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Arabidopsis Histone Deacetylase *HDA6* Is Required for Maintenance of Transcriptional Gene Silencing and Determines Nuclear Organization of rDNA Repeats

Aline V. Probst,[a,b,1] Mathilde Fagard,[c,2] Florence Proux,[c,3] Philippe Mourrain,[c,4] Stéphanie Boutet,[c,5] Keith Earley,[d] Richard J. Lawrence,[d] Craig S. Pikaard,[d] Jane Murfett,[e] Ian Furner,[f] Hervé Vaucheret,[c] and Ortrun Mittelsten Scheid[a,6]

[a] Friedrich Miescher Institute for Biomedical Research, CH-4058 Basel, Switzerland
[b] Laboratory of Plant Genetics, University of Geneva, CH-1211 Geneva 4, Switzerland
[c] Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, F-78026 Versailles Cedex, France
[d] Biology Department, Washington University, St. Louis, Missouri 63130
[e] Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211
[f] Department of Genetics, University of Cambridge, Cambridge, CB2 3EH, United Kingdom

Histone acetylation and deacetylation are connected with transcriptional activation and silencing in many eukaryotic organisms. Gene families for enzymes that accomplish these modifications show a surprising multiplicity in sequence and expression levels, suggesting a high specificity for different targets. We show that mutations in Arabidopsis (*Arabidopsis thaliana*) *HDA6*, a putative class I histone deacetylase gene, result in loss of transcriptional silencing from several repetitive transgenic and endogenous templates. Surprisingly, total levels of histone H4 acetylation are only slightly affected, whereas significant hyperacetylation is restricted to the nucleolus organizer regions that contain the rDNA repeats. This switch coincides with an increase of histone 3 methylation at Lys residue 4, a modified DNA methylation pattern, and a concomitant decondensation of the chromatin. These results indicate that HDA6 might play a role in regulating activity of rRNA genes, and this control might be functionally linked to silencing of other repetitive templates and to its previously assigned role in RNA-directed DNA methylation.

INTRODUCTION

Nuclear DNA is organized in a higher order structure, which overcomes the space constraints in the nucleus and facilitates the spatio-temporal regulation of gene expression. The first level of compaction is achieved by nucleosomal packaging of DNA. Each nucleosome comprises 147 bp of DNA wrapped around a histone octamer that consists of two molecules each of histone proteins H2A, H2B, H3, and H4 (Luger et al., 1997). The histone proteins are subject to various covalent modifications, particularly within their N-terminal tails. These modifications include methylation, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation (Jenuwein and Allis, 2001; Berger, 2002). In addition, DNA itself may be modified by methylation at cytosine residues. DNA methylation and histone tail modifications are believed to help organize chromatin into transcriptionally active (euchromatin) or transcriptionally inert (heterochromatin) regions by influencing the accessibility of DNA to the transcriptional machinery (Fischle et al., 2003).

Whereas heterochromatin is enriched in hypoacetylated histones, methylated DNA and histone H3 methylated at Lys residue 9 (H3K9met), euchromatin is characterized by highly acetylated histone H4, and histone H3 methylated at Lys residue 4 (H3K4met) (Nishioka et al., 2002; Peters et al., 2002).

Acetylation of Lys residues was one of the first histone modifications described to correlate with transcriptional activity (Allfrey et al., 1964). Acetylation was initially suggested to influence transcription by neutralizing the positive charge of the histone tails and decreasing their affinity for DNA; however, there is growing evidence that acetylation helps shape the binding surface for activators and repressors (Kurdistani and Grunstein, 2003).

[1] To whom correspondence should be addressed. E-mail aline.probst@bioveg.unige.ch; fax 41-22-379-31-07.
[2] Current address: Laboratoire de Pathologie Vegetale UMR 217, Institut National de la Recherche Agronomique/Institut National Agronomique Paris–Grignon, Université Paris 6, F-75005 Paris Cedex 05, France.
[3] Current address: Laboratoire de Génétique Moléculaire UMR 8541, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris Cedex 05, France.
[4] Current address: Groupe DANIO U368, Institut National de la Santé et de la Recherche Médicale, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris Cedex 05, France.
[5] Current address: Nutrition Azotée des Plantes, Institut National de la Recherche Agronomique, 78026 Versailles Cedex, France.
[6] Current address: Gregor Mendel-Institute of Molecular Plant Biology, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Aline V. Probst (aline.probst@bioveg.unige.ch).
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Histone acetylation of a particular region of chromatin is regulated by a balance between the activities of histone acetyltransferases and histone deacetylases (HDACs). Histone acetyltransferases are transcriptional coactivators and components of large multisubunit complexes (e.g., SAGA, NuA4; Grant et al., 1998; Sterner and Berger, 2000), and HDACs are found associated with sequence-specific regulatory factors (Sin3, NuRD, and CoREST; Ahringer, 2000; You et al., 2001). HDACs also can be recruited by high DNA methylation levels, via association with methyl-DNA binding domain (MBD) containing proteins such as MeCP2 and MBD2 (Bird and Wolffe, 1999), or directly via recruitment by the maintenance DNA methyltransferase itself (Fuks et al., 2000). Evaluation across kingdoms indicates that HDAC families comprise conserved as well as highly divergent members (Pandey et al., 2002). The large number of different HDACs suggests that they have evolved to have specific and/or overlapping roles concerning their targets. In addition, HDACs are regulated in various ways, including by subcellular compartmentalization, posttranscriptional modification, and interacting proteins (Yang and Seto, 2003). Although a few HDACs are relatively well characterized for their role in transcriptional regulation, it will take an enormous effort to decipher the biological function for each family member in every experimental system. Defined loss-of-function mutations in genes for individual HDACs have helped to elucidate the role in some model organisms. For example, RPD3 from bakers' yeast is required to maintain histone hypoacetylation levels in vivo. Through its interaction with the transcriptional repressors SIN3 and UME6, RPD3 can be targeted to promoters and repress genes containing UME6 binding sites (Rundlett et al., 1998). Coupled chromatin immunoprecipitation (ChIP) and DNA microarray analyses indicated that RPD3 affects the acetylation of genes in virtually all cellular pathways (Robyr et al., 2002) but preferentially associates with promoters that direct high transcriptional activity such as ribosomal protein genes or rRNA genes (Kurdistani et al., 2002).

In the Arabidopsis (*Arabidopsis thaliana*) genome, 16 potentially functional HDACs have been identified, and these can be classified into three families (Pandey et al., 2002; see also http://chromdb.biosci.arizona.edu): the RPD3/HDA1-like histone deacetylases, the members of the SIR2-like family, and the plant-specific HD2-like HDACs originally identified as acidic nucleolar phosphoproteins from maize (*Zea mays*) (Lusser et al., 1997). Interference of HDAC functions in plants has been studied using inhibitors such as trichostatin A, SAHA, or butyrate and using transgenic plants containing antisense or overexpressing constructs. These approaches have provided evidence that HDACs are involved in regulation of histone acetylation and thereby gene expression, with consequences for plant morphology and development (Chen and Pikaard, 1997; Wu et al., 2000; Tian and Chen, 2001). Dissecting the function of individual HDAC members is problematic in these studies; therefore, the analysis of loss-of-function mutations of individual *HDAC* genes should add valuable information on specific roles.

Mutants in an Arabidopsis *RPD3-like HDAC* gene, *AtHDA6*, were found in two independent mutant screens based on their effects on specific transgene expression (Murfett et al., 2001; Aufsatz et al., 2002). The *HDA6* mutant allele *axe1* lead to higher expression from a marker gene with an auxin-responsive promoter element (Murfett et al., 2001), whereas the *rts1* alleles of the locus interfere with double-stranded RNA-directed transcriptional silencing (Aufsatz et al., 2002).

The *sil1* (modifiers of silencing 1, Furner et al., 1998) mutation was selected as a monogenic recessive trait reactivating silent and methylated transgenes (Furner et al., 1998). Here, we report the identification of the gene mutated by the *sil1* mutation. The *sil1* mutant is a new allele of *AtHDA6*. We show that *sil1*, as well as *axe1-5*, reactivate transcriptionally silent transgenes and endogenous repeats. We further provide evidence that mutations in the Arabidopsis *HDA6* gene influence histone acetylation levels. Specifically, rDNA loci become enriched in acetylated histone H4, whereas total H4 acetylation levels are only slightly increased. The rDNA repeats in the mutant plants become locally hypermethylated at H3K4 and DNA hypomethylated, concomitant with significant changes in the structural organization of rDNA loci. The *AtHDA6* gene product is therefore implicated in determining transcription, DNA methylation, and structural organization of multiple classes of repetitive DNA.

