Selker, 1998). The concerted switch in both DNA and histone methylation suggests that these epigenetic marks are interdependent and integral to rRNA gene silencing.

A Plant-Specific Histone Deacetylase Is Required for Nucleolar Dominance

Trichostatin A-induced derepression of silenced rRNA genes in *A. suecica* indicates that one or more of the 16 predicted histone deacetylases in *Arabidopsis* is required in the silencing mechanism. A systematic attempt to knock down histone deacetylase activities in *A. suecica* using RNA interference (RNAi) identified *HDT1*, a member of a histone deacetylase gene family first identified in maize (maize *HD2*) and unique to plants (Lusser et al., 1997; Pandey et al., 2002), as a gene required for nucleolar dominance (Figure 5). RNAi-mediated degradation of *HDT1* mRNA was accomplished by expressing a transgene that encodes double-stranded *HDT1* RNA (Figure 5A). RT-PCR revealed reduced *HDT1* mRNA levels in all *HDT1*-RNAi plants (Figure 5B, lanes 6, 8, 10, 12, and 14) relative to nontransgenic control plants (lanes 2 and 4). *HDT1*-RNAi plants were vigorous and displayed no visible phenotypes. Upon examining nucleolar dominance using the S1 nuclease protection assay, *A. thaliana* rRNA gene transcripts were detected only in trace amounts whereas *A. arenosa* transcripts were abundant in nontransgenic *A. suecica* (Figure 5C, columns 4 and 5; compare results using the different probes). Treatment of *A. suecica* with TSA derepressed the silenced *A. thaliana*-derived rRNA genes and upregulated *A. arenosa* genes 2- to 3-fold (lane 3), consistent with previous results. Importantly, in all *HDT1*-RNAi lines, the underdominant *A. thaliana* rRNA genes were derepressed (columns 6–10), and the degree of derepression was consistent with the extent to which *HDT1* mRNA levels were reduced. We conclude that *HDT1* activity is required for rRNA gene silencing in nucleolar dominance.

The involvement of *HDT1* in the concerted promoter DNA methylation/histone methylation switch was tested by comparing wild-type and *HDT1*-RNAi plants using ChIP dot-blot and ChIP-chop-PCR assays (Figures 5D and 5E). In *HDT1*-RNAi plants, the *A. thaliana* rRNA genes switch from an association with $H3^{dimethyl}K9$ to and association with $H3^{trimethyl}K4$, accompanied by a loss of promoter DNA methylation. Loss of H3K9 dimethylation in *HDT1*-RNAi plants is also accompanied by H3K9 acetylation (Figure 5D, column 7). These data indicate that K9 methylation and K9 acetylation are mutually exclusive and that *HDT1* is required for H3K9 deacetylation, either directly or indirectly.

The subcellular location of *HDT1* was determined by transfecting a plasmid encoding a YFP-*HDT1* fusion protein into onion epidermal cells (Klein et al., 1988). A control vector plasmid that expressed YFP alone resulted in fluorescence throughout transformed cells (note that ~90% of the cell volume is vacuole), with the brightest signal corresponding to the nucleus (Figure 6A). By contrast, YFP-*HDT1* transformed cells showed two adjacent spots of fluorescence (Figure 6B), corresponding to two nucleoli per nucleus, as shown conclusively using differential interference contrast microscopy (Figures 6C–6E). The nucleolar localization of *HDT1* suggests that this histone deacetylase may act directly on rRNA gene chromatin.

Discussion

Ribosomal RNA gene transcription accounts for the majority of all nuclear transcription in an actively growing cell, dictating the pace of ribosome production and, in turn, establishing the protein synthetic capacity of the cell (Grummt, 2003; Moss and Stefanovsky, 2002). The role that DNA methylation plays in regulating rRNA transcription has long been controversial (reviewed in

