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Epigenetic silencing of clustered tRNA genes in Arabidopsis

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ABSTRACT

Beyond their key role in translation, cytosolic transfer RNAs (tRNAs) are involved in a wide range of other biological processes. Nuclear tRNA genes (tDNAs) are transcribed by the RNA polymerase III (RNAP III) and *cis*-elements, *trans*-factors as well as genomic features are known to influence their expression. In Arabidopsis, besides a predominant population of dispersed tDNAs spread along the 5 chromosomes, some clustered tDNAs have been identified. Here, we demonstrate that these tDNA clusters are transcriptionally silent and that pathways involved in the maintenance of DNA methylation play a predominant role in their repression. Moreover, we show that clustered tDNAs exhibit repressive chromatin features whilst their dispersed counterparts contain permissive euchromatic marks. This work demonstrates that both genomic and epigenomic contexts are key players in the regulation of tDNAs transcription. The conservation of most of these regulatory processes suggests that this pioneering work in Arabidopsis can provide new insights into the regulation of RNA Pol III transcription in other organisms, including vertebrates.

INTRODUCTION

In eukaryotes, transcription of nuclear genes is carried by three conserved RNA polymerases (RNAPs), named I, II and III. RNAP III transcribes a set of non-coding RNAs, including transfer RNAs (tRNAs), 5S ribosomal RNAs (rRNAs) and other small RNAs, such as the U6 small nuclear RNA (snRNA) or some small nucleolar RNAs (snoRNAs) (1). Classically, tRNAs serve as physical link between mRNAs and nascent polypeptides during translation.

Transfer RNAs are classified according to their anticodon and their cognate amino acid. For each amino acid, tRNAs with distinct anticodons are called tRNA isoacceptors (2). During evolution, nuclear tRNA genes (tDNAs) have been duplicated several times leading to multicopy tDNA isoacceptor families. Their members are dispersed along chromosomes (2–4). In addition, few single nucleotide polymorphisms exist at certain tDNA isoacceptors, which are defined as isodecoders (2). The number of tDNAs is highly variable within eukaryotes (2–6) and does not show correlation with their genome size. Nevertheless, it has been reported in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Chlamydomonas reinhardtii* and in some higher plants that the copy number of tDNA isoacceptors correlates with codon usage and likely translational needs (7–10). Indeed, to enable an effective and rapid protein translation, abundance of tRNAs cannot be the limiting factor. Therefore, under normal growth conditions, tDNAs were considered as 'housekeeping genes' and their transcription by the RNAP III was believed to be constitutive and high (11). However, several studies on the regulation of RNAP III transcription showed that the rate of tRNA transcription varies in response to multiple stress conditions (12) and that identical tDNAs can be differentially expressed in cell-/time-specific manners (for reviews, see (1,13)). Moreover, half of the predicted tDNAs are transcriptionally silent in human (14).

Transcription of eukaryotic tDNAs is regulated *via* different ways (for reviews see (12,13)). First, their sequences contain two intrinsic RNAP III internal promoter elements, called A and B boxes (15). These evolutionary conserved motifs allow the specific recruitment of the general transcription factor (TF) TFIIIC and subsequently TFIIIB for RNAP III pre-initiation complex assembly. While these TFs together with non-core *trans*-factors (e.g. Maf1) (16) play important roles in the regulation of tDNAs biosynthesis, other strategies to modulate tDNAs transcription have emerged during evolution. It includes the presence of other *cis*-elements on tDNAs such as the upstream AT-rich/TATA-like motifs (17) and the downstream short poly-T stretches (18). This later motif releases RNAP III and al-

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lows recruitment of another *trans*-factor stabilizing precursor tRNAs: the La protein (19). Moreover, additional *cis*-elements can exist. Indeed, in higher plants, a CAA triplet located between positions −1 and −20 seems to be essential for transcription initiation (20). The presence of several consecutive CAA motifs has also been identified (7).

In addition to the important roles of these *cis*-elements and *trans*-factors, some data suggest that RNAP III-mediated tDNAs transcription in eukaryotes is also subjected to a wider range of regulatory processes such as their genomic (e.g. (21)) and epigenomic environments as well as their 3D organization (e.g. (22)) within the nucleus (13,23). Indeed, nowadays, in eukaryotic organisms, it is well established that normal growth, development and differentiation of distinct cell lineages are governed by transcriptional but also epigenetic mechanisms (24).

In plants, epigenetic processes such as DNA methylation (5-methyl cytosine: 5-mC) and histone post-translational modifications (PTMs) are thought to contribute to the regulation of gene expression and the silencing of repeats (25–29). In mammals, although DNA methylation of cytosine residues (5-mC) is mainly present in the symmetric CG context, other non-CG methylation environments exist (CHG and CHH where H: A, C or T) (30–32). In Arabidopsis, 5-mC is deposited by three main types of DNA methyltransferases: METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 2 and 3 (CMT2 and CMT3) and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). These enzymes are responsible for maintaining methylation in the CG (MET1), CHG (CMT3) and CHH (CMT2 and DRM2) sequence contexts on newly synthetized DNA strands upon replication (27,33,34). Additionally, cytosines can be methylated *de novo* through the RNA-directed DNA methylation pathway (RdDM) (35) involving two plant-specific RNAPs evolutionarily related to RNAP II (36), RNAP IV (37–39) and RNAP V (40). RNAP IV, together with RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), produces double-stranded RNA (dsRNA) precursors that are diced into 24-nt siRNAs by DICER-LIKE 3 (DCL3) and loaded into ARGONAUTE 4 (AGO4) (27,33,34,36,41). These 24-nt siRNAs are thought to interact with scaffold transcripts produced by RNA POL V, thereby mediating *de novo* DNA methylation of cognate DNA sequences in the three cytosine contexts by DRM2 (27,41). Importantly, the SWI2/SNF2-related chromatin remodeling factor DECREASE IN DNA METHYLATION 1 (DDM1) provides DNA methyltransferases access in cooperation with the RdDM pathway (42–44).

In addition to DNA methylation, regulation of gene expression can be mediated by chromatin remodelers and histone modifying enzymes. In plants, the computational integration of high-throughput epigenomic data at the whole genome level has led to the identification of different epigenomic profiles named chromatin states (45–48). Chromatin states are meaningful in several biological functions and impact the activity of gene expression during developmental processes and in response to environmental cues (49).

In Arabidopsis, while most tDNAs are scattered throughout the nuclear genome, it was previously shown that part of the proline (Pro), serine (Ser) and tyrosine (Tyr) tDNAs are organized in clusters (7). Here, we identify a novel cluster formed by cysteine (Cys) tDNAs. Then, we demonstrate that while tDNAs dispersed along chromosomes are expressed, clustered tDNAs are transcriptionally silent although most of them possess the canonical RNAPIII *cis*-elements. Using *in silico* analyses, we unveil that these clustered tDNAs contain elevated CG methylation levels compared to their dispersed counterparts. Molecular approaches allowed identifying that mutant plants defective for the maintenance of DNA methylation pathways exhibit a release of the expression of clustered tDNAs. Concomitantly to DNA methylation, we reveal that clustered tDNAs display heterochromatic (repressive states) features whereas their dispersed equivalents show euchromatic (permissive states) characteristics. Collectively, our findings reveal that both genomic environment and epigenetic landscape act to fine tune the differential expression of dispersed and clustered tDNAs in Arabidopsis.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana mutant plants used in this study are summarized in Supplementary Table S4 and are in the Col0 background. Plants were sown in batches on soil (Hawita Gruppe) supplied by a fertilizer N–P–K: 12–7–19 (Osmocote, 1g per 1 l of compost) in 7 × 7 × 6.4 cm pots and cultured in growth chambers at 23°C/18°C for a photoperiod of 16 h/8 h (day/night).

For *in vitro* culture, after a 2-day stratification in darkness at 4°C, Col0 sterilized seeds were grown in Petri dishes (GBO, Reference: 664102) containing a MS222 (Duchefa Biochemie, Reference: M0222.0010) half-strength medium pH 5.8 supplemented with 0.5 g/l MES (Bio Basic Canada Inc., Reference: MB0341), 1% (w/v) sucrose, 0.68% (w/v) agar (Sigma Aldrich, Reference: 102067977), 250 μg/ml of sodium cefotaxime (Duchefa Biochemie, Reference: C0111) and 2 ml/l of Plant Preservative Mixture (Plant Cell Technology, PPM) in growth chambers 21°C/17°C for a photoperiod of 16 h/8 h (day/night).

5-azacytidine and zebularine treatments

5-Azacytidine (5-azaC) and zebularine assays (0, 5, 25, 50 and 100 μM) were performed using Col0 sterilized seeds and direct (DT) or postponed (PT) treatments as described in (50).

Total RNA extraction and northern blot analysis

Total RNAs were extracted from 22 days-old plants using TRI reagent (MRC) following the manufacturer instructions. For 4 days-old seedlings, roots, rosette leaves, inflorescences and green siliques of 7-week-old plants, total RNAs were prepared according to Chang *et al.* (51). Total tRNAs were enriched from total RNAs as described in (52). This protocol includes a LiCl precipitation step that allows enrichment, in the supernatant, of RNAs of a size smaller than 150 nt (i.e. 5S rRNA, tRNAs and small non-coding RNAs).