RESULTS

The *sil1* Mutation Is an Allele of *HDA6*

The mutant *sil1* was identified in a screen for mutations releasing silencing of the complex, rearranged transgenic locus C containing the chalcone synthase gene (*CHS*) and the resistance marker genes neomycin phosphotransferase and hygromycin phosphotransferase. The *sil1* mutation reactivates mainly the resistance marker genes, whereas the homology-dependent silencing of the endogenous and transgenic *CHS* copies are only weakly affected (Furner et al., 1998). In contrast with other mutations that alleviate silencing of the C locus (*hog1* and *ddm1*), *sil1* does not affect DNA methylation at the transgenes or at rDNA loci (Furner et al., 1998). The *sil1* mutation has been mapped to chromosome V between markers CER456030 and CER455379 (Figure 1). This region encompasses the putative *HDAC* gene *HDA6* (At5g63110), a homolog of yeast (*Saccharomyces cerevisiae*) *RPD3* (Rundlett et al., 1996) and human *HDAC1* (Taunton et al., 1996). Additional recessive mutant alleles of this gene (*axe1-1*, *axe1-2*, *axe1-3*, *axe1-4*, and *axe1-5*) were identified and shown to upregulate expression of other complex transgenic loci (produced by transformation with plasmids pDR5 and p2xD0) that contain marker genes under the control of an auxin-responsive promoter (Murfett et al., 2001). Crosses between wild-type plants and plants homozygous for *sil1* and the C locus yielded hygromycin-sensitive hybrids, indicating that the C locus is quickly resilenced in a *sil1*-heterozygous background. Crosses between plants homozygous for different alleles of transgene-free *axe1* mutant plants and homozygous *sil1* mutants containing the C locus resulted exclusively in hygromycin-resistant hybrids, indicating a lack of resilencing of the C locus, failure of complementation, and allelism between the *axe1* and *sil1* mutations. Sequencing of the *HDA6* coding region from *sil1* genomic DNA revealed a G-to-A transition in the

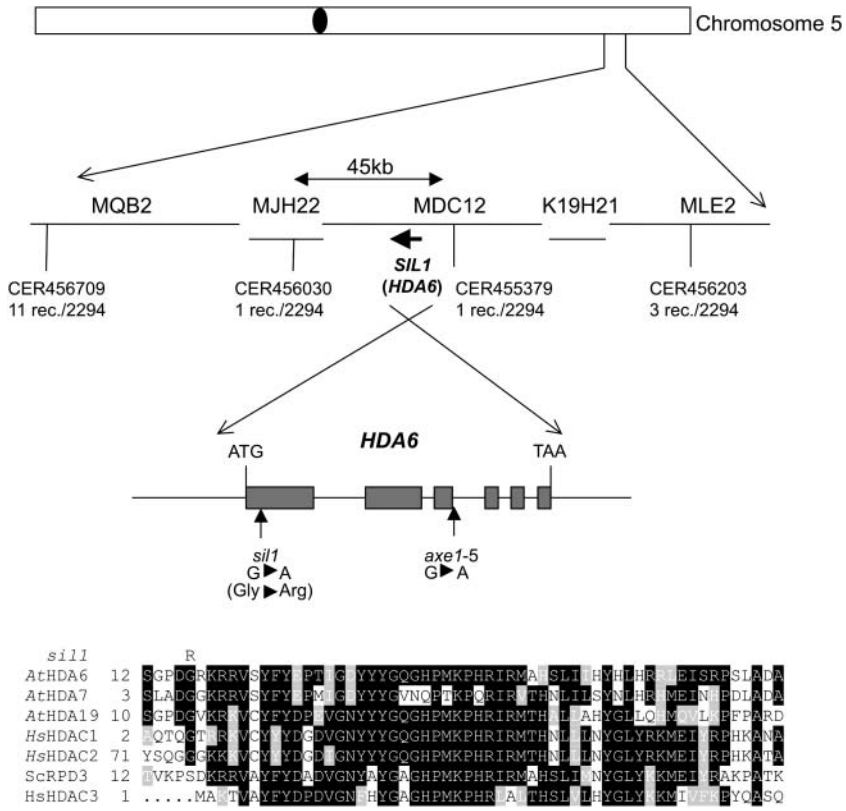

Figure 1. *sil1* Has a Mutation in the *AtHDA6* Gene.

The *sil1* mutation maps between markers CER456030 and CER455379 at the bottom of chromosome 5. Sequencing of the *HDA6* gene in the *sil1* mutant reveals a point mutation, 46 bases after the ATG initiation codon, leading to the replacement of Gly_{16} by Arg. The *axe1-5* mutant has a base substitution at position 1635 downstream of the ATG at the third exon-intron junction. Alignment of *AtHDA6* with Arabidopsis (At) and human (Hs) RPD3-like HDACs and yeast (Sc) RPD3 reveals a conservation of Gly_{16} in plant and human RPD3-like HDACs.

N-terminal part of the protein, resulting in replacement of Gly residue 16 by Arg (Figure 1). The allelism between *axe1* and *sil1* implies that the upregulation of DR5 and 2xD0 transgenes is based on alleviation of silencing because of an impaired function of HDA6 rather than an effect on auxin signaling (Murfett et al., 2001).

All *HDA6* Mutations Release Transcriptional Gene Silencing

The locus C (reactivated by the mutant *sil1*) and genomic insertions formed by pDR5 and p2xD0 integration (reactivated by the *axe1* mutants) are all very complex transgenic loci, consisting of multiple, rearranged, and methylated transgene copies. These features made it likely that they were transcriptionally inactivated and that the mutations interfered with transcriptional gene silencing (TGS). To verify this assumption, we crossed alleles of *HDA6* mutant plants (*sil1*, *axe1-1*, *axe1-3*, *axe1-4*, and *axe1-5)* to a well-established TGS test line. This transgenic line, L5 (Morel et al., 2000), is homozygous for an insert carrying multiple and methylated copies (Figure 2A) of a transgene consisting of the 35S promoter of *Cauliflower mosaic virus* and the ß-glucuronidase (*GUS*) marker gene. The 35S:GUS transgene is silenced at the transcriptional level, as determined by RNA gel blot analysis (Figure 2B) and transcriptional run-on assays (Figure 2C). F2 seeds derived from the crosses were grown under axenic conditions, and seedlings were stained for GUS activity 1 week after germination. Approximately 19% of each F2 progeny expressed the *GUS* marker gene. This corresponds to the expected 3:16 ratio of F2 plants homozygous for an *HDA6* mutation and carrying one or two copies of the L5 insert. Conversely, none of 150 F2 seedlings resulting from a cross between a wild-type plant and line L5 expressed GUS, indicating that the maintenance of TGS at the L5 insert requires the *HDA6* gene product and is unaffected by crossing.

We also tested the effect of the *HDA6* mutations on silencing of endogenous targets. A specific class of pericentromeric repeats termed *TSI* (transcriptionally silent information, not expressed in wild-type plants) is transcribed in plants homozygous for the *sil1* allele (Steimer et al., 2000). We analyzed the reactivation of *TSI* repeats by RNA gel blots in the *axe1-5* mutant, which does not express an *HDA6* transcript of the expected size but shorter and longer mRNAs because of a splice-site mutation (Murfett et al., 2001). RNA gel blots show that *axe1-5* plants express *TSI* (Figure 3). Interestingly, the point mutant *sil1* results in similar if not higher *TSI* expression compared with the splice-site mutant

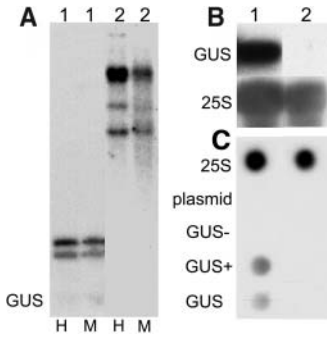

Figure 2. The 35S:GUS Transgene at the L5 Locus Is Methylated and Transcriptionally Silenced.

