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Interplay of RNA Pol IV and ROS1 During Post-Embryonic 5S rDNA Chromatin Remodeling

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We have investigated the chromatin structure of 5S rDNA, a heterochromatic pericentromeric tandemly repeated family, at 2, 3, 4 and 5 days post-germination. Our results revealed a large-scale reorganization of 5S rDNA chromatin that occurs during the first days of development. Unexpectedly, there is a decondensation followed by a 're'condensation of 5S rDNA chromatin, to obtain almost mature nuclei 5 d post-germination. The reorganization of 5S rDNA chromatin is accompanied by a rapid and active demethylation of 5S rDNA mediated by the ROS1 (repressor of silencing 1) demethylase, whereas the plant-specific RNA polymerase IV (Pol IV) is essential to the 5S chromatin 're'condensation. In conclusion, Pol IV and ROS1 collaborate to unlock the 5S rDNA chromatin inherited from the seed, and establish adult features.

Keywords: *Arabidopsis thaliana* — Chromatin — RNA polymerase IV — ROS1 — 5S rDNA.

Abbreviations: BAC, bacterial artificial chromosome; CC, chromocenter; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; NOR, nucleolar organizing region; Pol IV, RNA polymerase IV; RNAi, RNA interference; ROS1, repressor of silencing 1; siRNA, small interfering RNA; TSI, transcriptionally silent information; WT, wild type.

Introduction

In mammals, heterochromatin displays distinct characteristics in different cell types, reflecting the identity of the cell and the developmental stage (Probst and Almouzni 2007). The cell type-specific behaviors underline the particular plasticity of the heterochromatin domain and show that differentiation is associated with changes in heterochromatin features (Keohane et al. 1996, Kobayakawa et al. 2007, Puschendorf et al. 2008). The RNA interference (RNAi) machinery and unidentified RNAs (Maison et al. 2002, Kanellopoulou et al. 2005) have been implicated in the assembly of mammalian centromeric heterochromatin.

Recent work, especially in fission yeast, has shown that a low level of transcription and a functional RNAi pathway are required to maintain heterochromatin (Grewal and Jia 2007). Similarly, the plant-specific RNA polymerase IV (Pol IV) has recently been reported to contribute to small interfering RNA (siRNA) production and shown to be essential to RNA-directed DNA methylation (Herr et al. 2005, Kanno et al. 2005, Onodera et al. 2005, Pontier et al. 2005, Zhang et al. 2007). RNA-directed DNA methylation is the prominent nuclear silencing pathway which guides formation of transcriptionally silent heterochromatin at repeated loci (Brodersen and Voinnet 2006). *Arabidopsis thaliana* contains approximately 1,000 copies of 5S rRNA genes per haploid genome. 5S rDNA is arranged in tandem arrays (Campell et al. 1992) and is located within the pericentromeric heterochromatin of chromosomes 3, 4 and 5 (with a large locus on the left arm and a small locus on the right arm of chromosome 5) in the Columbia accession (Murata et al. 1997, Fransz et al. 1998). In a model proposed by Onodera et al. (2005), the AGO4-containing effector complex RISC (RNA-induced silencing complex) is guided by siRNA to drive DNA methylation and chromatin compaction at Arabidopsis pericentromeric sequences such as 5S rDNA (Pikaard 2006). The phenotypes observed in *pol IV* mutant nuclei, lacking 5S siRNA, revealed that 5S genes are typically decondensed, hypomethylated and show significantly less co-localization with heterochromatic centromeres.

We have reported that at 2 d post-germination, the heterochromatin fraction in nuclei of leaves is much smaller than in wild-type (WT) 3-week-old nuclei, i.e. the heterochromatin fraction is composed of pre-chromocenters (pre-CCs) that are much smaller than the CCs observed in 3-week-old nuclei. Fluorescence in situ hybridization (FISH) revealed that 2 days post-germination, the 5S rDNA co-localizes with pre-CCs. In 3-week-old nuclei, part of the 5S rDNA is located within heterochromatic CCs, whereas the other fraction forms loops with euchromatic features that emanate from CCs and were proposed to contain the transcribed 5S rRNA genes (Mathieu et al. 2003). This provides evidence that chromatin needs to be reorganized during development to obtain mature pericentromeric and constitutive heterochromatin.

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The plant epigenome, like mammals or other epigenomes, responds to developmental cues (Madlung and Comai 2004). It is likely that dynamic changes in DNA methylation status are a key to the plasticity and inheritance of the epigenome. Two opposing pathways, DNA methylation and demethylation, converge at numerous loci scattered throughout the genome (Penterman et al. 2007). This dynamic control may be important in keeping the plant epigenome plastic (Zhu et al. 2007). In Arabidopsis, heterochromatic repeated sequences such as 45S rDNA and many transposons represent loci affected by the DNA demethylase ROS1 (repressor of silencing 1) that has a role in erasing DNA methylation and preventing transcriptional gene silencing (Gong et al. 2002, Kapoor et al. 2005, Agius et al. 2006).