For tRNA northern blots, up to 6μg of total tRNAs were separated in 15% polyacrylamide gel, elecrotransferred

onto Hybond N+ membrane (GE Healthcare Life Sciences) and hybridized to ^{32}P radiolabeled oligonucleotides probes in 6X SSC, 0.5% (v/v) SDS at the following temperatures: Y14 P, Y32 P, P12 P, Ala P: 48°C, and P46 P: 44°C. Washing conditions were: 2 times 10 min in 2× SSC and 1 time 30 min in 2× SSC, 0.1% SDS, at hybridization temperature.

tDNA and tRF analysis

tRF analysis was performed as described in Cognat *et al.* (52) using small ncRNA libraries (Supplementary Table S2) retrieved from SRA (https://www.ncbi.nlm.nih.gov/sra/). Adapter sequence was trimmed for the short read libraries with cutadapt (version 1.18) (53). Data were mapped against the Arabidopsis tRNAs extracted from plantRNA database (http://plantrna.ibmp.cnrs.fr) (6) with patMaN (version 1.2.2) (54). The reads that can be assigned specifically to the clustered tRNAs and dispersed tRNAs have been counted to measure their expression.

The seqlogo of the upstream and downstream tDNA sequences were performed with WEBLOGO available at (https://weblogo.berkeley.edu).

DNA methylation levels analysis

Published BS-Seq datasets (Supplementary Table S2) have been used to determine cytosine methylation levels of clustered and dispersed tDNA loci using 50 bp upstream (promoter) to 25 bp downstream (terminator) windows. Cytosine methylation levels in CG, CHG and CHH contexts were determined according to the method described in Daccord *et al.* (55). Statistical analysis was performed using pairwise Wilcoxson tests. Graphical representations and pairwise Wilcoxson tests were done in R (https://www.r-project.org/).

Chromatin states and nucleosome analyses

Genomic coordinates of Arabidopsis tDNAs were extracted from plantRNA database (6) and used to address the distribution of tDNAs across chromatin states. Genomic coordinates of the nine chromatin states (CS) were downloaded from Sequeira-Mendes *et al.* (47). Data for nucleosome occupancy (NO) were downloaded from Liu *et al.* (56) and converted in bigwig format using the UCSC wigToBigWig tool. A matrix of scores for dispersed and clustered tDNA regions (regions spanning from 50 bp upstream and 25 bp downstream) was calculated with the program computeMatrix from deeptools (57) v. 3.1.3 (with parameter –binsize 5). The heatmaps were plotted with the plotHeatmap tool.

Miscellaneous

The sequence of oligonucleotides probes used in this study are:

Yd: 5′-CGGAAGACTGTAGATCTTTAGGTCGCTGG TTCGATTCCGGCAGG-3′
Yc: 5′-CGGAGGACTGTAGATCCTTAGGTCACTGG TTCGAATCCGGTAGG-3′
Pd: 5′GCGAGAGGTCCCGAGTTCGATTCTCGGA ACGCCCCCCA-3′
Pc: 5′-GCGAGAGGTCCCGAGTTCGATTCTCGGAA TGCCCCCCA-3′
Y16 P: 5′-TGCCGGAATCGAACCAGCG-3′
Y34 P: 5′-TACCGGATTCGAACCAGTG-3′
P12 P: 5′-GGGCGTTCCGAGAAT-3′
P45 P: 5′-GGGCATTCCGAGAAT-3′
Ala P: 5′-ACCATCTGAGCTACATCCCC-3′

RESULTS

Extra copies of tDNAs are organized into clusters in Arabidopsis

In various organisms, a positive correlation exists between the copy number of tDNA isoacceptors and the frequency of occurrence of the corresponding codons (8,10). We have previously shown that this correlation holds true in Arabidopsis except for the Pro, Ser and Tyr tDNA families (7). These families are in excess and lie clearly outside of the regression line correlating the number of genes for each tDNA families and the frequency of occurrence of each amino acid (Figure 1A). According to our previously published analyses (7,6), this excess of copies is organized in several clusters with adjacent tDNAs in same orientation (Figure 1C; Supplementary Table S1).

Two Pro tDNA clusters (i.e. one with 24 copies and one with six copies) are located on Arabidopsis chromosome (Chr) 1, nearby the centromeric/pericentromeric regions, and one cluster of nine copies on Chr2 long arm (Figure 1B). Clustered Ser and Tyr tDNAs are found on Chr1 arranged in interspaced Ser-Tyr-Tyr units tandemly repeated 27 times nearby the pericentromeric region (Figure 1B). Interestingly, we further identified an additional cluster on Chr5 formed by four Cys tDNAs oriented in the same direction (Figure 1B and C; Supplementary Table S1). Compared to other clusters, the low number of gene copies in the Cys tDNAs cluster does not drastically affect the positioning of the Cys point on the regression line (Figure 1A). Lastly, while Cys and Ser-Tyr-Tyr repeats are regularly interspaced, clustered Pro tDNAs are not (Figure 1C).

Unlike dispersed Pro tDNAs that are primarily intergenic, their clustered counterparts are predominantly located in long non-coding RNAs (LncRNAs) or in protein coding genes (PCGs), within introns or untranslated regions (UTRs), and always in opposite orientation of the transcriptional unit (Figure 1C and D). The Ser-Tyr-Tyr cluster location is exclusively restricted to a large intergenic region of around 40 kb, resembling the genetic environment predominant for dispersed tDNAs. Finally, the Cys cluster is positioned at three transposable elements (TEs) belonging to the Helitron superfamily (Figure 1C and D). Taken together, it emerges that Pro and Cys tDNA clusters stand in distinct genetic environments compared to dispersed tDNAs, while the Ser-Tyr-Tyr cluster does not.

RNAP III *cis*-acting elements are well conserved among clustered and dispersed tDNAs

Transcription efficiency of plant tDNAs by RNAP III relies on the presence of several upstream and downstream *cis*-acting elements (20,58). To further explore genetic architecture of Arabidopsis clustered tDNAs, their promoter and