The transcriptionally active transgenic line Hc1 (1) and the silenced line L5 (2) were characterized by a combination of DNA gel blot **(A)**, RNA gel blot **(B)**, and nuclear run-on analysis **(C)**. The presence of high molecular weight fragments observed after digestion by the methylation-sensitive restriction enzymes *Hpa*II (H) and *Msp*I (M) and hybridization with radiolabeled 35S and GUS probes indicate that the entire insert in line L5 is strongly methylated **(A)**. Hybridization of 10 μg of total RNA with a probe corresponding to the GUS coding region (top panel) or a 25S rDNA probe reveals the absence of GUS cytoplasmic transcript in line L5 **(B)**. Run-on experiments using labeled RNA extracted from leaf nuclei of adult plants for hybridization of dot blots demonstrate the lack of nascent GUS transcript in line L5. Dots contain 2 μg DNA each of the 25S rDNA-containing plasmid (25S), single-stranded pBluescript KS+ (plasmid), and GUS-containing plasmids (GUS−, sense single-stranded; GUS+, antisense single-stranded; GUS, double-stranded) **(C)**.

axe1-5 (Figure 3), but both mutants have lower *TSI* levels than *mom1*, a TGS mutant that causes significant but moderate transcription from TSI templates without changing their DNA methylation (Amedeo et al., 2000). The possibly nuclear nonpolyadenylated, 1250-nucleotide *TSI* fragment and the cytoplasmic, polyadenylated 2500-nucleotide RNA (Steimer et al., 2000) accumulate to similar levels in the mutant *mom1*. By contrast, the *HDA6* mutants predominantly express the shorter nonpolyadenylated fragment (Figure 3). As in *mom1* mutant plants, the release of silencing does not result in obvious phenotypic alterations, with the exception of a significant delay in flowering in both *sil1* and *axe1-5* plants.

rDNA Repeats Become Highly Acetylated in *HDA6* Mutants

Because the *HDA6* gene product has sequence homology with other nuclear proteins shown to have HDAC activity, we studied the effect of *HDA6* mutations on the nuclear distribution of histone H4 acetylation. Mesophyll protoplasts from wild-type Landsberg *erecta* (L*er*), the *sil1* mutant, DR5 (the transgenic background of the *axe1-5* mutant), and the *axe1-5* mutant were fixed, stained with an antibody detecting tetra-acetylated histone H4 (α-H4ac), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). DAPI staining of DNA in interphase nuclei of Arabidopsis distinguishes the nucleolus devoid of dye, the loosely packed euchromatin, and 8 to 10 condensed heterochromatic regions (Figure 4). The latter, known as chromocenters, contain centromeric and pericentromeric chromatin, and as many as four chromocenters also include rDNA repeats (Maluszynska and Heslop-Harrison, 1991). The DAPI-stained nuclei of mutants were indistinguishable from the wild type. Chromatin containing tetra-acetylated histones was found exclusively in euchromatin in all wild-type nuclei (Figures 4A and 4C). However, nuclei of *axe1-5* and *sil1* mutant plants contained chromocenters that were intensively stained with the H4ac antibody. This effect was more pronounced in the *axe1-5* mutant than in the *sil1* mutant (Figures 4B and 4D, Table 1). The labeled chromocenters were always in close association with the nucleolus (Figures 4B and 4D, arrow). Layer-by-layer analysis of mutant nuclei revealed that the number of highly acetylated heterochromatic regions never exceeds 4 in one nucleus and comprises only part of the chromocenter (Figures 4B and 4D). The tight association of the highly acetylated chromocenters with the nucleolus, together with the proposed role in rRNA gene repression described for the *HDA6* homolog *RPD3* in yeast (Sandmeier et al., 2002), suggested that these represent the rDNA loci. To examine a possible relationship between acetylated histones and rDNA, we combined immunodetection of modified histones with fluorescent in situ hybridization (FISH) for rDNA repeats. The rDNA loci of wild-type and mutant nuclei are localized close to the nucleolus. However, whereas all chromocenters, including those with the rDNA repeats, were free of any H4ac signal in DR5 (Figure 4E), the double labeling technique revealed an overlap between the bright H4ac immunosignals and rDNA FISH signals in mutant nuclei (Figure 4F). Therefore, the loss of functional HDA6 resulted in a drastic enrichment of histone acetylation specifically at rDNA repeats.

Total Histone H4 Acetylation Levels Are Not Increased in *HDA6* Mutant Plants

All *HDA6* alleles were originally isolated as mutations affecting loci other than rRNA genes. Intense H4ac immunosignals could indicate a global increase in histone acetylation that would

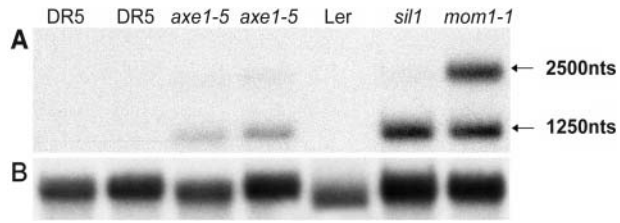

Figure 3. *sil1* and *axe1-5* Alleles Release Silencing of an Endogenous TSI Sequence.

(A) RNA gel blot analysis using the TSI pA2 fragment as probe reveals TSI transcripts in the two *HDA6* mutant alleles *axe1-5* and *sil1*. Lanes 1, 2, and 5 show silencing of the endogenous TSI repeats in the transgenic background of the *axe1-5* mutants (DR5) and the L*er* ecotype, whereas lanes 3, 4, 6, and 7 show reactivation of TSI in the two *HDA6* mutant alleles and the *mom1-1* mutant, respectively. Predominantly, two transcripts are expressed—a longer, polyadenylated one (Steimer et al., 2000) as well as a shorter transcript. nts, nucleotides.

(B) The blot was reprobed with RAN (small GTP binding protein) (Haizel et al., 1997) as a loading reference. Total RNA (20 μg per lane) was extracted from rosette leaves of adult plants.

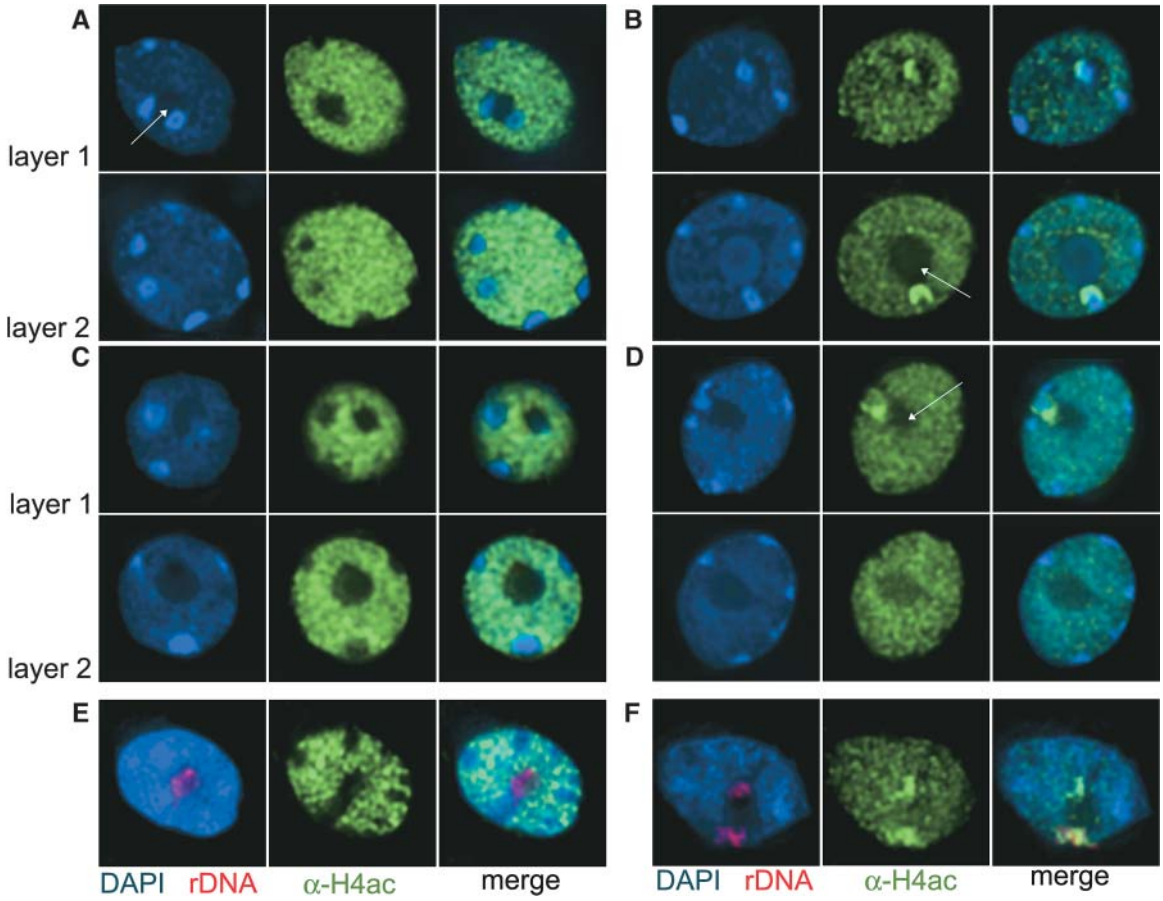

Figure 4. rDNA Repeats Are Hyperacetylated in Nuclei of HDA6 Mutants.

