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Title: Regulation of Pol I-Transcribed 45S rDNA and Pol III-Transcribed 5S rDNA in Arabidopsis

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ABSTRACT

The ribosomal RNAs 18S, 5.8S and 25S, which result from the 45S precursor, together with 5S rRNAs, are central components of the ribosome. The integration of one molecule of each ribosomal RNA per ribosome necessitates an elaborate coordination between transcriptions of the two ribosomal DNA (rDNA) families. Even though 5S rDNA is transcribed by RNA polymerase III and 45S rDNA by RNA polymerase I, the two ribosomal DNA families present certain similarities in their transcriptional regulation. This review aims to compare 5S and 45S ribosomal RNA genes in the plant model *Arabidopsis thaliana* in terms of organization, transcription and regulation, and draws parallels between the two rDNA families.

Keywords: Arabidopsis, 5S ribosomal DNA, 45S ribosomal DNA, Transcriptional Regulation

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Introduction

Biosynthesis of ribosomes monopolizes up to 80% of the cellular transcription activity**.** Ribosome biogenesis in eukaryotic cells requires the concerted synthesis of RNAs by three nuclear RNA polymerases. RNA polymerase II (Pol II) produces mRNAs that encode ribosomal proteins. The genes encoding the 5.8S, 18S and 25S ribosomal RNAs, known as 45S ribosomal DNA (rDNA) in plants are transcribed in the nucleolus by RNA polymerase I (Pol I), while 5S ribosomal RNAs are transcribed by RNA polymerase III (Pol III). Ribosomal RNAs (rRNA) associate with around 80 proteins to form the 40S and 60S ribosomal subunits (Figure 1A) (Korostelev and Noller 2007). Since one molecule of each ribosomal RNA is integrated in a ribosome, stoechiometric amounts of rRNA need to be produced which requires a tight coordination between synthesis of the RNA and protein moieties of the ribosome (Laferte et al. 2006).

This review aims to compare 5S and 45S ribosomal RNA genes (named 5S and 45S genes in this review) in the plant model *Arabidopsis thaliana* in terms of organization, transcription and regulation, and illustrates the similarities between the two rDNA families.

Structure and localization of 5S and 45S ribosomal RNA genes

The *Arabidopsis thaliana* genome contains approximately 1000 copies of 5S genes per haploid genome, which are arranged in tandem arrays located within the pericentromeric heterochromatin of chromosomes 3, 4 and 5 in the Columbia accession (Figures 1B and 2) (Campell et al. 1992). A typical 5S rDNA unit is 500 bp-long and consists of a 120 bp transcribed sequence, with an internal promoter and an approximately 380 bp intergenic spacer. Transcription by Pol III gives rise to a 120 nt 5S rRNA (Cloix et al. 2002) which is integrated into the large subunit of the ribosome. Its transcription requires the 5S rDNA-specific transcription factor IIIA (TFIIIA) which binds to the internal promoter composed of three conserved elements Box A, Intermediate Element and Box C (Bogenhagen et al. 1980;

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Cloix et al. 2000). The Arabidopsis internal promoter shows high sequence similarity with the one in Xenopus 5S RNA genes (Bogenhagen et al. 1980; Cloix et al. 2000).

The 45S genes are tandemly repeated at nucleolar organizer regions (NORs), termed as such because the nucleolus, the site of ribosome synthesis, is organized around rRNA genes during interphase (Lam et al. 2005; Shaw and Doonan 2005). Around 570-750 copies of 45S genes per haploid genome are arranged in head to tail tandem arrays located at the tips of the short arms of chromosomes 2 and 4 (NOR 2 and 4) in Arabidopsis (Figure 1B) (Copenhaver et al. 1995; Copenhaver and Pikaard 1996). Each rRNA gene transcription unit is ~10 kb-long and consists of sequences encoding a precursor transcript that includes the structural rRNAs (18S, 5.8S, 25S), the Internal Transcribed Spacers (ITS) and the External Transcribed Spacers (ETS) (Figure 2). The rRNA gene units are separated from the adjacent gene in the array by an Inter Genic Spacer (IGS) (Sáez-Vásquez and Echeverria 2006). Transcription by Pol I gives rise to a precursor or pre-rRNA, which is then processed into the 18S, 5.8S and 25S rRNA mature forms (Gruendler et al. 1991; Gruendler et al. 1989; Unfried et al. 1989), integrated in the ribosome (Figure 1A). Transcription starts from the promoter localized in the IGS. The sequences extending from –55 upstream to +6 downstream from the transcription start site (+1) are sufficient to program minimal and accurate Pol I transcription initiation in *A. thaliana* (Doelling et al. 1993; Doelling and Pikaard 1995). Nucleolin, a nucleolar protein, plays a role in Pol I transcription and processing of 45S pre-rRNA (Gaume et al. 2011; Mongelard and Bouvet 2007).

In Arabidopsis, 5S and 45S arrays are physically separated. Wicke et al. (2011) recently reported that the physical linkage of all rRNA genes observed in streptophyte algae, mosses and lycophytes may be regarded as the ancestral state of rDNA organization within land plants.

