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A Pol V–Mediated Silencing, Independent of RNA–Directed DNA Methylation, Applies to 5S rDNA

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Abstract

The plant-specific RNA polymerases Pol IV and Pol V are essential to RNA–directed DNA methylation (RdDM), which also requires activities from RDR2 (RNA–Dependent RNA Polymerase 2), DCL3 (Dicer-Like 3), AGO4 (Argonaute), and DRM2 (Domains Rearranged Methyltransferase 2). RdDM is dedicated to the methylation of target sequences which include transposable elements, regulatory regions of several protein-coding genes, and 5S rRNA–encoding DNA (rDNA) arrays. In this paper, we have studied the expression of the 5S-210 transcript, a marker of silencing release at 5S RNA genes, to show a differential impact of RNA polymerases IV and V on 5S rDNA arrays during early development of the plant. Using a combination of molecular and cytological assays, we show that Pol IV, RDR2, DRM2, and Pol V, actors of the RdDM, are required to maintain a transcriptional silencing of 5S RNA genes at chromosomes 4 and 5. Moreover, we have shown a derepression associated to chromatin decondensation specific to the 5S array from chromosome 4 and restricted to the Pol V–loss of function. In conclusion, our results highlight a new role for Pol V on 5S rDNA, which is RdDM–independent and comes specifically at chromosome 4, in addition to the RdDM pathway.

Introduction

The plant-specific RNA polymerases firstly named Pol IVa and Pol IVb and now referred as Pol IV and Pol V [1], contribute to siRNA production and are essential to RNA-directed DNA methylation (RdDM) [2–6]. The revised nomenclature denotes the largest subunits of Pol IV and Pol V as NRPD1 and NRPE1. Both Pol IV and Pol V share the second largest subunit NRPD2. In current models of RdDM silencing pathway [7–10], Pol IV is speculated to produce single-stranded RNA transcripts from heterochromatic repeated regions. These transcripts, converted onto double-stranded RNAs by RDR2 (RNA–DEPENDENT RNA POLYMERASE 2), are processed into siRNAs duplexes by DCL3 (Dicer-Like 3) [11–14]. The resulting siRNAs are methylated at 2′ hydroxyl groups of 3′-terminal nucleotides by HEN1 (HUA ENHANCER 1) prior to loading into AGO4/RISC complex [15]. Recently, Pol V and DRD1 (DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1) were found to be required to mediate production of non-coding transcripts which are necessary for steps downstream of siRNA biogenesis [1]. Recent evidence suggests that siRNAs/AGO4 complexes bind to Pol V transcripts, ultimately guiding the de novo DNA methyltransferase DRM2 [16] and histone modifying complexes to the target loci [17,18].

Targets of the Pol IV/Pol V–dependent RdDM include transposable elements, regulatory regions of several protein-coding genes and 5S rRNA-encoding DNA (rDNA) arrays [2–5,19–23]. We [24] and others [4,5,11] have reported changes in 5S rDNA methylation, 5S rDNA chromatin compaction and 5S siRNA accumulation in Pol IV/V mutants. However, in these reports, 5S rDNA arrays, which have separate functions and locations [For a review, [25]] were considered together.

Arabidopsis thaliana contains approximately 1000 copies of 5S RNA genes per haploid genome. 5S rDNA is arranged in tandem arrays [26] located within the pericentromeric heterochromatin of chromosomes 3, 4 and 5 in the Columbia accession [27,28]. Only 5S-repeat clusters located on chromosomes 4 and 5 are transcribed by Pol III to produce the 120 nucleotide (nt) transcripts which are integrated into ribosomes [29]. Nevertheless, it is not yet clear what proportion of these 5S genes is active at any one time. Indeed, both active 5S-repeat clusters contain transcribed and repressed 5S RNA genes in WT plants. Previous study revealed that in adult wild-type plants, only «major» 5S RNA genes were expressed whereas «minor» genes, which diverge from «major» ones at only one or several positions, are repressed [30]. In addition to «major» and «minor» 5S RNA species, we have previously identified an atypical 5S RNA (5S-210) composed of genic and intergenic regions which is a marker of silencing release at 5S RNA genes [31]. The presence of a chromosome-specific T stretch identifies the chromosome origin of 5S-210 transcripts [29].