(A) to **(D)** Distribution of histone H4 acetylation revealed by DAPI staining of DNA (blue, left panel) and immunodetection with an antibody specific for tetra-acetylated H4 (green, middle panel) in nuclei of control lines DR5 **(A)** and L*er* **(C)** and in *axe1-5* **(B)** and *sil1* **(D)** mutant nuclei. Right panels show merged images. For each nucleus, two layers were selected from deconvoluted image stacks, arrows mark the nucleolus.

(E) and **(F)** FISH using rDNA repeats (red, left panel) after immunostaining with α-H4ac antibodies (green, middle panels) shows that the rDNA loci indeed are devoid of H4ac staining in the wild type **(E)** but become highly enriched with H4ac in mutant nuclei **(F)**.

appear more prominent at rDNA loci because these include long stretches of silent rRNA genes that are highly condensed in comparison with euchromatic regions (Pontes et al., 2003). To test the possibility that reduced HDA6 activity affects histone acetylation levels globally, we isolated histones from DR5 and *axe1-5* plants and performed protein gel blot analysis. There is no obvious increase in tetra-acetylated H4 in mutant plants (Figure 5A). Also, the amount of methylation at position 4 of histone H3, another epigenetic mark for actively transcribed genes, is not significantly increased in the mutants (Figure 5A). Although we cannot exclude the possibility that the HDA6 protein in *axe1-5* is still partially functional and sufficient to maintain a basal level of hypoacetylation, it seems likely that HDA6 is not the major HDAC in Arabidopsis but may represent a type directed to selected targets, such as rDNA repeats or complex transgenes.

Table 1. Number of *HDA6* Mutant Nuclei with NOR-Specific Enrichment in Acetylated Histone H4 and Methylated H3K4

Mutant	H4ac		H3K4met	
axe1-5	98%	$n = 100$	62.4%	$n = 303$
sil1	39.6%	$n = 306$	19.1%	$n = 308$

HDA6 Mutations Affect Histone H3K4 Methylation Patterns

An increase in histone acetylation is often correlated with another specific modification—methylation at Lys residues at position 4 of histone H3 (H3K4) (Strahl and Allis, 2000). To investigate this correlation for the hyperacetylated rDNA loci in *HDA6* mutants, antibodies specific for H3K4 methylation were included in our immunostaining experiments. All chromocenters in the wild type were free of H3K4 methylation, but we observed an enrichment of H3K4 methylation in the *axe1-5* and *sil1* mutants at the chromocenters presumably containing the rDNA (Figures 5C and 5D, bottom panels). This change affects a significant number of nuclei in both mutants, although the proportion is lower in *sil1*

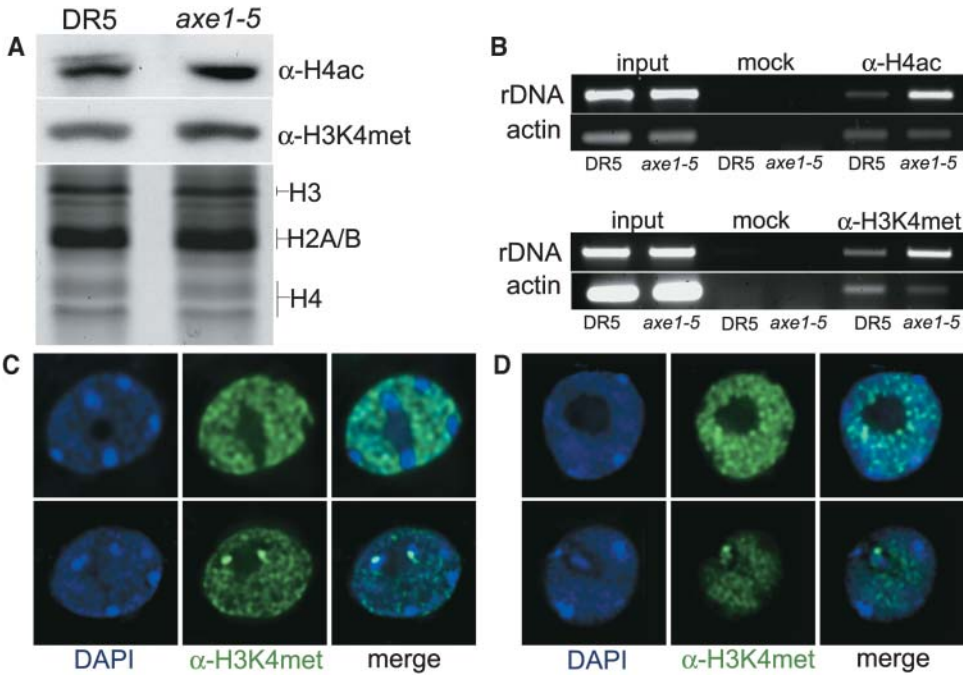

Figure 5. Changes in Levels of H4ac and H3K4met Are Limited to Specific Loci in *HDA6* Mutants.

(A) Protein gel blot analysis detecting H4ac (top panel) and H3K4met (middle panel) using α-H4ac and α-H3K4met antibodies, respectively, on protein extracts from wild-type (DR5) and *axe1-5* mutant plants. Bottom panel, Coomassie staining shows equal protein loading.
(B) ChIP performed in the control line DR5 and the mutant allele *axe1-5* reveals an increase in H4ac and H3K4met at rDNA repeats. The *Actin2/7* gene is equally present in mutant and control precipitates. If the antibodies are omitted during the procedure (mock), neither target is amplified, whereas the equal strength of bands after PCR with the input fraction indicates equal amounts of chromatin before immunoprecipitation.
(C) and **(D)** Distribution of histone H3 methylated at Lys 4 revealed by DAPI staining (blue, left panel) and immunodetection with an antibody specific for H3K4met (green, middle panel) in nuclei of control lines (top row) DR5 **(C)**, L*er* **(D)**, and mutants (bottom row) *axe1-5* **(C)** and *sil1* **(D)**. Right panels show merged images.

(Table 1). To confirm the local hyperacetylation at rDNA loci and the concomitant increase in H3K4 methylation at the molecular level, we performed ChIP on 3-week-old soil-grown plants of DR5 and the mutant allele *axe1-5*. Amplification with primers specific for a 280-bp region of the 25S rRNA gene showed that indeed rDNA repeats are enriched in both H4ac and H3K4met immunoprecipitates in the mutant compared with the control line DR5. These chromatin modifications at the *Actin2/7* gene, serving as reference (Johnson et al., 2002; Tariq et al., 2003), remain unaffected by the *HDA6* mutation (Figure 5B). Comparison of mutant and wild-type chromatin in the α-H3K4met–precipitated fraction by ChIP dot blot analysis confirmed the enrichment of rDNA to a similar extent as in the PCR-based assay (data not shown). Therefore, the irregular histone acetylation in the *HDA6* mutants at rDNA-comprising chromocenters is correlated with an increase in H3K4 methylation.

Hyperacetylation at rDNA Is Not Accompanied by Increased rRNA Expression in the Mutants

Only a subset of rDNA repeats in eukaryotic cells is transcribed at a given time (McKnight and Miller, 1976; Morgan et al., 1983; French et al., 2003). The hyperacetylation of rDNA repeats in the *HDA6* mutants and the increased histone H3K4 methylation at the repeats suggested that these changes may reflect an increase in rRNA transcription. However, comparison of rRNA levels relative to total RNA using semiquantitative RT-PCR (data not shown) or using an S1 nucelase protection assay to detect pre-rRNAs initiated directly at the gene promoter (Figure 6A) did not reveal any differences between mutant and the wild type. However, potential upregulation of rRNAs might be masked when normalized to total RNA because rRNA represents the major species of RNA. Therefore, a subsequent S1 nuclease protection experiment compared rRNA transcript levels relative to the mRNA levels for ubiquitin and actin (Figure 6B). The results of this experiment reveal that *axe1-5* mutant plants contain the same or even slightly reduced amounts of rRNA transcripts compared with the wild type (Figure 6B); thus, there is no indication for increased rRNA transcription concomitant with hyperacetylation at rDNA.

rDNA Repeats Become Decondensed in *HDA6* Mutants

With the exception of the 5S RNA genes, rRNA genes of Arabidopsis are arranged in long tandem arrays comprising the two nucleolus organizer regions (NORs) on chromosomes II and IV (Maluszynska and Heslop-Harrison, 1991). Both NORs adjoin the telomeres (Copenhaver and Pikaard, 1996). FISH with rDNA

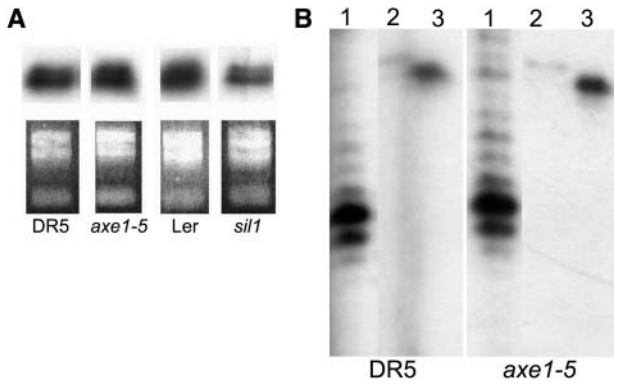

Figure 6. rDNA Expression Is Not Increased in HDA6 Mutant Plants.