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Only some of the ribosomal DNA loci are active

Eukaryotes contain thousands of rRNA genes, and it has been believed that the number of these genes far exceeds the number expected to be required to supply ample cytoplasmic rRNA (Rogers and Bendich 1987) and excess copies are transcriptionally repressed. Indeed, heterochromatic chromocenters (CC), that can easily be observed as bright DAPI-positive domains with fluorescence microscopy (Figure 2), and which are sites where heterochromatin coalesces (Fransz et al. 2002), include centromeric repeats, other heterochromatic repeats and silent rRNA genes (Costa-Nunes et al. 2010). From these observations, the following questions arose: what distinguishes active and silent rRNA genes, do they differ in primary sequence and how is their differential regulation achieved?

In the *A. thaliana* Columbia accession, only two 5S rDNA arrays are transcribed. *In vivo* and *in vitro* results failed to show expression of the 5S genes from chromosome 3 and from the small locus of chromosome 5. Only 5S genes from chromosome 4 and from the large locus on chromosome 5 are expressed (Figure 1B) (Cloix et al. 2002; Cloix et al. 2003).

Selective activation of ribosomal DNA variants

In addition to the presence of silent rDNA loci, the active loci themselves contain transcribed and repressed rRNA genes. Indeed, variants of both 5S and 45S genes have been identified in the genome of *A. thaliana*. These gene variants show length or sequence heterogeneity.

Both active 5S-repeat clusters, on chromosome 4 and the left arm of chromosome 5 in the Columbia accession, contain transcribed and repressed 5S genes. In most of the tissues from adult wild-type plants, only major 5S genes are expressed whereas minor/heterogenous genes, which diverge from major ones from one up to several nucleotides, are repressed (Mathieu et al. 2003a). The terms major and minor/heterogenous refer to the transcriptional activity. While major 5S genes contribute majorly to the 5S rRNA pool as opposed to the minor ones,

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they only represent 20% of the 5S genes in both active 5S-repeat clusters. This illustrates that only a fraction of the 5S genes is actually expressed. However, in the seed, a tissue in which large quantities of 5S rRNA accumulate, 5S rRNA heterogeneity is observed. Therefore, expression of the minor genes varies, depending on tissues and/or developmental stages, suggesting the existence of mechanisms which dictate the « on » and « off » state of the corresponding minor/heterogenous 5S genes.

Interestingly, 5S genes whose organization has been analyzed for the pericentromeric 5S locus on chromosome 5 are not randomly positioned along the 5S array. This analysis revealed an increasing number of mutations along the 5S locus (AGI 2000; Cloix et al. 2002; Vaillant et al. 2008). This suggests that 5S major genes, constitutively transcribed, reside at the euchromatic side of the 5S array. The most mutated 5S genes (containing 3 to 10 mutations in their coding sequence) at the centromere-proximal side of the 5S locus are repressed. Indeed, we never recovered 5S cDNA with more than 2 nucleotide substitutions among the thousands of 5S cDNA sequences we have analyzed (Tourmente, personal communication).

At least four 45S rDNA variants also exist in the Columbia accession. Sequencing analysis of 3'ETS sequences (Figure 2) indicates that *A. thaliana* contains three major 45S gene variants, *VAR1*, *VAR2* and *VAR3* representing respectively 48, 30 and 22% of total 45S genes, and the very low copy number *VAR4* (Pontvianne et al. 2010). Remarkably, *VAR2*, *3* and *4* are expressed in WT plants, however we do not know whether all copies are transcribed, whereas the most highly represented *VAR1* is inactive. However, *VAR1* is transcribed in germinating seeds or chromatin mutants (Pontvianne et al. 2010) demonstrating its transcription ability. This illustrates that only part of the 45S genes is expressed. Whether these variants localize to NOR2 and/or NOR4 is currently unknown.

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Taken together, for both rDNA families, some DNA variants are selectively activated while others are repressed. This raises the question of the biological relevance of these gene variants in the Arabidopsis genome.

In yeast, inactive ribosomal RNA genes are required as a landing platform for cohesion complexes and in this way they help to ensure genetic stability of the ribosomal locus (Ide et al. 2010). We also observed that a large fraction of 5S and 45S genes is never transcribed. Therefore, a similar role for the rDNA loci in genome stability can also be envisaged in Arabidopsis.

Loops of active, euchromatic, 5S and 45S genes

The work of Fransz et al. (2002) revealed a relatively simple organization of chromosomes within Arabidopsis nuclei with chromosome territories consisting of a single repeat-rich, heterochromatic CC, from which gene-rich, euchromatic loops emanate. The presence of transcribed and repressed rRNA genes inside a locus implies differential regulation acting at these two genes fractions. Fluorescence *in situ* hybridization (FISH) revealed that a large fraction of 5S rDNA colocalizes with heterochromatin whereas another fraction forms loops that emanate from the heterochromatic CC in leaf nuclei (Figure 2) (Mathieu et al. 2003a). Since only major 5S rRNA was recovered in this tissue, the transcribed major 5S genes presumably reside in the 5S loops whereas the fraction residing in the heterochromatic CC is considered silent. Coherently, immunosignals obtained with antibodies directed against dimethylated lysine 9 of histone H3 (H3K9me2), a repressive mark, are clustered at the CC. 5S rDNA loops are less labeled with H3K9me2, and enriched for histone H3 acetylated at lysine 9 and methylated at lysine 4 suggesting a euchromatic structure permissive for transcription (Mathieu et al. 2003a).