To determine the individual contribution of Pol IV and Pol V in the transcriptional silencing and heterochromatic state of each 5S array, we assayed 5S-210 transcript expression, chromatin compaction and DNA methylation during early development. We have shown that Pol IV, Pol V and several actors of the RdDM, contribute to maintain a transcriptional silencing of 5S RNA genes at chromosomes 4 and 5. Moreover, we showed an additional Pol V activity, Pol IV- and RdDM-independent which drives silencing of...
5S rDNA specifically at chromosome 4. The large silencing release observed at chromosome 4 in NRPE1 and NRPE5a (Pol V) mutants, is accompanied by a decompaction of the corresponding 5S rDNA locus, as well as decompaction of NOR loci.

Results

Different impact of Pol IV and Pol V on 5S RNA genes silencing

We previously identified a 5S transcript, 210 bases-long (5S-210) which is a marker of silencing release at 5S RNA genes. This 5S-210 transcript homologous to the 120 nt genic region and 90 nt from the adjacent intergenic region [31] contains the sequence of the chromosome-specific T-stretch which identifies its 5S array-origin (Figure 1).

In order to define the impact of Pol IV (formerly Pol IVa) and Pol V (formerly Pol IVb) on 5S RNA genes silencing, we analysed 5S-210 accumulation by RT-PCR experiments in WT, nrpd1 (mutant of the largest subunit of Pol IV), nrpd2 (mutant of the common subunit of Pol IV and Pol V), nrpe1 (mutant of the largest subunit of Pol V) and nrpe5a (mutant of a new Pol V-specific subunit; [32]) plants. RDR2 (RNA-DEPENDENT RNA POLYMERASE 2), DCL3 (DICER-LIKE 3), AGO4 (ARGONAUTE 4), DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2), DCL3 (DICER-LIKE 3), AGO4 (ARGONAUTE 4), DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2), HEN1 (HUA ENHANCER 1) and HDA6 (HISTONE DEACETYLASE 6) involved with Pol IV and POL V in the production of siRNAs and the associated DNA methylation and histone modifications, were also tested.

5S-210 transcripts overaccumulated by a factor between 2 and 2.5 in nrpe1, nrpd2 and nrpe5a compared to WT plants. On the contrary, nrpd1, rdr2, dcl3, hau1, ago4, drm2 and sil1 (mutant allele of HDA6) plants accumulate similar 5S-210 transcripts quantities than WT (Figure 2A and 2B).

The release of silencing in nrpe1 and nrpe5a indicates that Pol V is involved in the quantitative regulation of 5S-210 expression. The results obtained with nrpd2 confirm these observations. The absence of silencing release in nrpd1, rdr2, dcl3, hau1, ago4, drm2 and sil1 shows that they have no influence, at the quantitative level, on 5S-210 RNA transcription.

Pol V mediates repression of 5S RNA genes from chromosome 4

5S rDNA arrays are located within the pericentromeric heterochromatin of chromosomes 3, 4 and 5. We assessed whether this Pol V-mediated silencing operates on all 5S arrays or operates selectively. Using the T stretch signature [29], we analysed the origin of the 5S-210 transcripts in Pol IV/Pol V mutants and in two mutants of

Figure 1. Schematic representation of a 5S rDNA unit and 5S-210 transcript. (A) A 5S rDNA unit contains the 120 bp-genic region followed by the intergenic region containing the 5S array-specific T-stretch. Boxes represent differences between the T-stretch from chromosome 4 and chromosome 5 left arm. (B) The 5S-210 transcript is homologous to the 120 nt-genic region and 90 nt from the adjacent intergenic region.

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proteins involved in respectively upstream and downstream RdDM steps i.e. RDR2 and DRM2. Sequencing of RT-PCR products revealed that 5S-210 transcripts only originate from the transcriptionally active 5S-repeat clusters located on chromosomes 4 and 5. In WT conditions, the 5S array from chromosome 5 contributes for 70%, the 5S array from chromosome 4 contributing for the remaining 30% of the 5S transcripts (Figure 2C). The proportions and quantities are not significantly different in npd1, nrpe1, nrpd2, rdr2, dcl3, hen1, drm2, ago4, sil1, and nrpe5a plants. (A) Expression of ACTIN 2 was used to normalize the 5S-210 amounts. Negative controls were performed without reverse transcriptase (no RT). (B) A graphic representation of 5 to 7 independent experiments is given. The standard deviation of the mean is indicated on each bar. Asterisks indicate values significantly different from the WT value which is arbitrarily fixed as 1 (Mann-Whitney test, * P<0.05). (C) Proportion of 5S-210 transcripts from chromosomes 4 and 5. RT-PCR products from (B) were sequenced and the T-stretch signature analysed to determine the 5S-array origin of 5S-210 transcripts. A total of 70, 53, 68, 51, 39, and 52 5S-210 cDNA clones were sequenced for WT, npd1, nrpe1, nrpd2, rdr2 and drm2 respectively. Statistics were done to appreciate the participation of 5S array from chromosome 4. Asterisks denote significant differences to the WT value (Fisher’s exact test, ** P<0.001).