Total RNA from control lines DR5 and L*er* and mutants *axe1-5* and *sil1* was subjected to S1 nuclease protection using probes specific for the 5′ end of pre-rRNA transcripts and compared with total RNA amounts, as seen from ethidium bromide staining **(A)**. The signals obtained for rRNA (1) of DR5 and *axe1-5* were normalized against signals obtained with probes specific for protein-coding genes ubiquitin (2) or actin (3) **(B)**. All lanes are from the same exposure of the same autoradiogram.

probes on wild-type interphase nuclear spreads revealed the rDNA to be compactly organized in the chromocenter(s) close to the nucleolus, and only a few DNA repeats extend visibly into the nucleolus (Figure 7A). No obvious change in appearance occurred in the point mutation allele *sil1* (data not shown). However, in the splice-site mutation *axe1-5*, the tight organization was abolished. The rDNA appears less condensed, and rDNA enters the nucleolus and overlaps with adjacent euchromatin (Figure 7A, bottom panel). By contrast, the core centromeric regions, represented by the 180-bp tandem repeats, do not become disorganized in either mutant (Figure 7B). This result appears distinct from the drastic decondensation of all chromocenters observed in *ddm1* mutants (Mittelsten Scheid et al., 2002; Soppe et al., 2002; Probst et al., 2003) because of the general hypomethylation of heterochromatin. The decondensation of rDNA repeats is correlated with the high acetylation of histone H4 and possibly also with an increase in histone H3K4 methylation, suggesting a specific role for the HDA6 deacetylase in the regulation of chromatin structure at particular loci, such as the rDNA repeats.

HDA6 Mutations Affect DNA Methylation Levels Specifically at rDNA Loci

Inhibition of HDACs by trichostatin A (TSA) in *Neurospora crassa* results in reduced DNA methylation at specific transgenic loci (Selker, 1998). No significant changes in DNA methylation levels of either transgene or rDNA was reported in the initial study of the *sil1* mutation (Furner et al., 1998) or the *axe1* mutations (Murfett et al., 2001). By contrast, the *rts1* and *rts2* alleles caused limited demethylation at the target site analyzed (Aufsatz et al., 2002). We investigated whether the increased H4 acetylation at the rDNA loci in *HDA6* mutants was accompanied by changes in DNA methylation at these targets. We incubated DNA from wild-type plants (Columbia and L*er*), the transgenic line DR5, and the mutants *axe1-5* and *sil1* (devoid of the C locus) with different methylation-sensitive restriction enzymes and performed DNA gel blot analysis with an rDNA probe. Clear changes in the methylation pattern between the wild type and mutants were detected using *Cfo*I ($G^{m5}CGC$; Figures 8A and 8B). The hypomethylation in the *axe1-5* background was slightly stronger, and this line also showed more pronounced hyperacetylation at rDNA repeats (Figure 4B, Table 1). Other enzymes, which also are specifically inhibited by CG methylation, *Hpa*II ($C^{m5}CGG$) and *Mae*II ($A^{m5}CGT$), confirmed the rDNA hypomethylation in both mutants (Figures 8A and 8B). The digest with the enzyme *Ava*II, which is inhibited by either CG, CNG, or CNN methylation (GGW ^{m5}CC and GGWC^{m5}C; Figures 8A and 8B), showed only minor

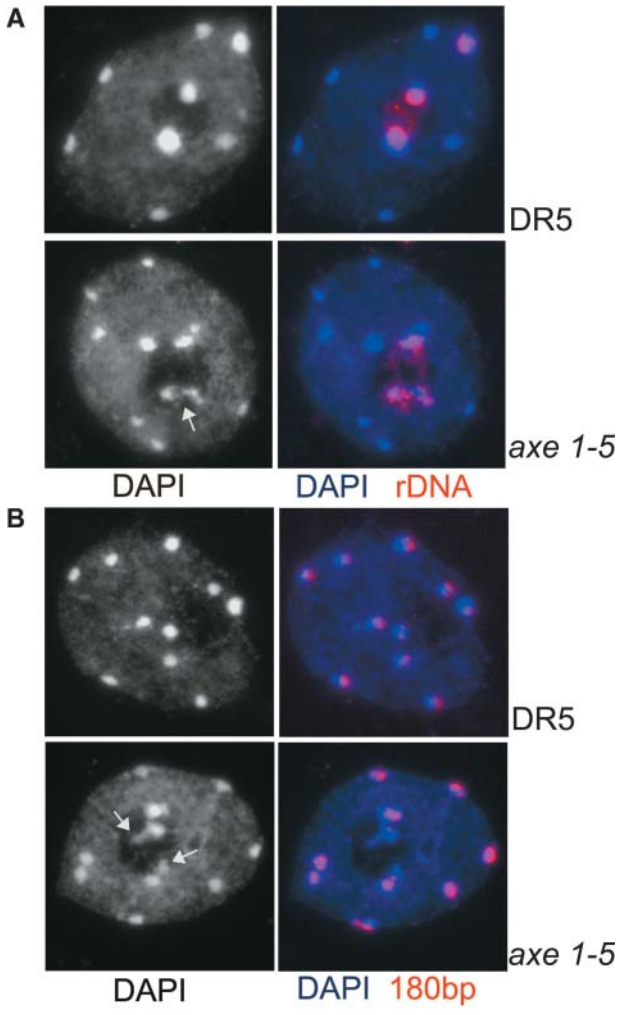

Figure 7. rDNA Loci, but Not Chromocenters in General, Are Decondensed in *HDA6* Mutant Nuclei.

Interphase nuclear spreads of control lines DR5 and L*er* and mutants *axe1-5* and *sil1* stained with DAPI (black and white in left panel, blue in merged images in the right panel) and FISH with biotin-labeled probes for rDNA repeats **(A)** and centromeric (180 bp) repeats **(B)**. Arrows in the black and white images point to decondensed rDNA repeats in mutant nuclei in **(A)** and **(B)**.

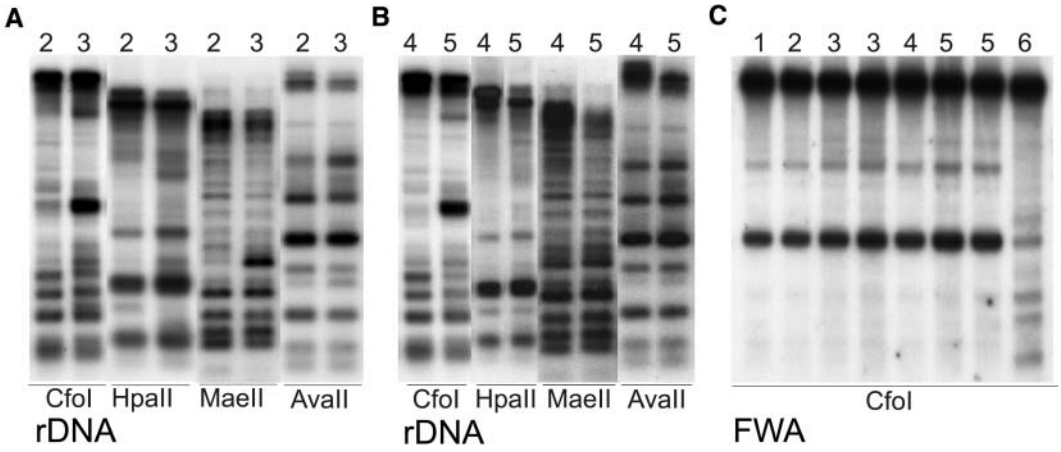

Figure 8. DNA Methylation Patterns at rDNA Repeats Are Affected in *HDA6* Mutants.

Genomic DNA samples from wild-type Columbia (1) and L*er* (4), the transgenic line DR5 (2), the *HDA6* mutant alleles *axe1-5* (3) and *sil1* (5), and from the DNA methylation mutant *ddm1-5* (6) (Jeddeloh et al., 1999) were analyzed. The DNA was digested with methylation-sensitive restriction enzymes *Cfo*I, *Hpa*II, *Mae*II, and *Ava*II (**[A]** and **[B]**), subjected to DNA gel blot analysis, and probed with rDNA (Vongs et al., 1993). A *Cfo*I digest hybridized with a *FWA* probe (Saze et al., 2003) is shown in **(C)**.

reductions in cytosine methylation at the rDNA repeats. Though the changes in CG methylation are significant and distinct, the demethylation is much less pronounced than in DNA of *ddm1-5* (Jeddeloh et al., 1999) used as demethylation control and strongly affected in methylation patterns at many repetitive sequences. The changes caused by the two *HDA6* mutant alleles are relatively subtle and may have been missed in our earlier studies (Furner et al., 1998).

