

RNA Polymerase V Functions in *Arabidopsis* Interphase Heterochromatin Organization Independently of the 24-nt siRNA-Directed DNA Methylation Pathway

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ABSTRACT In *Arabidopsis*, pericentromeric repeats, retroelements, and silenced rRNA genes are assembled into heterochromatin within nuclear structures known as chromocenters. The mechanisms governing higher-order heterochromatin organization are poorly understood but 24-nt small interfering RNAs (siRNAs) are known to play key roles in heterochromatin formation. Nuclear RNA polymerase IV (Pol IV), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), and DICER-LIKE 3 (DCL3) are required for biogenesis of 24-nt siRNAs that associate with ARGONAUTE 4 (AGO4). Nuclear RNA polymerase V (Pol V) collaborates with DRD1 (DEFICIENT IN RNA-DEPENDENT DNA METHYLATION 1) to generate transcripts at heterochromatic loci that are hypothesized to bind to siRNA-AGO4 complexes and subsequently recruit the de-novo DNA methylation and/or histone modifying machinery. Here, we report that decondensation of the major pericentromeric repeats and depletion of the heterochromatic mark histone H3 lysine 9 dimethylation at chromocenters occurs specifically in *pol V* and *drd1* mutants. Disruption of pericentromeric repeats condensation is coincident with transcriptional reactivation of specific classes of pericentromeric 180-bp repeats. We further demonstrate that Pol V functions independently of Pol IV, RDR2, and DCL3-mediated siRNA production to affect interphase heterochromatin organization, possibly by involving RNAs that recruit structural or chromatin-modifying proteins.

Key words: RNA polymerase V; RNA-directed DNA methylation; heterochromatin; centromere; gene expression.

INTRODUCTION

During interphase, the genome is differentially organized into regions of decondensed euchromatin, rich in actively transcribed genes, and highly condensed heterochromatin that is less frequently transcribed (Grewal and Moazed, 2003). So-called constitutive heterochromatin remains condensed throughout the cell cycle (Heitz, 1928) and coalesces into higher-order structures, known as chromocenters (Fransz et al., 2002; Soppe et al., 2002). Constitutive heterochromatin is rich in transposons, retrotransposons, and other repetitive DNA elements that are maintained in a transcriptionally repressed state by DNA hypermethylation and/or repressive histone post-translational modifications (Attwood et al., 2002; Bender, 2004; Vongs et al., 1993). The mechanisms by which heterochromatin clusters into chromocenters is mostly unknown; however, mutations that disrupt DNA methylation and Histone H3 lysine 9 dimethylation (H3K9me2) in *Arabidopsis thaliana* interfere with heterochromatin content and chromocenter formation (Soppe et al., 2002).

Transposons and repetitive elements that comprise constitutive heterochromatin tend to be enriched near centromeres, the highly specialized chromosome domains that are essential for chromosome segregation during mitosis and meiosis. In *A. thaliana*, centromeric regions include related, but distinct classes of ~177–180-bp repeats that are tandemly arranged in large clusters (Heslop-Harrison et al., 1999; Round et al., 1997) ranging from 1.4 to 3.3 Mb in length (Haupt et al., 2001). Additional copies, but not long arrays, of ~180-bp repeats are also found elsewhere in the genome (Lin et al., 1999; Round et al., 1997; The Arabidopsis Genome Initiative,

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2000). Interspersed among the centromeric and pericentromeric ~180-bp repeats are diverse classes of transposable elements and transposon-derived repeats (Hall et al., 2003b; Martinez-Zapater et al., 1986; Thompson et al., 1996).

Although centromeres evolve rapidly and display a remarkable lack of sequence conservation (Henikoff, 2002), a characteristic of their central core is the presence of centromeric Histone H3 (CENH3), known as CENP-A in mammals, centromere identifier (CID) in *Drosophila melanogaster*, and HTR12 in *Arabidopsis* (Talbert et al., 2002). These centromere-specific histone H3 variants help mediate the assembly of the kinetochore (Choo, 1997; Guenatri et al., 2004; Sullivan and Karpen, 2004). However, epigenetic modifications of pericentromeric repeats beyond the centromere core also contribute to centromere functions, including sister chromatid cohesion (Bernard et al., 2001; Choo, 2001; Nasmyth et al., 2000).

Until recently, centromeric regions were not thought to be actively transcribed. However, genes are present within centromeric regions (Appelgren et al., 2003; Kanellopoulou et al., 2005) and transcripts that traverse pericentromeric repeats contribute to heterochromatin formation in these regions (Gaubatz and Cutler, 1990; Nakano et al., 2003; Topp et al., 2004). As was first shown in *Schizosaccharomyces pombe*, pericentromeric transcripts play at least two roles, serving as precursors for short interfering RNA (siRNA) production and serving as the targets for siRNA action via the RNA interference (RNAi) pathway (Hall et al., 2003a; Fukagawa et al., 2004; Volpe et al., 2002). In this pathway, dsRNAs generated by bidirectional transcription, or by RNA-dependent RNA polymerases acting on single-stranded primary transcripts, are cleaved by one or more Dicer endonucleases to produce siRNAs (Hannon, 2002). The siRNAs are then incorporated into an Argonaute protein that comprises the core of an RNA-induced silencing complex (RISC) known as the RITS (RNA induced transcriptional silencing) complex (Noma et al., 2004). The siRNAs then guide the RITS complex to homologous loci by base-pairing with nascent RNA transcripts, promoting the recruitment of histone methyltransferases and deacetylases that bring about heterochromatin formation and transcriptional silencing of a pericentromeric repeats subset (Noma et al., 2004; Verdell et al., 2004; Volpe et al., 2003). Consequently, mutations in the RNAi pathway disrupt heterochromatin formation and gene silencing within the pericentromeric regions. Moreover, the association with centromeres of the Rad21/Scc1 regulatory subunit of cohesin is lost in RNAi mutants, causing defects in chromosome cohesion and aberrant chromosome segregation. However, CENH3 association at the centromere core is not disrupted in RNAi-deficient mutants (Fukagawa et al., 2004; Hall et al., 2003a; Kanellopoulou et al., 2005; Volpe et al., 2003).