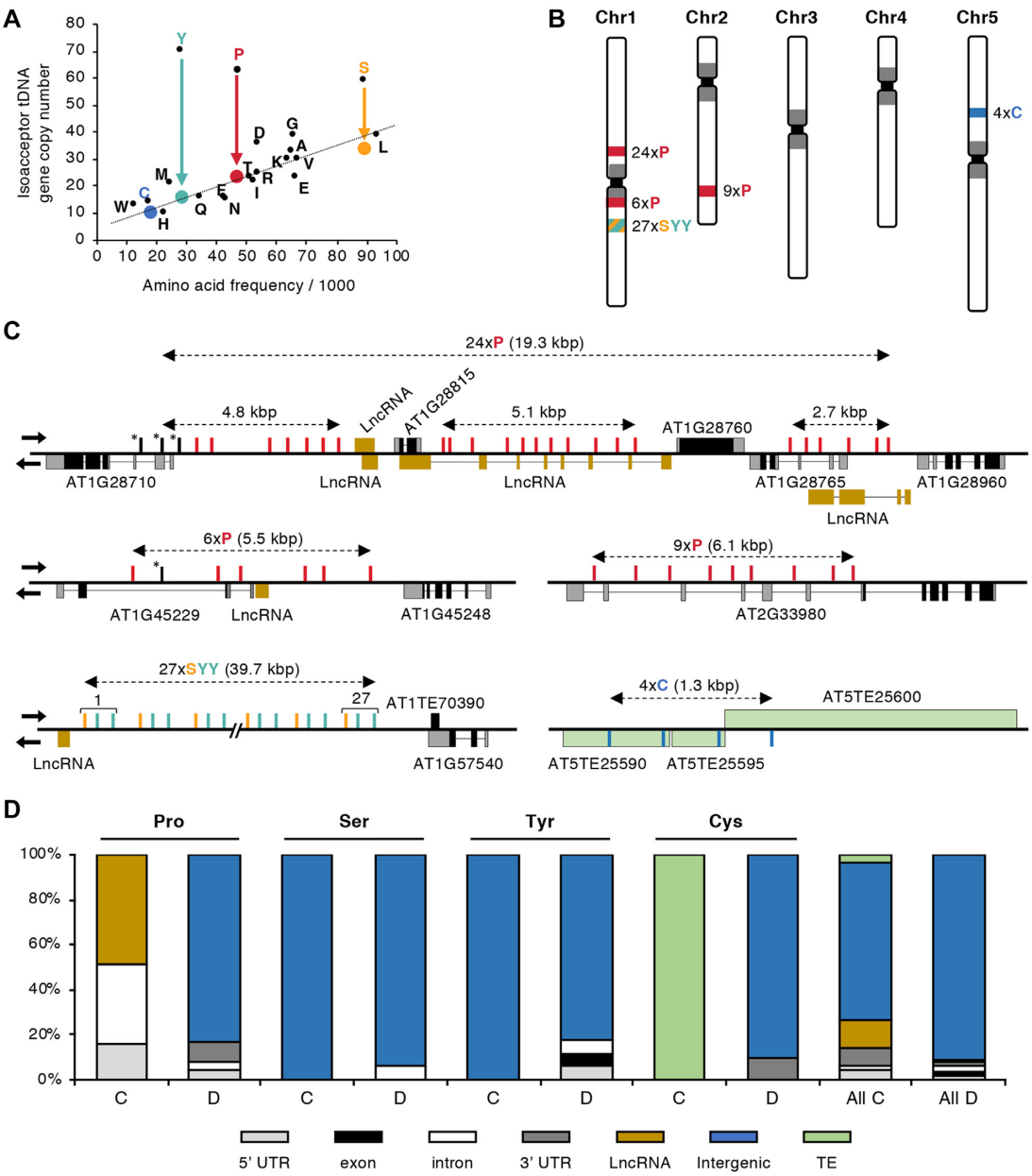

Figure 1. Characterization of tDNA clusters and their genomic context in Arabidopsis. (**A**) Correlation between the number of tDNAs specific for each amino acid and the frequency of occurrence of the same amino acid. For Ser (S yellow), Tyr (Y green), Pro (P red) and Cys (C blue) amino acids, the numbers corresponding to tDNAs found in clusters were subtracted and colored dots represent the new correlation. (**B**) Schematic representation of tDNA clusters loci and tDNA copies located on Arabidopsis Chr1, 2 and 5. Pericentromeric regions are in gray. (**C**) Schematic representation of genomic regions containing clustered tDNAs. tDNAs are indicated by colored vertical bars (Y: green, P: red, S: yellow and C: blue). Presence of tDNA pseudogenes is also indicated by black bars with an asterisk (see Supplementary Table S1 for details). Black boxes: exons in CDS; gray boxes exons in UTRs; lines: introns; brown boxes: putative long non coding RNAs (LncRNAs). Arrows indicate transcription orientation. (**D**) Distribution of the location of clustered (c) and dispersed (d) tDNAs on Arabidopsis genome (TAIR10.1).

terminator regions were analyzed and compared to those of dispersed tDNAs. Corresponding sequences were retrieved from PlantRNA database (6).

Promoters of clustered Pro, Tyr and Cys tDNAs are A/T-rich and present a CAA triplet in the −7 to −3 region (Supplementary Figure S1a) known to be important for efficient RNAP III transcription (20,58). Although promoters of clustered Ser tDNAs are also enriched in A and T residues, CAA motif is lacking (Supplementary Figure S1a, b). Downstream, both clustered and dispersed tDNAs contain short poly(T) tails (Supplementary Figure S1a). Generally, terminator regions of clustered tDNAs are less enriched in T residues as compared to their dispersed counterparts (Supplementary Figure S1c). Median values of the longest stretch of T residues is around six for dispersed tDNAs, and of five for clustered ones (Supplementary Figure S1d). Nevertheless, these shorter stretches of T residues found in clustered tDNAs as compared to dispersed ones

would be likely long enough to promote efficient RNAP III termination (14,59).

Altogether, these analyses demonstrate that almost all clustered tDNAs exhibit required core components to be potentially efficiently transcribed by RNAP III.

Clustered tDNAs are transcriptionally silent

Given that clustered tDNAs represent extra copies of tRNA genes and that most of them exhibit all features for RNAP III transcription, we were interested in assessing the level of expressed tRNAs originating from these clustered tDNAs. In Arabidopsis, as in other eukaryotes, a high level of conservation between tRNA sequences among each tRNA isoacceptor family exists (6) preventing a straightforward differential expression analysis. To overcome this impediment, clustered and dispersed tDNA sequences were analyzed into details (Supplementary Figure S2) to identify potential polymorphisms that would allow discriminating specifically their expression level by northern blotting. Interestingly, we found nucleotide polymorphisms between the most abundant clustered and dispersed tDNA Tyr and Pro copies (Supplementary Figures S2 and S3), allowing design of specific oligonucleotide probes (Figure 2).

Among Tyr tDNAs, three nucleotide polymorphisms (G49A, T59A and C65T) allows discriminating sequences of dispersed Tyr tDNAs from those of predominant clustered Tyr tDNA copies. Thereby, probe Y16 P would be specific to 16 dispersed Tyr tDNAs, while probe Y34 P would be specific to 34 Tyr tDNAs out of the 54 clustered Tyr tDNAs (Figure 2A and Supplementary Figure S2). Northern blot analyses using two synthetic oligonucleotides (Yd and Yc), representative of these dispersed and clustered Tyr tDNAs respectively (Figure 2A), confirm that Y34 P and Y16 P probes allow discriminating specifically between Tyr tRNA originating from clustered versus dispersed tDNAs (Figure 2B).

Similarly, a single-nucleotide polymorphism (C67T) is sufficient to distinguish by northern blot most of tRNAs expressed from clustered Pro tDNAs (i.e. 38 out of 39) from dispersed ones (Figure 2B and Supplementary Figure S2). Indeed, P45 P probe looks specific to 38 clustered Pro tDNAs, but also to 7 out of 24 dispersed ones since they share the same sequence. Conversely, P12 P probe can exclusively recognize 12 dispersed Pro tDNAs (Supplementary Figure S2a, b). Specificity of the two probes, P45 P and P12 P was confirmed by northern blot experiments using as controls two oligonucleotides, Pd and Pc, representative of major dispersed and clustered Pro tDNAs respectively (Figure 2B).

Unfortunately, the small numbers of gene copies with sequence polymorphisms prevented design of specific probes for the differential analysis of Ser and Cys tDNAs expression by northern blot (Supplementary Figure S2b).

While Tyr tRNA isodecoders expressed from dispersed copies are detectable using Y16 P probe, those originating from clustered copies (Y34 P probe) are below detection limits (Figure 2B). Pro tDNAs expression is also detected for dispersed copies using P12 P probe (Figure 2B). Although a weak signal is observed using P45 P probe (Figure 2B), we believe that it most likely reflects the ability of this probe to recognize not only expression of clustered Pro tDNAs, but also of few copies of dispersed ones.

Taken together, these differential molecular analyses suggest that clustered tDNAs are likely silenced in Arabidopsis.

To determine whether this lack of detection relies on a transcriptional or on a post-transcriptional regulatory process, we used publicly available small RNA libraries (52) to undertake a degradome analysis of tRNA-derived fragments (tRFs) bearing polymorphic ribonucleotides (Figure 2C, Supplementary Figure S2). To do so, we analyzed small RNA libraries from WT and post-transcriptional gene silencing (PTGS) mutant plants (*hen1*, *rdr6*, *dcl2/3/4*, *dcl4* and *ago1*). We assumed that tRF levels reflect tRNA abundance and that PTGS mutant plants may accumulate tRFs, although we cannot exclude that half-life between tRF species may differ. tRFs originating from tRNAs expressed from clustered Tyr tDNAs (Y53) are barely detectable, whereas those from dispersed Tyr tRNAs (Y15) are abundant (Figure 2c). Similarly, while tRFs derived from a single dispersed Ser tRNA (S1) are present in small RNA libraries prepared from WT plant, tRFs arising from expression of 9 clustered Ser tDNAs (S9) are absent (Figure 2C). For Pro tRNAs, the level of tRFs derived from expression of 12 dispersed tDNAs (P12) is higher than that derived from 40 clustered (P40) plus eight dispersed tDNAs (P8), (Figure 2C). Finally, tRFs potentially arising from expression of two clustered Cys tRNAs are not detected, whereas those arising from two dispersed Cys tRNAs exist (Figure 2C).

Importantly, in *hen1*, *rdr6*, *dcl2/3/4*, *dcl4* and *ago1* mutant plants, tRFs abundance remains low to undetectable (Supplementary Figure S7) highlighting that canonical PTGS pathway may not play a predominant role in silencing of clustered tDNAs.

Collectively, although we cannot fully exclude existence of complex/non canonical post-transcriptional regulatory processes, our analyses strongly suggest that expression of clustered Ser, Tyr, Pro and Cys tDNAs is predominantly transcriptionally repressed in Arabidopsis.

Clustered tDNA repeats display elevated DNA methylation levels

We assumed that the lack of expression characterizing clustered tDNAs could be due to the presence of repressive epigenetic marks. Conversely, dispersed tDNAs may contain permissive marks allowing efficient expression. Among repressive epigenetic marks, DNA methylation is well known to play a key role in plant transcriptional gene silencing (60). Therefore, DNA methylation landscape at both dispersed and clustered Ser, Tyr, Pro and Cys tDNAs in Arabidopsis plants was analyzed.