Here we have studied the 5S rDNA chromatin behavior between 2 and 5 d post-germination. A decondensation followed by a 're'condensation of the 5S chromatin is observed.The reorganization of the 5S rDNA chromatin is associated with an active demethylation, at asymmetrical cytosines, mediated by the demethylase ROS1, and the plant-specific Pol IV is essential to the 5S chromatin 're'condensation.

Results

5S rDNA chromatin remodeling occurs during early development

We were interested in investigating the remodeling events that lead to the progressive establishment of a mature heterochromatin organization. We therefore decided to investigate the CC fraction and the chromatin structure of 5S rDNA at 2, 3, 4 and 5 d post-germination.

In agreement with our previous results, in 2-day-old nuclei from cotyledons, the heterochromatic [4′,6-diamidino-2-phenylindole (DAPI)-stained] CCs were smaller than those in corresponding 3-week-old nuclei (Mathieu et al. 2003) and were considered to represent pre-CCs (Fig. 1A). In 2-day-old nuclei, the 5S rDNA probe predominantly hybridized to the heterochromatic pre-CCs. The 5S signals were compact (Fig. 1A) in 69% of the nuclei (Fig. 1C). Surprisingly, at 3 d post-germination, the 5S signals were strongly decondensed and clearly showed less co-localization with CCs compared with 2-day-old nuclei (Fig. 1A). Only 48% of the nuclei displayed compact 5S signals at this stage (Fig. 1C). At 4 d post-germination, 5S rDNA has begun to 're'condense, giving more compact FISH signals, and the fraction of nuclei which displayed compact 5S signals increased to 54% (Fig. 1A, C). 5S rDNA compaction at 5 d post-germination (Fig. 1C) was similar to that observed in 3-week-old nuclei (Mathieu et al. 2003), signifying that 5S rDNA chromatin structure is roughly fully developed at 5 d post-germination. At this stage, part of the 5S signal co-localizes with heterochromatin whereas the other fraction forms decompacted loops (supposed to contain the transcribed 5S rRNA genes) that emanate from CCs.

Whereas we were expecting a progressive decondensation (from a compacted 5S rDNA chromatin 2 d post-germination to a less compacted one in 5-day-old plants), the results show that starting from 2-day-old nuclei, heterochromatin, and 5S rDNA in particular, undergo a considerable decondensation followed by the 're'condensation of a 5S rDNA fraction to obtain almost mature nuclei 5 d post-germination.

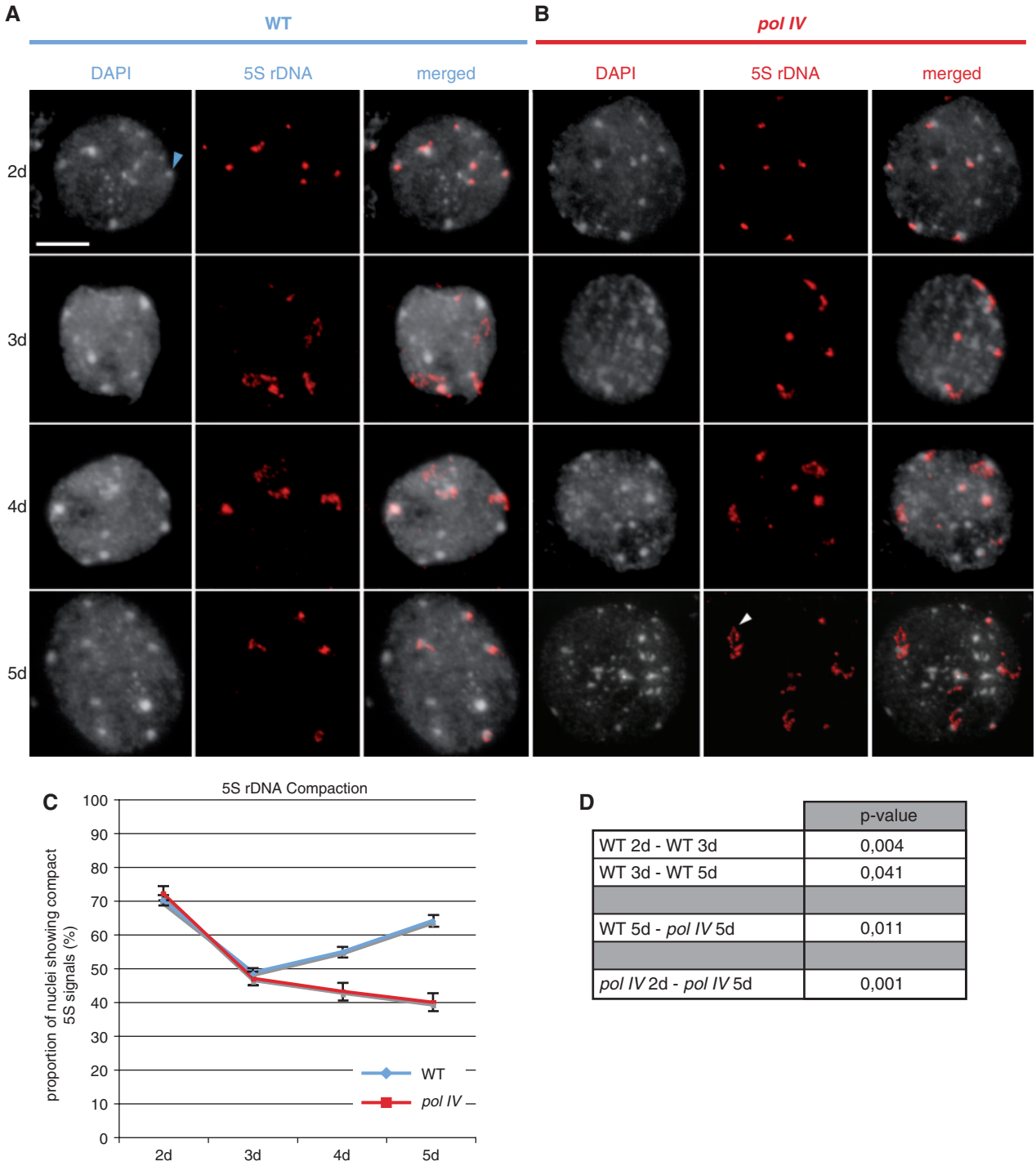
Pol IV is required for 5S rDNA 're'condensation