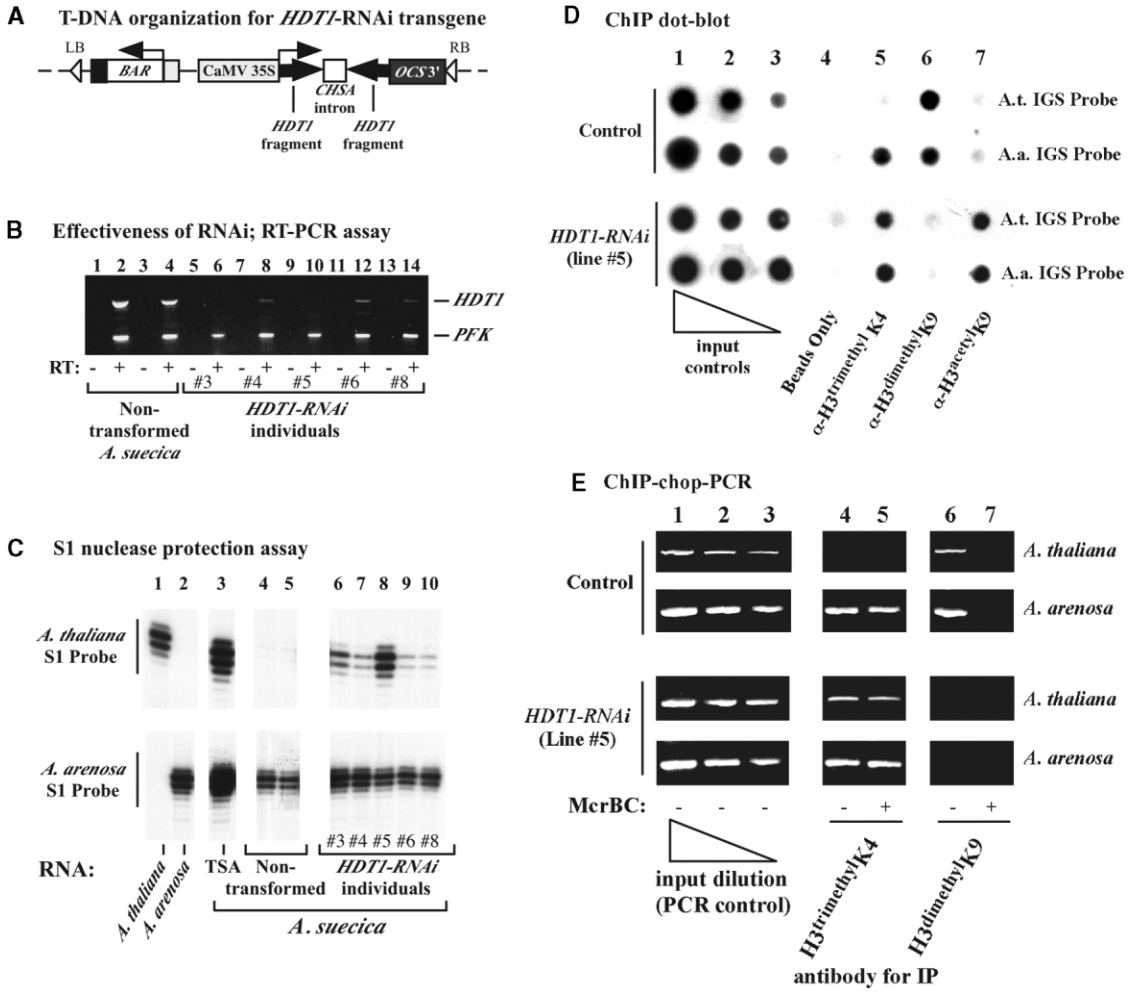

Figure 5. Histone Deacetylase *HDT1* Is Required for rRNA Gene Silencing and the Concerted DNA Methylation/Histone Methylation Switch in *A. suecica*

(A) Transferred DNA introduced into *A. suecica* to cause *HDT1* depletion by RNA interference. The transgene expresses double-stranded *HDT1* RNA from the cauliflower mosaic virus 35S promoter (CaMV 35S). A chalcone synthase (CHSA) intron separates the *HDT1* inverted repeats and 3′ sequences of the octopine synthase gene (OCS 3′) provided polyadenylation signals. The *BAR* selectable marker gene confers herbicide resistance.

(B) *HDT1* mRNA levels are knocked down in *HDT1*-RNAi *A. suecica* plants. RT-PCR was used to compare levels of *HDT1* mRNA in nontransgenic control plants (lanes 1–4) and five independent *HDT1*-RNAi transgenic plants (lanes 5–14). RT-PCR amplification of the phosphofructokinase (PFK) β subunit mRNA served as an internal control in each reaction. As controls for genomic DNA contamination, reverse transcriptase was omitted (−) from mock cDNA synthesis reactions prior to PCR.