Like fission yeast, *A. thaliana* makes use of siRNAs to direct DNA methylation and silencing of retroelements, transposons, and other repeats that are enriched in the pericentromeric regions (Zaratiegui et al., 2007). This RNA-directed DNA methylation (RdDM) process involves two plant-specific nuclear

RNA polymerases, Pol IV and Pol V (formerly Pol IVa and Pol IVb, respectively) (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005), that are structurally distinct and functionally non-redundant. Pol IV collaborates with RDR2 (RNA DEPENDENT RNA POLYMERASE 2) to generate double-stranded RNAs that are processed by DCL3 (DICER-LIKE 3) into 24-nt siRNAs (Xie et al., 2004; Chan et al., 2004) that bind to AGO4 (ARGONAUTE 4). Analogous to the fission yeast RITS complex, AGO4-RISC is thought to target heterochromatic modifications at loci complementary to 24-nt siRNAs (Qi et al., 2006; Zilberman et al., 2004). In a parallel process, Pol V generates transcripts at target loci (Wierzbicki et al., 2008) with the help of DRD1, a SWI2/SNF2-like chromatin remodeler (Kanno et al., 2004). Recent evidence suggests that siRNA-AGO4 complexes bind to Pol V transcripts (Wierzbicki et al., 2008), ultimately guiding the *de novo* DNA methyltransferase DRM2 (Chan et al., 2004) and histone modifying complexes to the target loci.

In this study, we tested components of the *A. thaliana* siRNA-directed DNA methylation pathway for effects on heterochromatin organization in chromocenters. Our study reveals that loss of Pol V or DRD1 function disrupts heterochromatin organization during interphase, which correlates with the transcriptional derepression of specific pericentromeric repeats. By contrast, loss of Pol IV, RDR2, or DCL3 functions, which are all required for 24-nt siRNA biogenesis, does not cause major disruptions in chromocenter organization. Our data suggest that Pol V contributes to heterochromatin formation and pericentromeric repeat silencing via a previously unrecognized ability to function outside of the currently defined siRNA-dependent DNA methylation pathway.

RESULTS

Nuclear Localization of Pericentromeric Repeats Is Disrupted in *pol V* and *drd1* Mutants

We demonstrated previously that the gene encoding the second largest subunit of both Pol IV and Pol V, *NRPD2/INRPE2*, is necessary for the compaction and association of pericentromeric 5S rRNA genes within chromocenters (Onodera et al., 2005). However, it has been unclear whether Pol IV, Pol V or both enzymes are needed for chromatin condensation or whether other proteins of the siRNA pathway are also involved. To address these questions, we compared wild-type and mutant plants for the co-localization of heterochromatic repeats within chromocenters using DNA fluorescence in-situ hybridization (DNA-FISH). In wild-type nuclei, 180-bp centromere repeats (Figure 1, green signals) partially co-localize with 5S genes, with abundant repeat classes present within pericentromeric BAC clone F28D6, and with the centromeric Histone H3, HTR12, within chromocenters that stain intensively with the fluorescent DNA-binding dye, DAPI (Figure 1, top row). In *met1* (DNA METHYLTRANSFERASE 1) mutants defective for the major CG maintenance methyltransferase (Kakutani et al., 1995), the centromere repeats and HTR12 remain

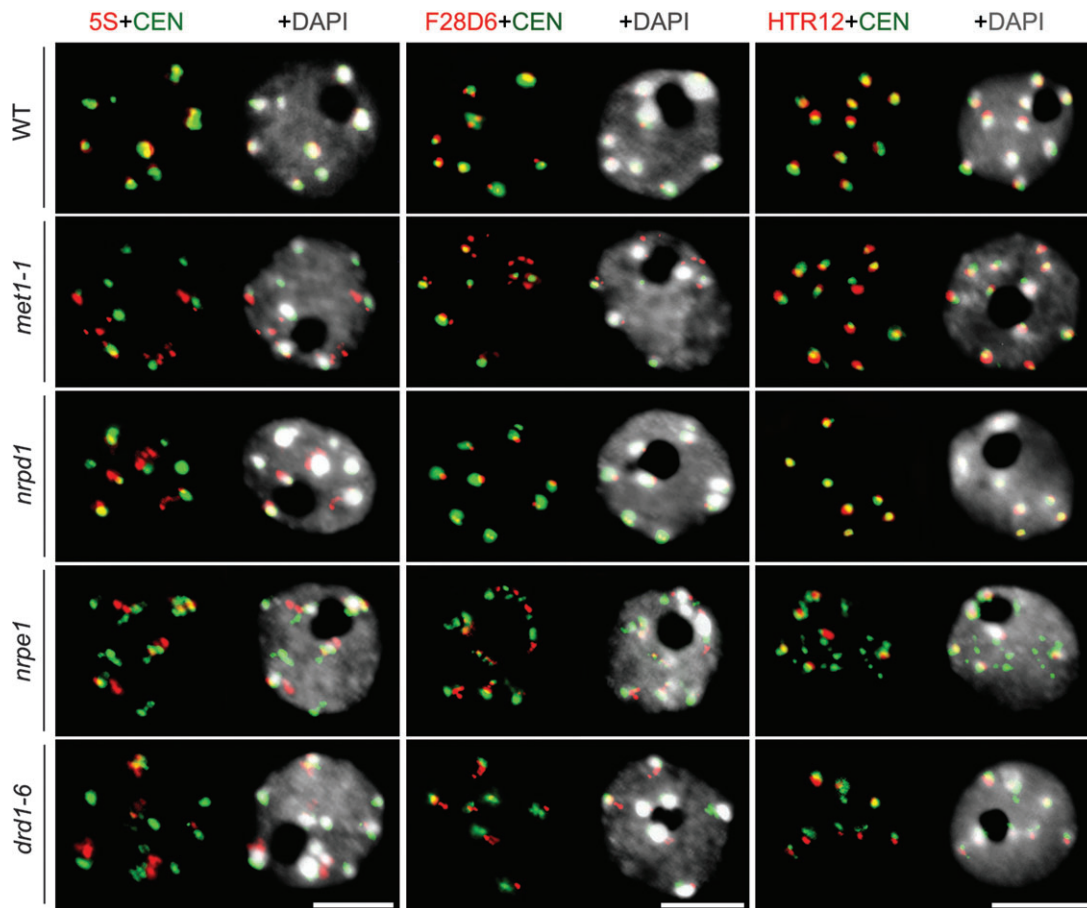


Figure 1. Major Pericentromere Repeat Organization at Interphase Is Disrupted in *nrpe1* and *drd1* But Not *nrpd1* Mutants.

DNA-FISH of 5S genes (red) and 180-bp centromeric repeats (green) (left panel).

Organization pattern of pericentromeric regions analyzed by FISH using the 180-bp centromere repeat (green) and the BAC F28D6 (red) DNA probes (middle panel).