Compared with WT adult leaf nuclei, the *pol IV* mutant shows an increased number and decreased size of DAPI-positive heterochromatic foci associated with a 5S rDNA decondensation (Onodera et al. 2005). In order to identify pathways implicated in the reorganization of heterochromatin observed in the WT plants, we have investigated the *nrpd2a* Pol IV mutant (NRPD2A is the subunit common to the two Pol IVa and Pol IVb forms). We therefore analyzed remodeling of 5S rDNA repeats and heterochromatin in the *nrpd2a* mutant at 2, 3, 4 and 5 d post-germination.

First, 2- and 3-day-old *nrpd2a* nuclei show the same CCs and 5S signals as WT homologs (Fig. 1B). This is clearly illustrated by an identical proportion of nuclei with compact 5S signals in the WT and the *nrpd2a* mutant (Fig. 1C). The difference between the WT and the *nrpd2a* mutant became obvious 4 d post-germination, with an absence of 're'condensation of 5S chromatin in the mutant, and so the retention of decondensed 5S clusters (Fig. 1B, C). These results show that Pol IV is necessary for the 5S chromatin reorganization observed in WT plants.

Young WT nuclei contain supernumerary heterochromatic foci

Arabidopsis chromosomes ($n = 5$) display small, conspicuous heterochromatin segments (CCs) that mark the position of each (peri-)centromere and of the nucleolus-organizing regions (NORs) of chromosomes 2 and 4 (Fransz et al. 1998, Fransz and de Jong 2002). Fourteen CCs are expected in diploid nuclei, but, due to close association of CCs, most of the nuclei contain <10 CCs (Fransz and de Jong 2002). Surprisingly, WT plants between 3 and 5 d post-germination contained >14 DAPI-stained foci. The excess heterochromatic foci, smaller and distinct from the CCs, termed supernumerary foci (s-foci, Fig. 2A, B), were transient since they were absent in mature WT nuclei (3% of nuclei with s-foci, not shown). A larger proportion of *nrpd2a* nuclei contained s-foci (Fig. 2B) and maintained them (Onodera et al. 2005). These results show



D

	p-value
WT 2d - WT 3d	0,004
WT 3d - WT 5d	0,041
WT 5d - *pol IV* 5d	0,011
pol IV 2d - *pol IV* 5d	0,001

Fig. 1 5S rDNA chromatin organization in early development. (A and B) Counterstaining with DAPI (left); FISH with a 5S rDNA probe (middle); and the merge of both (right) on nuclei from WT (A) and *pol IV* (*nrpd2a*) plants (B) (Columbia background) at 2, 3, 4 and 5 d post-germination. The blue arrow points to a pre-chromocenter. The white arrow shows a 5S rDNA loop. Bar = 5 µm. (C) Proportion (%) of WT and *pol IV* (*nrpd2a*) nuclei harboring compact 5S signals at different times of development. Nuclei with a maximum of one diffuse 5S rDNA signal were considered as compact whereas nuclei with at least two diffuse 5S signals were considered as decompacted. A compact signal is a punctate signal. For each stage, between 50 and 90 nuclei were observed. The confidence interval is indicated for each value. (D) Table presenting the significant *P*-values of the graph in C, obtained from the one-tailed Z-test.

