Regulation of *Arabidopsis thaliana* 5S rRNA Genes

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The *Arabidopsis thaliana* genome comprises around 1,000 copies of 5S rRNA genes encoding both major and minor 5S rRNAs. In mature wild-type leaves, the minor 5S rRNA genes are silent. Using different mutants of DNA methyltransferases (*met1*, *cmt3* and *met1 cmt3*), components of the RNAi pathway (*ago4*) or post-translational histone modifier (*hda6/sil1*), we show that the corresponding proteins are needed to maintain proper methylation patterns at heterochromatic 5S rDNA repeats. Using reverse transcription–PCR and cytological analyses, we report that a decrease of 5S rDNA methylation at CG or CNG sites in these mutants leads to the release of 5S rRNA gene silencing which occurred without detectable changes of the 5S rDNA chromatin structure. In spite of severely reduced DNA methylation, the *met1 cmt3* double mutant revealed no increase in minor 5S rRNA transcripts. Furthermore, the release of silencing of minor 5S rDNAs can be achieved without increased formation of euchromatic loops by 5S rDNA, and is independent from the global heterochromatin content. Additionally, fluorescence in situ hybridization with centromeric 180 bp repeats confirmed that these highly repetitive sequences, in spite of their elevated transcriptional activity in the DNA methyltransferase mutants (*met1*, *cmt3* and *met1 cmt3*), remain within chromocenters of the mutant nuclei.

**Keywords:** *Arabidopsis thaliana* — 5S rDNA — DNA methylation — Transcription.

Abbreviations: CC, chromocenter; DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; RdDM, RNA-directed DNA methylation; RISC, RNA-induced silencing complex; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; siRNA, small interfering RNA; WT, wild type.

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**Introduction**

*Arabidopsis thaliana* contains about 1,000 tandemly arranged copies of 5S rRNA genes per 1C genome (Campell et al. 1992). In the Landsberg *erecta* accession, these repeats are clustered at four 5S loci within the pericentromeric regions of chromosomes 3, 4 and 5 (with a large locus on the left arm and a smaller one on the right arm of chromosome 5) (Murata et al. 1997, Fransz et al. 1998). Fluorescence in situ hybridization (FISH) on interphase nuclei revealed 5S rDNA within heterochromatic chromocenters (CCs). Additionally, euchromatic 5S rDNA loops that apparently represent transcribed genes emanate from CCs (Mathieu et al. 2003). Transcriptionally active 5S rDNA units encoding major and minor 5S rRNA transcripts (that differ by one or two base substitutions) are only found on chromosome 4 and in the large locus of chromosome 5 (Cloix et al. 2002, Tutois et al. 2002). In wild-type (WT) mature leaves, only genes encoding major 5S rRNA are transcribed. Previously we have shown that minor 5S genes are transcribed upon genome-wide hypomethylation induced by the decrease in DNA methylation1 (*ddm1*) mutation or by 5-azacytidine treatment. Silencing of minor 5S genes is also relieved in WT roots and during early development when 5S rDNA methylation is reduced (Mathieu et al. 2003). When minor 5S genes are transcribed, 5S rDNA chromatin organization differs from that of WT mature leaves (Mathieu et al. 2003, O. Mathieu unpublished data).

Heterochromatin of *Arabidopsis* WT nuclei is marked by a high level of DNA methylation and dimethylation of Lys9 of the histone H3 (H3K9me2), whereas euchromatin is associated with dimethylation of H3 at Lys4 (H3K4me2) and histone acetylation (Soppe et al. 2002, Jasencakova et al. 2003, for an overview see Fuchs et al. 2006). Some of the proteins involved in establishment and maintenance of these epigenetic marks have been identified, and it has been reported that the *ddm1* mutation affects the patterns of all these marks at many targets, including 5S rDNA (Gendrel et al. 2002, Soppe et al. 2002, Mathieu et al. 2003).

It has been uncovered that non-coding small RNAs of 21–24 nucleotides processed from double-stranded RNAs by the RNA interference machinery (RNAi) are involved in heterochromatin assembly and gene silencing (Hall et al. 2002, Volpe et al. 2002, Zilberman et al. 2003, Verdel et al. 2004).