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The nucleolus is a substructure that results from expression of 45S genes. It is formed around the NORs during transcription by Pol I (Figure 2) (Saez-Vasquez and Medina 2008). Indeed, a loop of decondensed 45S rDNA repeats that originates from the NOR has been observed in the nucleolus (Probst et al. 2004). Excess, inactive 45S genes are highly condensed and sequestered in heterochromatin at the external periphery of the nucleolus (Raska et al. 2004; Saez-Vasquez and Medina 2008; Shaw and Jordan 1995).

These results provide evidence that for both rDNA families, a fraction of the genes "escapes" from heterochromatin to be transcribed.

Transcription by Pol I but also maturation steps of the precursor are performed in the nucleolus. There is now extensive evidence of 5S gene clusters at chromosomal locations distant from the 45S rDNA that preferentially associate with the nucleolus or nucleolar periphery (Haeusler and Engelke 2006; Montijn et al. 1999). Whether this is the case in Arabidopsis merits investigation.

Nucleolar dominance and repression of ribosomal RNA genes are mediated by epigenetic mechanisms

As reported above, the repression of rRNA genes is mediated by a heterochromatic structure in Arabidopsis. DNA in heterochromatin is heavily methylated and contains repressive histones marks.

One of the earliest recognized epigenetic phenomena, nucleolar dominance describes the transcription of 45S genes inherited from only one parent in genetic hybrids. The phenomenon is widespread, occuring in insects, amphibians, mammals and plants (reviewed in McStay 2006; Tucker et al. 2010). Therefore, nucleolus forms around rRNA genes inherited from only one progenitor, whereas the other progenitor's rRNA genes are silent (Chen and Pikaard 1997). Nucleolar dominance has been well described in *Arabidopsis*

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suecica, the allotetraploid hybrid of *A. thaliana* and *A. arenosa* (Chen et al. 1998). In this hybrid, the entire *A. thaliana*-derived NORs are selectively silenced, enriched for the heterochromatic mark H3K9me2 and depleted for the euchromatic mark H3K4me3. However, only a subset of the dominant *A. arenosa* 45S genes is active, decondensed and associated with H3K4me3 whereas the remaining excess, inactive fraction is heterochromatic and associated with H3K9me2 (Lawrence et al. 2004). HDT1 and HDA6, two histone deacetylases and DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2) a *de novo* methyltransferase, are required to maintain DNA hypermethylation at the promoters of silenced genes, whereas acetylated histones H3K9, H3K14 and tetra-acetylated histone H4 (K5, K8, K12 and K16) are associated with active promoters whose cytosines are hypomethylated (Earley et al. 2006; Lawrence et al. 2004). RNAi-mediated knockdown of RDR2 (RNA-DEPENDENT RNA POLYMERASE 2), DCL3 (DICER like protein) or DRM2, components of the RdDM (RNA-directed DNA methylation) pathway (Matzke et al. 2009), disrupts the silencing of the *A. thaliana*-derived rRNA genes in *A. suecica.* RNAi mediated knockdown of MBD (Methylcytosine Biding Domain) protein genes revealed that MBD6 and MBD10 are also required for *A. thaliana*-derived rRNA gene silencing in *A. suecica.* In agreement with these results, small RNAs corresponding to the rRNA gene promoter and intergenic regions are eliminated in DCL3-RNAi lines and are depleted in RDR2-RNAi plants, suggesting a role for the siRNAs in the selective silencing of *A. thaliana*-derived rRNA genes (Costa-Nunes et al. 2010; Preuss et al. 2008; Tucker et al. 2010).

Interestingly, when silent *A. thaliana*-derived rRNA genes subjected to nucleolar dominance are derepressed with aza-dC (an inhibitor of DNA methylation) or TSA (histone deacetylase inhibitor), or by knockdown of required chromatin modifying activities, transcription of the dominant set of *A. arenosa* rRNA genes also increases (Chen et al. 1998; Lawrence et al. 2004; Preuss et al. 2008). This suggests that the mechanisms responsible for the silencing of

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the underdominant genes are also silencing the subset of the dominant rRNA genes and that nucleolar dominance is a manifestation of dosage control. However, there are probably additional regulatory mechanisms. Indeed, knockdown of DDM1, which affects rDNA methylation, does not release silencing of both *A. thaliana*-derived and *A. arenosa*-derived rRNA genes in *A. suecica* (Preuss et al. 2008).

Such a dominance mechanism might also apply to 5S rDNA loci. However, the sequence of the transcribed region of 5S rDNA units is highly conserved between accessions and subspecies, preventing the analysis of a "potential 5S dominance" (Tutois et al. 2002).

In nonhybrid *Arabidopsis thaliana*, the RdDM silencing pathway (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005; Zhang et al. 2007) which mediates *de novo* DNA methylation at asymmetrical CHH sites (Huettel et al. 2007; Wassenegger 2000, 2005) participates to 5S and 45S genes repression involved in dosage control (Blevins et al. 2009; Douet et al. 2008; Douet et al. 2009; Earley et al. 2006; Preuss et al. 2008). The following section presents the common principles in transcriptional repression between 5S and 45S rDNA.