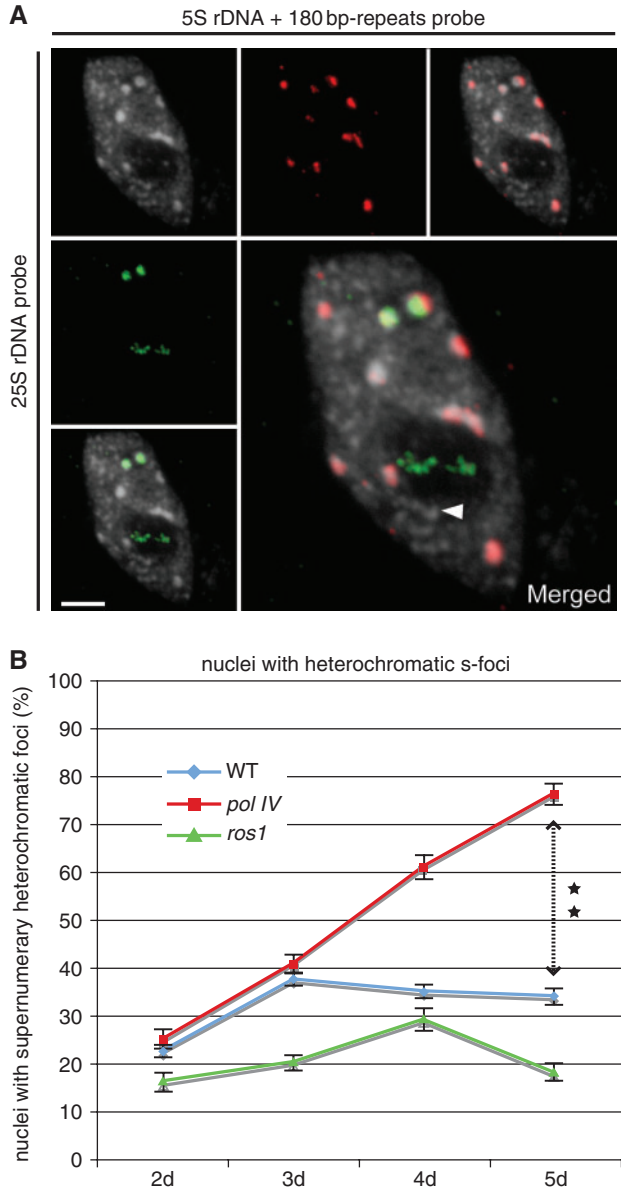


Fig. 2 Analysis of supernumerary foci. (A) Counterstaining with DAPI (top left); FISH with a 25S rDNA probe (green); FISH with a mix of 5S rDNA and 180 bp repeats probes (red); and the merged image on nuclei from 5-day-old WT nuclei. The white arrow shows s-foci, which hybridized with none of the probes. Bar = 5 µm. (B) Proportion (%) of WT, *pol IV* (*nrpd2a*) and *ros1* nuclei containing heterochromatic s-foci at different times of development i.e. the proportion of nuclei containing >14 heterochromatic foci. For each stage, between 50 and 90 nuclei were observed. Asterisks ($^{**}P<0.01$; one-tailed Z-test) indicate significant differences between the WT and *pol IV* values. The confidence interval is indicated for each value.

that Pol IV is necessary for the resorption of the s-foci observed in WT plants.

The s-foci hybridized neither with the 5S rDNA probe, nor with the 180 bp or 25S rDNA probe (Fig. 2A), which are repeated sequences present in the pericentromeric region of chromosomes 3, 4 and 5 (5S rDNA); on chromosomes 2 and 4 (25S rDNA); and in every chromosome centromere (180 bp repeats). (Peri)centromeric sequences such as TSI (transcriptionally silent information) (Steimer et al. 2000) or the Athila retrotransposon (Pelissier et al. 1995) co-localize with CCs in mature 3-week-old nuclei (Supplementary Fig. S1 online). They might provide FISH signals co-localizing with some of these s-foci in plantlet nuclei, therefore revealing a fragmentation of heterochromatin. The other possibility was that sequences which were normally euchromatic might transiently adopt a heterochromatic organization. To test this possibility, we used a probe composed of 13 bacterial artificial chromosomes (BACs) covering the euchromatic region of the chromosome 5 left arm. However, none of these probes (TSI, Athila or BACs) hybridized to these s-foci in 5-day-old nuclei (Supplementary Fig. S1) whose nature remains to be elucidated.

Post-germination changes of 5S rDNA methylation patterns

To determine whether the 5S rDNA chromatin reorganization observed was associated with modification of the 5S rDNA methylation pattern, we performed Southern blotting using methylation-sensitive restriction endonucleases. *Hpa*II cuts CCGG motifs but is inhibited by methylation of either cytosine, corresponding to contexts CG and CXG (with X different from G) (McClelland et al. 1994). *Hae*III recognizes GGCC but will not cut if the inner C is methylated. Digestion of 5S genes with these two enzymes reports on methylation at symmetrical CG/CXG contexts (*HpaII*), and asymmetrical CXX context (*HaeIII*). The Southern blots revealed ladders of bands at 500 bp intervals, the size of a 5S gene repeat (Campell et al. 1992). High levels of methylation cause most of the hybridization signal to be near the top of the ladder, whereas loss of methylation results in more signals near the bottom.

5S gene methylation at *Hpa*II (not shown) and *Hae*III sites was lower in *nrpd2a* mutants relative to their WT siblings, at each developmental stage (Fig. 3).

In WT plants, we observed a faint increase at symmetrical sites with *Hpa*II between 2 and 5 d post-germination, as already described in Mathieu et al. (2003). Interestingly, the *Hae*III digestion revealed a striking decrease of asymmetrical methylation between 2 and 5 d post-germination (Fig. 3).