Immunostaining followed by DNA-FISH performed respectively with an antibody recognizing the *A. thaliana* centromeric histone H3, HTR12 (red), and 180-bp centromeric repeats (green) (right panel). In all panels, DNA was visualized by DAPI (gray) and size bars indicate 5 μ m.

co-localized with the chromocenters, as in wild-type cells. In contrast, while we observed only a subtle decondensation of 5S gene loci, F28D6 repeats are significantly decondensed such that they no longer co-localize precisely with chromocenters (Figure 1; Supplemental Tables 1–3). Interestingly, the Pol IV mutant, *nrpd1*, causes a small degree of 5S gene decondensation but has no significant effect on F28D6 or HTR12 co-localization with 180-bp repeats or chromocenters. However, in *nrpe1* or *drd1* mutants, 180-bp centromeric repeats, 5S gene repeats, and F28D6 repeats are all decondensed and no longer co-localize precisely with chromocenters (Figure 1 and Supplemental Tables 1 and 2). Like *nrpd1* mutants, *rdr2* and *dcl3* mutants that disrupt 24-nt siRNA biogenesis have only subtle effects on pericentromeric repeat condensation (Supplemental Figure 1 and Supplemental Tables 1 and 2).

Despite the pericentromeric region decompaction apparent in *nrpe1* and *drd1* mutants, it is noteworthy that the centromere core is unaltered such that HTR12 signals remain at the chromocenters, similar to wild-type cells (Figure 1). We con-

clude that *nrpe1* and *drd1* are required for the association of pericentromeric heterochromatic repeats with chromocenters but are not required for the deposition of HTR12 at the centromere core.

Heterochromatin Content Is Reduced in *pol V* and *drd1* Mutants

DNA methyltransferase MET1, which functions primarily in the maintenance of methylation at CG motifs (Kakutani et al., 1995), and DDM1 (DECREASE IN DNA METHYLATION 1), a SWI2/SNF2 chromatin remodeler required for the maintenance of cytosine methylation in multiple sequence contexts (Vongs et al., 1993), are both required for the organization of pericentromeric heterochromatin within chromocenters (Soppe et al., 2002). As a consequence, heterochromatin content in interphase nuclei visualized by DAPI staining is diminished in *met1* and *ddm1* mutants (Soppe et al., 2002). Using the approach of Soppe et al. (2002), we determined the fraction of nuclear area that is occupied by chromocenters. In *nrpe1*,

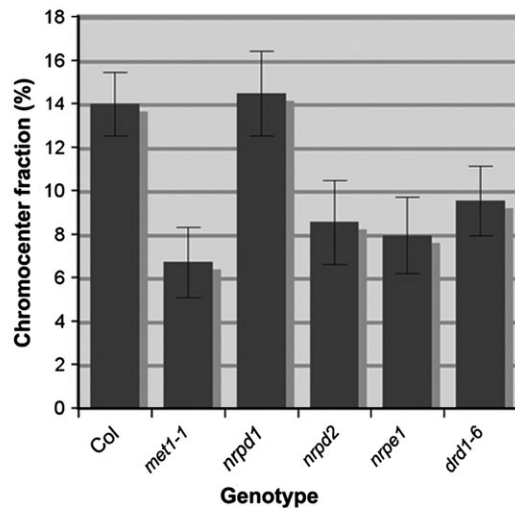
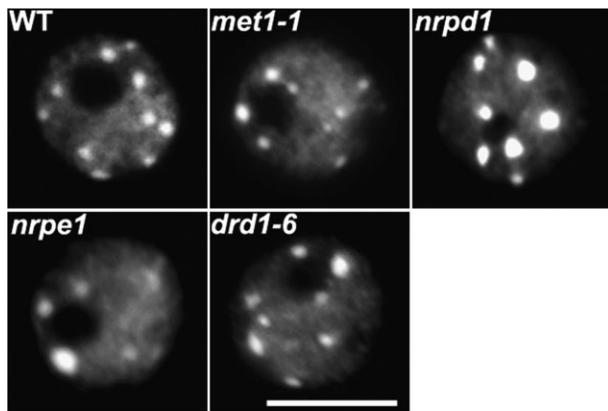


Figure 2. *Pol V*, *drd1*, and *met1* Mutations Result in Loss of Heterochromatin at Chromocenters.

In the upper panel, representative interphase nuclei of the different mutants are displayed. DNA was counterstained by DAPI (gray). The size bar corresponds to 5 μ m. The graph shows the heterochromatin content in wild-type, *nrpd1*, *met1*, *pol V* subunits and *drd1* mutants. The mean values and standard deviation are shown in the histogram calculated from Col ($n = 97$), *met1-1* ($n = 93$), *nrpd1* ($n = 127$), *nrpd2/nrpe2* ($n = 90$), *nrpe1* ($n = 171$), *drd1-6* ($n = 116$).

nrpd2/nrpe2 and *drd1* mutants, heterochromatic content is strongly reduced, similarly to *met1* and *ddm1* mutants (Figure 2 and Supplemental Figure 2). By contrast, chromocenter content in nuclei of the *Pol IV* mutant *nrpd1* resembles that of wild-type cells (ecotypes Col and Ler).

Decreased heterochromatin content is correlated with disruption of histone H3 lysine 9 dimethylation (H3K9me2) in interphase nuclei (Soppe et al., 2002). Immunolocalization of H3K9me2 combined with DNA-FISH shows that H3K9me2 foci co-localize with 180-bp centromeric repeats and DAPI-stained chromocenters in wild-type and *nrpd1* nuclei (Figure 3). However, in *met1* mutants, the H3K9me2 signals are no longer confined to the chromocenters but are dispersed into the decondensed euchromatic regions, with fainter signals detected