Earley et al. (2010) analysed the molecular basis for 45S rDNA repression in *A. thaliana*. In *hda6* mutants, symmetric methylation at CG/CNG motifs is reduced (Earley et al. 2010). Surprisingly, spurious Pol II transcription occurs throughout the intergenic spacers. The resulting sense and antisense spacer transcripts facilitate a massive overproduction of siRNA diced by two DICER proteins (DCL3 and DCL4) of the RdDM pathway that, in turn, direct *de novo* asymmetric cytosine methylation to the corresponding gene sequences. However, the resulting *de novo* DNA methylation fails to suppress Pol I or Pol II transcription in the absence of HDA6 activity, and euchromatic histone modifications typical of active genes, such as histone H3 acetylation and K4 methylation, accumulate. As a result, 45S rDNA is decompacted and the 45S gene variant (*VAR1*), normally inactivated, becomes transcribed.

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The results obtained for 5S rDNA allow to propose an analogous model of repression, coherent with the stoichiometric needs of 5S and 45S rRNA.

In mutants for the maintenance METHYLTRANSFERASE1 (*met1-1*) and CHROMOMETHYLASE3 (*cmt3-7*), but also in seedlings treated with 5-aza C or in the mutant of the chromatin remodeling factor DDM1, symmetric methylation at CG/CNG motifs is reduced, and a silencing release of minor 5S genes is observed (Mathieu et al. 2003a; Vaillant et al. 2007). In addition, a particular set of transcripts, termed 5S-210, that extend into the intergenic spacer (IGS) downstream of 5S RNA genes overaccumulate in *met1* and *ddm1* mutants (Vaillant et al. 2006). These transcripts are the precursors of 5S siRNA and consequently 5S siRNA which match to the IGS overaccumulate (Blevins et al. 2009), leading to enhanced RdDM and increased *de novo* methylation (Blevins et al. 2009) mediated by DRM2 (Mathieu et al. 2007) in these mutants. However, despite the 5S rDNA asymmetrical hypermethylation in these mutants, 5S rDNA is hypomethylated at symmetrical cytosines leading to derepression of minor 5S genes. In absence of HDA6, symmetrical CNG methylation is decreased at 5S rDNA and a silencing release of minor 5S genes is observed (Vaillant et al. 2007).

All the results obtained for 5S and 45S rDNA show that a combination of maintenance symmetrical methylation patterns and *de novo* asymmetrical methylation patterns, mediated by the RdDM pathway, together with histone modifications, as for instance deacetylation performed by HDA6, help to ensure the correct 5S and 45S genes repression and therefore the correct gene dosage. In addition, in all the mentioned mutants, the total amount of mature rRNA was not significantly affected, at least in nonhybrid *Arabidopsis thaliana.* This agrees with idea that the 5S rDNA-specific TFIIIA is the limiting factor in 5S rDNA transcription

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(Mathieu et al. 2003a) and suggests a redistribution of the Pol III complexes on the 5S genes (and eventually the Pol I complexes on 45S genes) in chromatin mutants.

A Pol V activity, RdDM- independent, drives compaction of both 5S and 45S rDNA

Each of the two 5S arrays from chromosome 4 and 5 is a target of RdDM, which contributes to the selective repression of genes. In mutants of the RdDM pathway, such as *nrpd1* (Pol IV subunit)*, rdr2, drm2, ago4* or *dcl3*, a reduction of asymmetric methylation at each 5S array is associated with the derepression of minor/heterogenous 5S genes at chromosomes 4 and 5; however without 5S rDNA decompaction (Douet et al. 2009).

Interestingly, the 5S array from chromosome 4 (but not those from chromosomes 3 and 5) as well as NOR loci (45S rDNA) are decompacted in cotyledons of *nrpe1* and *nrpe5a*, mutants of two Pol V-specific subunits, illustrating another common regulation process of 5S and 45S rDNA. This decompaction is restricted to the Pol V–loss of function (Douet et al. 2009), specific to the 5S array from chromosome 4 and is not associated with changes of 5S rDNA asymmetric methylation. Indeed, this additional role of Pol V is Pol IV- and RdDM-independent and therefore adds an additional level of regulation. This suggests that an alternative pathway *i.e.* without Pol IV and RdDM partners exists to regulate organization and expression of 5S (at chromosome 4) and 45S rDNA loci. It also suggests that the Pol V complex might recruit other repressive epigenetic marks in addition to DNA methylation and H3K9me2 which is dependent on DNA methylation at CG sites (Mathieu et al. 2005).

Since there is a need in the plant kingdom for rapid, reversible changes in gene expression to respond to growth demands or environmental changes, some of the rDNA repeats may be specifically targeted for silencing as a mechanism to modulate or fine-tune total cellular rDNA activity.

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Nucleolin controls 45S RNA gene transcription

The transcription of 45S rDNA by Pol I involves the transcription factor nucleolin which represents an additional layer of regulation (Figure 2).