In the *nrpd2a* mutant, two major observations can be made: first, the asymmetrical methylation revealed by *Hae*III at 5S genes is already lower at 2 days post-germination and remains lower, compared with WT homologs. This means that Pol IV is involved in setting DNA methylation patterns at 5S genes before 2 d of plant

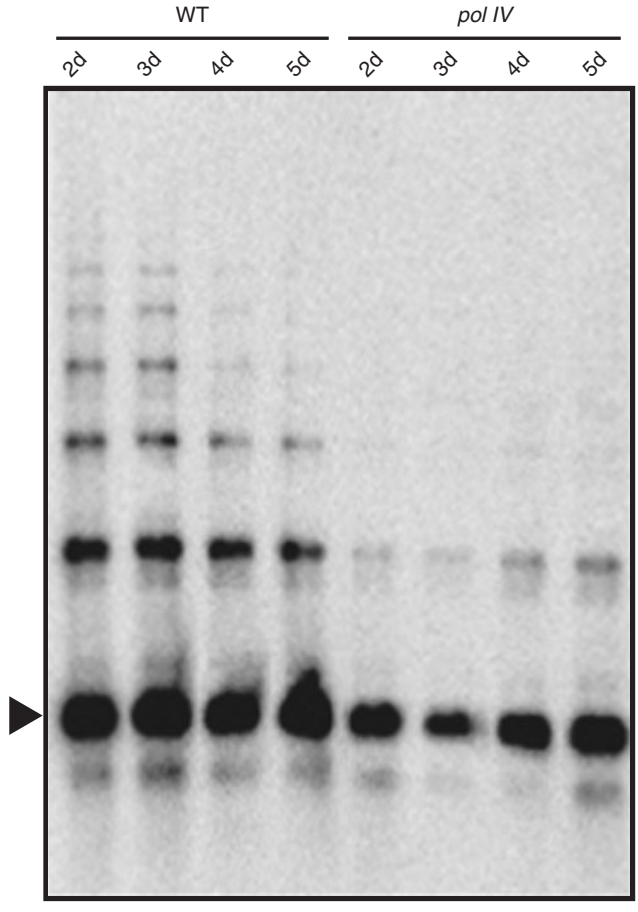


Fig. 3 5S rDNA methylation analysis in WT and *pol IV* plants. Genomic DNA (300 ng) of WT and *pol IV* (*nrpd2a*) genotypes was digested with *Hae*III. The DNA gel-blot was probed with 5S rDNA. Digestions were performed from plants at 2, 3, 4 and 5 d post-germination. The arrowhead shows a 0.5 kb 5S rDNA unit.

development, probably during the seed formation. Secondly, the decrease of asymmetrical DNA methylation observed in WT plants is absent in the *nrpd2a* mutant; however the demethylation is not mediated by Pol IV (see later).

These results show that important 5S rDNA methylation changes (particularly at the CXX position) accompany the major 5S rDNA chromatin remodeling observed between 2 and 5 d post-germination.

ROS1 mediates 5S rDNA demethylation

The absence of replication in the cotyledons between 2 and 5 d post-germination implies that the demethylation observed at 5S rDNA is not a passive process but an active one. Heterochromatic repetitive sequences such as 45S rDNA and many transposons represent targets for the DNA glycosylase/demethylase ROS1 (Gong et al. 2002, Kapoor et al. 2005, Agius et al. 2006). However, 5S rDNA has not been reported yet as a ROS1 target.

To assess the role of ROS1 in the demethylation of 5S rDNA at asymmetrical cytosines during the first days of development, we performed Southern blotting using the methylation-sensitive restriction enzyme *Hae*III and DNA from the WT and the *ros1* mutant at 2 and 5 d post-germination. As shown in Fig. 4A, in the *ros1* mutant the decrease of asymmetrical methylation is absent or nearly absent. We cannot exclude a slight decrease of methylation at the 5 d stage which could be performed by other glycosylases of the DEMETER family strongly expressed at that time (Ortega-Galisteo et al. 2008). These results show that ROS1 is necessary to erase 5S rDNA methylation at CXX positions during the first days of development.

Since methylation and compaction of highly repeated sequences are often correlated, an absence of 5S rDNA chromatin decompaction was expected in *ros1* plants. To confirm the role of ROS1 in 5S rDNA chromatin remodeling during early development, we performed 5S rDNA FISH experiments on *ros1* plants at 2, 3, 4 and 5 d post-germination. First, in contrast to the *pol IV* mutant, the cytological analysis revealed a comparable evolution of the s-foci in *ros1* and WT plants (Fig. 2B). Secondly, FISH experiments support the methylation results obtained by Southern blot. Indeed, the number of nuclei displaying compact 5S rDNA signals was equivalent in WT and *ros1* plants at 2 d post-germination (Fig. 4B, C). However, in 3-day-old nuclei, whereas 5S rDNA underwent a decondensation event in WT seedlings, the decondensation step did not occur in the *ros1* background (65% of nuclei at 2 d vs. 73% in 3-day-old nuclei displayed compact 5S rDNA). Naturally, no 're'condensation event was observed in *ros1* nuclei. Five-day-old *ros1* plants displayed the same proportion of nuclei with compact 5S rDNA as their WT counterpart and showed the same mature features (i.e. part of the signal is euchromatic and the other part is heterochromatic).

