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REVIEW

# Transcription of the 5S rRNA heterochromatic genes is epigenetically controlled in *Arabidopsis thaliana* and *Xenopus laevis*

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5S ribosomal DNA is a highly conserved tandemly repeated multigenic family. As suggested for a long time, we have shown that only a fraction of the 5S rRNA genes are expressed in *Arabidopsis thaliana.* In *Xenopus laevis*, there is a developmental control of the expression of the 5S rRNA genes with only one of the two 5S rDNA families expressed during oogenesis. For both Arabidopsis and Xenopus, the strongest transcription of 5S rRNA, respectively in the seed and during oogenesis is correlated with heterogeneity in the transcribed 5S rRNAs. Epigenetic mechanisms such as modification of the chromatin structure are involved in the transcriptional regulation of the 5S rRNA genes in both organisms. In Arabidopsis, two silencing pathways, methylation-dependent (RNAi) and methylation-independent (MOM pathway), are involved in the silencing of a 5S rDNA fraction.
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## Introduction

Higher eukaryotes contain thousands of rRNA genes, and it has been believed for some time that only a fraction of rRNA genes are active, since the number of these genes far exceeds that expected to be required to supply ample cytoplasmic rRNA (reviewed in Rogers and Bendich, 1987).

Eukaryotes regulate the effective dosage of their 45S rRNA genes with fewer than half of the genes being transcribed by polymerase I at any one time. rRNA gene dosage control utilizes a common mechanism with nuclear dominance, a phenomenon in which genetic hybrids transcribe the rRNA genes inherited from one parent, whereas the rRNA genes of the other parent are inactive (Pikaard, 2000). Central to this mechanism is an epigenetic switch in which concerted changes in promoter cytosine methylation density and specific histone modifications dictate the on and off states of rRNA genes (Lawrence *et al*., 2004).

In most eukaryotes, the 5S rRNA genes are organized as separate clusters of tandem repeats transcribed by RNA polymerase III. There is a substantial literature on 5S rDNA but most reports concern the size of the repeat, its copy number and chromosomal localization obtained from *in situ* hybridizations. In the yeast model, considerable work concerns the characterization of polymerase III transcription machinery components (Geiduschek and Kassavetis, 2001; Ducrot *et al*., 2006). The first results on the regulation of 5S rDNA transcription were obtained in *Xenopus laevis*, where a developmental control of the 5S rRNA genes expression occurs. The somatic gene family (400 copies organized in tandem) is expressed in both oocytes and somatic cells, whereas the oocyte-type genes (20 000 copies, tandemly arranged on several chromosomes) are only expressed during oogenesis and early embryogenesis when the larger amount of 5S rRNA is synthesized (Peterson *et al*., 1980). Both families encode a 120 nucleotides (nt) 5S rRNA with a five-nucleotide difference between the oocyte and somatic 5S rRNAs (Ford, 1973; Wolffe, 1994).

In the plant kingdom, results obtained in rice suggest a developmental control of the 5S rRNA genes expression. Two species of 5S rRNA were recovered in 48 h germinated embryos whereas only one was present in ungerminated embryos (Hariharan *et al*., 1987). In *Arabidopsis thaliana*, different species of 5S rRNA were recovered in tissues like seeds and roots, but this heterogeneity is absent in adult leaves and stems (Mathieu *et al*., 2003a) implying silencing of the corresponding genes.

For several years our work has focused on the transcriptional regulation of 5S rDNA in *A. thaliana*. This review demonstrates that 5S rDNA is highly regulated at different levels and that epigenetic mechanisms are involved in both *A. thaliana* and *X. laevis*.

### Arabidopsis 5S rDNA loci

*A. thaliana* has approximately thousands of 5S rRNA genes per haploid genome and these occur in tandem arrays (Campell *et al*., 1992), localized in the pericentromeric heterochromatin of chromosomes 3, 4 and 5 in the Columbia ecotype (with a large locus on the left arm and a small locus on the right arm of chromosome 5)

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(Murata *et al*., 1997; Fransz *et al*., 1998; Tutois *et al*., 1999; Cloix *et al*., 2000). A typical 5S rDNA unit is 0.5 kb long (Figure 1). It contains a 120 bp transcribed sequence, with an internal promoter and an approximately 380 bp intergenic spacer. The latter contains a TATA-like motif 28 bp upstream the transcribed region, necessary for transcription, and a simple cluster of T residues downstream the transcribed region, used as terminator signal (Cloix *et al*., 2003). Transcription by RNA polymerase III gives rise to a 120 nt 5S rRNA (Cloix *et al*., 2002).