(C) *A. thaliana* rRNA genes are derepressed in *HDT1*-RNAi lines. RNA of nontransgenic (columns 4 and 5), TSA-treated (column 3), and *HDT1*-RNAi *A. suecica* plants (columns 6–10) was analyzed for *A. thaliana*- and *A. arenosa*-derived rRNA gene transcripts using the S1 nuclease protection assay. Note that *A. thaliana* rRNA gene transcripts are detected in all *HDT1*-RNAi lines, but not in nontransgenic plants, unless they are treated with TSA. All data are from the same autoradiogram.

(D) ChIP dot-blot assay comparing control and *HDT1*-RNAi plant (line #5) chromatin immunoprecipitated using antibodies specific for $H3^{trimethyl}K4$, $H3^{dimethyl}K9$, or $H3^{acetyl}K9$. RNAi-induced knockdown of *HDT1* causes the loss of $H3^{dimethyl}K9$ and gain of $H3^{trimethyl}K4$ and $H3^{acetyl}K9$ association. Columns 1–3 are controls showing hybridization signals for 5%, 2.5%, and 1.25% of the input chromatin used for ChIP.

(E) ChIP-chop-PCR assay comparing chromatin of control and *HDT1*-RNAi plants (line #5), showing loss of promoter methylation in *HDT1*-RNAi plants coincident with the loss of $H3^{dimethyl}K9$ and gain of $H3^{trimethyl}K4$ association. Input controls in lanes 1–3 represent 0.08%, 0.04%, and 0.02%, respectively, of the chromatin subjected to immunoprecipitation. The data of (D) and (E) were obtained as part of the same experiments shown in Figures 2C and 4, respectively; thus, the controls are the same.

Grummt and Pikaard, 2003), a controversy which we feel is now substantially resolved. Promoters of silenced genes are heavily methylated and are associated with histone H3 dimethylated on lysine 9, both in *A. thaliana* and in the allotetraploid hybrid, *A. suecica*. By contrast, those promoters that are hypomethylated are associated with histone H3 trimethylated on lysine 4 and with pol I, showing that these are the actively transcribed subclass of rRNA genes. Likewise, loss of silencing is accompanied by loss of promoter cytosine methylation, loss of $H3^{dimethyl}K9$, and gain of $H3^{trimethyl}K4$ association. Collectively, these data suggest that DNA methylation plays an important role in the epigenetic switch regulating rRNA gene dosage control and nucleolar dominance.

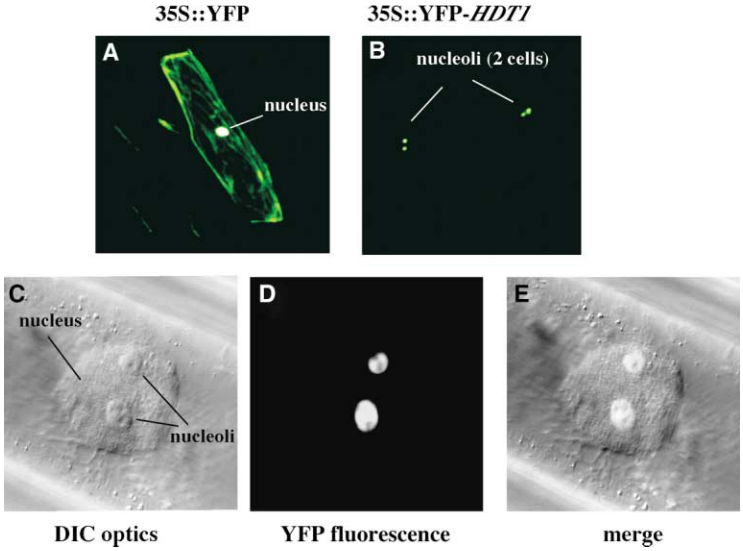

Figure 6. Histone Deacetylase *HDT1* Localizes to the Nucleolus

Plasmids engineered to express YFP (A) or a YFP-*HDT1* fusion protein (B) were transfected into cells by particle bombardment. Cells expressing YFP were detected by fluorescence microscopy ([A] and [B]; ~60×, magnification). Differential interference contrast (DIC) microscopy of a YFP-*HDT1* transformed cell nucleus (600×, magnification) reveals two nucleoli (C). Overlaying the fluorescence signal for YFP (D) and the DIC image reveals that YFP-HDT1 localizes to nucleoli (E).