In *Arabidopsis thaliana*, silencing of endogenous repeat loci involves H3K9 methylation and RNA-directed DNA methylation (RdDM) (Pelissier et al. 1999, Mathieu and...
Bender 2004) which is correlated with the generation of homologous small interfering RNAs (siRNAs). 5S rDNA is highly methylated in the CNN context (Mathieu et al. 2002) which is a hallmark of RdDM, and also 5S siRNA was detected in WT plants (Xie et al. 2004). A recent model regarding the maintenance of the heterochromatic state at pericentromeric sequences including 5S rDNA (Pontes et al. 2006) proposes that transcripts trailing from polymerases that are stalled or slowed by DRM (domains rearranged DNA methylation)-mediated methylation are sensed as aberrant and directly or indirectly become templates for the plant-specific RNA polymerase IV (Herr et al. 2005, Kanno et al. 2005, Onodera et al. 2005, Pontier et al. 2005). Polymerase IV generates precursor RNA at the endogenous repeats that is transferred into the nucleolus. siRNAs are then produced and loaded into the AGO4-containing effector complex RISC (RNA-induced silencing complex) within the siRNA processing centers of the nucleolus. How AGO4–RISC–PolIV complexes mediate their effect on chromatin modification at target loci, resulting in compacted chromatin and silencing, is unclear.

To elucidate further the epigenetic regulation of 5S rRNA gene expression, we assessed DNA methylation, transcription (including the proportion of minor 5S rRNAs) and nuclear localization of 5S rDNA in several mutants known either to affect distinct epigenetic marks directly or to be deficient for RNAi. The mutants for the DNA methyltransferases METHYLTRANSFERASE1 (met1-1), and CHROMOMETHYLASE3 (cmt3-7), for the histone deacetylase HDA6 (sil1) and for the ARGONAUTE4 protein (ago4-1) all display fewer pleiotropic effects on chromatin organization than the ddm1 mutant analyzed previously (Mathieu et al. 2003).

The centromere region of the Arabidopsis chromosomes consists of the core regions and of flanking pericentromeric heterochromatic domains. The latter contain 5S rDNA among transposons and other dispersed repeats. The centromere core which accommodates the functional centromere mainly consists of the 180 bp tandem repeats spanning about 1.3–2.1 Mbp (Haupt et al. 2001). In the ddm1 mutant, the 180 bp repeats may form loops extending into euchromatin territories (Mittelsten Scheid et al. 2002, Probst et al. 2003) and their transcriptional derepression was observed (May et al. 2005). We used DNA methyltransferase mutants (met1, cmt3 and the met1-cmt3 double mutant) to trace the subnuclear localization of the 180 bp satellite under conditions of their elevated transcription (May et al. 2005, Vaillant et al. 2006).

Together our results show that minor 5S genes silencing is relieved in the context of 5S rDNA hypomethylation without detectable changes in the chromatin structure of (peri)centromeric regions. It is likely that the centromeric tandem repeats remain in CCs irrespective of their expression.

**Results**

5S rDNA methylation is decreased in met1, cmt3, met1 cmt3, sil1 and ago4 mutants

We assessed 5S rDNA methylation in the different mutants by DNA gel blot analysis of genomic DNA after digestion with HpaII (inhibited by the methylation of either cytosine in the sequence CCGG) and MspI (inhibited by the methylation of the first cytosine in this CCGG sequence). 5S rDNA methylation was estimated with the intensity of the 0.5 kb monomeric band of 5S rDNA which is inversely proportional to the methylation frequency (Fig. 1). The met1-1 mutation strongly decreased CG methylation at 5S rRNA genes and to a lesser extent non-CG methylation, whereas cmt3-7 specifically decreased CNG methylation. The sil1 mutant, affected for the histone deacetylase HDA6, and the ago4 mutant also exhibited reduced CNG methylation at 5S rDNA (Fig. 1). In the met1-1 cmt3-7 double mutant, 5S rDNA methylation was strongly reduced in CG and in non-CG contexts, as previously shown (Vaillant et al. 2006). The severe hypomethylation of 5S rDNA in met1-1, cmt3-7 and met1-1 cmt3-7 is illustrated by the strong intensity of a 430 bp fragment resulting from cleavage at a second HpaII/MspI site within some