Using publicly available data (61), 5-mC levels in CG, CHG and CHH contexts were calculated for each single clustered tDNA locus within a genomic window starting 50 bp upstream (promoter) and ending 25 bp downstream (terminator) of each tDNA (Figure 3A). Importantly, cytosine content is homogeneous between all studied tDNAs, thus allowing comparative studies (Figure 3B and Supplementary Figure S4a). All clustered tDNAs exhibit higher methylation levels in CG context compared to their dispersed counterparts (Figure 3C). CHG and CHH methyla-

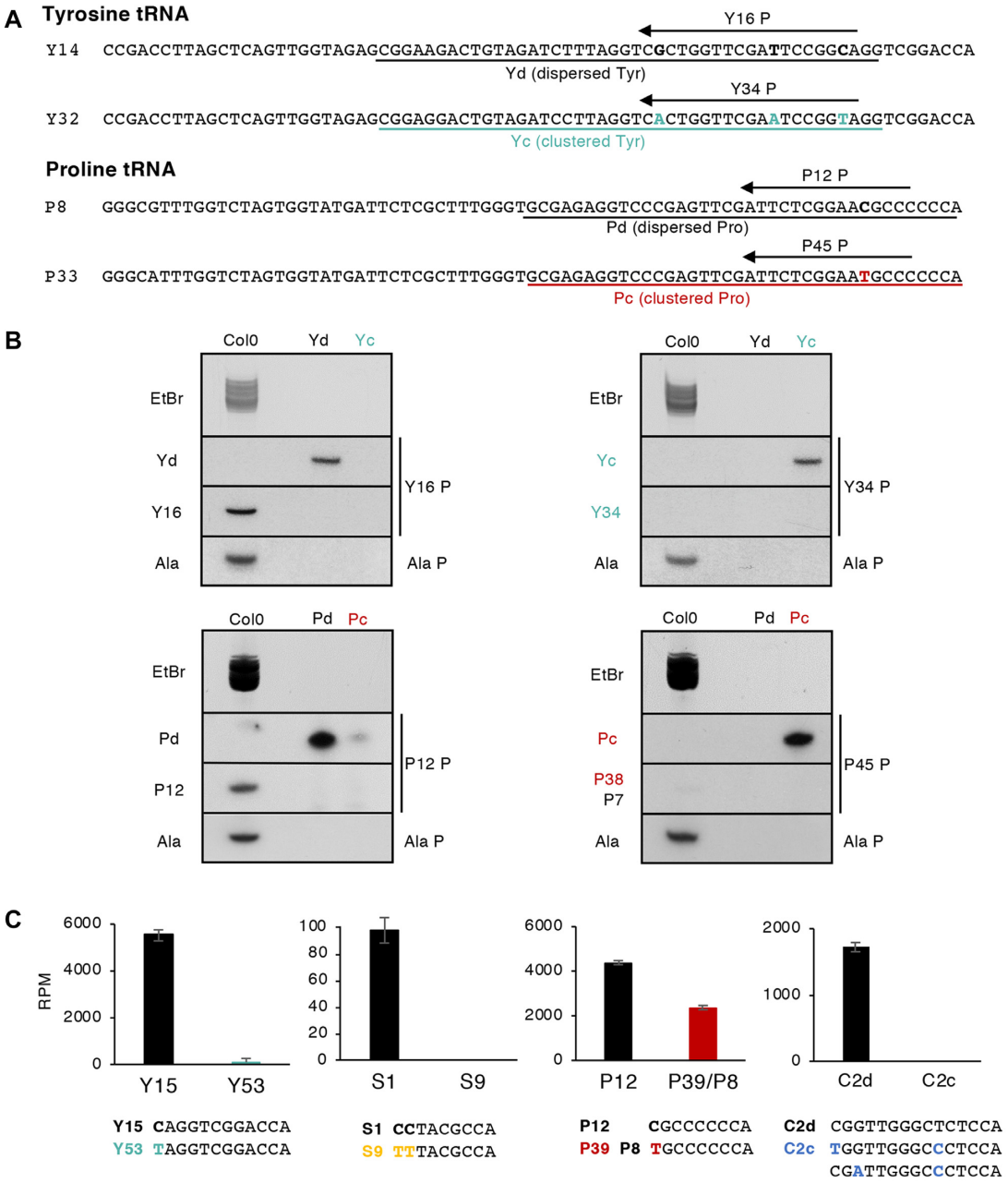

Figure 2. Expression analysis of Arabidopsis tDNA clusters. (**A**) Predominant sequences of dispersed (Y14 and P8) and clustered (Y32 and P33) Tyr and Pro tDNAs. Nucleotide differences are in bold for Tyr (green) and Pro (red) clustered tDNAs. Underlined sequences (Yd: dispersed Tyr, Yc: clustered Tyr, Pd: dispersed Pro and Pc: clustered Pro) were used as controls. Sequences indicated by arrows (Y16 P, Y34 P, P12 P and P45 P) were used as oligonucleotide probes for northern blot. Note that P45 P probe designed for clustered Pro tDNAs (P38) is also complementary to 7 dispersed Pro tDNAs (P7; Supplementary Figure S2). (**B**) Northern blot detection of dispersed (upper left) and clustered (upper right) Tyr plus dispersed (lower left) and clustered (lower right) Pro tRNAs in WT plants. Nomenclatures of the left and right sides refer to controls and probes as depicted in (A). Ethidium bromide (EtBr) staining and hybridization with an Arabidopsis cytosolic Alanine tRNA probe (Ala P) were used as loading controls. Original uncropped blots are available in Supplementary Figure S11. (**C**) Histograms showing the average abundance of tRFs (±SE) specific to dispersed Tyr (Y15), Serine (S1), Pro (P12) and Cys (C2d) tRNAs and clustered Tyr (Y53), Ser (S9) and Cys (C2c) tRNAs. The tRF sequence 'TGCCCCCCA' is specific to 40 clustered Pro tRNAs (P40) and eight dispersed Pro tRNAs (P8). tRFs sequences are indicated below each graph with nucleotide differences in bold and colored. tRFs with lengths ranging from 19 to 26 nt were analyzed.

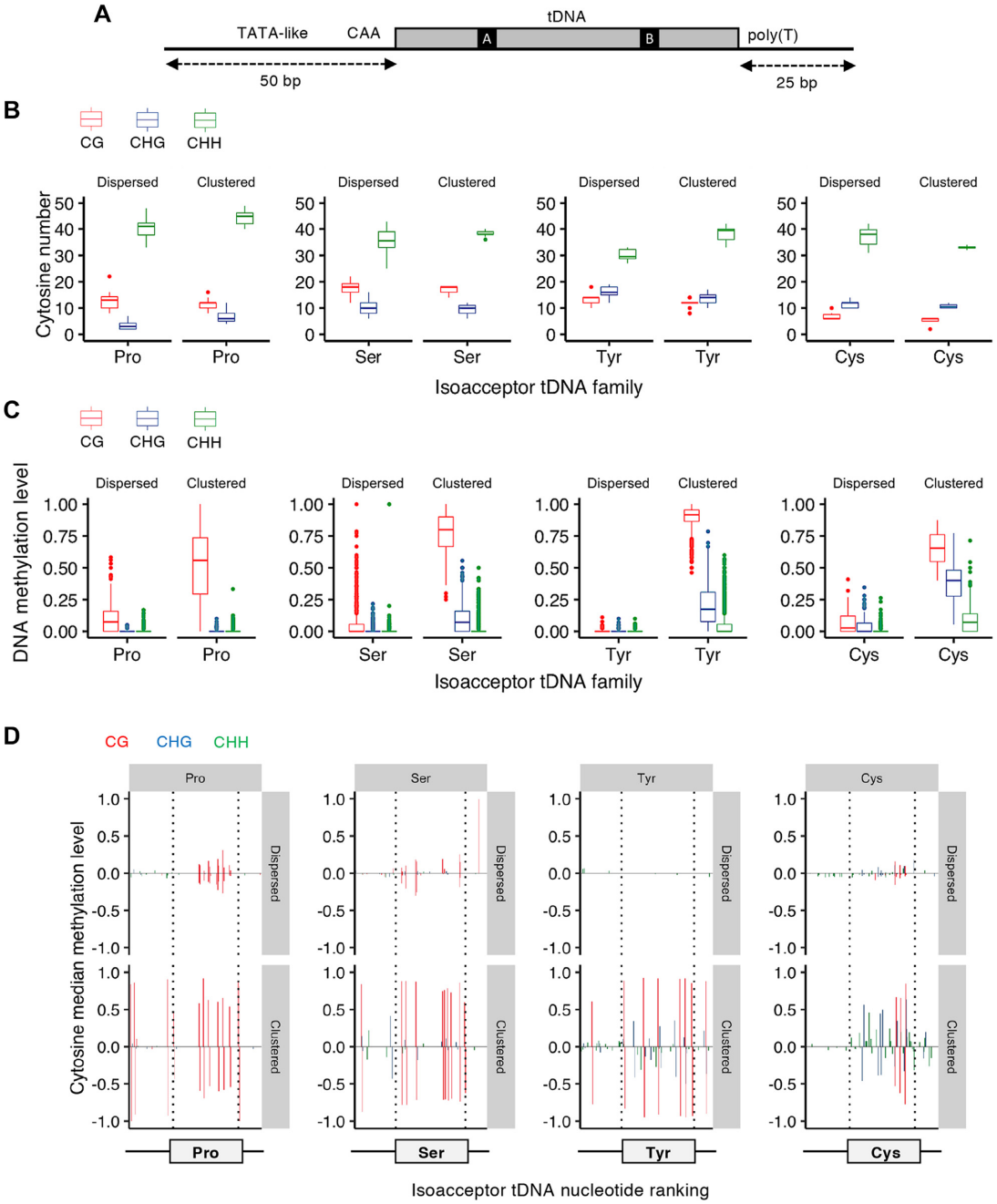

Figure 3. Methylation landscape at dispersed and clustered tDNA loci. (**A**) Schematic representation of a tDNA locus with transcriptional internal control A and B boxes, upstream TATA-like elements and CAA motif and downstream poly-T tract (7). For each tDNA, the region spanning from −50 bp to +25 bp was examined for DNA methylation level in CG (red), CHG (green) and CHH (blue) contexts. (**B**) Boxplots representing DNA cytosine counts in dispersed and clustered Pro, Ser, Tyr and Cys tDNAs. (**C**) Boxplots representing DNA methylation levels on dispersed and clustered Pro, Ser, Tyr and Cys tDNAs. (**D**) DNA methylation levels at single nucleotide resolution on dispersed and clustered Pro, Ser, Tyr and Cys tDNAs. Only predominant polymorphic sequences of identical length have been used for these representations. Positive and negative values are referring to DNA strands. Regions corresponding to tDNA sequences are delimited by dotted lines. Statistical analysis (pairwise Wilcoxson tests) are provided in Data S1.