### In *A. thaliana*, only two 5S rDNA arrays are transcribed

Our *in vivo* and *in vitro* results have revealed that 5S rRNA genes from chromosome 3 and from the small locus of chromosome 5 are not expressed (Figure 2). The presence of numerous mutations in the internal promoter of these genes probably prevents their transcription (Cloix *et al*., 2003). 5S rRNA genes from chromosome 3 are dispensable since some ecotypes lack these loci without deleterious effects for the plant (Fransz *et al*., 1998; Tutois *et al*., 2002).

*In vivo* and *in vitro* experiments have shown that only two 5S rDNA loci are transcribed in the cell: the locus on chromosome 4 and the large locus on the left arm of chromosome 5 (Figure 2; Cloix *et al*., 2002, 2003). These two 5S blocks are around 150 kb, each containing around 300 tandemly repeated 5S rDNA units.

### 5S rRNA genes and 5S rRNA heterogeneity

In Arabidopsis, we identified two types of 5S rRNA genes in each of the two transcribed 5S loci (chromosome 4 and large locus on the left arm of chromosome 5). The first class corresponds to the major genes (which represent 15–20% of the potentially transcribed 5S genes) coding for one 5S rRNA species, whereas the second class contains what we have named the minor 5S rRNA genes (80–85% of the potentially transcribed 5S genes; Figure 2). These latter genes harbor one to three nucleotide substitutions in their transcribed region compared to the major genes (Cloix *et al*., 2002). We observed that only the major 5S rRNA genes are transcribed in wild-type (WT) 3-week-old leaves (Mathieu *et al*., 2003a). Since *in vitro* experiments have demonstrated that minor genes have the potential to be transcribed, their silent state must result from an active process. This indicates that inside the two transcribed 5S blocks, only a fraction of the 5S rRNA genes are transcribed (the major genes) with therefore a silencing of the rest of the genes (the minor genes) in WT leaf nuclei.

In the Xenopus model, 5S gene heterogeneity results from the presence of two distinct, physically separated, 5S gene families: the constitutively expressed somatic one and the oocyte one whose expression is restricted to oogenesis. They encode a 120 nt 5S rRNA with a five-nucleotide difference (Ford, 1973).

When the greatest quantities of 5S rRNA are accumulated, that is in the seed and during oogenesis, in Arabidopsis and Xenopus respectively, 5S rRNA heterogeneity is observed. The 5S rRNA heterogeneity depends on tissues and/or developmental stages and implies mechanisms which dictate the on and off states of the corresponding 5S genes.

### Methylation of 5S rRNA genes

DNA methylation is one of the major events causing gene repression. In plants, 5–25% of all cytosines are methylated (Rangwala and Richards, 2004).

The tandemly repeated 5S rDNA has been shown to be highly methylated in different plant species: maize (Mascia *et al*., 1981), wheat (Grellet and Penon, 1984), lupin (Rafalski *et al*., 1982), pea (Ellis *et al*., 1988), flax (Goldsbrough *et al*., 1982) and tobacco (Fulnecek *et al*., 1998). Using genomic sequencing, we studied the methylation of *A. thaliana* 5S rDNA and found it to be highly methylated (79% of methylated cytosines) at CpG,