These results show the ROS1 independence of s-foci and the role of ROS1 in the decondensation event occurring at 3 d post-germination.

Discussion

The centromere region of Arabidopsis chromosomes consists of the core region which is mainly composed of 180 bp tandem repeats (Haupt et al. 2001) and flanking pericentromeric domains, which contain 5S rDNA, transposons and other dispersed repeats (Fransz et al. 1998, Tutois et al. 1999).

Our previous work prompted us to think that 5S rDNA chromatin had to undergo a progressive decondensation during post-embryonic development to establish its

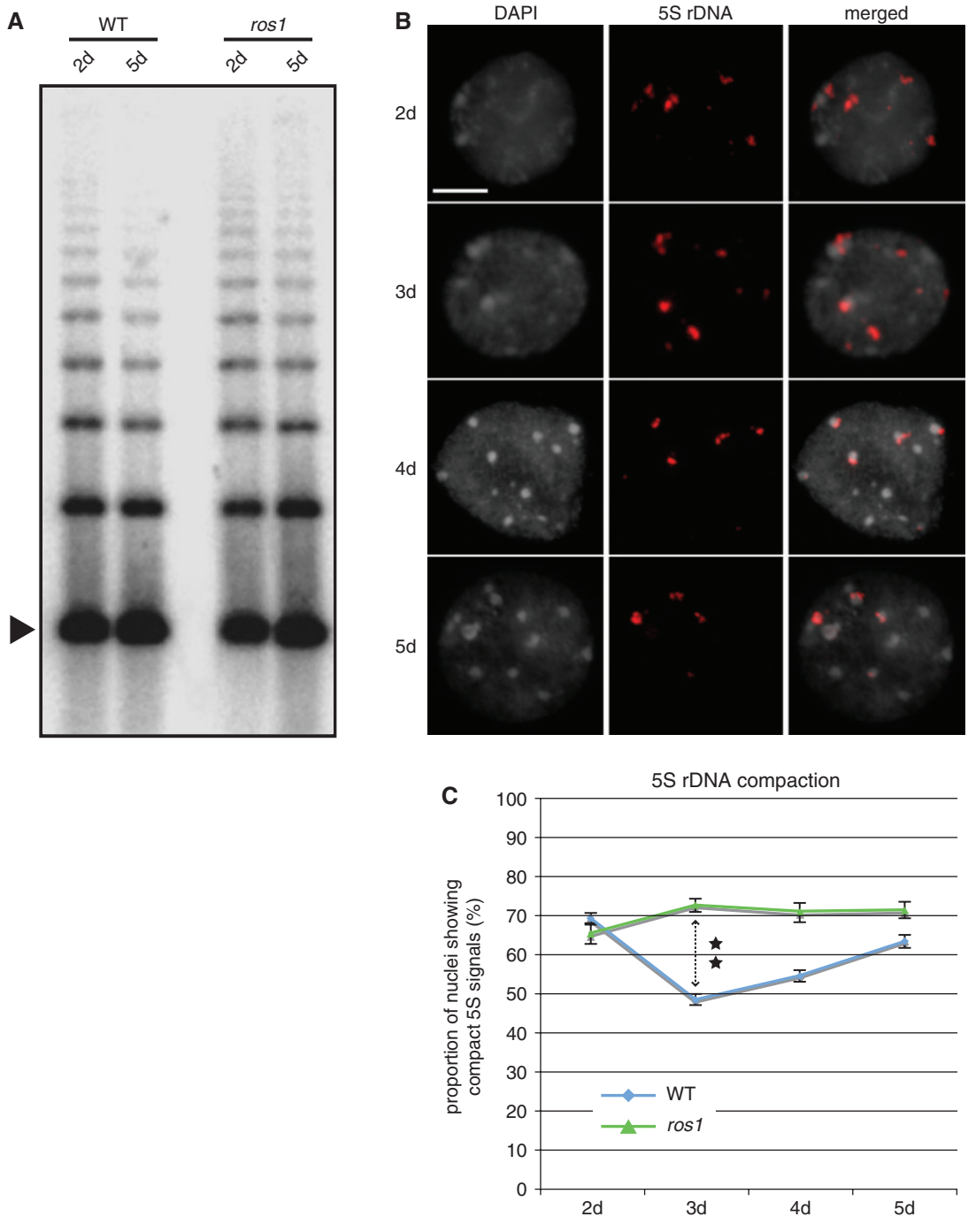


Fig. 4 ROS1 mediates 5S rDNA demethylation. (A) Genomic DNA (300 ng) of WT and *ros1* plants was digested with *Hae*III. The DNA gel-blot was probed with 5S rDNA. Digestions were performed from plants at 2 and 5 d post-germination. The arrowhead shows a 0.5 kb 5S rDNA unit. (B) Counterstaining with DAPI (left); FISH with a 5S rDNA probe (middle); and the merge of both (right) on nuclei from *ros1* plants at 2, 3, 4 and 5 d post-germination. Bar = 5 µm. (C) Proportion (%) of WT and *ros1* nuclei harboring compact 5S signals at different times of development. Nuclei with a maximum of one diffuse 5S rDNA signal were considered as compact, whereas nuclei with at least two diffuse 5S signals were considered as decompacted. A compact signal is a punctate signal. For each stage, between 50 and 90 nuclei were observed. The confidence interval is indicated for each value. Asterisks ($^{**}P < 0.01$; one-tailed Z-test) indicate a significant difference between the WT and *ros1* values. The confidence interval is indicated for each value.