tion levels are slightly higher for clustered Ser, Tyr and Cys tDNAs compared to their dispersed equivalents, whereas this is not the case for clustered Pro tDNAs (Figure 3C). Additionally, while analyzing methylome at single-nucleotide-resolution we found that DNA methylation occurs mainly in body of clustered tRNA genes (Figure 3D). Cytosines at promoter and terminator regions are also methylated but to a low extent (Figure 3D). Importantly, dispersed Ala tDNAs used as control display low DNA methylation levels in all contexts (Supplementary Figure S4b, c).

To test whether these DNA methylation profiles are modulated during plant development, we analyzed DNA methylation landscapes of dispersed and clustered tDNAs in different tissues and organs using publicly available methy-

lomes (33,62–68), (Supplementary Figure S5, Table S2). CG context shows the higher methylation level in clustered tDNAs compared to dispersed tDNAs in all tested samples. Reproductive tissues contain higher CHG methylation levels for Ser, Tyr and Cys tDNA clusters compared to somatic tissues (Supplementary Figure S5). Clustered Ser and Tyr tDNAs exhibit increased CHH methylation levels in central cell compared to the other tissues (Supplementary Figure S5). In contrast, control dispersed Ala tDNAs do not display any strong variations. Furthermore, using northern blot analyses, we found that tRNA levels of clustered Ser, Tyr and Pro tDNAs were below detection limits in all tested organs, while tRNAs originating from dispersed tDNAs were detectable (Supplementary Figure S6).

Collectively, these data reflect that a negative correlation likely exists between CG methylation levels and the expression of clustered tDNAs.

Effectors of maintenance of DNA methylation regulate methylation landscape at clustered tDNAs

Methylome analyses of tDNA families reveal that clustered tDNAs contain high levels of DNA methylation compared to dispersed ones. To determine the involvement of particular DNA methylation pathways in these profiles, we re-analyzed DNA methylation levels of clustered and dispersed tDNAs in mutant plants defective for expression of the main Arabidopsis DNA methylation processes (61) (Supplementary Table S2 and Figure S4). In this way, as expected, we show that for all clustered tDNAs, CG methylation level relies on MET1 DNA methyltransferase (Figure 4). In addition, CG methylation pattern is strongly affected in *ddm1* mutant plants for Ser, Tyr and Cys clusters but not for Pro ones (Figure 4), consistent with the ability of DDM1 to predominantly control DNA methylation at TEs and repeats compared to PCGs (43). Enhanced CHG and CHH DNA methylation levels are observed in *ddm1* and *met1* for Ser and Tyr cluster respectively (Figure 4), in agreement with ectopic gain of CHH methylation already reported in both mutant plants (61).

Our analyses further show that for Ser, Tyr and Cys clusters, CHG methylation relies on CMT3 and KYP. For all clustered tDNAs, CHH methylation levels are poorly affected in both *cmt2* and *drm2* mutant plants (Figure 4), suggesting that both CMT2 and RdDM may act synergistically. Importantly, our data also show that Pro tDNA clusters are under control of histone H3K9 demethylase INCREASE IN BONSAI METHYLATION 1 (IBM1), known to indirectly modulate CHG methylation levels (69). Indeed, *ibm1* mutant plants exhibit enhanced CHG methylation level at clustered Pro tDNAs (Figure 4). Interestingly, among Pro clusters, one of them is located within coding sequence of AT2G33980 (Figure 1C), reported to be an IBM1 target (69). Thus, our analyses suggest that dynamics of histone PTMs (i.e. H3K9me2) may also influence DNA methylation levels at this particular locus (Figure 4).

Together, our data show that DDM1, MET1, CMT2, CMT3, KYP, IBM1 and DRM2 maintain high level of DNA methylation at clustered tDNAs.

In order to determine whether DNA methylation restricts clustered tDNAs expression, we experimentally analyzed tRNA steady state levels by northern blots using different strategies. First, we challenged the effect of a global loss of cytosine methylation on clustered Tyr and Pro tDNAs expression by assaying the effect of the cytidine analog 5-azacytidine (5-azaC) (70). 5-azaC treatment significantly released clustered Tyr tDNAs expression whilst no effect was detected for Pro ones (Figure 5A). Another analog of cytidine, zebularine (50), confirmed the release of clustered Tyr tDNAs expression (Figure 5A). This pharmacological approach highlights that DNA methylation represses clustered Tyr tDNAs expression. In a second approach, we used mutant plants deficient for expression of RdDM factors and of maintenance of DNA methylation pathways. In RdDM mutant plants, no release of clustered Tyr and Pro tDNAs expression could be detected (Supplementary Figure S6), suggesting that this pathway does not play a predominant role in their silencing. Moreover, both *ddm1* and *met1* mutant plants display re-expression of clustered Tyr tDNAs (Figure 5B), in agreement with reduced CG methylation levels observed in these mutants (Figure 4). Surprisingly, clustered Pro tDNAs do not display release of expression in *ddm1* and *met1* mutant plants, suggesting that additional factors may repress their expression (Figure 5B). Importantly, no transcriptional reactivation is observed for clustered Tyr and Pro tDNAs in *cmt2*, *cmt3* and *kyp* plants (Figure 5C), demonstrating that both CHG and CHH methylation are not predominant contexts repressing clustered tDNAs expression.

In addition to all tRNAs, RNAP III also transcribes 5S rRNA (1). In Arabidopsis, the helicase MORPHEUS MOLECULE 1 (MOM1) is known to mediate silencing of 5S rDNA repeats in a DNA methylation independent manner (71–73). We therefore explored putative roles of MOM1 on the silencing of clustered tRNA genes by northern analysis using Y34 P and Y16 P for Tyr and P45 P and P12 P for Pro (Figure 2A). Tyr and Pro clustered tDNAs, were not re-activated in the *mom1* mutant plants (Supplementary Figure S6c), strengthening the idea that DNA methylation plays a predominant role in the silencing of clustered tDNAs.

Given that DNA methylation must be efficiently maintained through mitosis and meiosis, we tested whether inheritance/maintenance of DNA methylation would repress expression of clustered tDNAs through a directional parental effect. For that purpose, we analyzed clustered Tyr tDNAs expression in progeny of reciprocal crosses between WT and *ddm1* mutant plants. As above reported, expression of clustered Tyr tDNAs is released in *ddm1* self-crossing plants (S1; Figure 5D). In both F1 plants *ddm1*/Col0 and Col0/*ddm1*, expression of clustered Tyr tDNAs could still be detected, albeit weaker than in *ddm1* parent plants (Figure 5D). These data reflect that maintenance of DNA methylation is important to efficiently repress expression of clustered tDNAs and demonstrate its bi-parental inheritance.

As a whole, the combination of *in silico* and molecular approaches allowed determining that silencing of clustered tDNA repeats relies on DNA methylation. Moreover, our analyses suggest that pathways involved in maintenance of cytosine methylation may act synergistically to repress clustered tDNA expression.