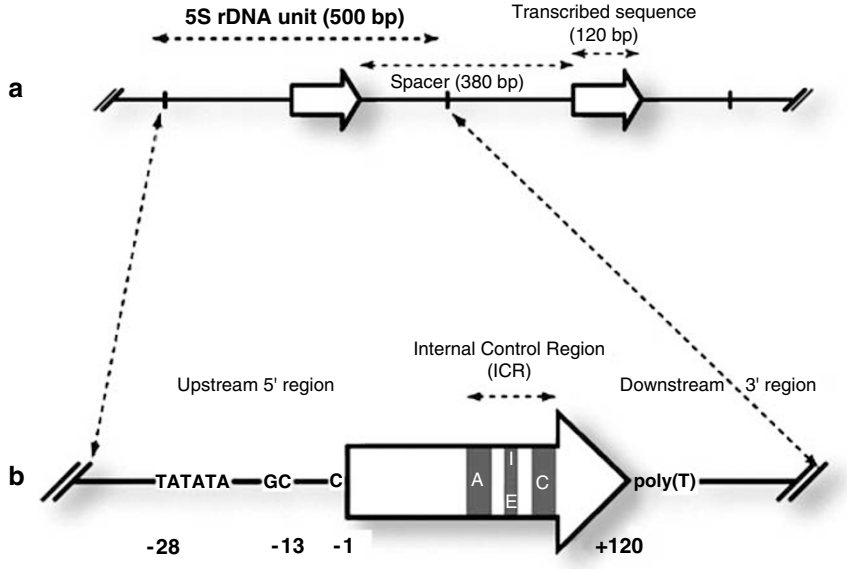


**Figure 1** Structure of *A. thaliana* 5S rDNA units. (**a**) Two tandemly organized 5S rDNA units. (**b**) One 5S rDNA unit with the 120 bp-transcribed sequence containing the internal promoter composed of box A (A), intermediate element (IE) and box C (C). The upstream region contains three motifs necessary for transcription at −28, −13 and −1. The downstream region contains the poly-T cluster used as transcription terminator.

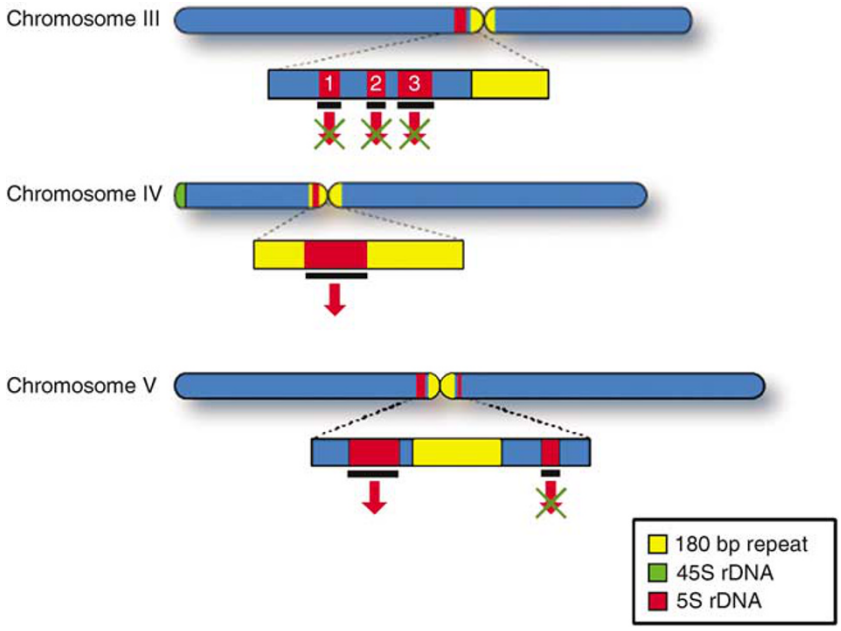


**Figure 2** Localization of transcribed and not transcribed 5S rDNA loci. 5S loci (red) are localized in the pericentromeric region (enlarged on the figure) of chromosomes 3, 4 and 5 composed of the 180 bp-satellite (yellow) and other sequences (blue). The loci 1, 2, 3 of chromosome 3 and the small locus of chromosome 5 are not transcribed (crossed arrow). The 5S locus of chromosome 4 and the large locus of chromosome 5 are the only transcribed loci (red arrow). Both contain major and minor 5S genes, which have the ability to be transcribed.

CpNpG and asymmetrical contexts. We also identified a small fraction of less methylated 5S rDNA units (Mathieu *et al*., 2002), thought to be the transcribed fraction (see later). However, we did not find any nonmethylated 5S genes, suggesting that all 5S genes contain methylated cytosines, probably at degrees depending on their transcriptional activity. We found an inverse correlation between 5S rDNA methylation, studied with methylation-sensitive enzymes, and the proportion of minor 5S rRNA. Minor 5S rRNAs were recovered in roots (13%), in plants treated with the cytosine methylation inhibitor compound 5-azacytidine (22.7%) and in *ddm1* mutant plants (22%, see later), where 5S rDNA is hypomethylated (Mathieu *et al*., 2003a).

DNA methylation is a stable epigenetic mark for transcriptional gene silencing (TGS). Miller *et al*. (1978) reported that Xenopus oocyte-type 5S rDNA is highly methylated in erythrocytes where it is repressed, although no direct correlation between methylation and transcription has been made. To assess whether methylation impaired transcription, the expression of an *in vitro* methylated 5S gene was checked after injection into Xenopus oocytes. However, the absence of transcription inhibition (Besser *et al*., 1990) probably revealed that methylation alone was not sufficient to repress transcription, rather than an absence of influence of the methylation on Xenopus 5S rDNA transcription. According to Santoro and Grummt (2001), no transcription repression was observed presumably because the DNA was not properly packaged into nucleosomes. Similarly, *in vitro* transcription of an Arabidopsis naked 5S rDNA gene or mouse 45S rDNA gene was not impaired by DNA methylation (Santoro and Grummt, 2001; Mathieu *et al*., 2002). This suggests the requirement for a specific chromatin state for the repression of these genes in the cell.