In eukaryotes, nucleolin is one of the most abundant non-ribosomal proteins in the nucleus. Nucleolin plays a role in different steps of ribosome biogenesis, including Pol I transcription and processing of 45S pre-rRNA (Gaume et al. 2011; Mongelard and Bouvet 2007). In *Arabidopsis*, AtNUC-L1 (a nucleolin-like protein) localizes in the nucleolus and plays a role in 45S rDNA chromatin condensation and in controlling homeostatic rRNA gene expression (Pontvianne et al. 2007). For that, AtNUC-L1 specifically binds rRNA gene promoter sequences and directs rRNA transcription from the transcription initiation site (Pontvianne et al. 2007). To demonstrate that AtNUC-L1 interacts with transcriptionally active 45S genes, *hda6* mutants have been used (Pontvianne et al. 2010). In *hda6* plants, 45S rDNA chromatin decondensation induces expression of *VAR1* (normally repressed in WT plants). ChIP assays revealed a specific and direct interaction of AtNUC-L1 with 5' and 3' ETS sequences of *VAR1* in the *hda6* mutant only. However, AtNUC-L1 does not co-precipitate with Pol I subunits. Interestingly, AtNUC-L1 seems to be required for rDNA symmetric methylation of transcribed but not promoter sequences. Indeed, in *Atnuc-L1* plants methylation at CG (and in some extent at CHG) decreases specifically in the 5'ETS sequences (Pontvianne et al. 2010).

Considering the nucleosome remodeling activity of nucleolin proteins in mammals (Angelov et al. 2006; Erard et al. 1988; Kharrat et al. 1991), it is rational to propose that binding of AtNUC-L1 to rRNA genes may be required to position nucleosomes in specific transcriptional frames that determine the "on" or "off" state of rRNA genes. Many questions remain, including mutual dependency and complex crosstalk among nucleolin proteins and other functionally related protein factors, such as RdDM partners and HDA6.

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Finally, in animals nucleolin localizes also in the nucleoplasm and might control Pol II transcription (Mongelard and Bouvet 2007). In Arabidopsis plants, the transcriptomal analysis of *Atnuc-L1* mutant revealed accumulation and/or reduction of the level of different transcripts transcribed by Pol II, including AtNUC-L2 (a second nucleolin-like protein) and other small RNA (Pontvianne et al. 2007). The *Atnuc-L1* mutant also showed a significantly reduced sugar-induced expression of ribosomal protein genes (Kojima et al. 2007). Therefore we can expect that nucleolin protein from plants might also control expression of Pol III genes. However to date whether or not Pol III transcription of 5S rRNA is affected in *Atnuc-L1* has not been established. Consequently, it will be interesting to determine whether the expression of major or minor/heterogenous 5S gene variants is modified in *Atnuc-L1*.

Transcriptional regulation of 5S RNA genes by TFIIIA

Transcription factor IIIA (TFIIIA) which was first isolated from Xenopus oocytes, contains nine zinc finger domains and is required for transcription of the 5S RNA gene by Pol III (Ginsberg et al. 1984). The protein binds specifically within the internal control region (ICR) of the 5S RNA gene and to the 5S RNA product. Zinc fingers 4 to 7 are responsible for the binding to 5S rRNA (Clemens et al. 1993) whereas fingers 1 to 3 are critical for the binding to the 3'-portion of the internal promoter (C-box element) of the Xenopus 5S gene (Clemens et al. 1992). In yeast, a second species where TFIIIA has been extensively studied, the ICR is a surprisingly short control region relative to the ICRs of Xenopus and Arabidopsis which are highly similar (Challice and Segall 1989; Cloix et al. 2000*)*. Later, the TFIIIA protein, present in every organism, has been characterized in several other species including *Arabidopsis thaliana* where it binds both 5S rDNA and 5S rRNA. It shows remarkably poor conservation of sequence (Mathieu et al. 2003b) and references therein) but except TFIIIA from *S. pombe*, which contains a tenth finger, all proteins analyzed carry nine C2H2-zinc fingers.

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The Arabidopsis TFIIIA gene consists of seven exons, the third of which results from a 5S RNA exonization in the gene that occured early in evolution of angiosperms. This exon, which bears considerable similarity in its secondary structure to plant 5S rRNA (Fu et al. 2009; Hammond et al. 2009)**,** is alternatively skipped or included to produce either of the two transcript isoforms (Yoine et al. 2006). The ES (Exon-skipped) isoform encodes the fully functional TFIIIA protein. Instead, the EI (Exon-inclusion) isoform is eliminated by the NMD (nonsense mediated decay) pathway. Two groups (Fu et al. 2009; Hammond et al. 2009) proposed a post-transcriptional negative feedback auto-regulation model specific to plant TFIIIA genes. The model stipulates that when TFIIIA protein levels are low, the alternative exon is skipped, which results in an increase in the ES isoform and a consequent increase in production of new TFIIIA protein. When in abundance, a greater fraction of the TFIIIA transcripts is spliced into the unproductive isoform and then degraded through NMD, thereby downregulating TFIIIA production. Hammond et al. (2009) demonstrated that ribosomal protein L5 directly binds the 5S rRNA-like structure of the alternative exon, which parallels a known binding interaction between L5 and 5S rRNA (Szymanski et al. 2002). This binding promotes exon skipping and is expected to promote production of the TFIIIA protein.

This model stipulates a quantitative regulation of the 5S rRNA transcription by TFIIIA and illustrates the importance to control functional TFIIIA amounts in the cell. Given that during development the needs for 5S rRNAs vary considerably, e.g. they strongly accumulate in the seed, it will be interesting to test this model during plant development.