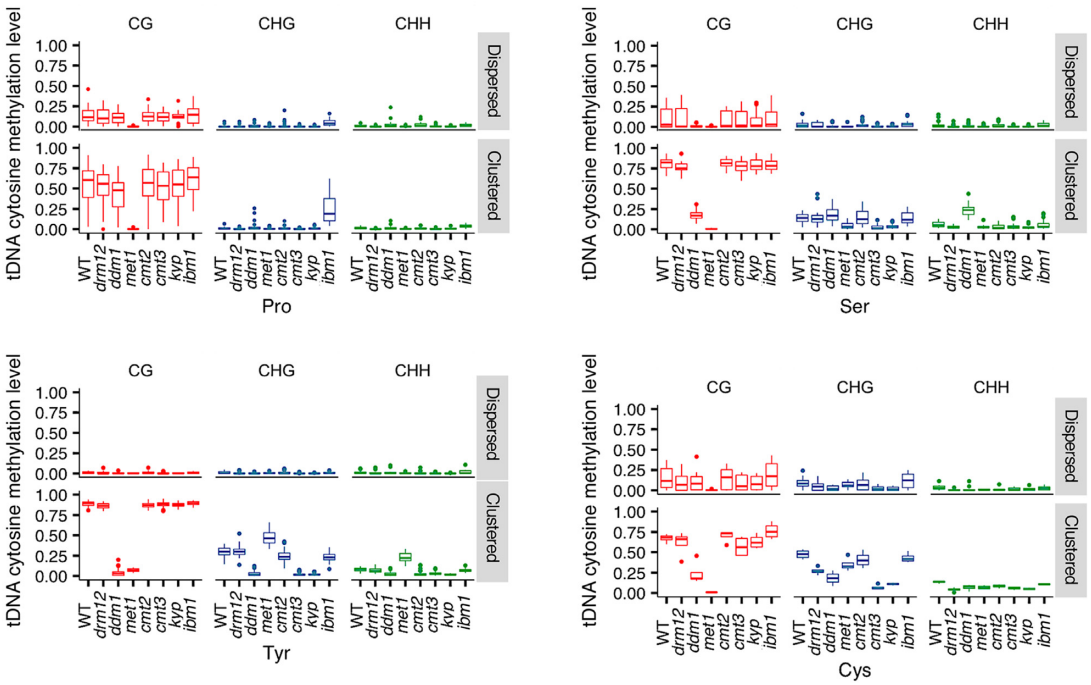

Figure 4. DNA methylation landscape at clustered tDNAs in DNA methylation mutant plants. Box plots representing DNA methylation levels in various mutant lines for dispersed and clustered Pro, Ser, Tyr and Cys tDNAs. CG (red), CHG (green) and CHH (blue) contexts. Statistical analysis (pairwise Wilcoxson tests) are provided in Data S2.

Clustered tDNAs display particular chromatin features compared to their dispersed equivalents

Besides DNA methylation, histone modifications and histone variants are integral part of molecular features that characterize chromatin landscape in eukaryotic cells. As such, certain chromatin signatures of genomic elements establish particular gene expression patterns. Here, we examined the relationship between clustered tDNAs and each of the nine chromatin states (CS) previously described in Arabidopsis (47).

Dispersed Pro, Ser and Tyr tDNAs, as well as Ala control, are similarly enriched in CS2 (Figure 6A), a state characterized by coexistence of active/repressive marks (e.g. H3K4me3 and H3K27me3) in proximal promoter regions and considered as the second most active state according to the first principal component analysis (47). This enrichment in CS2 is significantly higher than the average distribution of CS observed over the entire Arabidopsis genome, with all P value < 0.005 (Supplementary Table S3).

For dispersed Cys tDNAs, CS distribution is roughly similar to the average one, with CS1, CS2, CS4 and CS6 being the most abundant states. While dispersed tDNAs are mainly enriched in euchromatin states, Ser, Tyr and Cys clustered tDNAs are enriched in heterochromatin state 8 (CS8), a repressive state found within intergenic regions and TEs with high levels of histone variant H3.1, H3K9me2, H3K27me1 and CG methylation (47). Interestingly, while CS8 characterizes chromatin of Ser, Tyr and Cys tDNA clusters, CS7 is predominantly present along chromatin of the three Pro tDNA clusters (Figure 6A). This state appears almost exclusively related to intragenic regions, as it colocalizes with coding sequences and introns. Moreover, CS7 strongly associates with transcription units longer than average (47). Dichotomy in chromatin states between CS8 of Ser, Tyr and Cys tDNA clusters and CS7 of Pro tDNA clusters is further reinforced through exploiting RNAP II ChIP-seq data (56). Indeed, tDNA clusters colocalizing with intragenic CS7 are enriched in RNAP II, while clusters colocalizing with intergenic CS8 are not (Supplementary Figure S8).

Nucleosomes occupancy is another key factor impacting DNA accessibility in nucleus. Methods combining digestion of unbound double-stranded DNA using enzymes such as micrococcal nuclease (MNase) and high throughput sequencing of remaining protected nucleosome-bound DNA allow the generation of genome-wide maps of nucleosome positions. Furthermore, while being enriched at heterochromatin states, nucleosome positioning was also reported to influence DNA methylation patterning throughout genome. Indeed, DNA methyltransferases were found to preferentially target nucleosome-bound DNA (74). We therefore decided to explore NO at clustered tDNAs in comparison to non-clustered ones using public MNase-seq data (75). For Pro, NO was similar between clustered and non-clustered tDNAs (Figure 6b, Supplementary Figure S9). In agreement with their heterochromatin state, NO at Ser and Tyr tDNA clusters was the highest. Considering clustered Ser and Tyr as interspaced Ser-Tyr-Tyr units tandemly repeated, we found that while nucleosomes often localized at the center of body of Ser and of the second Tyr tDNA repeats (Tyr C2), they are located toward 3′ end of the first Tyr tDNAs (Tyr C1; Figure 6B, Supplementary Figure S9). Surprisingly, despite the primarily heterochromatin state of

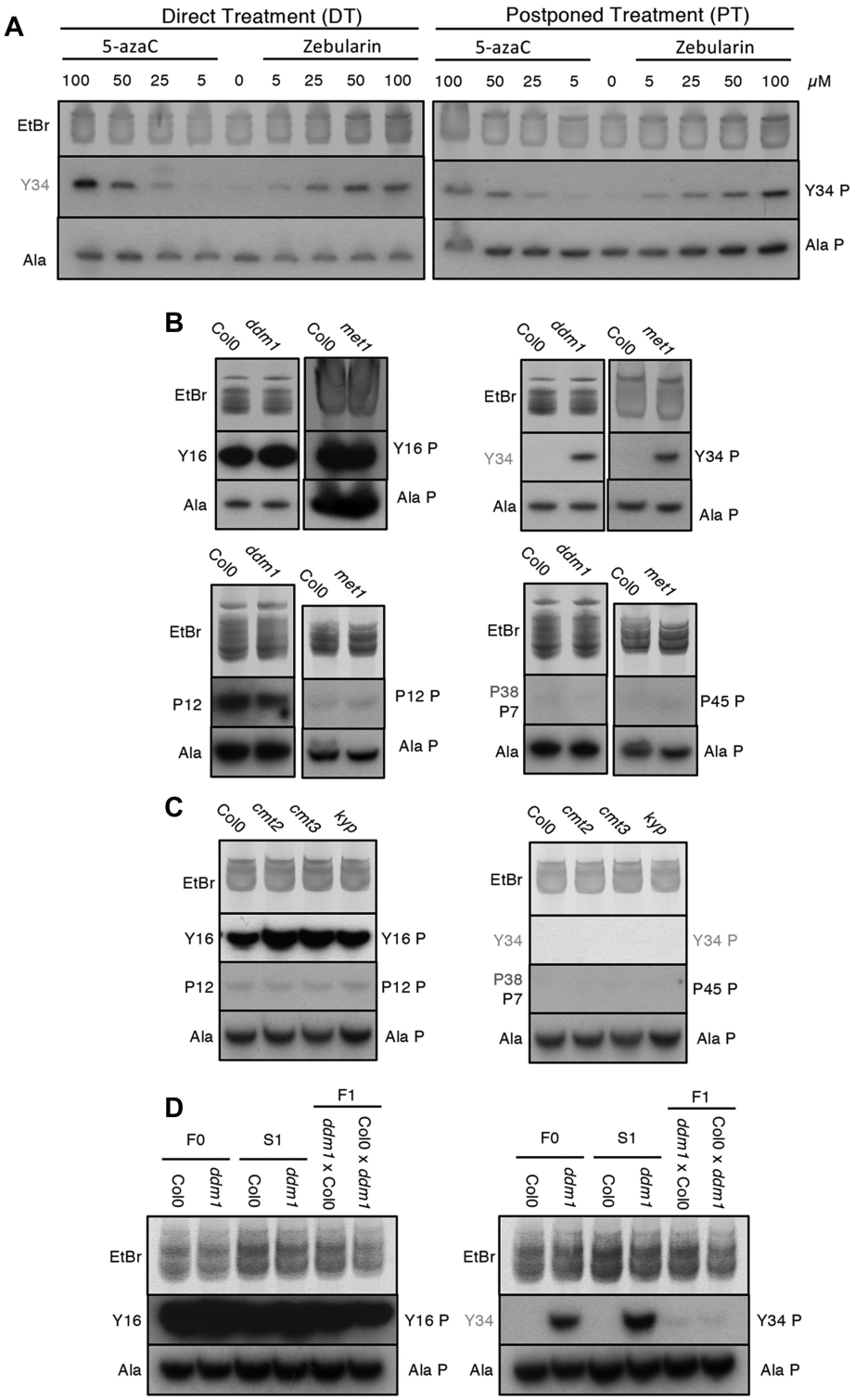

Figure 5. Effect of DNA hypomethylation on tDNAs cluster expression. (**A**) Northern blots analysis of total tRNAs of untreated (0), 5-azaC or zebularine treated WT Arabidopsis plants. Two conditions were used: direct treatment (DT) or postponed treatment (PT) as described in (50). (**B** and **C**) Northern blots analysis of total tRNAs of the indicated mutant genotypes. Experimental conditions and probes are similar to those described in Figure 2B. (**D**) Northern blot analysis in S1 plants (selfing) and in F1 hybrids (reciprocal crosses) between Col0 and *ddm1* mutant plants. Names of probes are as in Figure 2: Y16P and P12P probes are specific to dispersed Tyr and Pro tRNAs, respectively. Y34P and P45P are mainly specific to clustered Tyr and Pro tRNAs, respectively. Original uncropped blots are available in Supplementary Figure S11.