These results refine our previous conclusions indicating that the silencing release observed in nrpe1 concerns the 5S array from chromosome 4; the results obtained with nrpd2 confirm these observations. Therefore, the Pol V-mediated repression operates on the 5S array from chromosome 4.

Pol V drives compaction of 5S rDNA from chromosome 4

In order to determine whether chromatin decompaction is associated to the release of silencing observed at chromosome 4, we performed FISH experiments with both 5S rDNA and 45S rDNA probes on WT, npd1, nrpe1 and nrpd2 plants. The chromosome 4 is the only chromosome to carry both rDNA species. 5S rDNA from
chromosome 4 colocalizes with 45S rDNA signals in almost all nuclei [33]. 5S signals outside chromosome 4, i.e. coming from chromosomes 3 and 5 were considered together (Figure 3A and 3B).

FISH analysis revealed that 36% of the WT nuclei contain one or two decondensed 5S signals at chromosome 4. The proportions are similar in WT and nrpd1 mutant whereas a significant larger proportion (59%) of the nrpe1 and (77%) of nrpd2 nuclei harbor decondensed 5S signals at chromosome 4. From these analyses, we conclude that 5S arrays from chromosome 4 are decondensed in nrpe1, and these results are confirmed with nrpd2 observations, whereas nrpd1 has no visible effect on this compaction. Therefore, NRPE1/Pol V has the ability to act on the chromatin of 5S rDNA from chromosome 4 in a NRPD1/Pol IV- independent manner.

We observed no significant variation in the proportion of nuclei with decompacted 5S signals at chromosomes 3 and 5 in WT, nrpd1, nrpe1 and nrpd2 plants (Figure 3B). These results show that nrpd1 and nrpe1 mutations have no visible effect on chromatin compaction of 5S arrays from chromosomes 3 and 5. From these analyses, we conclude there is a correlation between the large silencing release and the decompaction of 5S rDNA at chromosome 4 in nrpe1 mutant.

Pol V drives compaction of 45S rDNA

A question arises from these results: how and why is the 5S rDNA array from chromosome 4 concerned by this particular Pol V regulation? The main difference between 5S arrays from chromosomes 4 and 5 is the close proximity of NOR4 (Nucleolar Organizing Region) with the 5S array from chromosome 4. Therefore, we hypothesized that both rDNA arrays (5S and 45S) might be co-regulated and be the site of common decompaction events.

We analyzed the NOR condensation in WT, nrpd1, nrpe1 and nrpd2 mutants (Figure 3A and 3C). There are four NORs in diploid A. thaliana, but they tend to coalesce and in agreement with Pontes et al. [34] we detected two to four NORs in 79% of WT nuclei and more than four signals in 21% of them. Similar results were obtained in nrpd1 nuclei. Contrarily, more than four NORs FISH signals were observed in 37% and 39% of nrpe1 and nrpd2 nuclei reflecting a significant increase of NOR decompaction compared to WT and nrpd1 nuclei.

These results show that no detectable chromatin decompaction is observed for NOR loci and 5S array from chromosome 4 in nrpd1 mutant whereas a concomitant decompaction event is observed for 5S rDNA from chromosome 4 and 45S loci in nrpe1 and nrpd2 mutants. This might illustrate a common regulation, Pol V-mediated, at 45S and adjacent 5S rDNA.

RdDM qualitatively controls 5S-210 RNA transcription

The quantitative 5S RNA derepression associated with chromatin decompaction is unambiguously limited to the 5S array from chromosome 4 in PolV-loss of function mutants. However, previous results have unequivocally shown that 5S rDNA is hypomethylated in NRPD1, RDR2 and DRM2 mutants of the RdDM pathway [4,5,11,24,35]. 5S rDNA loci consist of both active and heterogenous copies of the 5S RNA gene. We previously showed that the release of silencing of 5S RNA genes illustrated by the increase in the proportion of “heterogenous” 5S transcripts (i.e. containing some mutations in the genomic region) can occur without changes of the 5S RNAs quantity [30,36].