Post-germination chromatin remodeling of both 5S and 45S rDNA

The observation that the heterochromatin fraction in nuclei of cotyledons is much smaller at 2 day post-germination compared to 3 week-leaves provided evidence that chromatin is reorganized during development to obtain mature heterochromatin (Mathieu et al. 2003a).

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Investigation of the chromatin structure by FISH, revealed a large-scale reorganization of 5S rDNA chromatin during the first days of development, to obtain almost mature nuclei 5 days post-germination (Douet et al. 2008).

Similarly, FISH experiments revealed that 45S rDNA is decompacted in nuclei of 2 day-germinating seeds (Pontvianne et al. 2010), illustrating that 45S rDNA chromatin also reorganizes during post-germination. Interestingly, the progressive chromatin reorganization during the post-germination period leads to the mature rRNA transcription *i.e*. transcription and repression of major and minor 5S genes respectively, as well as transcription of 45S variants 2, 3, and 4 and repression of *VAR1*.

TOR controls rRNA expression

The eukaryotic TOR (Target Of Rapamycin) pathway controls translation, growth and the cell cycle in response to environmental signals such as nutrients or growth stimulating factors (Hay and Sonenberg 2004; Menand et al. 2002; Schmelzle and Hall 2000). Studies in yeast and animal cells have shown that TOR acts positively on the activity of the eIF4F translation initiation complex and on the transcription of ribosomal protein and RNA genes therefore promoting growth in nutrient sufficient conditions (Beretta et al. 1996; Berset et al. 1998; Martin et al. 2004). In starvation conditions, TOR regulates the utilization of alternative energy resources, allows autophagy and generally drives the cell towards survival pathways (Beck and Hall 1999; Kamada et al. 2004). The antibiotic rapamycin was found to mimic starvation responses in yeast through TOR inactivation and cell cycle arrest (Barbet et al. 1996). Rapamycin leads to the formation of a ternary complex by binding simultaneously to TOR and to the FKBP12 protein (Choi et al. 1996).

However, *Arabidopsis thaliana*, and more generally land plants were found to be resistant to rapamycin due to mutations in the FKBP12 protein, suggesting TOR specificities in plants in

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addition to conserved activities (Sormani et al. 2007). Indeed, TOR controls embryogenesis, post-embryonic development and 45S rRNA production by phosphorylation of various downstream targets in *Arabidopsis*. ChIP experiments have shown that TOR activates the expression of 45S rRNA through binding to its promoter and mainly to its 5'ETS region (Ren et al. 2011). Binding of TOR to the 5'ETS suggests a functional link between TOR and AtNUC-L1. The kinase domain of TOR is essential for 45S rDNA transcription and AtNUC-L1 might be a potential target. Although its phosphorylation by TOR has not been demonstrated, nucleolin is a phosphoprotein that can be targeted by multiple kinases (Tuteia and Tuteia 1998). Thus it is tempting to speculate that phosphorylation of AtNUC-L1 by TOR might be required for 5'ETS binding and activation of rRNA gene expression in *Arabidopsis.*

Binding of TOR to 5S rDNA has been reported in yeast and animals (Li et al. 2006; Wei et al. 2009) but not yet in *Arabidopsis*, even though the conservation of TOR suggests that TOR regulates also Pol III transcription in plants, to coordinate the transcription of the different rRNA species.

Although postulated, there is no clear evidence indicating whether the maintenance of an equimolar supply of ribosomal components reflects communication between the nuclear transcriptional machineries such as Pol I and Pol III in Arabidopsis. This interesting question deserves further investigation.

In conclusion, although 45S and 5S RNA genes are transcribed by two different RNA polymerases, Pol I and Pol III respectively, and involve different transcription factors, parallels concerning their organization, function and transcription regulation can be drawn. The following model is proposed: in the Columbia accession, 5S major genes and 45S rDNA variants 2, 3, and 4 are transcribed. Inversely, 5S minor/heterogenous genes and 45S rDNA variant 1 are repressed. In addition, depending on the tissues, the total 5S and 45S rRNA

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quantities vary. This illustrates a level of qualitative regulation, with the selective transcription or repression of a subset of genes, and a level of quantitative regulation depending on the need of ribosomes for protein synthesis.

The qualitative regulation is achieved by a combination of symmetrical methylation maintenance and *de novo* deposition of asymmetrical methylation, mediated by the RdDM pathway. Together with specific histone modifications this leads to repression of certain 5S and 45S genes, *i.e*. the correct choice of the gene variants for transcription.

Tightly regulated amounts of the specific transcription factor TFIIIA control the quantitative regulation of 5S rRNA transcription. Similarly, phosphorylation or other post-traductional modifications of the nucleolin protein are supposed to be required for activation of 45S RNA gene expression, suggesting that the level of 45S rRNA transcription might be regulated by the amount of this modified/activated nucleolin protein.