![Fig 1](image)

5S rDNA methylation. Genomic DNA prepared from WT (Landsberg erecta accession), met1-1, cmt3-7, met1-1 cmt3-7, sil1 and ago4-1 plants digested by HpaII (H) or MspI (M) and probed by 5S rDNA on gel blots. Arrowheads indicate the 0.5 kb monomeric band. For quantification of 5S rDNA methylation in ago 4-1, the 0.5 kb band radioactivity (which corresponds to monomeric units of 5S rDNA) was compared with the whole line radioactivity. From five experiments, the 0.5 kb band, in the MspI line, is 1.7-fold (SD = 0.19) more intense in ago4-1 compared with the corresponding band in the WT.
5S rDNA units (Cloix et al. 2000, Tutois et al. 2002) compared with the WT. The impact of the met1-1, cmt3-7, met1-1 cmt3-7, ago4-1 and sil1 mutations on 5S rDNA methylation suggests that the corresponding proteins are involved in establishing appropriate 5S rDNA methylation patterns.

Minor 5S transcripts occur in met1 and cmt3 but not in met1-1 cmt3-7 double mutant plants

Previously, minor 5S rRNA transcripts were found (in addition to the major 5S rRNA transcripts) in ddm1, in 5-azacytidine-treated plants, in roots and in young seedlings where 5S rDNA methylation is reduced (Mathieu et al. 2003). Here the occurrence of minor transcripts in the mutants was analyzed by sequencing 5S-specific reverse transcription–PCR (RT–PCR) products. The proportion of minor 5S rRNAs in each mutant genotype was compared with that of the WT (Table 1). Three to four independent RNA extractions were performed per genotype, and a pool of plants was used for each extraction. The met1-1 (P < 0.01), cmt3-7 (P < 0.05), sil1 (P < 0.001) and ago4-1 (P < 0.01) mutants showed significantly more transcripts than the WT, suggesting that these genes are involved in the silencing of minor 5S rRNA genes. Notably, the release of transcription of minor 5S rRNA genes in the ago4-1 mutant confirms that AGO4, the RNA-binding compound of the RNAi pathway, is involved in 5S rDNA silencing. Unexpectedly, no significant increase in minor 5S transcripts was observed in the met1-1 cmt3-7 double mutant (P > 0.1).

RNA gel blot analysis revealed that the total amount of 5S rRNA was not significantly affected in met1-1, cmt3-7, met1-1 cmt3-7, sil1 and ago4-1 when compared with the WT value arbitrarily fixed as 1 (U-test, P > 0.05) (Fig. 2). These results support our previous conclusions that the total amount of 5S rRNAs is not dependent on the 5S rDNA methylation level (Mathieu et al. 2003) and that despite the derepression of minor 5S rRNA genes in the different mutants, the total amount of 5S rRNA is maintained to satisfy the appropriate ribosome production. They also show that the release of transcription of minor 5S rRNA genes is observed when 5S rDNA is hypomethylated at CG or CNG sites.

Expression of minor 5S genes is not correlated with the global heterochromatin content

In Arabidopsis interphase nuclei, heterochromatin is visualized as bright conspicuous CCs after 4',6-diamidino-2-phenylindole (DAPI) staining. To find out whether the presence of minor 5S rRNAs is correlated with a global decrease in heterochromatin content, we quantified the heterochromatin fraction by measuring the area and intensity of DAPI-stained CCs in relation to the entire nucleus for mutant vs. WT nuclei (Fig. 3).

Our analysis showed that the cmt3-7 single mutation had no significant influence on the heterochromatin content (P > 0.1), whereas in sil1 and met1 nuclei significantly less heterochromatin was found than in WT (Landsberg erecta) nuclei (8.26 and 7.22%, respectively, vs. 10.47%; P < 0.025 and P < 0.001). Also nuclei of the double mutant met1-1 cmt3-7 revealed a heterochromatin fraction significantly lower than that of WT nuclei (7.67; P < 0.001), and