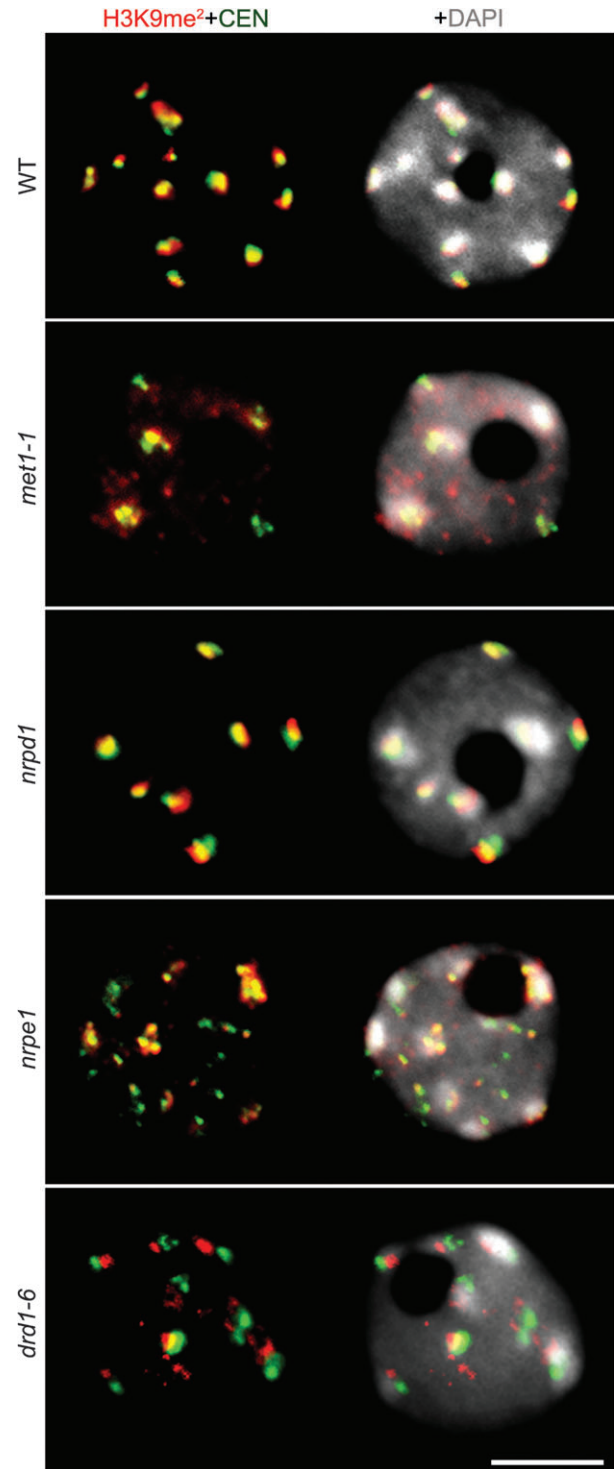


Figure 3. H3K9me2 Clustering at the Chromocenters Is Disrupted by *nrpe1*, *drd1*, and *met1* Loss-of-Function Mutants.

The panel shows representative images of immuno-stained interphase nuclei with H3K9me2 (red) combined with DNA-FISH to detect the 180-bp centromeric repeats (green). DNA was visualized by DAPI (gray) and the size bar indicates 5 μ m.

in the chromocenters. A similar phenotype was observed in *ddm1* mutants (Supplemental Figure 3 and Supplemental Table 4), in agreement with a prior report (Soppe et al., 2002). In *nrpe1* and *drd1* nuclei, dissociation of H3K9me2 signals from the chromocenters is also observed (Figure 3), although not as dramatically as in *met1* mutants. Like the Pol IV mutant, *nrpd1*, other mutants disrupting the 24-nt siRNA-dependent DNA pathway, including *rdr2*, *dcl3*, and *ago4* mutants, had no significant effect on H3K9me2 signals compared to wild-type (Figure 3, Supplemental Figure 3 and Supplemental Table 4).

Specific Pericentromeric Transcription Units Are Suppressed by MET1, DDM1, Pol V, and DRD1, Independently of the 24-nt siRNA Biogenesis Pathway

To investigate potential links between heterochromatin disruption and pericentromeric transcription, we used RT-PCR to detect RNAs corresponding to pericentromeric 180-bp repeats. Two sets of strand-specific primers that distinguish be-

tween conserved and non-conserved 180-bp repeat families (May et al., 2005) were tested. These primers were designated CEN-Fc and CEN-Rc, and CEN-F and CEN-R, where F denotes forward-strand transcripts, R denotes reverse-strand transcripts, and c denotes conserved repeats (supplemental Figure 4 and May et al., 2005). In wild-type plants (ecotypes Col, Ler, and WS), CEN-Fc repeats are not significantly expressed. However, in *met1* and *ddm1* mutants, as well in all mutants that define the 24-nt siRNA-dependent DNA methylation pathway (*nrpd1*, *nrpe1*, *rdr2*, *dcl3*, *ago4*, *drd1*, and *drm2*), CEN-Fc transcripts are readily observed, suggesting that a combination of maintenance and de-novo cytosine methylation suppresses these transcription units. In contrast, CEN-Rc transcripts are up-regulated specifically in *met1* and *ddm1* mutants (Figure 4A), suggesting that maintenance cytosine methylation is primarily responsible for silencing these transcription units. Interestingly, CEN-F and CEN-R transcripts derived from non-conserved centromeric repeats were selectively de-repressed