## The RNAi pathway is proposed to be involved in the silencing of 5S rRNA genes in Arabidopsis

Like almost every eukaryote, plants have the potential to neutralize invading nucleic acids and to repress repetitive sequences to prevent deleterious effects on genome stability and expression. These sequences can be repressed transcriptionally (TGS) through DNA methylation, histone methylation and/or chromatin remodeling. Short interfering RNAs (siRNAs) of 21–24 nt, processed from double-stranded RNAs (dsRNAs) derived from the controlled sequences, have a key role in determining the mode and sequence specificity.

In *A. thaliana*, silencing at endogenous repeat loci involves histone H3 lysine 9 (H3K9) methylation and RNA-directed DNA methylation (RdDM) (Pelissier *et al*., 1999; Mathieu and Bender, 2004). RdDM is accomplished by the *de novo* cytosine methyltransferase domains rearranged methylase 2 (DRM2) (Cao *et al*., 2003) and defective in RNA-directed methylation 1 (DRD1) (Kanno *et al*., 2004), a SWI2/SNF2-related protein. RdDM is correlated with the production of homologous siRNAs. 5S siRNAs are detected in WT plants (Xie *et al*., 2004) and 5S rDNA is highly methylated at CNN (Mathieu *et al*., 2002), which is a hallmark of RdDM.

Recent works have proposed the following model for the maintenance of the heterochromatic state at Arabidopsis pericentromeric sequences such as *AtSN1* (small retroelements), 45S and 5S rDNA: transcripts trailing from polymerases (I or III in these cases) that are stalled or slowed by DRM-mediated *de novo* methylation are sensed as aberrant and directly or indirectly become

templates for RNA polymerase IVa (Pol IVa), one of the two forms of the plant-specific nuclear RNA polymerase IV (Pol IV) (Herr *et al*., 2005; Kanno *et al*., 2005; Onodera *et al*., 2005; Pontier *et al*., 2005; Vaucheret, 2005). Pol IVa would generate precursor RNA at the endogenous repeats and these transcripts then move to the nucleolus where their complements are generated by RNA-dependent RNA polymerase 2 (RDR2) transcription. The resulting double-stranded RNA are then diced by dicer-like 3 (DCL3) and loaded into an argonaute 4 (AGO4)-containing effector complex or RNA-induced silencing complex (RISC) within the siRNA processing centers in the nucleolus. Then a subunit of the second Pol IV form, Pol IVb, would associate with AGO4-RISC (Pontes *et al*., 2006).

How AGO4-RISC-Pol IVb complexes mediate their effect on chromatin modification at target loci is unclear. The resulting compacted chromatin is methylated at DNA, methylated and deacetylated at histones. *De novo* DNA methylation by DRM2, which is predicted to contribute to aberrant RNA production, would provide for positive feedback (Pontes *et al*., 2006). Although this model is highly probable (5S siRNA accumulation is decreased in *pol IV*, *rdr2*, *dcl3* mutants), it needs to be checked for 5S rDNA transcription level (derepression of minor genes in RNAi pathway mutants). In addition, if RNAi mediates silencing, the initiating trigger RNA needs to be identified. Recently, Mayer *et al*. (2006) reported that long intergenic spacer transcripts, which are processed into smaller intermediate RNAs, are required for heterochromatin and silencing of a subset of mouse 45S rDNA arrays. Whether such transcripts have similar function for 5S rDNA needs to be investigated.

Finally, it is reasonable to assume that the 21–24 nt 5S siRNAs cannot discriminate the major and minor 5S rRNA genes. However, we postulate the existence of a chromatin condensation gradient along the 5S rDNA arrays. It remains to be found whether some structural features of the loci can favor this model and explain the selective repression of the minor 5S rRNA genes.

## Involvement of the chromatin structure

Both *in vitro* transcription experiments (Mathieu *et al*., 2002) and the Pol IV model (Onodera *et al*., 2005; Pontes *et al*., 2006) suggest the involvement of the chromatin structure in 5S rDNA regulation.