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total clonesa</th>
<th>No. of minor 5S rRNAs</th>
<th>Percentage of minor 5S rRNAs</th>
<th>Significance levelb</th>
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<td>WT (L)</td>
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<td>1</td>
<td>1.0</td>
<td>Reference</td>
</tr>
<tr>
<td>met1 (L)</td>
<td>93</td>
<td>9</td>
<td>9.6</td>
<td>0.008**</td>
</tr>
<tr>
<td>cmt3 (L)</td>
<td>91</td>
<td>8</td>
<td>8.8</td>
<td>0.014*</td>
</tr>
<tr>
<td>met1cmt3</td>
<td>96</td>
<td>3</td>
<td>3.1</td>
<td>0.31</td>
</tr>
<tr>
<td>(L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sil1 (L)</td>
<td>71</td>
<td>10</td>
<td>14.1</td>
<td>0.0009**</td>
</tr>
<tr>
<td>ago4 (L)</td>
<td>64</td>
<td>7</td>
<td>10.9</td>
<td>0.007**</td>
</tr>
</tbody>
</table>

aTotal clones = number of minor 5S rRNA clones + number of major 5S rRNA clones recovered.
bSignificance levels are as follows: ‘*’0.05; ‘**’0.01 (Fisher’s exact test). (L), Landsberg-erecta accession.

Fig. 2 5S rRNA amounts of wild-type and mutant plants. 5S rRNA amounts in relation to WT (Landsberg erecta accession) arbitrarily fixed as 1, of met1-1, cmt3-7, met1-1 cmt3-7, sil1 and ago4-1 plants. Total RNA was hybridized using a 5S rRNA probe. RNA quantities were normalized using a RAS-related nuclear protein probe (Haizel et al. 1997), and 5S rRNA amounts were quantified accordingly. The results of at least three independent experiments are represented in the histogram; a pool of plants was used for each extraction. The standard deviation of the mean is indicated on each bar.
comparable with that observed for met1-1. This result supports the idea that heterochromatin compaction is mainly under the control of MET1-mediated CG methylation (Soppe et al. 2002, Fransz et al. 2006). No significant deviation in the heterochromatin fraction was observed for ago4-1 (P > 0.1) nuclei.

The lower heterochromatin fraction in met1-1 cmt3-7 did not mediate an increased proportion of minor 5S rRNAs, whereas release of silencing of minor 5S genes in ago4-1 nuclei was not linked to a lower heterochromatin content. Together, these observations show that expression of minor 5S rRNA genes is not tightly correlated with the global heterochromatin content, and therefore has its own regulation.

Transcription of minor 5S rRNAs does not require larger 5S rDNA loops

Previously we observed 5S rDNA loops emanating from CCs in WT nuclei (Columbia accession) and an increased loop formation of 5S rDNA in ddm1 nuclei which express minor 5S rRNAs (Mathieu et al. 2003). These results led us to hypothesize that the additional decondensed 5S rDNA fraction in ddm1 nuclei encompasses the transcribed 5S minor genes.

To test this hypothesis further, we assessed whether the increased proportion of minor 5S rRNAs is correlated with a larger fraction of 5S rDNA outside the CCs, in the mutants analyzed in the present study which display less pleiotropic effects on chromatin organization than the ddm1 mutant. FISH on 3-week-old leaf nuclei with a 5S rDNA probe revealed no significant differences in the proportion of 5S rDNA signals outside CCs between WT (~70%, Landsberg erecta accession) and met1-1, cmt3-7, met1-1 cmt3-7, sil1 and ago4-1 mutants (67–79%, P > 0.1) (Fig. 4). The presence of ~70% of 5S rDNA signals outside the CCs in Landsberg erecta nuclei indicates that the majority of 5S rDNA units do not co-localize with the DAPI-stained heterochromatin and reveals a general situation and not an
accession-specific one. Even though subtle changes cannot be ruled out because of the limited microscopic resolution, the results suggest that the release of silencing of the minor 5S rDNA does not result from relocation of 5S rRNA genes outside heterochromatin.

Centromeric tandem repeats remain in chromocenters
To extend our analysis about the correlation between the release of silencing of highly repeated sequences and their subnuclear localization, FISH with 180 bp centromeric repeats was performed on met1-1, cmt3-7, met1-1 cmt3-7 mutant nuclei (Fig. 5). In spite of severe demethylation on 180 bp and 5S rDNA repeats (Johnson et al. 2002, Vaillant et al. 2006) and notwithstanding the elevated transcriptional activity of 180 bp repeats in cmt3 mutants (May et al. 2005, Vaillant et al. 2006), cmt3 nuclei revealed WT-like CCs, and 180 bp FISH signals only co-localizing with CC (Fig. 5).