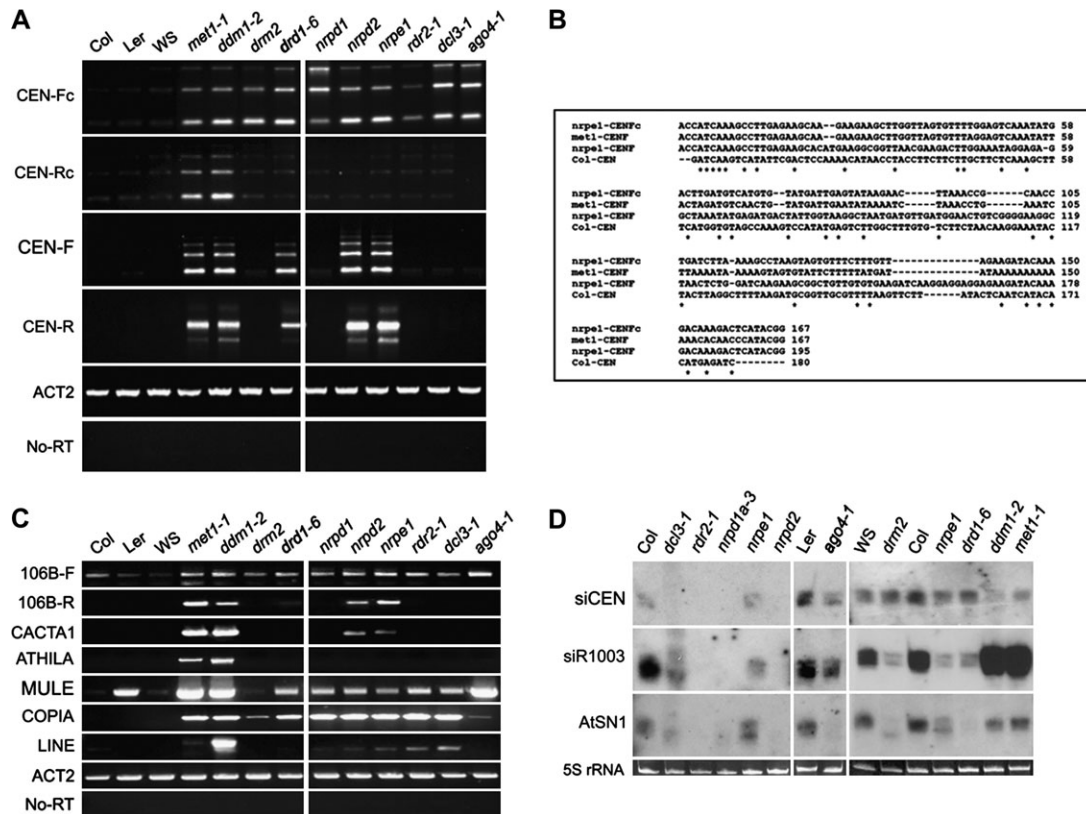


Figure 4. Specific Centromere Sequences Are De-Repressed in *pol V* and *drd1* Mutants.

(A) Strand-specific centromere transcription was evaluated by RT-PCR using primers specific for different subsets of 180-bp repeat families: forward conserved (CEN-Fc), reverse conserved (CEN-Rc), forward non-conserved (CEN-F), and reverse non-conserved (CEN-R) 180-bp repeats.

(B) Different centromere sequences are up-regulated in *nrpe1*. The figure displays aligned centromere transcript consensus sequences obtained by cloning RT-PCR products from the following mutants: *nrpe1* CEN-Fc 180-bp ($n = 12$), *nrpe1* CEN-F ($n = 15$), and *met1* CEN-F 180-bp ($n = 10$).

(C) Transposable element transcription detected by RT-PCR.

(D) Northern blot analysis of centromere-derived siRNAs (siCEN). Ethidium bromide-stained 5S rRNA resolved by agarose gene electrophoresis serves as a loading control.

in *met1*, *ddm1*, *drd1*, and Pol V (*nrpe1* and *nrdp2/nrpe2*) mutants, but not in mutants required for 24-nt siRNA-dependent DNA methylation pathway (Figure 4A). Sequencing revealed that the 180-bp, 360-bp, and 520-bp RT-PCR products correspond to transcripts that spanned one, two, or three repeats. Interestingly, the sequencing data also revealed that 180-bp repeats reactivated in *pol V* mutants differ in sequence from repeats de-repressed in *met1* (Figure 4B).

Next, we examined several classes of transposable elements and repeats enriched in pericentromeric heterochromatin to compare the genetic requirements for their suppression (Figure 4C). Strand-specific RT-PCR of 106B-dispersed repeats (May et al., 2005) indicates that forward-strand transcription is detectable, even in wild-type plants, but is up-regulated in *met1* and *ddm1* mutants as well as in mutants that define the 24-nt siRNA-dependent DNA methylation pathway (Figure 4C). However, transcripts corresponding to the reverse-strand of the 106B repeats were abundant in *met1*, *ddm1*, and *pol V* mutants (*nrpe1* and *nrdp2/nrpe2*) and were detectable in the *drd1* mutant, but were not observed in the other mutants tested. Similarly, CACTA1-like transposons are de-repressed in *met1* and *ddm1* plants, in agreement with prior studies (Mathieu et al., 2005; Kato et al., 2003), and are also de-repressed in the Pol V catalytic subunit mutants (*nrpe1* and *nrdp2/nrpe2*) but not in Pol IV, *rdr2*, *dcl3*, *ago4*, or *drm2* mutants (Figure 4C). In contrast, transcripts of Mutator-like DNA transposons (MULE), Copia4 transposons, and LINE transcripts are detected in the majority of the mutants analyzed (Figure 4C). Athila retroelement transcription was only detected in *met1* and *ddm1* mutants. Collectively, these results indicate that Pol V collaborates with MET1 and DDM1-dependent cytosine methylation in the suppression of specific classes of pericentromeric repeats, but can function independently of the 24-nt siRNA biogenesis or RNA-directed DNA methylation pathway.

Transcription or Silencing of Pericentromeric Regions Is Not Correlated with siRNA Accumulation

Although mutations disrupting the 24-nt siRNA biogenesis pathway do not phenocopy Pol V mutants with respect to chromocenter organization or the de-repression of CEN-F, CEN-R, 106B-R, or CACTA1 elements (Figure 4A and 4C), siRNAs generated by other pathways might be involved. Using small RNA Northern blotting, we examined the abundance of siRNAs corresponding to 180-bp centromere repeats in mutants that have differential effects on pericentromeric repeat silencing (Figure 4D). No strict correlation between CEN siRNA abundance and silencing was observed. For instance, CEN siRNAs are undetectable in *nrdp1*, *nrdp2/nrpe1*, and *rdr2* mutants, in which CEN-F, CEN-R, 106B-R, or CACTA1 elements remain silenced, yet siRNAs are also reduced in *met1* and *ddm1* mutants, in which the elements are de-repressed (Figure 4D). Likewise, in Pol V mutants (*nrpe1*), siRNA levels persist at near wild-type levels, yet CEN-F, CEN-R, 106B-R, and CACTA1 elements are de-repressed. A smear of differently sized siRNAs is present in *dcl3*

mutants, likely attributed to the redundant action of the three remaining functional DCL enzymes present in *A. thaliana* (Gascioli et al., 2005). Collectively, the RT-PCR and siRNA data suggest a lack of correlation between transcription or silencing and siRNA abundance, at least with respect to siRNAs that correspond to 180-bp centromere repeats.