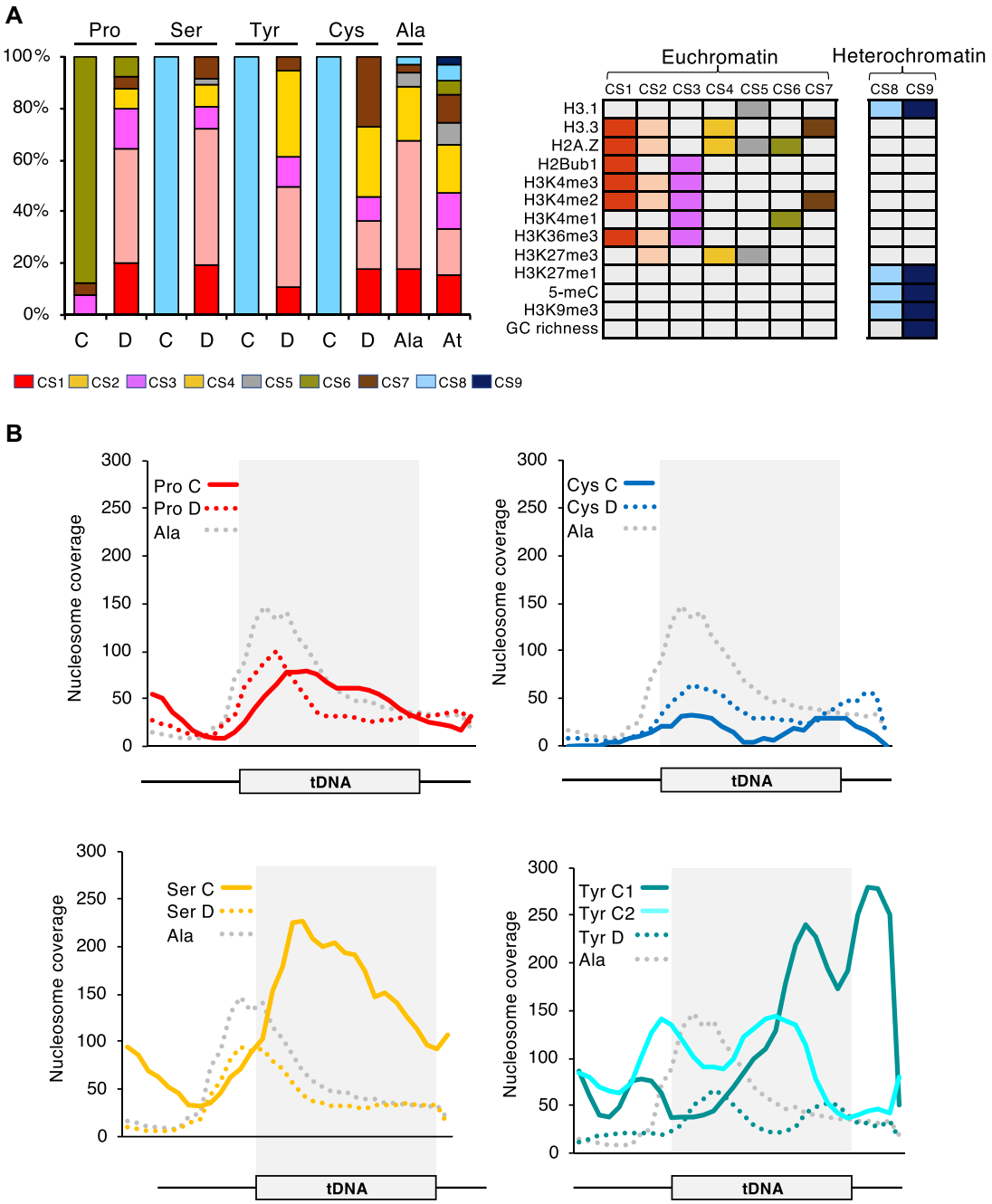

Figure 6. Chromatin states (CS) of dispersed and clustered tDNAs loci. (**A**) Histogram (left panel) showing proportions of the nine chromatin states (CS) (29) at dispersed (D), clustered (C) and Ala tDNAs and for Arabidopsis genome (At). Table (right panel) featuring characteristic signatures for each CS (adapted from (47)). (**B**) Nucleosome occupancy at Ser, Tyr, Pro, Ala, dispersed (D) and clustered (C) tDNA loci. The two Tyr tDNAs in the Ser-Tyr-Tyr tandemly repeated units are numbered 1 and 2, respectively.

Cys tDNA clusters (i.e. CS8 like Ser and Tyr tDNA clusters), NOs were similar between clustered and dispersed Cys tDNAs.

Together, our analysis demonstrates that clustered tDNAs display particular chromatin features compared to their dispersed equivalents. Moreover, clustered tDNAs can be split into three categories based on their chromatin organization, (i) Pro with a predominant euchromatic state mainly associated with long genes and intronic regions, (ii) Ser and Tyr with their typical heterochromatic and repressive environment and (iii) Cys with its heterochromatic state and low NO.

DISCUSSION

Beyond the major role of tRNAs in protein synthesis and their multiple functions in other biological processes, transcriptional modulation of the whole repertoire of tDNAs

by RNAP III remains poorly documented (13). Taken together, our findings provide evidence that DNA methylation and histone PTMs negatively affect RNAP III transcription of clustered tDNAs loci in Arabidopsis and demonstrate the influence of epigenetic environment on expression of tDNAs.

We report the differential expression levels of clustered *versus* dispersed copies of Ser, Tyr, Pro and Cys tDNAs in Arabidopsis. Indeed, under normal growth conditions, clustered tDNAs are repressed whilst their non-clustered equivalents are expressed. Most of these clustered tDNA loci contain the conserved *cis*-elements (A and B internal boxes, TA-rich and CAA upstream motifs, downstream poly-T stretches) involved in RNAP III transcriptional complex recognition, ruling out regulatory genetic alterations. Indeed, Yukawa *et al.* (76) demonstrated that single Pro, Ser and Tyr tDNAs organized in clusters on Arabidopsis Chr1 have the potentiality to be fully transcribed giving raise to mature and stable tRNAs. Furthermore, Stange *et al.* (77) showed that clustered Tyr tDNAs can be efficiently transcribed and processed in HeLa cells. However, it is to note that lack of a CCA upstream motif in clustered Ser tDNAs would likely affect their transcription as shown by (20). On the opposite, presence of two consecutive CCA triplets upstream of many clustered Pro tDNAs would increase initiation of transcription once optimal conditions of RNAP III accessibility are met. Importantly, given that clustered tRNAs (i) exhibit canonical cloverleaf tRNA structures (e.g. Supplementary Figure S2) and (ii) possess all signatures required for efficient tRNA biogenesis (78), their lack of detection is unlikely caused by their rapid degradation following post-transcriptional events.

In silico analyses of epigenomic landscapes show that silencing of clustered tDNAs is correlated with particular epigenetic states involving DNA methylation and repressive histone marks. As compared to dispersed tDNAs scattered in euchromatic environment, clustered tDNAs display high CG methylation levels suggesting that DNA methylation triggers their transcriptional repression as described for most repeats and TEs in Arabidopsis (79,80). Indeed, epigenetic silencing at tDNA clusters is under the complex interplay of DNA methyltransferases MET1, CMT2 and CMT3, as well as chromatin remodeling factor DDM1 (Figure 7). Such process shares some similarities with the transcriptional control of heterochromatic 5S rDNA repeats (81), strengthening the idea that DNA methylation efficiently prevents RNAP III transcription at several loci including clustered tDNAs. Conversely to 5S rDNAs, we did not identify a predominant role neither for the RdDM pathway nor for the DNA methylation-independent pathway involving MOM1 in the transcriptional repression of clustered tDNAs. The particular interplay between genomic environments, epigenetic profiles and RNAP III transcription at clustered tDNAs loci would thus correspond to distinct, complex and yet undetermined mechanisms (Figure 7).

The Ser-Tyr-Tyr cluster is located like its dispersed counterparts in a large intergenic region. This region spans over about 40 Kbp on Chr1 and DNA methylation seems to rely on DNA methylation maintenance effectors MET1, CMT2 and CMT3 (Figure 7). In addition, the Ser-Tyr-Tyr cluster stands in a heterochromatic context enriched in nucleosomes and referred as CS8 (i.e. DNA methylation, H3K9me2 and H3K27me1), a chromatin state reported to map at intergenic regions (47). Therefore, repression of Ser-Tyr-Tyr cluster may rely notably on H3K9 histone methyltransferase KYP. Finally, transcriptional release of clustered Tyr tDNAs in both *ddm1* and *met1* mutant plants, despite ectopic gains of CHG and CHH methylations, indicates that RNAP III can overcome a high non-CG methylation context.