Furthermore, TOR might emerge as a central player in the coordination of 5S and 45S rRNA expression. Indeed, TOR directly interacts with 45S rDNA *in vivo* to regulate rRNA expression and phosphorylation of AtNUC-L1 by TOR might be a way to link rRNA transcription with nutrient availability. Coordination of 5S rRNA transcription with 45S rRNA transcription might result from binding of TOR proteins to 5S RNA genes, but the nucleolin protein might also control expression of Pol III-transcribed genes. This highly coordinated regulation of 5S and 45S is very likely motivated by the requirement for stoechiometric amounts of the different ribosomal RNA families.

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Legends:

Figure 1: Localisation and function of rRNA genes in *Arabidopsis thaliana*.

A. The plant 80S ribosome is a large ribonucleoprotein complex. It consists of one molecule of each rRNA and around 80 ribosomal proteins. The small 40S subunit contains the 18S rRNA together with 33 proteins. The large 60S subunit consists of 50 proteins and 25S, 5,8S and 5S rRNA. Transcription by RNA polymerase III gives rise to a 120 nt 5S rRNA. Transcription by RNA polymerase I gives rise to a 45S rRNA, which is then processed into the 1806 nt 18S rRNA, 163 nt 5.8S rRNA, and 3384 nt 25S rRNA mature forms.

B. Localization of 5S and 45S rDNA arrays, in *Arabidopsis thaliana* Columbia accession.
The 5S arrays are in red and the NOR (nucleolus organizer region) containing the 45S arrays in green. NOR 2 and 4 are similar in size, each spanning 3,5-4,0 Mbp. The grey boxes symbolize the centromeric 180 bp repeats. Transcriptionally active 5S arrays on chromosome 4 and left arm of chromosome 5 are surrounded in blue. These two 5S blocks are around 150-250 kb-long, each containing about 300-400 tandemly repeated 5S rDNA units.
5S arrays on chromosome 3 and right arm of chromosome 5 are not transcribed. The presence of numerous mutations in the internal promoter of these genes probably prevents their transcription (Cloix et al. 2003). 5S genes from chromosome 3 are dispensable since some accessions lack these loci (Cloix et al. 2000; Fransz et al. 1998; Tutois et al. 2002).
Chromosomes and rDNA loci are drawn approximately to scale.

Figure 2: Schematic representation of *Arabidopsis thaliana* chromosome 4.

Chromosome 4 contains clustered tandemly repeated 5S (red) and 45S (green) ribosomal RNA genes at centromere (grey) and Nucleolar Organizer Region (NOR) respectively.

Top: Enlarged 5S rDNA unit showing the 5' and 3' flanking sequences containing TATA-like sequence, GC sequence, C residue at -1, necessary for transcription, and a simple cluster of T residues downstream the transcribed region, used as Pol III terminator signal and specific for each 5S locus (Cloix et al. 2000; Cloix et al. 2002; Cloix et al. 2003) and 5S transcribed region containing the promoter region (Box A, Intermediate element (IE) and Box C). 5S rDNA is transcribed by Pol III and requires Transcription Factor IIIA activity (TFIIIA). Disruption of Histone Deacetylase 6 (HDA6) gene induces expression of silent minor 5S genes.

Down: Enlarged 45S rDNA unit showing Intergenic Spacer (IGS) Region containing Sal I repeats, Spacer (SP1 and SP2) and Gene (GP) Promoter sequences. The transcribed region contains External (5'ETS and 3'ETS) and Internal (ITS1 and ITS2) Transcribed Spacer and structural rRNA (18S, 5.8S and 25S) sequences. 45S rDNA is transcribed by Pol I from GP, and requires Nucleolin-Like1 (AtNUC-L1). Disruption of Histone Deacetylase 6 (HDA6) gene induces expression of silent 45S rDNA *VAR1*.

The 5S (in *chr3*, *4* and *5*) and the 45S (in *chr2* and *chr4*) arrays can be visualized by FISH (red and green signals respectively). Heterochromatic chromocenters are observed as DAPI stained structures (arrowhead). 45S loops are too thin to be visualized in the nucleolus (N). A 5S loop extending from a chromocenter is shown (arrow).

