

A Duplicated NUCLEOLIN Gene with Antagonistic Activity Is Required for Chromatin Organization of Silent 45S rDNA in *Arabidopsis*^{C1WJOPEN}

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In plants as well as in animals, hundreds to thousands of 45S rRNA gene copies localize in Nucleolus Organizer Regions (NORs), and the activation or repression of specific sets of rDNA depends on epigenetic mechanisms. Previously, we reported that the *Arabidopsis thaliana* nucleolin protein NUC1, an abundant and evolutionarily conserved nucleolar protein in eukaryotic organisms, is required for maintaining DNA methylation levels and for controlling the expression of specific rDNA variants in *Arabidopsis*. Interestingly, in contrast with animal or yeast cells, plants contain a second nucleolin gene. Here, we report that *Arabidopsis* NUC1 and NUC2 nucleolin genes are both required for plant growth and survival and that NUC2 disruption represses flowering. However, these genes seem to be functionally antagonistic. In contrast with NUC1, disruption of NUC2 induces CG hypermethylation of rDNA and NOR association with the nucleolus. Moreover, NUC2 loss of function triggers major changes in rDNA spatial organization, expression, and transgenerational stability. Our analyses indicate that silencing of specific rRNA genes is mostly determined by the active or repressed state of the NORs and that nucleolin proteins play a key role in the developmental control of this process.

INTRODUCTION

In eukaryotes, 45S rDNA (encoding 18S, 5.8S, and 25S rRNA) is arranged in tandem arrays, with copy numbers ranging from hundreds to several thousands in plants. However, only a small proportion of rRNA genes are transcribed. The active fraction forms loops that emanate from knobs of heterochromatin to form the nucleolus (Sáez-Vásquez and Gadal, 2010; Layat et al., 2012; Grummt and Längst, 2013). Although the epigenetic pathways to silence rDNA have largely been studied in *Arabidopsis thaliana* hybrid plants (Lawrence et al., 2004), the mechanisms that activate and/or repress hundreds of rDNA units have not yet been fully elucidated in a nonhybrid plant. Recent reports reveal that proteins involved in histone and DNA modifications might play major roles in the activation and/or

repression of specific subsets of rRNA genes in *Arabidopsis* plants (Earley et al., 2010; Pontvianne et al., 2012).

Nucleolin is one of the most abundant nucleolar proteins and is expected to play a major role in the multiple functions of the nucleolus. Moreover, it has been implicated in DNA replication, recombination, and repair (Tuteja and Tuteja, 1998), nucleolar localization of telomerase (Khurts et al., 2004), and chromatin remodeling (Angelov et al., 2006; Mongelard and Bouvet, 2007). However, in contrast with animals and yeast, the genomes of different plant species encode at least two nucleolin-like proteins (Supplemental Figure 1). This suggests that duplicated nucleolin genes might play redundant and/or specific roles during plant growth and development or in response to environmental conditions.

In *Arabidopsis*, two genes encoding nucleolin proteins have been described: NUC1 and NUC2 (formerly named NUC-L1 and NUC-L2). The first is highly and ubiquitously expressed in normal growth conditions (Kojima et al., 2007; Petricka and Nelson, 2007; Pontvianne et al., 2007). Knockout mutations of the gene are not lethal but lead to severe defects in plant growth and development (Petricka and Nelson, 2007; Pontvianne et al., 2007). Moreover, the absence of this protein in *nuc1* (formally named *nuc-L1*) mutant plants induces nucleolar disorganization, Nucleolus Organizer Region (NOR) decondensation, pre-rRNA accumulation (Petricka and Nelson, 2007; Pontvianne et al., 2007), and the derepression of specific 45S

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rDNA variants (Kojima et al., 2007; Pontvianne et al., 2010). By contrast, little is known about NUC2 and its role in rDNA chromatin organization and expression. This led us to investigate more precisely the biological significance of the second nucleolin gene in *Arabidopsis*. We report here that *Arabidopsis* plants require the expression of both *NUC1* and *NUC2* for survival. *NUC1* and *NUC2* both bind chromatin and control rDNA variant expression, but they act through different and antagonistic molecular mechanisms. In *nuc2* loss-of-function plants, rRNA genes are globally hypermethylated, and the copy number of rDNA variants is affected. Finally, the data suggest that delocalization of silent 45S rDNA from inactive to active NORs is able to induce transcriptional activation of normally repressed rRNA genes.

RESULTS

NUC2 Is a Functional Protein-Coding Gene

To address the role and biological significance of the second nucleolin gene in plants, we characterized two homozygous *nuc2* mutant lines, inducing partial (*nuc2-1*) or complete (*nuc2-2*) loss of *NUC2* gene expression (Figure 1; Supplemental Figure 2).

Under long-day conditions (16-h day/8-h night), *nuc2-2* mutant seedlings emerge and grow quite similarly to wild-type plants (Figure 1A), while *nuc1* plants display growth defects (Kojima et al., 2007; Petricka and Nelson, 2007; Pontvianne et al., 2007, 2010). We further observed that *nuc1 nuc2* double mutants are not viable (Figure 1A). Indeed, homozygous *nuc1* and *nuc2-2* double mutations are seedling lethal, indicating that *NUC2* is a functional gene and that both nucleolin genes are required for plant survival. Similar results were obtained when the allele *nuc1-3* was introduced into *nuc2-1* mutant plants. We also observed that *nuc1* mutant plants heterozygous for *NUC2* are viable but display stronger growth and developmental defects than homozygous *nuc1* or *nuc2* single mutant plants (Supplemental Figure 3).