Silenced Pro clusters are located within introns of PCGs and/or overlapping with LncRNAs, while their expressed dispersed counterparts are predominantly found in intergenic regions. Pro clusters differ from other silenced tDNA clusters regarding their euchromatic environment (i.e. primarily CS7) typically found in intragenic regions of RNAP II transcription units longer than average (47). In addition, Pro clusters are enriched in RNAP II, which is not the case for other silenced tDNA clusters. Previously, expression level of the PCG AT2G33980 (Figure 1C) was suggested to negatively correlate with expression of intronic Pro cluster encoded on opposing DNA strands (82). In this sense and further supporting an impact of genomic environment on transcription of clustered tDNAs, intronic tRNAs were characterized by a lower RNAP III occupancy in comparison with non-intronic ones in *C. elegans* (83). More recently, RNAP II passage through tRNA genes was reported to negatively interfere with their transcription in human cells (84). Together, transcription of PCGs and LncRNA genes by RNAP II may lead to non-transcription of a set of overlapping RNAP III-transcribed Pro tDNA despite their permissive chromatin environment.

In addition, Pro clusters display high CG DNA methylation levels and, conversely to the Ser-Tyr-Tyr cluster, both *ddm1* and *met1* mutations did not lead to the release of their expression. Thus, our finding suggests that additional epigenetic mechanisms may act to regulate silencing of Pro clusters. In this sense, histone H3K9 demethylase IBM1, known to prevent CHG and to some extent CHH methylation, was reported to bind to the long PCG (AT2G33980) in which one of the three Pro clusters is located (69). Interestingly, this PCG displays several features characterizing IBM1/IBM2-EDM2 targets such as a long transcriptional unit (> 2 kbp), an alternative poly(A) site and intronic heterochromatic marks (69). Control of H3K9me2 homeostasis by antagonistic action of IBM1 and KYP may therefore prevent gain of CHG and CHH methylation, thus establishing an epigenetic signature distinguishable from those of other tDNA clusters. Recent reports revealed existence of a trade-off between heterochromatic TEs and gene functionality (85,86). Also, heterochromatic introns containing clustered tDNAs behave similarly to well characterized heterochromatic introns containing TE allowing the correct production of full transcripts (85). Therefore, it is tempting to speculate that, at clustered Pro tDNAs, the fine tuning of H3K9me2 homeostasis involving KYP and IBM1 may help to balance the transcription of different mRNA isoforms and tRNA under particular growth conditions.

Cys cluster is located on Chr5 within class II DNA transposons from the Helitron family, while their dispersed counterparts are predominantly found in intergenic

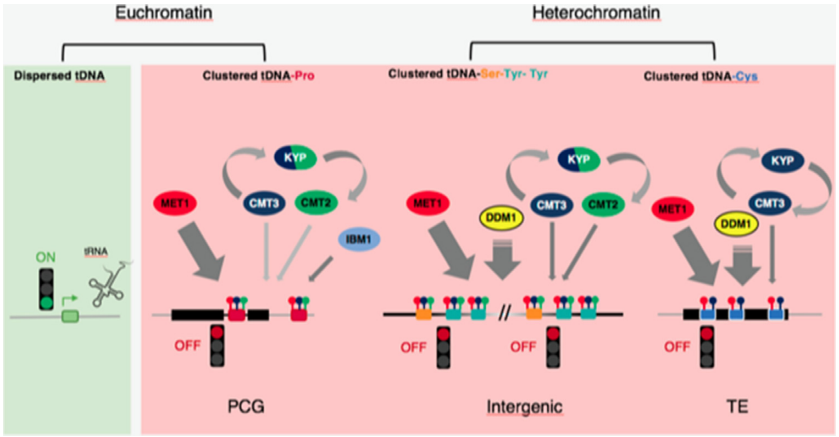

Figure 7. Model of epigenetic regulation of clustered tDNAs. Dispersed tDNAs are spread along Arabidopsis chromosomes and clustered tDNAs repeats are located in distinct genomic (Chr 1, 2 and 5) and epigenomic (heterochromatic and euchromatic) environments. Dispersed tDNAs are found in a euchromatic permissive context allowing their expression (ON: Green light). Ser-Tyr-Tyr and Cys clusters overlap with heterochromatic state and are found within intergenic and TE genomic regions, respectively. Pro clusters overlap with euchromatic state and are located in the vicinity of PCGs or within intron (black line) of PCGs. These clustered tDNAs are found in a repressive chromatin context preventing their expression (OFF: red light). In intergenic genomic regions, DNA methylation levels at Ser-Tyr-Tyr clusters is efficiently maintained through the combined actions of the DNA methyltransferases MET1 (CG: red circle), CMT3 (CHG: blue circle) and CMT2 (CHH: green circle). H3K9 histone methyltransferase KYP allows directing DNA methylation in both CHG and CHH contexts via CMT3 and CMT2, respectively. In TE genomic regions, DNA methylation levels at Cys cluster is maintained through the predominant action of DNA methyltransferases MET1 and CMT3. H3K9 histone methyltransferase KYP allows directing DNA methylation in the CHG context via CMT3. In these heterochromatic clusters, chromatin remodeling factor DDM1 plays an important role to allow efficient maintenance of DNA methylation. In PCG region, DNA methylation levels at Pro clusters is maintained through combined actions of DNA methyltransferases MET1, and to a lower extent CMT2 and CMT3. In this case, KYP would direct DNA methylation in both CHG and CHH contexts. Interestingly, H3K9 histone demethylase, IBM1, likely acts to properly balance H3K9me2 level and thus CHG and CHH DNA methylation controlled by KYP, CMT3 and CMT2.

regions. Compared to retrotransposons, Helitron transposons present a particular chromatin signature. They preferentially associate with the heterochromatin state CS8 (87) and have a low nucleosome occupancy (88,89), (Supplementary Figure S10), in agreement with their preferential location in AT rich regions.

Regarding DNA methylation, Helitron transposons have a high CG and CHG methylation levels (90) and are targeted by MET1, CMT3 and KYP (91). Thus, it is very likely that repression of Cys cluster is a direct consequence of the local Helitron heterochromatic environment and must therefore rely on DNA methyltransferases MET1 and CMT3, as well as on histone methyltransferase KYP. However, we cannot exclude the involvement of other mechanisms. For instance, in Arabidopsis, plant mobile domains MAIN and MAIL1 were shown to be required for silencing of TEs (92). Whether such proteins are involved in the regulation of tDNA expression remains to be established.

Another clue is also the close genomic relationship of tDNAs with TEs as observed now for decades (93–96). TEs mobilization/insertion in addition to recombination between repeats (e.g. tDNAs) allow speculating that these TE-tDNA pairs could presumably be at the origin of both numerous multi-copy of dispersed isoacceptor families and of clusters outstands (97). Such genomic organization may reflect ancient transposition/recombination events followed by the establishment of a repressive chromatin context leading to silencing of both transposons and tDNAs.

Molecular mechanisms underlying non-coding RNA gene clusters formation and location are quite elusive. Genomic location of 5S rDNA loci are variable as well as their copy numbers (79). Particular chromosomal rearrangements, such as unequal crossing overs and DNA break/repairs may have contributed to the formation and shaping of tDNA clusters (98). Moreover, considering the high variability of rRNA gene copy numbers through cell divisions in human and in plant clusters (99,100), it seems that these particular genomic arrangements are submitted to a high flexibility. Interestingly, Arabidopsis plants defective for the cross link repair factor RTEL1 exhibit significant reduction of 45S rDNA copies (101) highlighting that DNA repair factors also contribute to maintain repeats integrity. Additionally, *ddm1* Arabidopsis plants display higher somatic and meiotic recombination frequencies (102) suggesting that maintenance of both genome and methylome integrities are interconnected. Yet, it would be worth to know to which extend this dynamic could be applied to Arabidopsis tDNA repeat clusters. In *S. cerevisiae*, the role of tDNAs in genome instability through replication forks pausing was demonstrated, suggesting that such tDNA clusters might serve as motors for genome fitness and evolution (103). Also, considering the link between chromatin status on replication properties (104), this mechanism might also accompany genome instability or in contrary gently manage sensible genomic breakpoints at tDNA loci.

Biological relevance of the presence of repressed tDNA clusters may also reflect a role in chromosome architecture. For example, certain tDNA clusters surrounding pericentromeres act as insulators preventing the spreading of silent heterochromatin in animal and yeast (105). Given the epigenomics and chromatin dynamics during plant development and upon stress conditions (106–108) we can postulate that under certain developmental and/or growth conditions these tDNA clusters might be re-activated to sustain

yet unknown functions related, e.g. to adaptation to specific codon usage, regulation of protein synthesis or production of specific proteins such as proline-rich proteins (109). Indeed, as the codon-tRNA balance is likely a major factor determining translation efficiency (110), we can hypothesize that a shortage of a tRNA (e.g. Pro tRNA) would be a limiting factor for an optimal translation process to occur. Increasing the concentration of this tRNA by expressing clustered tDNAs would be a way to overcome this deleterious situation. Thus, it would be important to unveil the growth conditions releasing clustered tDNAs expression, if any.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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