To examine whether the mutations induce specifically rDNA demethylation or a more general genome-wide demethylation, DNA gel blots were reprobed for other potential candidate genes containing appropriate restriction sites. Suggested by the delay in flowering time in the *HDA6* mutants, the membrane with the *Cfo*I digest was hybridized with the promoter of the *FWA* gene (Saze et al., 2003), a positive regulator of flowering. We also analyzed the *Hpa*II digest for methylation changes at the (weakly expressed) *TSI* genes and at the 180-bp centromeric repeats. Only very subtle changes could be detected with the *FWA* and the *TSI* probes, whereas the methylation at the 180-bp repeats appeared unaffected by the *HDA6* mutations (Figure 8C; data not shown).

DISCUSSION

The Arabidopsis gene *HDA6* is a putative HDAC based on its close homology to yeast *RPD3* and mouse *HDAC1* and previously has been identified by seven different mutated alleles; the first five were isolated as recessive mutations increasing expression of transgenes with auxin-responsive promoters. A further two alleles were recovered in a screen for interference with transcriptional silencing acting in *trans* via RNA-directed DNA methylation (Aufsatz et al., 2002). The *sil1* mutant was isolated as a modifier of transgene silencing (Furner et al., 1998). Here, we report that *sil1* is another allele of *HDA6*. By crossing five of these alleles to a line having a well-characterized *cis*-transcriptionally silenced locus, we observed a release of silencing, indicating that HDA6 is involved in epigenetic regulation. The *HDA6* mutants also express noncoding RNA from endogenous repetitive templates (Steimer et al., 2000). This indicates that functional HDA6 is required to maintain TGS at certain, probably repetitive target sequences. All of the *HDA6* mutants analyzed accumulate less TSI transcripts than other TGS mutants that affect global DNA methylation, such as *met1* (Saze et al., 2003) or *ddm1* (Steimer et al., 2000). This could either be because of the fact that none of the *sil* or *axe* alleles are true null mutations or that there might be redundancy with other members of the HDAC family (Pandey et al., 2002). An interesting peculiarity of the *HDA6* mutations is the predominant accumulation of the smaller nonpolyadenylated TSI transcript (cf. with *mom1*, *ddm1*, and *met1* mutants). The origin and/or the processing of the *TSI* transcript family are not yet well understood; however, this might reflect release of expression from particular transcriptional initiation sites or from templates that lack appropriate polyadenylation signals. The changes of chromatin modifications at rDNA loci that also produce nonpolyadenylated transcripts suggest further studies to investigate whether HDA6 has a specific role for regulation of transcripts lacking this 3′ end modification.

The known *HDA6* mutations occur throughout the coding region of the gene. With the exception of the Gly mutated in *sil1* that is not conserved in the yeast counterpart RPD3, all amino acid exchange mutations affect residues that are highly conserved between plant, animal, and yeast HDACs (Murfett et al., 2001). Although the mutations cause different degrees of transcriptional reactivation, histone H4 acetylation, and histone H3K4 methylation at rDNA loci, these differences are not directly correlated with either single amino acid exchanges or splice-site mutations. HDA6 activity might be very sensitive to any structural changes of the protein.

Even though it cannot be excluded that HDA6 has an effect on acetylation of other, yet unidentified, target proteins, the significant increase of histone acetylation at the rDNA loci in *HDA6* mutants strongly suggests that HDA6 is indeed a functional HDAC. Because the protein gel blot analysis indicated no significant increase in the total level of acetylated H4, HDA6 might remove acetyl residues only from specific targets, whereas other related family members are responsible for a more general control of histone deacetylation. *HDAC* genes form a large family, and many members were already shown to be responsible for the reversible and dynamic acetylation changes of histone tails (Kurdistani and Grunstein, 2003). Both yeast RPD3 (Rundlett et al., 1998) and mouse HDAC1 (Doetzlhofer et al., 1999) are required for transcriptional repression of reporter genes, and RPD3 is involved in the deacetylation of large chromosomal domains throughout the yeast genome (Vogelauer et al., 2000; Kurdistani et al., 2002; Robyr et al., 2002). There are 10 members of the *RPD3*/*HDA1* gene family with complete HDAC domains in Arabidopsis falling into subgroups (Pandey et al., 2002) with varying and tissue-specific expression levels. Evidence for their role in histone modification and gene regulation so far was limited to *HDA19* (synonyms *AtRPD3A* and *AtHDA1*), the closest homolog of *HDA6* with detectable expression levels (Tian and Chen, 2001). Other members are weakly expressed or have yet to be analyzed for their transcript levels (http://www.chromdb.org/). Transcripts from *HDA19* are highly abundant in leaves, stem, and flowers, and expression as a GAL4 fusion protein was shown to downregulate a reporter gene (Wu et al., 2000). Antisense-based downregulation of *HDA19* resulted in a 10-fold increase in tetra-acetylated histone 4 (Tian and Chen, 2001). Therefore, HDA6 shares sequence homology and very likely enzymatic activity with its homolog HDA19 but seems to be responsible more for specific rather than for general deacetylation.

A knockout of mouse *HDAC1* leads to embryonic lethality because of severe proliferation defects (Lagger et al., 2002). Clr3 (for cryptic loci regulator; RPD3-like) and Clr6 (HDAC1-like) HDACs in fission yeast (*Schizosaccharomyces cerevisiae*) are involved in maintenance of silent mating type and centromeric heterochromatin (Grewal et al., 1998), and mutants for *clr3* and *clr6* show defective mitotic segregation (Grewal et al., 1998). No Arabidopsis mutant affected in any other *HDA* gene has been described, but downregulation of the *HDA19* by antisense RNA expression resulted in strong pleiotropic effects in transgenic plants, including some that are attributable to secondary deregulation of genes controlling development (Tian and Chen, 2001). By contrast, *HDA6* mutants do not have any drastic phenotype even after several generations of inbreeding (Furner et al., 1998; Murfett et al., 2001; Aufsatz et al., 2002), except a significant delay in flowering time. The fact that morphology is largely unaffected in all plants with a mutated *HDA6* is further evidence that the regulatory role of this protein is restricted to very specific target genes.

Our FISH analysis on interphase chromosome spreads indicated that the organization of rRNA genes into chromocenters is affected in the *HDA6* mutants. Decondensation of chromocenters also has been observed in plants mutated in a chromatin remodeling factor that shapes heterochromatin more generally and is required for proper DNA methylation and histone modification (Mittelsten Scheid et al., 2002; Soppe et al., 2002). Interestingly, in *ddm1-5* mutant nuclei, mainly centromeric and pericentromeric repeats undergo decondensation, whereas in *axe1-5* nuclei, specifically rDNA repeats are affected (Probst et al., 2003). Therefore, histone deacetylation by HDA6 may be required to establish a heterochromatin-like structure at the rDNA repeats, whereas downregulation of transcription may well be achieved also by other mechanisms, such as modulation of the initiation frequency at active decondensed rRNA genes (Sandmeier et al., 2002; Grummt and Pikaard, 2003). The number of active rDNA repeats can be variable, as evident by ultrastructural analysis in *Drosophila melanogaster*, *Xenopus laevis*, and yeast (McKnight and Miller, 1976; Morgan et al., 1983; French et al., 2003). A very drastic specific regulation of rRNA gene activity in numerous eukaryotes is known as nucleolar dominance (Pikaard, 2002a, 2002b), a phenomenon observed upon the formation of genetic hybrids between related but different species when one set of parental rDNA is suppressed while the other is active. Nucleolar dominance in interspecific hybrids of Brassica and Arabidopsis can be overcome by treatment with TSA, a general inhibitor of histone deacetylation, or by the DNA methyltransferase inhibitor 5-azacytidine (Chen and Pikaard, 1997). Suppressed rDNA in allotetraploid hybrids between Arabidopsis and *A. arenosa* also is characterized by DNA and histone modifications characteristic for heterochromatin, and nucleolar dominance is released by the same inhibitors as in Brassica (Lawrence et al., 2004). rRNA silencing was further shown to depend on HDAC HDT1, a member of the plant-specific class II HDAC family with nucleolar localization. Interference with HDT1 expression by RNA interference technology caused expression of the otherwise suppressed Arabidopsis rRNA, an increase in histone H3K4 methylation, and loss of cytosine methylation at rDNA (Lawrence et al., 2004). By contrast, the changes of chromatin features at rDNA in the *HDA6* mutants seem to occur without major changes in transcription rates. This suggests several layers of regulation: a general control of transcription potential via accessibility of the templates and a secondary control of actual transcription by polymerase loading or activity of the polymerase complex. The existence of additional rDNA loci in the allopolyploid hybrids might feed back on both regulatory systems, whereas transcriptional activity in an inbred diploid background is unaffected even if functional HDA6 is missing. This assumption is further supported by the observation that RNA interference downregulation of HDA6 in allopolyploid hybrids does indeed interfere with the selective uniparental transcription of rDNA repeats (R.L. Lawrence and C.S. Pikaard, personal communication).