Pericentromere-Derived Transcripts Accumulate in the Nucleus

To determine if we could visualize 180-bp centromere repeat transcripts and determine their spatial distribution, we used RNA-FISH with probes specific for pericentromeric CEN-F or CEN-R transcripts (Figure 5). Interestingly, CEN-F and CEN-R transcripts were found to have different spatial distributions within the nucleus. CEN-R signals are dispersed throughout the nucleoplasm and nucleolus and this pattern is not affected by any of the mutants analyzed (data not shown). However, CEN-F transcript localization patterns differed in the mutants relative to wild-type. In wild-type nuclei (ecotype Col-0), we detected weak punctuate CEN-F signals in the nucleoplasm and a conspicuous strong signal associated with the nucleolus, which appears as a prominent dark region in DAPI-stained nuclei due to the relative paucity of nucleolar DNA in this nuclear territory (Figure 5, top row nuclei in red; see also Supplemental Table 4). By contrast, multiple strong punctuate CEN-F signals, located at the edges of the chromocenters, are observed in the *met1*, *nrpe1*, and *drd1* mutants (Figure 5 and supplemental quantitative data in Supplemental Table 5), which correlates with the de-repression of CEN-F transcripts determined by RT-PCR (see Figure 4). The Pol IV mutant, *nrdp1*, displays a CEN-F RNA phenotype similar to wild-type nuclei, consistent with the fact that CEN-F transcripts are not de-repressed in *nrdp1*, except that the nucleolar signal is markedly reduced. RNase treatment prior to in-situ hybridization abolished the signals detected using CEN-F or CEN-R probes whereas DNase I treatment had no effect, indicating that only RNA is detected (Figure 5, bottom-most nuclei).

The increased RNA-FISH signals at the periphery of the chromocenters may correspond to pericentromeric CEN-F transcripts at the sites of transcription, in agreement with the RT-PCR analyses (Figure 4A). The reduced nucleolar localization of CEN RNA signals in mutants involved in siRNA formation, such as *nrdp1* and *rdr2*, strongly suggest that these nucleolar signals reflect the process of siRNA biogenesis, consistent with evidence that 24-nt siRNAs and siRNA pathway proteins localize to a nucleolar compartment bearing markers of Cajal Bodies (Li et al., 2006; Pontes et al., 2006).

RNA Is Required for the Topology of Pericentromeric Heterochromatic Marks

In mouse, pericentromeric heterochromatin is RNase-sensitive, as assayed by histones bearing heterochromatin marks, such as H3K9me2 or the presence of non-histone proteins such as Heterochromatin Protein 1; HP1 (Maison et al. 2002; Muchardt et al., 2002). These observations indicate that RNA is necessary

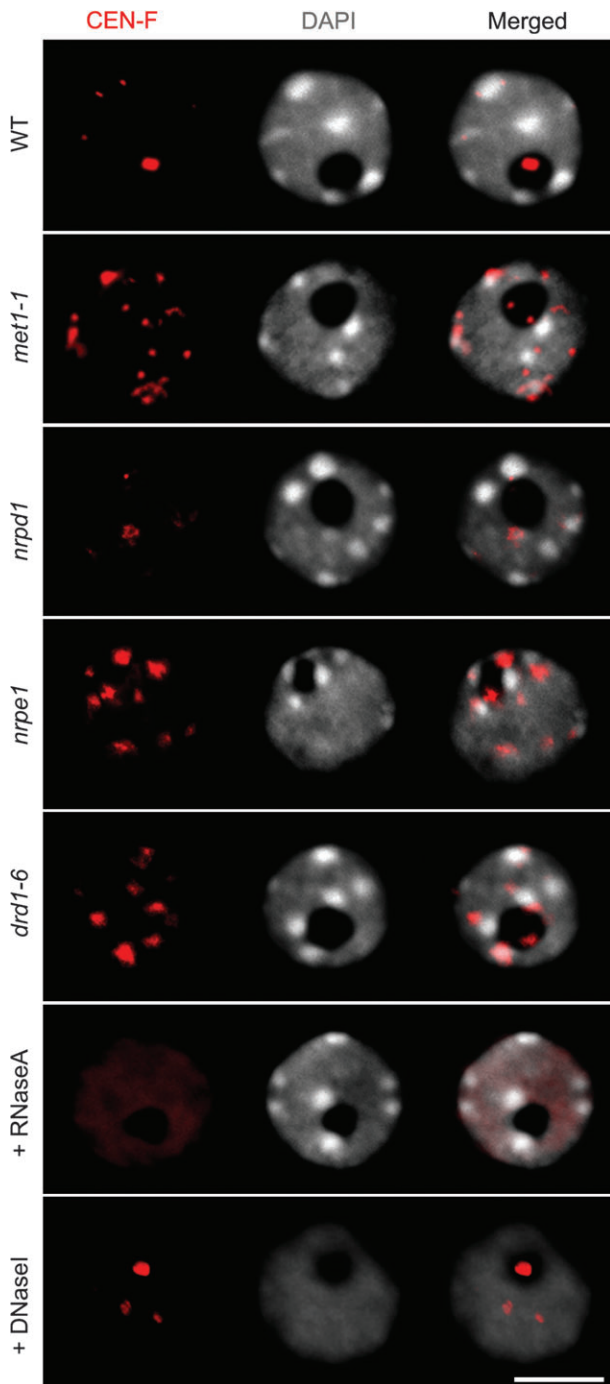


Figure 5. Pericentromere-Repeat RNAs Localized by RNA-FISH during Interphase.

The probe specifically detects forward transcripts (CEN-F, red). RNase A treatment prior to in-situ hybridization eliminates the FISH signals, indicating that the method predominantly detects RNA (bottom nuclei). DNA was DAPI-counterstained (gray). The size bar corresponds to 5 μ m.

for the large-scale organization of heterochromatin. To test the hypothesis that RNA is also essential for assembly of *A. thaliana* pericentromeric heterochromatin, immunolocaliza-

tion of H3K9me2 and 5-methylcytosine (5-mC), combined with DNA-FISH localization of 180-bp centromere repeats, was performed in RNase A-treated and untreated nuclei. As shown in Figure 6 (top row), H3K9me2, 5-mC, and 180-bp centromere repeats co-localize within discrete nuclear foci that correspond to the DAPI-enriched heterochromatic chromocenters in non-treated interphase nuclei. In contrast, RNase A treatment causes H3K9me2 to become distributed throughout the nucleoplasm (Figure 6, bottom-left panel), and causes 5-mC and centromere repeats to disperse into smaller foci (Figure 6, middle panel). Interestingly, DAPI-stained chromocenters persist in RNase-treated nuclei and HTR12, the *A. thaliana* centromeric histone H3 (Talbert et al., 2002), largely maintains its co-localization with these chromocenters and a fraction of the 180-bp centromeric repeats.