In Arabidopsis, the involvement of chromatin structure in the transcriptional regulation of 5S genes was demonstrated by cytological experiments using the *ddm1* mutant. The *decrease in DNA methylation 1* gene (DDM1) encodes a SWI/SNF2-like chromatin remodeling protein (Jeddeloh *et al*., 1999; Brzeski and Jerzmanowski, 2003). The *ddm1* mutation is known to cause a substantial demethylation of the genome and a striking decondensation of the chromocenters (Soppe *et al*., 2002). In leaf nuclei of 3-week-old WT plants, fluorescent *in situ* hybridization (FISH) with a 5S rDNA probe revealed that a fraction of 5S rDNA forms loops that emanate from the chromocenters (Figure 3). In this tissue, only the major 5S rRNA was recovered and the transcribed major 5S genes presumably reside in the 5S loops. The 5S rDNA fraction residing in the heterochromatic chromocenters is considered to be silent. In the *ddm1* mutant, larger 5S rDNA loops were observed and some 5S minor genes were expressed with 22% minor 5S rRNA transcripts recovered in 3-week-old leaves (minor 5S rRNAs contain 1–2 nucleotide substitutions compared to the major 5S rRNAs; Mathieu *et al*., 2003a).

In Xenopus, the involvement of chromatin structure was shown by the appearance of the linker histone H1 in embryonic chromatin, which correlates with the establishment of repression of oocyte-type 5S genes. Histone H1 is available during embryogenesis but not during oogenesis where it is replaced by the B4 variant. Histone H1 may direct the correct positioning of nucleosomes over the oocyte promoter (Chipev and Wolffe, 1992), which suggests significant phasing of nucleosomes influencing transcription factor IIIA (TFIIIA) binding over the promoters (Gottesfeld and Bloomer, 1980; Hayes and Clemens, 1992; Lee *et al*., 1993; Yang and Hayes, 2003). Changes in chromatin composition, as embryogenesis proceeds, resulting in a compacted chromatin in the presence of histone H1 and leading to the final repression of the oocyte 5S rRNA genes, had a major role in differential gene activity between the two 5S gene families (Wolffe, 1989; Dimitrov *et al*., 1993; Bouvet *et al*., 1994; Sera and Wolffe, 1998). It is likely, in plants, that chromatin condensation might be influenced by the position of nucleosomes along the DNA fiber (Martinez-Balbas *et al*., 1990). In tobacco, 5S rDNA units can be wrapped in a number of alternative nucleosome frames; thus potentially varying accessibility of transcription factors across the 5S rDNA tandem array (Fulnecek *et al*., 2006).

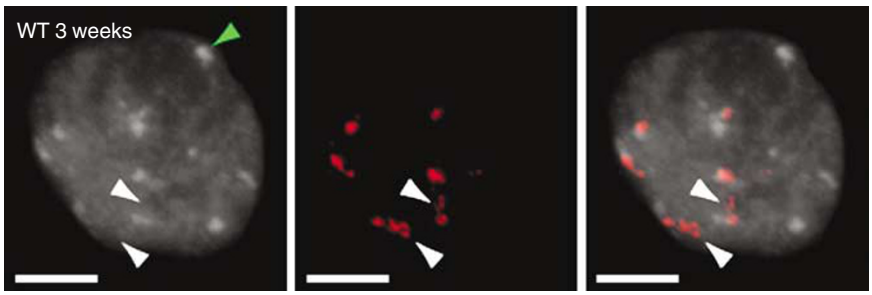


**Figure 3** 5S rDNA loops emanate from chromocenters in nuclei of 3-week-old plants. The heterochromatic chromocenters are DAPI-stained structures (green arrow). FISH with a 5S rDNA probe (red signal) on 3-week-old nuclei from WT plants. Bar = 5 $\mu$m. A fraction of the 5S rDNA signal is located in the heterochromatic chromocenters, while the other fraction forms loops within euchromatin (white arrows). Counterstaining with DAPI (left), FISH with the 5S rDNA probe (middle), and the merge of both (right) are shown.

## 5S rDNA histone modifications

The combination of DNA and histone modifications (methylation, acetylation, etc.) specifies chromatin structure and determines its transcriptional competence (Jenuwein and Allis, 2001). Transcribed and silent 5S rDNA fractions are therefore expected to contain different histone modifications specific for active and inactive transcriptional states, respectively.