The similar effects of chemical or genetic interference with DNA methyltransferase or HDAC in nucleolar dominance suggest that DNA methylation and hypoacetylation collaborate in gene silencing mechanisms at rDNA loci. TSA treatment also resulted in derepression of two silenced loci in *N. crassa* (Selker, 1998) and induced a specific reduction of DNA methylation at these two silenced loci without affecting overall methylation levels. This also indicates a reinforcing relationship between acetylation and DNA methylation, although an actual histone hyperacetylation at the affected loci was not demonstrated. It

has been well established that DNA methylation can lead to the recruitment of HDACs (Feng and Zhang, 2001), but our data suggest that histone H4 hyperacetylation also can affect DNA methylation levels. We observed clear differences in CG methylation of rDNA genes between wild-type and *sil1/axe1-5* mutants upon digestion with several methylation-sensitive enzymes. These differences were not observed previously in the *sil1* or *rts1* mutants (Furner et al., 1998; Aufsatz et al., 2002), possibly because they are most obvious with restriction enzymes *Cfo*I and *Mae*II that were not used in earlier studies. The reductions in rDNA methylation levels in *sil1/axe1-5* mutants, compared with wild-type plants, were much less than those observed in other DNA methylation mutants, such as *ddm1* and *hog1* (Furner et al., 1998; Jeddeloh et al., 1999). Furthermore, we observed a more significant effect on cytosines followed by G residues than on cytosines in other contexts. This is in accordance with the results of Aufsatz and coworkers (2002), who used bisulfite sequencing to measure cytosine methylation levels in the promoter region silenced by RNA-directed TGS. The highest reductions in methylation levels between mutant *rts1* and wild-type plants were observed in CG sites, and a lesser effect was observed in CNG sites. Nonsymmetrical CNN sites showed no significant decrease in cytosine methylation in the mutants. These results led Aufsatz and coworkers to propose a model for HDA6 function, in which HDA6 plays a role in reinforcing CG methylation after primary and intermediate de novo C(N)G methylation by other components, thus helping to lock in the silent state of the target gene. Our results are consistent with this model, which might explain why the *HDA6* mutations discovered to date show only moderate reactivation of silenced target genes. Because methylation of rDNA and centromeric repeats was not affected in *rts1* mutants and the *HDA6* gene had not been identified in other screens for DNA hypomethylation or TGS mutants, it was suggested that HDA6 might be specifically involved in RNA-directed pathways of gene silencing (Aufsatz et al., 2002). However, we have now shown that the *sil1/axe1* mutants not only alleviate silencing of a well-characterized TGS locus and endogenous transcriptionally silenced repeats, but also affect acetylation of histones and maintenance of chromatin structure at rDNA loci. These observations indicate that HDA6 is not restricted to its role in an RNA-dependent epigenetic regulation, but acts with a certain level of specificity on other selective targets.

The recent identification of a nucleolar remodeling complex (NoRC) in mouse (Santoro et al., 2002) might allow connecting DNA methylation and histone acetylation activities at the rDNA locus on a biochemical basis. NoRC, consisting of the large nucleolar proteins Tip5 and SNF2, can induce nucleosomal movement on chromatin templates in vitro that depends not only on ATP, but also specifically on the presence of the histone H4 tail (Strohner et al., 2001). Tip5 was shown to interact in vitro with the DNA methyltransferases DNMT1 and DNMT3b as well as with the deacetylase HDAC1. Being recruited to acetylated histone H4 tails via the bromodomain of Tip5, the complex might establish a repressive state by means of histone and DNA modifications. Interestingly, the failure to deacetylate histones also abolished DNA methylation of transfected rRNA gene templates, therefore supporting our observation that histone deacetylation can be required to maintain wild-type DNA methylation levels. It remains to be seen whether a NoRC-like complex with HDA6 (and/or HDT1) as a component exists in plants, or whether some other mechanism controls the equilibrium between decondensed active rDNA and condensed inactive rDNA repeats.

Hypoacetylation of rDNA repeats and DNA methylation also can be reinforced by methylation of histone H3K9, another hallmark of heterochromatin and gene silencing in eukaryotes (Zhang and Reinberg, 2001; Lawrence et al., 2004). Interestingly, recent studies in fission yeast have revealed that a mutation in the *clr3* HDAC impairs methylation of histone H3K9 (Nakayama et al., 2001). It will be interesting to investigate if *HDA6* mutants in Arabidopsis affect H3K9met on particular heterochromatic targets or have additional effects on other histone modifications. In our immunostaining experiments, we could not observe a clear reduction of H3K9 methylation at chromocenters close to the nucleolus (data not shown).

In spite of the general correlation between hyperacetylation and active transcription, recent studies in barley (*Hordeum vulgare*) and *Vicia faba* indicated that histone acetylation correlates more with timing of replication during the S phase of the cell cycle than with transcriptional activity (Jasencakova et al., 2000, 2001). The question of whether a shift in replication timing because of nonremoval of acetyl groups in *HDA6* mutants also occurs and under what conditions it is coupled with transcriptional activity remains for future analysis.

METHODS

Plant Material

Plants were grown in soil in a growth chamber under short-day conditions (12 h light, 21°C, 12 h dark, 16°C) or on MS medium (Ducheta Biochemie, Haarlem, The Netherlands) with or without appropriate selection.

Line L5 was obtained by transformation of wild-type Arabidopsis plants of the Columbia ecotype (Col-0) with a T-DNA composed of a *GUS* reporter gene driven by the 35S promoter of the *Cauliflower mosaic virus* and an *NptII* gene conferring resistance to kanamycin (Elmayan et al., 1998). GUS expression was detected in the hemizygous L5 primary transformant and in its homozygous progeny. Silencing took place between the second and third generation and was stably maintained to subsequent generations, whereas nonsilenced lines, such as the Hc1, continued to express the GUS transgene. Line L5 was previously cited as line 6b5 (Amedeo et al., 2000; Morel et al., 2000) and was renamed L5 to avoid confusion with a widely distributed posttranscriptionally silenced tobacco line named 6b5 (Elmayan and Vaucheret, 1996).

Transgene Expression Analysis

The *sil1* and *axe1* mutants were crossed to line L5, and the F1 progenies were allowed to self-fertilize. F2 plants homozygous for the TGS mutations and carrying the L5 insert were selected by staining with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid.

Map-Based Cloning of the *SIL1* Gene

Mapping of the *sil1* mutation was performed by crossing the *sil1* mutant homozygous for the C insert in the L*er* background with line L5 in the Col-0 background. The hygromycin-resistant F2 individuals that

were homozygous for the *sil1* mutation and carry the C insert were selected for mapping. The *sil1* mutation was mapped at the bottom of chromosome 5. The insertion/deletion markers CER456709, CER456030, CER455379, and CER456203 were derived from the Cereon database (http://www.Arabidopsis.org/cereon/). The analysis of 2294 recombinant chromosomes allowed us to map the *sil1* mutation between markers CER456030 and CER455379. Sequencing of the *HDA6* gene was performed on PCR products and repeated several times to confirm the point mutation in the *sil1* mutant.

RNA Gel Blot, Run-On, and DNA Gel Blot Analyses

Run-on and RNA gel blot analyses of the nonsilenced Hc1 control line and the silenced line L5 were performed as described (Mourrain et al., 2000). Genomic DNA of Hc1 and L5 was extracted by standard cetyl-trimethylammonium bromide method, and their analysis by DNA gel blots was performed as described (Mourrain et al., 2000). RNA isolation and RNA gel blot analysis for *TSI* expression in *sil1* and *axe1-5* mutants and isolation of genomic DNA from *sil1* and *axe1* mutants for DNA gel blot analysis were performed as described (Mittelsten Scheid et al., 2002). The pA2 probe (Steimer et al., 2000) was used for *TSI* detection in RNA gel blot and DNA gel blots, an *Eco*RI fragment of the rRNA gene (Vongs et al., 1993) was used for hybridization to rDNA repeats, and the FWA promoter region (amplified with primers 5′-CAGCGTCTACCAAATCTACACT-3′ and 5′-TAGTGTCTCGACAACGAACAAG-3′) was used for the methylation analysis (Saze et al., 2003). The small GTP binding protein RAN was used as a loading control for RNA gel blots.