We therefore decided to analyze the heterogeneity of 5S-210 transcripts produced by 5S arrays from chromosomes 4 and 5 to test whether NRPD1, RDR2, DRM2 and NRPE1 act on each 5S array. As shown Table 1, the proportion of heterogenous 5S RNA from chromosomes 4 and 5 is enhanced in nrpd1, nrpe1, nrpd2, rdr2 and drd2 compared to the WT. It demonstrates the impact of Pol IV, RDR2, DRM2 and Pol V on the 5S array from both chromosomes.

These results refine our previous conclusions indicating that silencing of 5S RNA genes from chromosome 3 is controlled by Pol IV, RDR2, DRM2 and Pol V at the qualitative level. Their mutation has an equivalent effect i.e. a derepression of heterogenous 5S RNA genes is observed without increasing the total
amount of 5S-210 RNA. Silencing of 5S RNA genes from chromosome 4 is controlled by Pol IV, RDR2, DRM2 and Pol V at the qualitative level, and Pol V exerts an additional role acting at the quantitative level.

The results show that Pol IV, RDR2, DRM2 and Pol V act in the RdDM pathway, to maintain the repression of heterogenous 5S RNA genes from chromosomes 4 and 5. They also show a specific and additional role of Pol V, Pol IV-, RDR2- and DRM2-independent and therefore RdDM-independent, on 5S array from chromosome 4. There is therefore a differential Pol V impact on 5S arrays from chromosomes 4 and 5.

Pol V--additional activity is not associated with changes in asymmetric DNA methylation

To confirm that Pol V-additional activity at chromosome 4 is RdDM-independent, we assayed cytosine methylation in asymmetric sequence context (CHH), which largely results from RdDM. Indeed, if the Pol V-additional activity is RdDM-independent, a lower DNA methylation in *nrpd1* and *nrpd2* compared to RdDM mutants is not expected [3,37,38]. Digestion with NlaIII for which the same restriction site is present in the intergenic spacer of every 5S rDNA unit of chromosomes 4 and 5 and PCR amplification with primers hybridizing to the chromosome-specific T-stretch were performed. As shown Figure 4, there is a reduction of CHH (CAT for NlaIII) methylation in *nrpd1*, *nrpd2*, *rdr2* and *drm2* mutants compared to the WT for both 5S rDNA arrays. Moreover, the same reduction of methylation is observed at chromosome 4 in all the mutants. These results reveal that derepression and decompaction of the 5S array at chromosome 4 in *nrpd1* is not associated with specific changes of 5S rDNA asymmetric methylation. They also show that the similar reduction of methylation observed at both arrays results from the loss of RdDM pathway.

Discussion

Previous results have shown that 5S rDNA is subject to a variety of overlapping regulation pathways, such as the limiting amount of TFIIIA (TRANSCRIPTION FACTOR IIIA; 5S rDNA specific) [30], the methylation-independent MOMI pathway [31], the DDM1/MET1- pathway [30,39], as well as the Pol IV/Pol V RdDM [4,24,39]. Although the impact of the Polymerases IV and V on 5S rDNA was previously demonstrated on the basis of DNA hypomethylation, decrease of 5S small RNA accumulation and chromatin decompaction [4,24,39], the relative impact of Pol IV and Pol V, their potential selective action on the different 5S arrays and the consequence of their mutation on 5S rDNA silencing of each 5S array were unknown.

In this paper, we have shown that silencing of 5S RNA genes from chromosomes 4 and 5 is controlled at the qualitative level by RdDM including Pol IV, RDR2, DRM2 and Pol V activities. Loss of this silencing pathway leads to the derepression of heterogenous 5S RNA genes, without an increase of total 5S RNA amount and without a detectable chromatin decompaction. These results are consistent with the previously reported reduction or elimination of
5S siRNAs (the 1003 siRNA) as well as with the 5S rDNA hypomethylation observed in npd1, rd2, dm2, npel, ago4 or dcl3 mutants [4,5,11,24,40]. Our results demonstrate that each of the two 5S arrays is a target of RdDM. Derepression of heterogeneous 5S RNA genes at chromosomes 4 and 5 in mutants of the RdDM pathway is associated with reduction of asymmetric methylation at each array.