mature organization (i.e. part of the 5S rDNA is located within heterochromatic CCs whereas the other fraction forms loops with euchromatic features, considered to contain the transcribed 5S genes, that emanate from CCs) (Mathieu et al. 2003). Surprisingly, FISH results showed that, starting from 2-day-old nuclei, a large and rapid 5S rDNA chromatin decondensation occurs, which precedes a 're'condensation of a 5S rDNA fraction to obtain almost mature nuclei 5 d post-germination. In this study, we provide evidence that ROS1 is responsible for the decondensation event and Pol IV is necessary for the reorganization phenomenon. We observed the same decondensation event between 2 and 3 d post-germination in WT and *nrpd2a* plants. However, the difference between them became obvious at 4 d post-germination. The absence of 're'condensation of 5S rDNA in the *nrpd2a* mutant clearly demonstrates the role of Pol IV in the establishment of the 5S rDNA heterochromatin fraction during early development.

Several publications demonstrated a correlation between the formation of pericentromeric heterochromatin and cell differentiation during mammalian early development (Cammas et al. 2002, Rangasamy et al. 2003, Probst et al. 2007, Puschendorf et al. 2008). For example, the first rearrangement of the pericentromeric heterochromatin into somatic CCs takes place at the 2-cell stage in the mouse. According to Probst and Almouzni (2007), this 2-cell stage might be the first opportunity to express an RNA component that could contribute to CC organization. In *Arabidopsis thaliana*, the two plant-specific nuclear Pol IV forms, Pol IVa and Pol IVb (Herr et al. 2005, Kanno et al. 2005, Pontier et al. 2005, Vaucheret 2005), mediate siRNA- and DNA methylation-dependent heterochromatin formation (Onodera et al. 2005).

In the same window of time (i.e. 2–5 d post-germination), we noticed changes in the methylation pattern of 5S rDNA in WT plants. We observed a faint increase of 5S rDNA methylation at symmetrical sites in accordance with Mathieu et al. (2003). We also observed a marked decrease of methylation at asymmetrical cytosines mediated by the demethylase ROS1. Most of the loci affected by *ros1* are affected in non-CG contexts (Zhu et al. 2007). The observed absence of asymmetrical demethylation in *nrpd2a* mutant results from the down-regulation of *ROS1* in the *pol IV* mutant (Huettel et al. 2006, Mathieu et al. 2007). 5S rDNA is strongly methylated in the seed (Mathieu et al. 2003) and at 2 d post-germination (this work) in WT plants. In *nrpd2a* plants, the 5S rDNA methylation is lower compared with WT plants whatever the methylation context or the age of the plants, confirming the role of Pol IV in the 5S rDNA methylation. The lower methylation of 5S rDNA in *nrpd2a* plants compared with WT plants 2 days post-germination suggests that Pol IV activities are already needed during seed formation. Accordingly, Pontier et al. (2005) reported a very abundant accumulation of NRPD1a and NRPD1b proteins, two Pol IV subunits, in the reproductive phase (flowers) compared with the vegetative phase (leaves). However, 5S rDNA compaction is not affected in 2-day-old *nrpd2a* nuclei. This shows that mechanisms other than methylation are involved in DNA compaction in the quiescent seed. Seeds are characterized by a high degree of dehydration known to play a significant role in DNA conformational structures (Zluvova et al. 2001).

Maturation of (hetero)chromatin goes through the presence of heterochromatic foci which disappear in mature WT nuclei but remain in *nrpd2a* nuclei. *nrpd2a* nuclei retain at least two characteristics of immature nuclei: the decompaction of pericentromeric sequences such as 5S rDNA and the maintenance of heterochromatic foci. Although ROS1, together with Pol IV, participates in the maturation of the 5S rDNA chromatin, maturation of the transient heterochromatic foci does not seem to depend on ROS1.

Nuclear reorganization should be considered as an important driving force in the regulation of developmental gene expression. During early mammalian development, chromatin remodeling and, hence, nuclear reorganization is thought to be functionally linked to transcriptional activation during embryo pre-implantation (De La Fuente et al. 2004, Martin et al. 2006). In plants, conformational changes of DNA (Boubriak and Osborne 1994) and large global demethylation events (Drozdenyuk and Vanyushin 1976, Follmann and Schleicher 1990) take place during germination and obviously reflect a transition from the metabolically quiescent seed to the actively growing and developing seed (Zluvova et al. 2001). In the *Arabidopsis suecica* allotetraploid hybrid, changes in NOR condensation and chromatin modifications correlate with changes in 45S rDNA gene expression during post-germination early development (Pontes et al. 2007).