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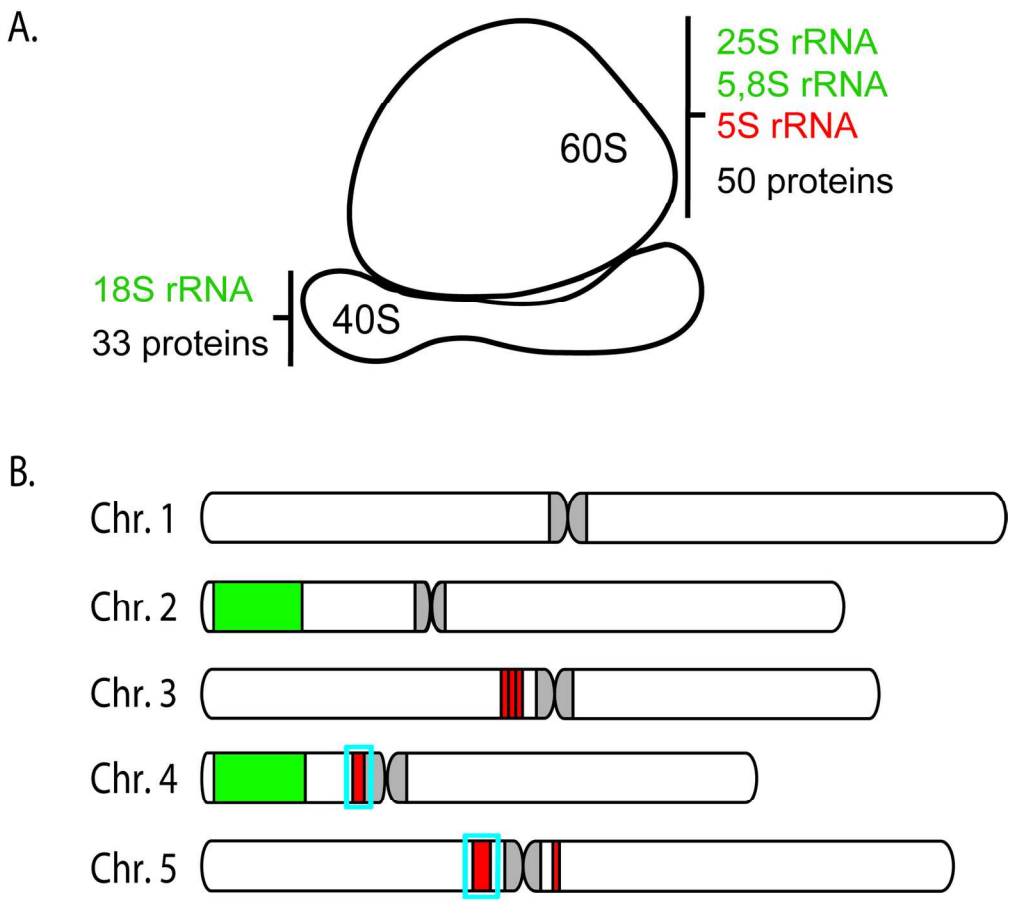

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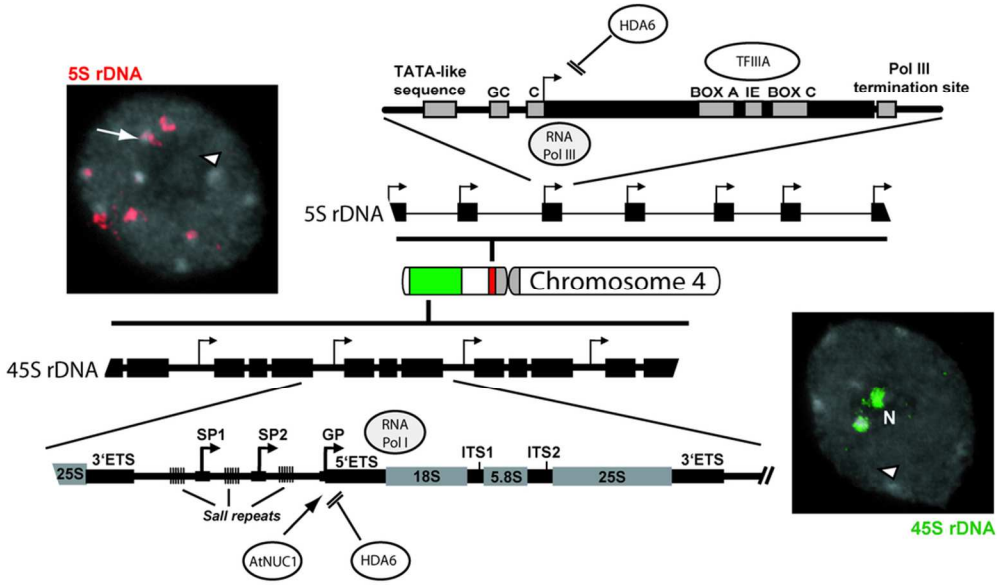

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Top: Enlarged 5S rDNA unit showing the 5' and 3' flanking sequences containing TATA-like sequence, GC sequence, C residue at -1, necessary for transcription, and a simple cluster of T residues downstream the transcribed region, used as Pol III terminator signal and specific for each 5S locus (Cloix et al. 2000; Cloix et al. 2002; Cloix et al. 2003) and 5S transcribed region containing the promoter region (Box A, Intermediate element (IE) and Box C). 5S rDNA is transcribed by Pol III and requires Transcription Factor IIIA activity (TFIIIA). Disruption of Histone Deacetylase 6 (HDA6) gene induces expression of silent minor 5S genes.
Down: Enlarged 45S rDNA unit showing Intergenic Spacer (IGS) Region containing Sal I repeats, Spacer (SP1 and SP2) and Gene (GP) Promoter sequences. The transcribed region contains External (5'ETS and 3'ETS) and Internal (ITS1 and ITS2) Transcribed Spacer and structural rRNA (18S, 5.8S and 25S) sequences. 45S rDNA is transcribed by Pol I from GP, and requires Nucleolin-Like1 (AtNUC-L1). Disruption of Histone Deacetylase 6 (HDA6) gene induces expression of silent 45S rDNA VAR1.
The 5S (in chr3, 4 and 5) and the 45S (in chr2 and chr4) arrays can be visualized by FISH (red and green signals respectively). Heterochromatic chromocenters are observed as DAPI stained structures (arrowhead). 45S loops are too thin to be visualized in the nucleolus (N). A 5S loop extending from a chromocenter is shown (arrow).

90x53mm (300 x 300 DPI)

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