Our results provide a rationale for why nucleolar dominance occurs; namely, as a manifestation of dosage control. However, dosage control in a hybrid could be achieved by adjusting the number of rRNA genes expressed from both parents. Therefore, additional, but unknown, mechanisms must choose only one parental set of rRNA genes for complete inactivation. It is also not clear what physiological signals dictate the proper rRNA gene dosage per cell. In yeast, rRNA synthesis is similar in wild-type cells with ~150 rRNA genes and in mutant mutants strains with only ~40 rRNA genes (French et al., 2003). This is due, in part, to transcription of only half (~75) of the rRNA genes in wild-type yeast but essentially 100% of the rRNA genes in the deleted strain. However, rRNA synthesis per gene is also increased in the deleted strain. Conversely, transcription per gene is reportedly downregulated in yeast strains impaired for growth-regulated conversion of rRNA genes from an open (transcriptionally active) to a closed conformation due to deletion of the histone deacetylase *RPD3* (Sandmeier et al., 2002). Collectively, these results suggest that feedback mechanisms in yeast balance the number of active genes with the transcription rate per gene to achieve homeostasis. However, our data show that inhibiting cytosine methylation or histone deacetylation in *Arabidopsis* increases the number of active genes and also upregulates the amount of rRNA synthesis. These results suggest that plants, unlike yeast, may lack the mechanism(s) that downregulate the amount of transcription per rRNA gene when more genes are active. If so, a prediction is that plants regulate rRNA synthesis only by controlling the number of rRNA genes that are active.

Interestingly, blocking DNA methylation with aza-dC causes a change in histone modification. Likewise, blocking histone deacetylation with TSA or *HDT1*-RNAi induces DNA demethylation. These results are inconsistent with models that would propose linear pathways placing either histone deacetylation or DNA methylation upstream of the other modification. Instead, the data support a self-reinforcing pathway for rRNA gene silencing in which both DNA methylation and H3K9 methylation are upstream of one another (Figure 7). According to the model, H3K9 methylation and H3K9 acetylation are mutually exclusive, suggesting that K9 deacetylation needs to precede K9 methylation (Nakayama et al., 2001). Our results suggest that *HDT1* is a potential candidate to be a H3K9 deacetylase (see Figure 5D). H3K9 methylases, whose action follows K9 deacetylation in the model, have been identified in numerous eukaryotes (for reviews see Dobosy and Selker, 2001; Grewal and Moazed, 2003; Richards and Elgin, 2002). Heterochromatin protein 1 (HP1) or related chromodomain-containing proteins are then known to bind to methylated H3K9 (see Grewal and Moazed, 2003; Richards and Elgin, 2002). DNA methyltransferases can interact with HP1, suggesting a mechanism by which cytosine methylation can be specified by histone H3K9 methylation (Fuks et al., 2003a; Lehnertz et al., 2003). Alternatively, plants have DNA methylases with built-in chromodomains (chromomethylases, e.g., CMT3) (Henikoff and Comai, 1998), such that an HP1-like intermediary may be unnecessary. Regardless, it is clear that H3K9 methylases are required for some or all cytosine methylation in plants and *Neurospora*, respectively (Johnson et al., 2002; Malagnac et al., 2002; Tamaru et al., 2003). In mammals, methylcytosine binding proteins (denoted as MBD in Figure 7) associate with histone deacetylases (Jones et al., 1998; Nan et al., 1998) and with histone methylases (Fuks et al., 2003b) such that cytosine methylation can act as a signal that brings about H3K9 deacetylation and histone methylation to complete the repression cycle depicted in Figure 7. According to the model, blocking cytosine methylation with aza-dC or blocking histone deacetylation by TSA treatment or by *HDT1*-RNAi would prevent both cytosine methylation and H3K9 deacetylation, consistent with our results. RNAi-mediated knockdown of *Arabidopsis* cytosine methyltransferases is in progress to test the model and rule out the possibility that aza-dC might affect rRNA gene silencing by a mechanism other than cytosine demethylation.