In contrast to the situation observed for 5S rDNA, we previously found a synergistic effect of met1-1 and cmt3-7 mutations on 180 bp repeat transcription with the presence of new transcripts of intermediate size in the met1-1 cmt3-7 double mutant (Vaillant et al. 2006). Since in met1-1 and met1-1 cmt3-7 nuclei, despite much smaller CCs, all 180 bp signals co-localized with DAPI-stained heterochromatin (Fig. 5) the relief of silencing is mediated without delocalization of the centromeric repeats outside the CCs in met1-1, cmt3-7 and met1-1 cmt3-7 mutants.

Discussion
HDA6 and AGO4 are required for repression of minor 5S genes
RPD3, a HDA6-like protein of the budding yeast, is required to inactivate rDNA repeats (Sandmeier et al. 2002). In Arabidopsis, HDA6 is needed for transcriptional silencing of TSI repeats (Probst et al. 2004) and transgenes (Murfett et al. 2001, Aussetz et al. 2002) and CG methylation of rDNA is reduced in hda6 plants (Probst et al. 2004). We found 5S rDNA demethylation at CNG sites mediating a derepression of minor 5S genes in this mutant (sil1 allele). These results confirm a HDA6 function in silencing repetitive sequences encoding non-polyadenylated transcripts (Probst et al. 2004). In accordance with the assumption that histone deacetylation precedes CG methylation and heterochromatin condensation (Richards and Elgin, 2002, Soppe et al. 2002), we observed a decreased heterochromatin fraction in sil1 nuclei.

AGO4 is involved in RdDM driven by direct repeats, and one of the 15 distinct 5S siRNAs isolated by Xie et al. (2004) was strongly reduced in ago4-1 (Zilberman et al. 2004). It correlates with a slight decrease of 5S rDNA methylation at CNG and release of silencing of minor 5S rRNAs genes, according to Pontes et al. (2006) who proposed that siRNAs are loaded into the AGO4-containing effector complex or RISC which mediates chromatin modification at target loci. The WT-like size of CCs in ago4-1 nuclei could reflect the restricted action of AGO4 on pericentromeric heterochromatin (Soppe et al. 2002).

No silencing relief of minor 5S genes in the met1-1 cmt3-7 double mutant
All mutations analyzed in the present study that impair 5S rDNA methylation at CG or CNG also alleviated silencing of the minor 5S rRNA genes, except for met1-1 cmt3-7 for which, despite a strong 5S rDNA hypomethylation at both CG and CNG sites, almost only major 5S rRNAs were recovered. This contrasts with the results obtained for the single mutants for which we found a release of silencing. This also contrasts with the additive effect of the met1 and cmt3 mutations on the transcription of the retrotransposons Ta2 and Ta3 (Johnson et al. 2002) and of the 180 bp repeats (Vaillant et al. 2006). However, it agrees with the previously observed lower accumulation of the 210 base long 5S rRNA transcripts which originate from the intergenic spacer, in the double mutant compared with the
met1-1 single mutant (Vaillant et al. 2006). Together these results indicate a specific regulation of 5S rRNA genes.

Previous studies have identified MOM1 as component of a silencing mechanism independent of DNA methylation marks (Amedeo et al. 2000, Mittelsten Scheid et al. 2002, Probst et al. 2003, Steimer et al. 2000) and is involved in silencing of 5S minor genes (Vaillant et al. 2006). MOM1 transcription was slightly up-regulated in met1-1 cmt3-7 and proposed to counteract the release of silencing of 5S-210 transcripts in the double mutant (Vaillant et al. 2006). Possibly, MOM1 also counteracts the release of silencing of the minor 5S genes. MOM1 could contribute to limit the proportion of minor 5S rRNA, perhaps not beneficial for the cell, in highly hypomethylated contexts, like the met1-1 cmt3-7 double mutant where both CG and CNG methylation are strongly reduced, and where the largest release of minor genes was expected. MOM1 has been proposed to prevent extremely rapid epigenetic deregulation in plants with DNA methylation deficiencies (Mittelsten Scheid et al. 2002) and could participate to maintain the function of 5S rDNA in largely hypomethylated plants.