Acetylation of the N-terminal domain of histones weakens the interaction of histones with DNA and facilitates the recognition of binding sites by transcription factors. In Xenopus, *in vitro* experiments from Tse *et al*. (1998) showed that high levels of acetylation completely inhibited higher order folding of 12-mer nucleosomal arrays assembled onto a DNA template consisting of twelve 5S tandem repeats. This was correlated with a large enhancement in the ability of RNA polymerase III to transcribe through the 12-mer nucleosomal arrays. Chromatin immunoprecipitation (ChIP) experiments conducted by Howe *et al*. (1998) confirmed that the upstream, non-transcribed region of the transcriptionally active Xenopus-somatic 5S genes is packaged with acetylated histone H4, whereas repressed oocyte 5S genes were not.

As already mentioned, silencing at endogenous repeat loci in *A. thaliana* involves RdDM and histone modifications. Lysines of histones H3 and H4 can appear in mono-, di- and trimethylated states (Paik and Kim, 1971; Turner, 2005), which have been recently proposed to reflect functional differences (Dutnall, 2003). In general, methylation of histone H3 lysine 4 has been associated with transcriptionally active genes (Sims *et al*., 2003). Conversely, methylation of H3K9 has been linked to heterochromatin and gene silencing (Lippman and Martienssen, 2004). Immunocytology has shown that Arabidopsis 5S loops, expected to contain the transcribed fraction, have euchromatic features such as dimethylation of lysine 4 of histone H3 (H3K4me2) and acetylation of lysine 9 of histone H3 (H3K9Ac), compared to the heterochromatic status (5-methylcytosine and H3K9me2) of the silenced 5S rDNA units colocalizing with the chromocenters (Mathieu *et al*., 2003a). ChIP experiments have shown that 5S rRNA genes become enriched in the euchromatic marks H3K4me2 and trimethylation of lysine 27 of histone H3 (H3K27me3) in *ddm1*, resulting from loss of CpG methylation and correlating with the release of silencing of some minor 5S rRNA genes and the larger 5S loops (Mathieu *et al*., 2003a, 2005) (Table 1).

ChIP experiments revealed that 5S rDNA is also associated with the heterochromatic marks H3K27me1 (monomethylation) and H3K27me2. However these 5S rDNA methylation patterns were both unaffected by CG hypomethylation in *ddm1* and *met1* (CG maintenance DNA methyltransferase) mutants, demonstrating their independence of CG methylation (Mathieu *et al*., 2005).

**Table 1** Histone modifications at *A. thaliana* 5S rDNA

| *Euchromatin* | *Heterochromatin* |
|---|---|
| H3K4me2 | H3K9me2[a] |
| H3K27me3 | H3K27me1[b] |
| H3K9Ac | H3K27me2[b] |

CG methylation directly restrains H3K27me3 (Mathieu *et al*., 2005).
[a]CG methylation-dependent (Tariq *et al*., 2003).
[b]CG methylation-independent (Mathieu *et al*., 2005).

The silenced 5S rDNA fraction is expected to be preferentially associated with these modifications.

Histone methylation depends on histone methyltransferases and the Arabidopsis genome encodes nine H3K9 methyltransferases (Baumbusch *et al*., 2001), referred as SuvH proteins. Chromomethylase3 (CMT3)-mediated non-CG maintenance methylation (see later) depends on H3K9 methylation. Ebbs and Bender (2006) have shown that the SuvH proteins 4, 5 and 6 make different relative contributions to the maintenance of H3K9 and DNA methylation at different loci, suggesting locus-specific mechanisms for their recruitment or activation. At 5S repeats, SuvH4/kryptonite, SuvH5 and SuvH6 together control CMT3-mediated DNA methylation with the hierarchy SuvH4 > SuvH5 and SuvH5 = SuvH6. They are therefore believed to methylate histones H3 at 5S rDNA, although histone methylation patterns have not been studied in the mutants.

Proteins considered to be involved in epigenetic pathways acting on 5S rDNA are presented in Table 2. In most of the cases, the impact of the proteins has been tested through the analysis of 5S rDNA methylation or accumulation of 5S siRNAs in the corresponding mutants. In some of them, we have also studied the impact of the mutation on 5S rDNA transcription. This is an accurate way to show the impact of a protein when DNA methylation and siRNA accumulation are unaffected in the corresponding mutant.