Histone deacetylation and histone acetylation are opposing reactions whose relative activity establishes a

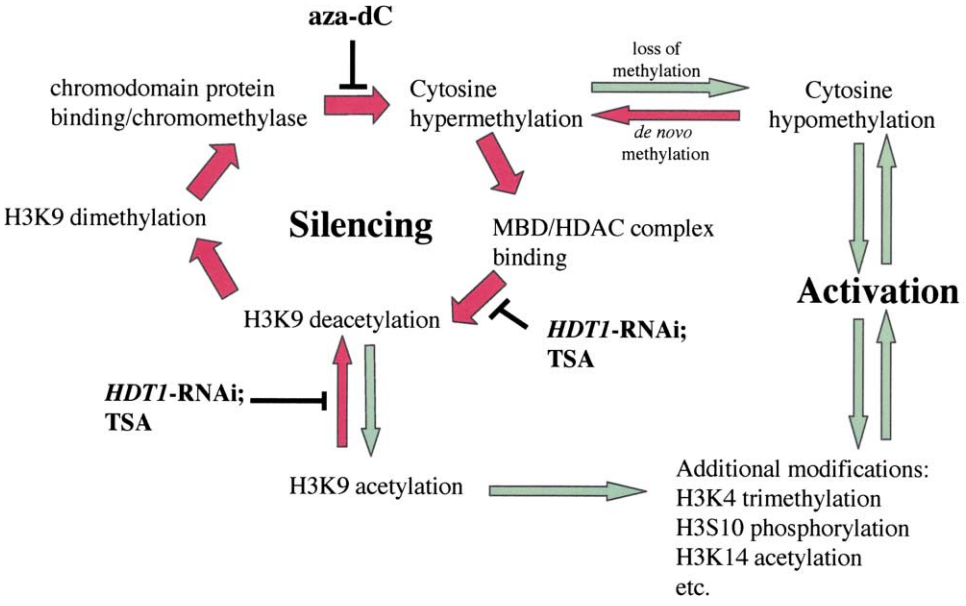

Figure 7. A Model for rRNA Gene Silencing and Activation

DNA methylation and silencing-inducing histone modifications are each upstream of one another in a self-reinforcing repression cycle. Histone acetylation/deacetylation and DNA methylation/demethylation are hypothesized to be the likely control points for switching between the silenced and active states. Abbreviations: MBD, methylcytosine binding domain protein; HDAC, histone deacetylase.

steady state, explaining why blocking histone deacetylation can cause histone hyperacetylation. If so, regulating the relative activity of histone acetyltransferases and deacetylases that modify a key target, such as H3K9, could flip the on/off switch. Shifting the balance toward H3K9 acetylation might, in turn, specify other chromatin modifications that bring about the switch to the activated state, including synergistic H3 serine 10 phosphorylation and H3 lysine 14 acetylation (Cheung et al., 2000; Lo et al., 2000) as well as H3K4 methylation (Berger, 2002; Jenuwein and Allis, 2001). Alternatively, cytosine methylation/demethylation could be a key regulatory event for the on/off switch. If so, passive (replication-dependent) or active (DNA glycosylase-mediated) DNA demethylation may be sufficient for pol I transcription to ensue, causing histones bearing heterochromatic marks to be exchanged for replacement histones (Ahmad and Henikoff, 2002) that then display the $H3^{trimethyl}K4$ modification. In the absence of promoter DNA methylation, genes might circumvent the circular silencing pathway depicted in Figure 7; thus, the activated state might also tend to be self-reinforcing.

Our finding of an epigenetic switch regulating *Arabidopsis* rRNA genes in their native chromosomal context is generally consistent with recent studies of rRNA gene silencing using rRNA minigenes transfected into mammalian cells. TIP5, a subunit of a protein complex, NoRC (Strohner et al., 2001), represses transcription from a cotransfected rRNA minigene when overexpressed (Santoro et al., 2002) and causes the silenced minigene to become methylated and associated with histone H3 methylated on lysine 9 (Santoro et al., 2002). The interdependence of cytosine methylation and histone methylation we observe in the regulation of endogenous *Arabidopsis* rRNA genes could involve a NoRC-like activity.