In addition to the RdDM process common to 5S arrays from chromosomes 4 and 5, an additional Pol V activity specifically applies to chromosome 4. Higher amounts of 5S-210 transcripts from chromosome 4 are observed in npel and npel5a, two Pol V-specific subunits, correlating with a specific decompaction of this 5S rDNA array in npel and npel2. On the contrary, similar amounts of transcripts were observed in WT and all the tested RdDM mutants, and chromatin decompaction is absent in npel1. Therefore, the additional role of Pol V on chromosome 4 is Pol IV- and RdDM-independent. Moreover, the Pol V activity observed specifically at chromosome 4 is not associated with changes of 5S rDNA asymmetric methylation, in agreement with the similar global 5S rDNA methylation observed in npd1 and npel [symmetric and asymmetric methylation; [5]. It suggests that Pol V complex might recruit other repressive epigenetic marks than DNA methylation and probably also other than H3K9me2, since 5S rDNA is not depacked in met1 mutants [36,41] despite a decrease of H3K9me2 [42]. H3K27me2, a repressive mark methylation-independent, which labels 5S rDNA [41], is a potential candidate.

The release of silencing of 5S RNA genes that we observed in RdDM mutants without changes of the 5S RNA quantities might be surprising. However, we previously observed this phenomenon in several mutants [36]. Moreover, in S. cerevisiae the number of active rRNA genes can change more than twofold without changing steady-state rRNA transcript levels [43]. On the contrary, higher amounts of 5S-210 transcripts from chromosome 4 are observed in npel1 and npel5a, suggesting that the large 5S rDNA decompaction enhances the transcription i.e. it might facilitate the access to transcription factors and/or the reinitiation process.

The needs for stoichiometric amounts of 5S and 45S rRNA implies that co-regulation events apply to 5S and 45S rDNA. We have shown that NOR loci and 5S array from chromosome 4 are decompacted in npel and npel2 mutants illustrating a common regulation process. This Pol V activity, NRPD1 and RdDM-independent, suggests that an alternative pathway i.e. without NRPD1 and RdDM partners, exists to target 5S and 45S rDNA loci.

Our results open the question about the interest for the plant to have a 5S array whose regulation is more dependent on Pol V. There is a need of 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 gene activity.

In conclusion, our results provide new insights on Pol V function in 5S rDNA regulation. On the basis of previous results [4,5,11,24] it was supposed that RdDM (including Pol IV, RDR2 and Pol V activities) was responsible for both silencing and compaction of 5S RNA genes. The current study confirms the participation of both Pol IV and Pol V in the 5S RNA gene silencing of each array, and clearly shows that 5S RNA silencing and compaction are not necessarily linked. It demonstrates that 5S rDNA decompaction is due to the sole Pol V’s loss of function, and not to that of both Pol IV and Pol V. Finally, it shows that Pol V has two different activities on the same 5S array, one in the RdDM pathway and the other one, RdDM-independent and chromatin-based which is not associated with methylation changes in early development.

**Materials and Methods**

**Plant material**

*Arabidopsis thaliana* npd1a-1 (npd1), npd1b-1 (npel1), npd2a-1 (npd2), rd2-1, dcl3-1, dm2-2 and the corresponding wild type young plants were from Columbia ecotype, npd1a-1, npd1b-1, rd2-1, dcl3-1 seeds were obtained from Dr T. Lagrange (University of Perpignan, France), npd2a-1 and dm2-2 seeds were obtained from the Arabidopsis Biological Resource Center (Stock # SALK 095689 and 150863 respectively). *sill*, *ago4-1* and WT plants were in *Landsberg erecta* background. Seeds of WT, *sill* and *ago4-1* plants were obtained from the NASC (stock numbers NW20 for Ler-0, N1894 for *sill* and N6364 for *ago4-1*). *npel5a-1* and WT WS seeds were obtained from Dr T. Lagrange. After synchronization 2 days at 4°C, seeds were grown on a germination medium (MS Salt [Duchefa Biochemie] supplemented with 3% sucrose and 0.8% BactoAgar) in a growth chamber using a 16 h light (120 µE.m⁻².sec⁻¹)/8 h dark regime at 23°C. Plantlets at 4 days post-germination were used.