FISH

FISH was performed as described previously (Fransz et al., 1998; Probst et al., 2003). Young rosette leaves (1 to 1.5 cm) were fixed in 3:1 ethanol-acetic acid and stored at −20°C. After digestion with a combination of cellulase, pectolyase, and cytohelicase in citrate buffer, the suspension was stirred for 1 min at 45°C in 60% acetic acid, and the nuclei were then spread on glass slides and fixed in ethanol-acetic acid. After a postfixation in 2% paraformaldehyde in PBS, the slides were air-dried. Subsequently, the slides were baked at 60°C, treated with RNase (100 μg/mL in 2× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate]) at 37°C for 1 h, then with pepsin (10 μg/mL in water, pH 2) at 37°C for 20 min. The pAL1 (180 bp) repeat was cloned into pBluescript KS+ vector, and labeled probes were generated by PCR with 0.1 mM dATP, dCTP, and dGTP, 0.065 mM dTTP, and 0.035 mM biotin-dUTP (Roche, Indianapolis, IN). rDNA probes were obtained with the biotin nick translation kit (Roche) using 18S- and 25S-rDNA–containing plasmids. Next, 1 μL of the PCR reaction or 3 μL of the nick translation mix were added to 20 μL of hybridization mix. After hybridization for ~15 h in a wet chamber, slides were washed for 5 min in 2× SSC, 5 min in 0.1× SSC, 3 min in 2× SSC at 42°C, and 5 min in 2× SSC/0.1% Tween 20 at room temperature. The biotin-labeled probe was detected with Texas Red conjugated avidin (5 μg/mL, Vector Laboratories, Burlingame, CA), followed by a biotinylated goat-anti-avidin antibody (5 μg/mL; Vector Laboratories) and once more Texas Red avidin. DNA was counterstained with DAPI (2 μg/mL) in Vectashield mounting medium (Vector Laboratories). Images were analyzed with a Leitz DMR fluorescence microscope and documented with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Images were merged and processed using Adobe Photoshop 7.0 (Mountain View, CA).

Immunostaining

Immunostaining experiments were performed as described (Probst et al., 2003). Protoplasts were isolated from young leaves of DR5, *axe1-5*, L*er*, and *sil1* plants by digestion with 1% cellulase and 0.25% macerozyme in Mes buffer (10 mM Mes, pH 5.7, 0.4 M mannitol, 30 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.1% BSA), washed in wash solution (4 mM Mes, pH 5.7, 2 mM KCl, and 0.5 M mannitol), attached to poly-Lys coated slides, fixed in 2% paraformaldehyde in Phem buffer (6 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9) for 10 min, permeabilized in 0.5% Nonidet P-40 in PHEM buffer, and postfixed in methanol:acetone 1:1 at −20°C. After rehydration in PBS, slides were blocked in 2% BSA in PBS (30 min, 37°C) and incubated with antibodies (Upstate, Charlottesville, VA) against tetra-acetylated H4 (dilution 1:100) or Lys4-dimethylated H3 (1:500) in blocking solution or 1% BSA in PBS (1 h, 37°C or overnight at 4°C). Detection was performed with an anti-rabbit fluorescein isothiocyanate–coupled antibody (1:100, 37°C, 45 min; Molecular Probes, Eugene, OR) in 0.5% BSA in PBS. DNA was counterstained with DAPI in Vectashield mounting medium.

For the combination of immunostaining and FISH, the slides were first processed as for immunostaining experiments. After incubation with the secondary antibody, the slides were dehydrated in an ethanol series (2 min in 70%, 2 min in 90%, and 2 min in 100%), air-dried, and baked at 60°C for 30 min. After an RNase treatment (100 μg/mL in 2× SSC) for 1 h at 37°C, the slides were washed in PBS, postfixed in 4% paraformaldehyde in PBS for 20 min at 4°C, washed again in PBS, dehydrated in an ethanol series, and air-dried. Hybridization, washing, and detection of the labeled probe were performed as for FISH on spread nuclei.

Images were analyzed with a Deltavision deconvolution microscope (Applied Precision, Issaquah, WA). The WoRx software (Applied Precision) was applied for deconvolution of the image stacks, and single layers were chosen for illustration.

Protein Gel Blot Analysis

Fresh leaf tissue (5 g) was ground in liquid nitrogen, transferred to extraction buffer (0.25 N HCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 20 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and treated with ultrasound. After centrifugation (10 min, 10,000 rpm), the supernatant was precipitated with TCA (25% final concentration). After centrifugation at 17000*g* for 20 min, the pellet was washed two times in acetone, dried, and resuspended in 1× SDS loading buffer (75 mM Tris-HCl, pH 6.8, 0.6% SDS, 15% glycerol, and 1.075 M β-mercaptoethanol). The proteins were separated on a 14% SDS page and blotted to a Hybond ECL membrane (Amersham, Buckinghamshire, UK). The membrane was blocked with 3% dry milk in protein gel blot basic buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and incubated overnight at 4°C with α-tetra-acetylated H4 or α-H3K4met (Upstate; 1:2000 in protein gel blot basic buffer supplemented with 1% BSA). After washing, the primary antibody was detected with secondary anti-rabbit horseradish peroxidase coupled antibody (1:7500; Amersham) at room temperature for 45 min. Visualization was achieved using the ECL system (Amersham).

ChIP

ChIP and PCR analysis was performed as described (Gendrel et al., 2002; Johnson et al., 2002) with minor modifications. In brief, leaves of 3-week-old plants of DR5 and *axe1-5* mutant plants grown in soil were vacuum-infiltrated with 1% formaldehyde in buffer 1 (0.4 M sucrose and 10 mM Tris-HCl, pH 8), ground to powder in liquid nitrogen, resuspended in buffer 1 supplemented with 5 mM β-mercaptoethanol, 1 mM PMSF, and protein inhibitors, filtered, and centrifuged. The pellet was dissolved in buffer 2 (0.25 M sucrose, 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1% Triton X-100, and 5 mM β-mercaptoethanol), centrifuged again, resuspended in buffer 3 (1.7 M sucrose, 10 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, and 1 mM PMSF), and layered on top of an equal amount of buffer 3. The pellet was finally resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, and 1% SDS), diluted with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM

Tris-HCl, pH 8, and 167 mM NaCl), and sonicated. The sheared chromatin was precleared with salmon sperm DNA/protein-A agarose (Upstate), and the histone-DNA complexes were immunoprecipitated with α-H4ac and α-H3K4met antibodies (Upstate). After a wash in wash buffer 1 (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, and 2 mM EDTA), wash buffer 2 (20 mM Tris-HCl, pH 8, 500 mM NaCl, 1% Triton X-100, and 2 mM EDTA), wash buffer 3 (10 mM Tris-HCl, pH 8, 250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA), and two washes in Tris-EDTA (TE) buffer, the chromatin was eluted with 0.1 M $NaHCO_3$ and 1% SDS. Cross-linking was reversed overnight at 65°C in the presence of 0.2 M NaCl, and samples were treated with proteinase K for 3 h. After phenol-chloroform extraction, the DNA was resuspended in TE supplemented with RNase A to 10 μg/mL. The immunoprecipitated DNA was analyzed by PCR as described (Johnson et al., 2002). Amplification of the *Actin2/7* gene (Tariq et al., 2003) was performed for 40 cycles, the rDNA repeats for 25 cycles using primers 5′-GATTCCCTTAGTAACGGCG-3′ and 5′-CGGTACTTGTTCGCTATCGG-3′.

S1 Nuclease Protection

Ten micrograms of total RNA was hybridized to oligonucleotide probes corresponding to the non-RNA (antisense) strands of Arabidopsis genes. Probes were 5′ end–labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and γ-^{32}P-ATP. The rRNA probe, which spans the transcription start site, was 5′-GGGTTCCCCACGGACTGCCCA-GACTCCCTCAACACCCACCCCCCTATATAGCTGCC-3′; the ubiquitin probe, matching internal UBQ10 transcript sequences, was 5′-GGA-ACGGAAACATAGTAGAACACTTATTCATCAGGGATTATACAAGGCCC-CCCGG-3′; and the actin probe, matching internal ACT2 sequences, was 5′-GCTCGTTGTAGAAAGTGTGATGCCATATCTTTTCCATGTCAT-GGGCCCCCC-3′. Three to six nucleotides at the 3′ termini of the oligonucleotides probes were purposely designed to be noncomplementary to the target RNAs so that undigested probe could be discriminated from S1 digested products. Hybridization, nuclease treatment, and anaylsis of products was done essentially as described by Lawrence et al. (2004).

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