The genome of *A. thaliana* includes 16 predicted histone deacetylases whose functions are mostly unknown. Interestingly, the HDT histone deacetylase family occurs only in plants and in *Arabidopsis* consists of four genes, *HDT1–4* (Pandey et al., 2002), also known as *AtHD2a–d* (Wu et al., 2000). Our study establishes a function for *HDT1(AtHD2a)* in rRNA gene silencing. RNAi-mediated knockdown of *HDT3* and *HDT4* has no effect on nucleolar dominance, indicating that the four HDT genes are likely to have distinct functions. By contrast, preliminary evidence based on the RNAi-mediated knockdown of 13 of the 16 *Arabidopsis* histone deacetylases reveals that a histone deacetylase related to yeast *RPD3* also plays a role in rRNA gene silencing in nucleolar dominance (R.J.L. and C.S.P., unpublished data). Because RNAi-inducing transgenes create dominant loss-of-function phenotypes, this approach circumvents the problems of gene redundancy inherent to an allopolyploid hybrid such as *A. suecica*, making RNAi a breakthrough technology for the genetic analysis of complex genomes.

Experimental Procedures

Plant Growth

For immunoprecipitation, *A. thaliana* (ecotype Columbia) and *A. suecica* (strain LC1) seeds were sown on sterile semisolid MS medium (Sigma-Aldrich), 1% sucrose (pH 5.8) supplemented with 10 μg/ml aza-dC or 4 μM TSA (no aberrant phenotypes are induced at these concentrations). Plates were incubated in a growth chamber under continuous light for 14 days, 22°C, at which time seedlings were harvested. Plants for cytogenetic examinations were grown in a greenhouse (20 hr photoperiod, 25°C ± 2°C).

Bisulfite-Mediated Methylcytosine Mapping

Bisulfite-treatment was performed according to the Jacobsen protocol (http://www.mcdb.ucla.edu/Research/Jacobsen/BisulfiteGenomic

Sequencing.html), starting with 4 μg of genomic DNA digested with SacI, DraI, EcoRI, EcoRV, and StuI (New England Biolabs), which do not cut the rRNA gene promoter region. Promoter regions were amplified using the primers 5′-ACCGGGTCCGAGGATT-3′ and 5′-ATCCCTCGATCGCTACCCA-3′. PCR conditions were: 2 min, 95°C; 35 cycles of 95°C, 30 s; 55°C, 45 s; 72°C, 45 s; 72°C, 10 min. PCR products from three independent reactions were pooled and cloned into a TOPO TA plasmid (Invitrogen). Independent clones were sequenced using an ABI3700 sequencer and Big Dye Terminator reagents (Applied Biosystems).

Chromatin Immunoprecipitation

ChIP was performed according to published methods (Ascenzi and Gantt, 1999; Gendrel et al., 2002) using seedlings crosslinked in 1% formaldehyde. Nuclei were subjected to four cycles of sonication, 10 s each, using a Branson sonifier (output setting 2, 40% duty cycle). Soluble chromatin was subjected to ChIP using anti-histone $H3^{dimethyl}K9$ (Abcam or Upstate Biotechnology; both yielded the same results), anti-histone $H3^{trimethyl}K4$ (Abcam), anti-histone $H3^{acetyl}K9$ (Upstate Biotechnology), or anti-pol I antibodies. The pol I antibodies were raised in rabbits against amino acids 292–831 of *A. thaliana* RPA2 expressed in *E. coli*. Chromatin-antibody complexes collected on protein A-agarose beads (Upstate Biotechnology) were washed extensively before eluting with 1% SDS, 0.1 M $NaHC0_3$. DNA-protein crosslinks were reversed at 65°C overnight. Purified DNA was precipitated with ethanol and resuspended in TE (pH 8.0). Immunoprecipitated chromatin assayed by dot blot was applied to Genescreen Plus membrane (Perkin-Elmer). Filters were hybridized to an NsiI fragment of intergenic spacer clone pCaIGS for *A. arenosa* rRNA genes or an EcoR I-KpnI fragment of *A. thaliana* intergenic spacer clone pAt4 (Pontes et al., 2003). For ChIP-chop-PCR, 10% of the immunoprecipitated DNA was digested with 10 units of McrBC (New England Biolabs) in reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 1 mM GTP [pH 7.9]) at 37°C for 2–3 hr. Ten percent of the McrBC digestion reaction (equivalent to 1.0% of the total immunoprecipitated material) was then amplified by 28 cycles of PCR using the same promoter primers and PCR conditions used for bisulfite sequencing. All ChIP and ChIP-chop-PCR experiments were repeated at least three times, yielding highly reproducible results.