## A new 5S-210 transcript arises from 5S rDNA CG-hypomethylation

In plants, DNA methylation patterns at CG sites are maintained by methyltransferase 1 (MET1), while CMT3 is the major methyltransferase that maintains non-CG methylation at transposable elements and centromeric repeats (Finnegan and Kovac, 2000; Bartee *et al*., 2001; Lindroth *et al*., 2001; Tompa *et al*., 2002; Lippman *et al*., 2003; Tran *et al*., 2005). We have investigated 5S rRNA gene silencing in mutants of MET1 and CMT3. The release of 5S gene silencing was tested through the detection of 210 nt-long 5S transcripts (named 5S-210). This extends beyond the 120 bp-transcribed sequence and contains part of the spacer, up to now considered as nontranscribed. The sequencing of 5S-210 transcripts revealed that they only originate from the transcriptionally active 5S repeat clusters (chromosome 4 and large locus on chromosome 5). 5S-210 is observed in *met1* but not in the *cmt3* mutant, indicating that it is mainly under the control of MET1-mediated CG methylation whereas non-CG methylation has little or no influence (Table 2; Vaillant *et al*., 2006). We actually do not know the function of this transcript and whether the 5S siRNAs derive from this longer transcript.

Surprisingly, combination of *met1* and *cmt3* mutations in the double-mutant releases 5S gene silencing (at 5S-210) to a lesser extent than the *met1* mutation alone, although 5S rDNA methylation was strongly reduced at both CGs and non-CGs (Vaillant *et al*., 2006). This result points to the existence of additional regulatory pathways acting on 5S rDNA transcription.

## 5S rDNA is a target of the methylation-independent MOM pathway

Previous studies have identified Morpheus' molecule 1 (MOM1) as a component of a silencing mechanism

**Table 2** Mutants of epigenetic pathways acting on 5S rDNA methylation, accumulation of 5S siRNAs or 5S rDNA transcription

| *Protein/function* | *5S rDNA methylation* | *5S siRNA accumulation* | *Minor 5S/210-5S rRNA* | *References* |
|---|---|---|---|---|
| Pol IVa (RNA polymerase IV) | Reduced at CG, CNG, CNN | Decreased | ND | Onodera *et al*. (2005), Pontes *et al*. (2006) |
| RDR2 (RNA-dependent RNA polymerase) | Reduced at CG, CNG, CNN | Decreased | ND | Xie *et al*. (2004), Pontes *et al*. (2006) |
| DCL3 (dicer-like) | Reduced at CG, CNG, CNN | Decreased | ND | Xie *et al*. (2004), Pontes *et al*. (2006) |
| AGO4 (argonaute) | Reduced at CG, CNG | Decreased | ND | Xie *et al*. (2004), Zilberman *et al*. (2004), Zilberman *et al*. (2003) |
| Pol IVb (RNA polymerase IV) | Reduced at CG, CNG, CNN | Decreased | ND | Onodera *et al*. (2005), Pontes *et al*. (2006) |
| DRM2 (domains rearranged methylase) | Reduced at CG, CNG, CNN | Decreased | ND | Elmayan *et al*. (2005), Zilberman *et al*. (2004), Onodera *et al*. (2005) |
| SuvH 4, 5, 6 (histone H3K9 methyltransferase) | Reduced at CNG | Increased (1) Unchanged (2) | ND | Ebbs and Bender (2006), (1) Elmayan *et al*. (2005), (2) Zilberman *et al*. (2004) |
| MET1 (DNA methyltransferase) | Reduced at CG, CNG | Increased[a] | 5S-210 increased | Onodera *et al*. (2005), Vaillant *et al*. (2006), Elmayan *et al*. (2005) |
| CMT3 (chromomethyltransferase) | Reduced at CNG | Increased | 5S-210 unchanged | Elmayan *et al*. (2005), Vaillant *et al*. (2006) |
| DDM1 (chromatin remodelling) | Reduced at CG, CNG | Increased[a] | 5S-210 increased, minor 5S RNA increased | Mathieu *et al*. (2003a, b), Vaillant *et al*. (2006) |
| HDA6/SIL1 (histone deacetylase) | Reduced at CNG | Increased | ND | Elmayan *et al*. (2005) |
| MOM (chromatin remodelling) | Unchanged | Unchanged | 5S-210 increased, minor 5S RNA increase | Onodera *et al*. (2005), Vaillant *et al*. (2006), Elmayan *et al*. (2005) |

Abbreviation: ND, not determined.
[a]Derepression of 5S-repeat transcription results in the amplification of 5S siRNAs in these mutants.

independent of DNA methylation marks (Amedeo *et al*., 2000; Steimer *et al*., 2000; Mittelsten Scheid *et al*., 2002; Probst *et al*., 2003). They indicated that MOM1 is essential for silencing of heterochromatic genes and that the methylation-independent silencing MOM1 mechanism reinforces the methylation-based system and prevents extremely rapid epigenetic deregulation in plants with DNA methylation deficiencies (Mittelsten Scheid *et al*., 2002).