**CG methylation mediates heterochromatin compaction**

Heterochromatin formation does not appear to depend on CNG methylation. Pericentromeric regions with dispersed repeats were found predominantly outside the CCs in met1 and ddm1 nuclei (Soppe et al. 2002), and a methylated CG mark has been proposed to be required to maintain heterochromatin at pericentric repeats (Fransz et al. 2006). The wild-type heterochromatin levels of cmt3 nuclei and the same size of CCs in met1 and met1cmt3 confirm that heterochromatin compaction is independent of CNG methylation. In addition, the smaller size of CCs in both DNA methyltransferase (met1, met1cmt3) and chromatin remodeling (ddm1; Soppe et al. 2002) mutants confirms that CG hypomethylation is responsible for the decreased size of the CCs. The loss of CG methylation in the met1-3 null mutant leads to very small but detectable CCs (Tariq et al. 2003), confirming the correlation between the level of DNA methylation and the heterochromatin content.

The global heterochromatin content changes result from the dispersion of pericentromeric sequences from heterochromatic CCs, except 5S rDNA whose proportion inside/outside CCs is maintained. Therefore, we found that the derepression of minor 5S genes does not correlate with the global heterochromatin content. These results highlight the specific behavior of 5S rRNA genes.

**Highly repeated tandem repeats remain in chromocenters**

Spatial nuclear organization is considered as an important factor influencing epigenetic states of gene expression. However, little information linking the expression of highly repeated sequences together with their subnuclear localization is available. Expressed genes from euchromatin domains can contain methylation within transcribed or promoter regions (Zhang et al. 2006). The majority of genes localize outside visible heterochromatin regardless of their transcriptional activity (Fransz et al. 2006). In contrast, transposable elements such as the low copy transposon CAC1 are generally in CCs. Loss of DNA methylation in met1 results in massive transcriptional reactivation of pseudogenes and transposons particularly at (peri)centromeric regions (Zhang et al. 2006) and in a relocation of low copy pericentromeric sequences and activated transposons away from CCs (Fransz et al. 2006).

In the mutants we analyzed, the centromeric 180 bp repeat remained densely packed, irrespective of the methylation levels of its DNA. Its co-localization with heterochromatin is therefore compatible with the release of silencing observed in met1-1 and cmt3-7, and even with the synergistic release observed in met1-1 cmt3-7 (May et al. 2005, Vaillant et al. 2006) without detectable chromatin changes.

Consequently, for 5S rDNA and for 180 bp repeats, we did not observe obvious alterations of the chromatin structure linked to the transcriptional activity of these sequences in methyltransferases mutants.

The transcription of 5S rDNA and of centromeric 180 bp repeats is derepressed in ddm1 (May et al. 2005) and a decondensation of the centromeric heterochromatin was observed (Mittelsten Scheid et al. 2002, Probst et al. 2003). Therefore, the structural alterations observed in the chromatin remodeling mutant ddm1, but absent in DNA methyltransferase mutants, for both 5S rDNA and 180 bp repeats are not directly linked to the DNA methylation.

**Conclusion**

Based on results obtained with the ddm1 mutant, our previous results led us to hypothesize that transcribed 5S rDNA units loop out from heterochromatin (Mathieu et al. 2003). Similarly, previous results have shown that 180 bp repeats may form loops into euchromatin territories in the ddm1 mutant (Mittelsten Scheid et al. 2002, Probst et al. 2003).

Here, using different mutants displaying fewer pleiotropic effects than ddm1, we found that mutant plants maintain proportions of 5S rDNA outside CCs similar to WT plants regardless of the percentage of minor transcripts which does not exceed the maximal proportion encountered in WT tissues (14% in roots and seeds). Furthermore, the WT total amount of 5S rRNA is maintained to satisfy the appropriate ribosome production.