RNA Isolation and S1 Nuclease Protection

RNA isolation and S1 nuclease protection were as described (Chen et al., 1998) except that radioactive 5′ end-labeled oligonucleotides were used as S1 probes in Figure 5. The *A. thaliana* probe was 5′-GGGTTCCCCACGGACTGCCCAGACTCCCTCAACACCCACCCCCCTATATAGCTGCC-3′; the *A. arenosa* probe was 5′-GGAACCGAGTAGGGAGGTACCCTCGGTCTGCCCAGACTTCACCAACACCCACCCCCTATATAGCTTTTT-3′. Following initial denaturation at 99°C, 15 min, probe-RNA hybridization occurred overnight at 50°C. Probe-RNA hybrids were subjected to S1 nuclease (Invitrogen) digestion (750 units/ml) at 50°C for 45 min. Resulting digestion products were resolved on a 10% urea-polyacrylamide sequencing gel, dried onto filter paper, and exposed to X-ray film.

Immunodetection and FISH

Immunolocalization in paraformaldehyde-fixed root tip cells was according to published methods (Houben et al., 1996). Primary antibodies (Abcam Ltd, UK) were diluted 1:2000 in PBS, 1% BSA. Secondary antibodies were conjugated to Cy3. DNA was counterstained with DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) in CITI-FLUOR antifade buffer (AF1; Agar Scientific). Epifluorescence microscopy (Zeiss Axioskop2) images were obtained using a Zeiss AxioCam digital camera. Following histone immunodetection, fluorescent in situ hybridization was performed using *A. thaliana*- or *A. arenosa*-specific intergenic spacer probes labeled with digoxygenin-dUTP (Roche) as described (Pontes et al., 2003).

Agrobacterium-Mediated Transformation

A. suecica plants were transformed with the *HDT1*-RNAi vector pFGC6362 (www.chromdb.org) by using the floral dip method, as adapted for *A. suecica* (Lawrence and Pikaard, 2003).

RT-PCR Analysis

RT-PCR was carried out as described (Lewis and Pikaard, 2001) using primers 5′-TCTCAACCTTGATTCTTAGCC-3′ and 5′-GCTCTAATAAGAAACCACTTCACTT-3′, which amplify a region of *HDT1* not present in the transgene. PCR detection of PFK used the primers 5′-GCCACGAAAACCAAACAGAC-3′ and 5′-CCGGAATTTCGATCAATCCT-3′. PCR reaction conditions were 95°C, 2 min; 38 cycles of 95°C, 30 s; 60°C, 30 s; 72°C, 90 s; 72°C, 10 min.

Transient Expression and Localization of YFP and YFP-HDT1

The *HDT1* (GenBank AF195545) coding region, amplified by PCR using Pfu polymerase (Invitrogen) and primers 5′-GCCGAGCTCTCATGGAGTTCTGGGGAATTG-3′ and 5′-GCCGGTACCTCACTTGGCAGCAGCG-3′, was cloned as a SacI-KpnI fragment to engineer a YFP-*HDT1* fusion expressed from the CaMV35S promoter. Ten micrograms of plasmid (in 10 μl water) was precipitated onto 3 mg tungsten particles (in 50 μl 50% glycerol) by addition of 50 μl of 2.5 M $CaCl_2$ and 20 μl of 100 mM spermidine. Coated particles were collected by centrifugation, washed with 300 μl each 70% ethanol and 100% ethanol, and resuspended in 50 μl of 100% ethanol. Ten microliters (0.6 mg particles) was dried onto a macrocarrier disk (Bio-Rad) and bombarded into an ~5 cm^3 cube of onion bulb using a Bio-Rad Biolistic Particle Delivery System (model PDS-1000). Bombarded tissue was incubated in the dark for 24 hr and imaged using a Zeiss M^2Bio microscope equipped with a Zeiss Axiocam digital camera and a Nikon Eclipse E600 fluorescence microscope with a Q Imaging Retiga EX digital camera.

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