The MOM1 mutation releases silencing of *transcriptionally silent information* (*TSI*) repeats at chromocenters without altering their DNA methylation status (Steimer *et al*., 2000). The impact of *mom1* on 5S gene silencing was tested and confirmed by the higher proportion of both minor and 5S-210 transcripts in *mom1* than in WT plants, although the dense cytosine methylation of 5S genes remained unaffected (Table 2). Nor were the heterochromatic-specific (H3K9me2, H3K27me2) and euchromatic-specific (H3K4me2, H3K27me3) histone H3 marks at 5S rDNA modified in *mom1* plants (Vaillant *et al*., 2006). Reverse transcription-polymerase chain reaction and RNA gel blot analyses revealed that the *MOM1* transcript was slightly upregulated in *cmt3* and *met1 cmt3* but not in *met1*, suggesting that *MOM1* transcription is influenced by non-CG methylation. Given that *MOM1* participates in the control of 5S gene silencing, we hypothesized that the upregulation of *MOM1* in *met1 cmt3* might counteract the release of silencing induced by *met1* at 5S genes. In contrast to the situation observed for 5S genes, the *TSI* and the 180 bp (two MOM1 targets) transcripts accumulate to higher levels in *met1 cmt3* than in either single mutant, indicating a synergistic effect of the mutations on release of silencing at these repeats and therefore a specific behavior of 5S rRNA genes (Vaillant *et al*., 2006).

### Other forms of *A. thaliana* 5S rDNA regulation

These epigenetic mechanisms as well as other mechanisms participate in the control of 5S rDNA transcription.

**TFIIIA is a limiting factor for 5S rDNA transcription:** TFIIIA is a 5S rDNA-specific transcription factor (Engelke *et al*., 1980). 5S rRNA, subsequently integrated in the large ribosomal subunit, needs to be produced in stoichiometric amounts with 45S rRNA. Our results revealed a good correlation between levels of AtTFIIIA mRNA and 5S rRNA (Mathieu *et al*., 2003b). TFIIIA could act as a limiting factor; for example, the transcription of additional minor 5S genes in *ddm1* does not result in enhanced quantities of the 120 nt 5S rRNA. Therefore, TFIIIA seems to be an additional level of 5S rDNA transcriptional regulation (Mathieu *et al*., 2003a).

**Facilitated recycling pathway for RNA polymerase III:** The tRNA and 5S rDNA transcription complexes are known to be highly stable and can initiate multiple

rounds of transcription (Sprague, 1995). In yeast, Dieci and Sentenac (1996) have shown that the terminator element appears to be required for polymerase III to enter the reinitiation pathway. Polymerase III preferentially recycles on the same template, in a way that allows it to complete new cycles more rapidly than the initial one. Accordingly, Jahn *et al*. (1987) provided some evidence that human Pol III is retained in the original transcription complex during the normal reinitiation process. Using *in vitro* transcription experiments, we (Cloix *et al*., 2003) and Yukawa *et al*. (2000) have shown that the 5′ TATA motif is important for reinitiation of respectively 5S rRNA and tRNA genes both transcribed by the RNA polymerase III. Whether this is also the case *in vivo* is not yet known.

## Conclusion

In conclusion, this review shows that 5S rDNA is a subject of specific epigenetic regulations. The high conservation of the 5S genes in terms of repetitivity, structure, DNA methylation, etc. suggests that epigenetic mechanisms are also involved in 5S rDNA regulation in other organisms.

Numerous experiments remain to be done to characterize new regulating pathways acting on 5S rDNA. The DNA repair pathway is one of them. Proteins acting in DNA repair pathway are required for maintenance of TGS at targets like *Athila* and *TSI* retrotransposons. The proteins BRU1, RPA2, FAS1 and 2 (chromatin-associated factors) do not affect DNA methylation, but *bru1*, *fas1* and *fas2* mutants increased 5S siRNA accumulation suggesting they may participate in 5S gene silencing (Elmayan *et al*., 2005). The finding that methyl-CpG-binding domain proteins (MBDs) associate with histone deacetylases in plants (Zemach and Grafi, 2003) suggests that MBDs may induce heterochromatin formation by coordinating the activities of histone deacetylases and histone methyltransferases. Experiments need to be carried out to show the binding of MBD proteins on 5S rDNA (at least the repressed heterochromatic fraction).

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