Since major 5S genes represent only ~10% of the 5S repeats in the Columbia accession (Cloix et al. 2002), silent minor 5S rRNA genes should be part of the 5S units outside
heterochromatin in WT nuclei while the centromeric 180 bp repeat remained densely packed and co-localized with heterochromatin even when transcriptioned. The decrease of 5S rDNA methylation at CG or CNG sites in the mutants analyzed led to the release of silencing of 5S rRNA genes. However, no significant increase in minor 5S transcripts was observed in the met1-1 cmt3-7 double mutant, and MOM1 could contribute to limit the proportion of minor 5S rRNA in highly hypomethylated contexts. This shows that 5S rRNA genes exhibit some specific features. 5S rDNA serves a vital function: to provide 5S rRNA to satisfy the demand for ribosome production and protein synthesis. This probably explains its high and specific regulation. Thus the fine regulation of silencing at highly repeated pericentromeric sequences deserves further investigation.

Materials and Methods

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes.

Plant materials

Arabidopsis thaliana met1-1, cmt3-7, met1-1 cmt3-7, sll, ago4-1 plants in Landsberg erecta background as well as WT plants were used. Seeds of WT, sll and ago4-1 plants were obtained from the NASC (stock numbers NW20 for Ler-0, N1894 for sll and N6364 for ago4-1). Steven E. Jacobsen provided seeds of met1-1 and cmt3-7 (which have been backcrossed several times), and provided the double mutant met1-1 cmt3-7 which has been obtained by crossing met1-1 to cmt3-7 single mutant plants (Steven E. Jacobsen, personal communication). All plants were grown in a greenhouse during winter and spring under standard conditions, with an 18 h photoperiod provided by SON-T AGRO 400 W lamps, at 22 °C (±4 °C; the temperature was controlled using an aerotherm system). The leaves from 3-week-old plants were used for the experiments.

Nucleic acid isolation and gel blot analysis

Total genomic DNA was isolated from a pool of plants for each genotype according to the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1987). Genomic DNA (500 ng) was digested with 20 U of restriction enzyme in the recommended buffer (New England Biolabs, Beverly, MA, USA). Digested DNA was electrophoresed on 0.8% (w/v) agarose gels overnight, depurinated in 0.25 N HCl, and capillary blotted onto Hybond-N+ membranes (Amersham). Total RNA was extracted according to Mathieu et al. (2003) and for RNA gel blot analysis, 2 μg of total RNA per lane was fractionated on 1% (w/v) agarose/1.9% (v/v) formaldehyde gels and capillary blotted onto Hybond-N membranes (Amersham).

DNA probes were labeled with [α-32P]dCTP using random hexamer priming (Megaprime DNA labeling system; Amersham). For quantifications a phosphoimager (Molecular Imager FX; Bio-Rad) was used.

Reverse transcription–PCR and sequencing

The reverse transcription reactions and 5S cDNA amplifications were performed as described (Mathieu et al. 2003).

Statistics

Minor 5S rRNA frequencies were compared with Fisher’s exact test for a 2 × 2 contingency table. The probabilities were calculated using a one-tailed test. Statistical analyses of 5S rRNA amounts were performed using the non-parametric Mann–Whitney U-test with mean value comparison. The 5S rDNA fractions outside the CCs, as well as the heterochromatic fractions, were compared with the non-parametric Kolmogorov–Smirnov test.

Fluorescent in situ hybridization (FISH)

Generation of nuclear suspensions, DAPI staining, measurement of CC fractions, FISH and microscopic analysis were performed as described (Mathieu et al. 2003). Digital images of nuclei after DAPI staining and with 5S or 180 bp repeat FISH signals were processed and merged using Photoshop software (Adobe Systems). Gray scale images were then analyzed with the freeware program NIH-image 1.62. A special macro was written to measure the volume and intensity of 5S rDNA signals outside CCs vs. signals within the CCs.

Acknowledgments

We thank Steven Jacobsen for providing seeds of met1 and cmt3 mutants, and Julien Douet for technical assistance. This work was supported by the Center National de la Recherche Scientifique (CNRS), the Université Blaise Pascal, the Ministère de l’Enseignement Supérieur et de la Recherche (ACI/BCMS) and the INCA (Institut National du Cancer, réseau Epipro). I.S. was supported by a grant from the Land Sachsen-Anhalt (3233A/0020T). I.V. was supported by a fellowship from the Ministère de l’Enseignement Supérieur et de la Recherche.

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(Received February 20, 2007; Accepted April 4, 2007)