In conclusion, we have shown that ROS1 and Pol IV act together to remodel the 5S chromatin via DNA methylation modifications. The viability of *ros1* plants shows that 5S RNA transcription can occur without the major 5S rDNA chromatin decondensation event. We cannot exclude that *ros1* mutant has developed alternative pathways to ensure an appropriate ribosome production (a situation encountered several times; Vaillant et al. 2007, Vaillant et al. 2008), or that these remodeling events facilitate an efficient transcription. However, according to Matzke et al. (2007), the elaboration of the Pol IV pathway in the plant kingdom might reflect the need for rapid, reversible changes in gene expression. Thus it is reasonable to think that in the WT context, the decondensation might allow unlocking of a fraction of 5S rDNA units able to respond to environmental changes. In this case, the 5S rDNA cytological difference in *ros1* and WT 5-day-old nuclei is expected to

be subtle. The Pol IV/ROS pathway might control a fraction of the 5S rRNA genes, i.e. the plastic 5S rDNA fraction.

Materials and Methods

Plant material

Arabidopsis thaliana WT, *pol IV* (*nrpd2a-1*) and *ros1-1* plants were from the Columbia ecotype. *nrpd2a-1* seeds were obtained from the Arabidopsis Biological Resource Center (Stock # SALK 095689) and *ros1-1* seeds were obtained from Dr. J. K. Zhu (University of California, Riverside, California, USA). After synchronization for 2 d at 4°C, seeds were grown on a germination medium [MS Salt (Duchefa biochimie, Haarlem, The Netherlands) supplemented with 3% sucrose and 0.8% BactoAgar] in a growth chamber using a 16 h light (120 μE m^{-2} s^{-1})/8 h dark regime at 23°C, and cotyledons (first leaves developed during embryogenesis) were collected at 2–5 d post-germination.

Nucleic acid isolation and gel-blot analysis

Total genomic DNA was isolated using the DNeasy kit (Qiagen, Courtaboeuf, France). Digestions were realized with 300 ng of Arabidopsis genomic DNA and 20 U of restriction enzyme in the recommended buffer (New England Biolabs, UK). Digested DNA was electrophoresed in 0.8% agarose gels overnight, depurinated in 0.25 N HCl and capillary blotted onto Hybond-N+ membranes (Amersham, GE Healthcare, Saclay, France).

DNA probes were labeled with [α-^{32}P]dCTP using a random hexamer priming method (Megaprime DNA labelling system, Amersham). Quantifications were done on a PhosphorImager (Molecular Imager FX, Bio-Rad). For quantification of 5S rDNA methylation, the radioactivity of the 0.5 kb band (which corresponds to monomeric units of 5S rDNA) was compared with the radioactivity of the whole lane.

Fluorescent in situ hybridization

Prior to use, tissues were fixed in ethanol/acetic (3 : 1) solution. Probes were labeled by PCR using gene-specific primers with biotin-16-UTP (Roche, Meylan, France) or digoxigenin-11-UTP (Roche). FISH experiments were performed according to Schubert et al. (2001). When biotin-labeled probes (5S rDNA or 5S rDNA mixed with 180 bp repeats) were used, avidin conjugated with Texas Red (1 : 500; (Vector Laboratories, Burlingame, CA, USA) followed by goat anti-avidin conjugated with biotin (1 : 100; Vector Laboratories) and avidin–Texas Red (1 : 500) were used for the detection. When biotin-labeled (5S rDNA mixed with 180 bp repeats) and digoxigenin-labeled (45S rDNA) probes were used together, avidin–Texas Red (1 : 500) followed by goat anti-avidin conjugated with biotin (1 : 100) and avidin–Texas Red (1 : 500) were used for the detection of the biotin-labeled probe, and mouse anti-digoxigenin (1 : 125; Roche) followed by rabbit anti-mouse fluorescein isothiocyanate (FITC) (1 : 500; Sigma) and Alexa 488-conjugated goat anti-rabbit (Molecular Probe, Eugene, OR, USA) were used for the detection of the digoxigenin-labeled probe. Before microscopic analysis, nuclei were stained with DAPI.

Microscopy and image processing

For microscopic analysis, an epifluorescence Imager Z1 microscope (Zeiss) with an Axiocam MRm camera (Zeiss) was used. Fluorescence images for each fluorochrome were captured separately through the appropriate excitation filters. The images were pseudocolored, merged and processed with the Adobe Photoshop software (Adobe Systems). Between 50 and 90 nuclei were analyzed for each developmental stage.

Statistical analysis

Statistical analyses were performed on cytological results for 5S rDNA compaction and s-foci. In both cases a comparison of proportions Z-test was used. The probabilities were calculated from a one-tailed test. The confidence interval was calculated for each proportion with a confidence level of 99%. Between 50 and 90 nuclei were analyzed for each developmental stage.

Supplementary data

Supplementary data are available at PCP online.

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