**Fluorescent in situ hybridization (FISH)**

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) or digoxigenin-11-UTP (Roche). FISH experiments were performed according to Mathieu et al. (2003). Biotin-labeled (5S rDNA) and digoxigenin-labeled (25S rDNA) probes were used. Avidin conjugated with Texas Red (1:500; Vector Laboratories) followed by goat anti-avidin conjugated with biotin (1:100; Vector Laboratories) and avidin–Texas Red (1:500) were used for the detection of the biotin-labeled probe; mouse antidi-goxigenin (1:125; Roche) followed by rabbit anti-mouse fluorescein isothiocyanate (FITC) (1:150; Sigma) and Alexa 488-conjugated goat-anti-rabbit (Molecular Probes) were used for the detection of the digoxigenin-labeled probe. Before microscopic analysis, nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole).

**Microscopy and image processing**

For microscopic analysis, an epifluorescence Imager Z1 microscope (Zeiss) with an Axioskop 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). 45 to 62 nuclei were analyzed for each genotype.

Compaction of 5S arrays from chromosomes 4 and chromosomes 3+5 were considered separately. Each group of 5S array (4 or 3+5) was considered as decompacted when at least one signal was decondensed. The number of NOR signals was analyzed in 45 to 62 nuclei for each genotype.

**Statistical analysis**

Proportion of 5S-210 transcripts from chromosomes 4 and 5 and percentage of heterogeneous 5S-210 transcripts were compared with Fisher’s exact test for a 2×2 contingency table. The probabilities were calculated from a one-tailed test. Statistical analysis of 5S-210 transcripts amounts were performed using the nonparametric Mann-Whitney U-test with mean values comparison. For statistical analyses of 5S rDNA and NOR compaction, a comparison of proportions Z-test was used. The probabilities were
calculated from a one-tailed test. Interval confidence (IC) was calculated for each proportion with a confidence level of 99%.

RT–PCR analysis

Aliquot of 1 μg of total RNA was treated with DNA-free™ Kit (Ambion) and 100 ng of DNase-treated total RNA was used as input in semi-quantitative RT-PCR reactions using the OneStep RT-PCR Kit (Qiagen). Controls were performed without reverse transcription step to detect contaminating DNA. Amplification of ACTIN2 RNA was used as an internal control and to normalize RNA amounts. Detection of 5S-210 and ACTIN2 transcripts was performed in the same reaction tube. Amplification conditions: 50°C for 30 min (reverse transcriptase inactivation step); 30 cycles [95°C for 30 s; 51°C for 30 s; 72°C for 45 s]; 72°C for 10 min. 5S-210 transcripts were amplified using primers RTPCR5S1 (5′-GGATGGCAT-CATACCAG-3′) and 5SUNIV2 (5′-CGAAAAAGTGATCG-ATGCC-3′). ACTIN2 transcripts were amplified using primers ACT12-F and ACT2-R according to Vaillant et al. [36].

Amounts of amplicons were estimated using a Versadoc coupled to the QuantityOne software (Biorad).

Subcloning and sequencing

PCR products were subcloned in the pGem-T easy plasmid using the pGem-T vector system (Promega). Sequencing was performed using the CEQ 2000 Dye terminator cycle sequencer (Beckman). Computer sequence analysis was performed with the Clustalw program (www.infobiogen.fr).

Genomic DNA extraction and methylation detection assays

Genomic DNA was extracted from seedlings according to the cetyltrimethylammonium bromide (CTAB) method [44]. 200 ng of DNA was digested with NlaIII, which recognizes the sequence C for 30 s; 72°C for 45 s; 72°C for 10 min. 5S-210 transcripts were amplified using primers CTTTTTATGTTTAACC specific for chromosome 5 and CATCCCTC(T)17 specific for chromosome 4, CATCCC- digested and undigested DNA using the following primers: CTCGAAAACAATGCTTGAACAAG used for both arrays.

The chromosome-specific T-stretch is in grey. Nucleotide positions diverging from the reference are in black box. The chromosome-specific T-stretch is in grey. Found at: doi:10.1371/journal.pgen.1000690.s001 (2.91 MB EPS)

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Author Contributions

Conceived and designed the experiments: J Douet, S Tourmente. Performed the experiments: J Douet, S Tutois. Analyzed the data: J Douet, S Tutois, S Tourmente. Wrote the paper: J Douet, S Tourmente.

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