DISCUSSION

Our study shows that Pol V has functions in heterochromatin organization and gene silencing that are independent of Pol IV-dependent 24-nt siRNA production and DRM2-dependent de-novo cytosine methylation. In this role, Pol V and DRD1, a chromatin remodeler required for Pol V transcription (Wierzbicki et al., 2008), apparently act in partnership with MET1 and DDM1, whose activities are required to maintain the majority of the methylation in the *Arabidopsis* genome. The correspondence between heterochromatin disruption and the de-repression of specific classes of 180-bp pericentromeric repeats, which accumulate within the nucleus in *met1*, *ddm1*, *nrpe1*, and *drd1* mutants, suggests that suppression of a subset of transcription units is important for pericentromeric repeat clustering within chromocenters. However, the transcription machinery that modulates the transcription of centromere repeats is not yet defined in plants. It is not yet clear how *met1*, *ddm1*, *nrpe1*, and *drd1* might work together within a discrete pathway to suppress these transcription units. Importantly, *nrpe1* and *drd1* mutants do not cause losses of methylation at 180-bp centromere repeats, unlike *met1* and *ddm1* mutants (Kanno et al., 2008, 2005), suggesting that NRPE1 and DRD1 act independently or downstream of MET1- and DDM1-dependent DNA methylation.

An apparent paradox revealed by our study is that despite the correlation between transcriptional silencing and large-scale pericentromeric heterochromatin, formation of the resulting heterochromatic structures is RNase A-sensitive. This observation suggests that RNA is an important structural component of pericentromeric heterochromatin in *A. thaliana*. To this end, Pol V or Pol V-generated transcripts might serve as structural RNAs that are important for long-range heterochromatin interactions, possibly forming a structural network or complex that retains or recruits heterochromatin-forming complexes. Similar roles for non-coding RNAs have been proposed in the control of sex chromosome dosage in mammals and *Drosophila* (Wutz, 2003), in the formation of the yeast telomerase complex (Zappulla and Cech, 2004), and in the

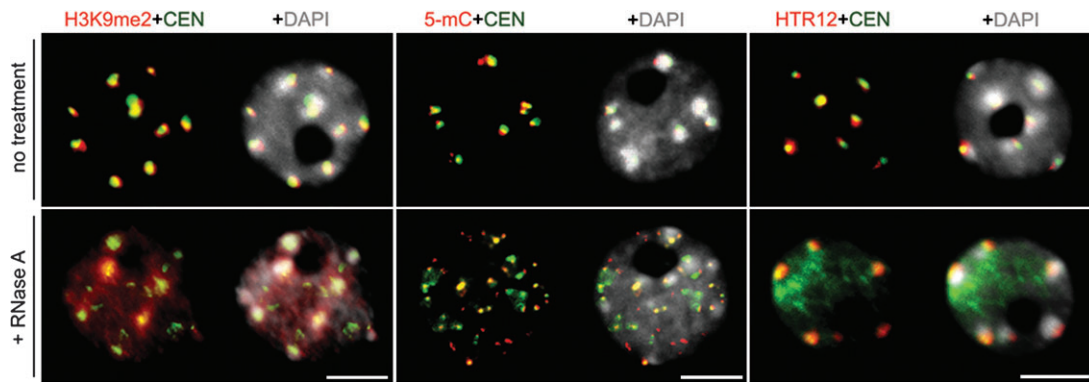


Figure 6. Effects of RNase A Treatment on the Interphase Organization of Centromere Repeats (Green), H3K9me2 (Red), and 5-mC (Red) Detected by DNA-FISH and Immunostaining.

Representative images of untreated and nuclease treated (+RNase) nuclei are shown. The right-most nuclei depict immunostaining of HTR12 (red) and 180-bp pericentromeric repeats (green) in non-treated and RNase A-treated nuclei. DNA was visualized by DAPI (gray). Size bars indicate 5 μ m.

organization of human pericentromeric heterochromatin (Maison et al., 2002). Importantly, RNase A treatment does not affect the organization and architecture of the core centromere domain, as indicated by the persistence of an HTR12 centromeric histone signal, which marks a domain that is structurally distinct from the flanking pericentromere (Choo, 2001).

In addition to potentially generating structural RNAs, Pol V, DRD1, MET1, and DDM1 could play a direct role in silencing specific pericentromeric repeats, such as AtSN1 and solo LTR retrotransposon elements that are subject to siRNA-directed DNA methylation (Wierzbicki et al., 2008). However, silencing of these latter elements requires the canonical 24-nt siRNA-directed DNA methylation pathway that includes Pol IV, RDR2, DCL3, AGO4, and DRM2. Pol V generates non-coding transcripts at AtSN1 and solo LTR loci, leading to the hypothesis that Pol V transcripts serve as scaffolds for the binding of siRNA-AGO4 silencing complexes (Wierzbicki et al., 2008). Importantly, Pol IV, RDR2, DCL3, AGO4, and DRM2 are not required for silencing CEN-F, CEN-R, 106B, and CACTA elements, whose de-repression specifically in Pol V (*nrpe1*), *drd1*, *met1*, and *ddm1* mutants correlates with long-range heterochromatin disruption. Interestingly, in an *nrpe1* mutant background, solo LTR transcription is increased relative to WT levels (Huettel et al., 2006)—an increase that correlates with a higher occupancy of Pol II at the *locus* (Wierzbicki et al., 2008). Additionally, transcriptional analysis of two other intergenic regions located in the pericentromeric regions showed their transcription to be dependent on Pol V (Wierzbicki et al., 2008). These previous findings, together with our new data concerning expression patterns of CEN-F, CEN-R, 106B, and CACTA elements, indicate an extensive alteration in pericentromeric region transcription in the absence of Pol V.

Collectively, the available evidence suggests that Pol V may play a role in gene silencing via one or more pathways distinct from the 24-nt siRNA-directed pathway; however, the possibility that other small RNA pathways may be involved cannot be

excluded. Knowing more about how pericentromere repeats are transcribed, the nature of these non-coding RNAs, and the identification of any interacting proteins likely reveals new insights into the epigenetic regulation of pericentromeric regions.

METHODS

Plant Material and Growth Conditions

Jim Carrington provided *Arabidopsis thaliana* mutants *rdr2-1*, *dcl3-1*, and *hen1-1*. *drd1-6* was provided by Marjori Matzke; *drm2-1* and *ago4-1* were provided by Steve Jacobsen. *nrpd1* (*nrpd1a-3*), *nrpd2* (*nrpd2a-2 nrpd2b-1*), and *nrpe1* (*nrpd1b-11*) were described previously (Kanno et al., 2005; Onodera et al., 2005). *drm2-1* is in the WS genetic background; *ago4-1* is in the Landsberg (Ler) genetic background. Other mutants are in the Columbia (Col-0) genetic background. Plants were grown either in sterile conditions on Murashigi-Skoog medium or on soil under long-day conditions (16 h light/8 h dark) at 22°C.

Isolation and RNA Blot Analysis of Small RNA

RNA was extracted from immature inflorescence using the mirVana[®] miRNA Isolation Kit (Ambion). Small RNA (smRNA) blot hybridization was performed as described (Onodera et al., 2005). Probes for smRNA blot hybridizations were derived from custom-made DNA oligonucleotides designed according to the mirVana[®] miRNA probe construction kit (Ambion). A T7-driven transcription reaction was used to generate labeled RNA probes with α -³²P CTP, which were subsequently used for RNA:RNA hybridization. The probes used to detect 5S genes smRNAs (siR1003) and AtSN1 were described previously (Pontes et al., 2006; Xi et al., 2006) and the following oligonucleotide was used to analyze 180-bp centromeric repeats

smRNAs: 5'-TGTATGATTGAGTATAAGAACTTAAAACGCAACCG-CATCTTAAAAGCCTAAGTAGTATTTTC-3'.

Transcript Analysis by Reverse Transcription PCR (RT-PCR)

To detect strand-specific pericentromeric transcripts, total RNA extracted as described above was treated with RQ1-DNase (Promega) and further purified using Trizol (Sigma). 100 ng was used in RT-PCR reactions using conditions and primers described previously (May et al., 2005). RT-PCR conditions and primers used to specifically amplify different elements are described in Supplemental Table 6. Negative controls were performed without reverse transcriptase to detect contaminant DNA and the constitutively expressed *ACTIN2* gene was used as internal control.

Nuclease Sensitivity Assays

For RNase sensitivity analysis, unfixed leaf nuclei were released into isolation buffer (25 mM HEPES, 1 mM MgCl₂, 1 mM DTT, 10 mM KCl, 25 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg ml⁻¹ pepstatin, pH 7.4) (Abranches et al., 1998; Jasencakova et al., 2000), washed in PBS and permeabilized in the nuclei isolation buffer containing 0.05% Triton-X100 for 2 min. An incubation of 10 min at RT was performed with RNase A (100 µg ml⁻¹, Roche) or without RNase A plus 100 U ml⁻¹ RNase inhibitor (RNA Guard, Pharmacia) as a control. After washing in nuclei isolation buffer and PBS, cells were fixed for 20 min in 4% paraformaldehyde/PBS, centrifuged, and applied to the slides for immunolocalization and DNA FISH.

Probe Labeling

55 and 180-bp pericentromeric gene probes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) by PCR as previously described (Pontes et al., 2003). BAC F28D6 was obtained from the *Arabidopsis* Biological Resource Center (ABRC, Accession No. AF147262) and labeled with biotin-Nick Translation Mix (Roche). For the detection of strand specific pericentromere transcripts, digoxigenin-11-UTP and biotin-16-UTP (Roche) labeled sense and antisense probes were prepared by *in-vitro* transcription of the 180-bp repeat and the custom-made oligonucleotide specific to centromeric repeats used for smRNA blot hybridization using T7 and SP6 polymerase (Ambion).

Immunofluorescence

Root meristem nuclei were isolated as described previously (Jasencakova et al., 2000). Upon 4% paraformaldehyde postfixation, the nuclei were incubated overnight at 4°C with primary antibodies for anti-H3K9me2 (1:200, AbCam) and anti-HTR12 (1:200, kindly provided by Steve Henikoff). Secondary antibody anti-rabbit Alexa 594 (Invitrogen) was diluted at 1:500 in PBS and incubated for 2 h at 37°C. DNA was counterstained with 1 µg ml⁻¹ DAPI in Vectashield mounting medium (Vector Laboratories). For immuno-FISH experiments, immunolocalization was performed first; cells were post-fixed in

4% paraformaldehyde and the standard DNA in-situ hybridization protocol was then followed.

RNA and DNA Fluorescence In-Situ Hybridization

Meristem nuclei were processed for DNA fluorescence in-situ hybridization (FISH) as described previously (Pontes et al., 2003), using as hybridization stringency conditions 50% formamide and 2 SSC. Post-hybridization washes were performed in 50% formamide and 0.1 SSC at 42°C. RNA in-situ hybridization was carried out at 50°C overnight using a probe solution containing 500 ng RNA probe, 5 µg yeast tRNA (Roche), 50% dextran sulphate, 100 mM PIPES, pH 8.0, 10 mM EDTA, and 3 M NaCl as described previously (Pontes et al., 2006). Slides were washed sequentially in 2 SSC, 50% formamide at 50°C followed by 1 SSC, 50% formamide. Digoxigenin-labeled probes were detected using mouse anti-digoxigenin antibody (1:250, Roche) followed by rabbit anti-mouse antibody conjugated to Alexa 488 (1:200, Molecular Probes). Biotin labeled probes were detected using goat anti-biotin conjugated with avidin (1:200, Vector Laboratories) followed by streptavidin-Alexa 546 (1:200, Molecular Probes). Nuclear DNA was counterstained with DAPI in Vectashield anti-fade medium (Vector Laboratories).

5-Methylcytosine Detection

Root tips were harvested, fixed in ethanol/acetic acid (3:1) and nuclei isolated as described (Pontes et al., 2003). Slide preparations were baked at 60°C, denatured in 70% formamide, 2 SSC, 50 mM sodium phosphate at 80°C for 3 min, washed in ice-cold PBS, blocked with 1% BSA, and incubated with the 5-methylcytosine specific antibody (1:100, AbD-Serotec). Methylated DNA was detected by incubation in rabbit anti-mouse antibody Alexa 546 (1:200, Molecular Probes) secondary antibody.

Measurement of the Heterochromatin Fraction

Nuclei were isolated from ethanol/acetic acid (3:1)-fixed material and stained with DAPI. Digital grayscale raw images were analyzed with the freeware software NIH-ImageJ. The chromocenter/heterochromatin ratio was correlated from the area and DAPI-fluorescence intensity of chromocenters relative to the whole nucleus (Soppe et al., 2002).

Microscopy and Imaging

The preparations were inspected with a Nikon Eclipse E800i epifluorescence microscope equipped with a Photometrics Coolsnap ES Mono digital camera. Images were acquired by the Phylum software and pseudocolored and merged in Adobe Photoshop.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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O.P. performed all microscopy and RT-PCR analysis; P.C.N. generated the siRNA blot; P.V. performed the centromere repeat cloning and sequencing. O.P. and C.S.P. wrote the paper. We thank Jeremy Haag and Thomas Ream for suggestions to improve the manuscript. Any opinions, findings, conclusions, or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of NIH or the Edward Mallinckrodt Foundation. No conflict of interest declared.

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