Interestingly, *nuc2-2* mutants flower later than wild-type plants (Figure 1B). After 53 d of growth, the wild type displays ~17 flowers per plant, in contrast with ~1 flower per plant for *nuc2-2*. The number of rosette leaves at the time of bolting is ~10 and ~20 in wild-type and *nuc2-2* plants, respectively. We do not observe any obvious floral and/or silique phenotype in *nuc2-2* plants. Late flowering is related to the disruption of the *NUC2* gene, since complementation of *nuc2-2* plants with the *Arabidopsis NUC2* genomic sequence restores wild-type flowering time (Figures 1B and 1C). Moreover, quantitative RT-PCR (RT-qPCR) analyses reveal a higher accumulation of *FLOWERING LOCUS C (FLC)* transcripts in *nuc2-2* than in wild-type plants (Figure 1D), which is restored in *nuc2-2* plants carrying the *gNUC2* transgene. These observations are further supported by RNA sequencing analysis showing that *FLC* is upregulated in the *nuc2-2* plants. No other genes related to flowering timing or development were detected among the 33 upregulated or 17 downregulated genes in *nuc2-2* compared with wild-type plants (Supplemental Figure 4).

The *Arabidopsis* genome encodes at least four variants of 45S rDNA. While rDNA VAR1 copies are globally transcriptionally

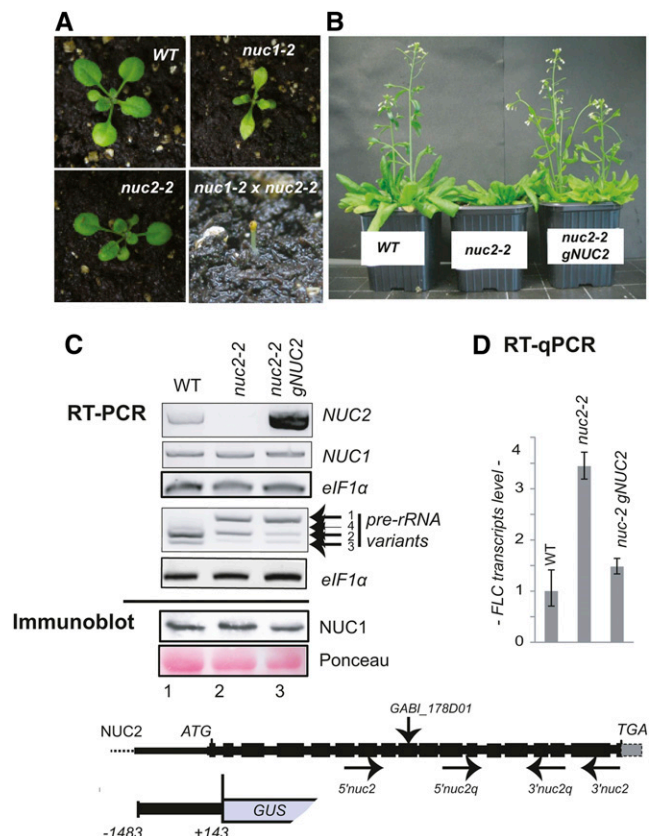


Figure 1. *Arabidopsis NUC2* Is a Functional Protein Gene.

(A) Photographs of *Arabidopsis* wild-type, *nuc2-2* (GABI_178D01), *nuc1-2* (SALK_002764), and *nuc1 nuc2* plants.

(B) *Arabidopsis* wild-type, *nuc2-2*, and *nuc2-2 gNUC2* plants were grown on soil under a 16-h/8-h light/dark cycle. Late flowering is observed in *nuc2-2* mutants compared with wild-type and *nuc2-2 gNUC2* plants.

(C) Top panel, RT-PCR using cDNA prepared from 15-d-old wild-type (lane 1), *nuc2-2* (lane 2), and *nuc2-2 gNUC2* (lane 3) seedlings to detect *NUC2*, *NUC1*, and 3' ETS pre-rRNA transcripts, respectively. Amplification of *eIF1α* transcripts was performed to verify similar amounts of cDNA in each sample. Bottom panel, immunoblot reaction using α -At-*NUC1*. The membrane was stained with Ponceau S before hybridization to verify similar amounts of proteins.

(D) RT-qPCR analysis to determine relative levels of *FLC* transcripts in wild-type, *nuc2-2*, and *nuc2-2 gNUC2* plants. The drawing at bottom shows the *Arabidopsis NUC2* gene from the ATG start codon to the TGA stop codon. Black boxes correspond to exons separated by introns. The positions of primers 5'*nuc2*/3'*nuc2* and 5'*nuc2q*/3'*nuc2q* used in RT-PCR experiments are shown. A sequence upstream from ATG (nucleotide 1483), the first intron, and the exon from the *NUC2* gene fused to GUS are represented. The positions of primers 5'*nuc1*/3'*nuc1* and rRNA 5'3ets/3'3ets are shown in Figure 4 and Supplemental Figure 3. The T-DNA insertion in the *nuc2-2* mutant plant GABI_178D01 is indicated.

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inactive, rDNA VAR2, VAR3, and VAR4 units are permissive to transcription in adult plants (Pontvianne et al., 2010; Abou-Elail et al., 2011). Disruption of the *NUC1* gene induces the derepression of rDNA VAR1 copies (Pontvianne et al., 2010). Interestingly, strong accumulation of pre-rRNA VAR1 and a decrease of pre-rRNA VAR2 and VAR3 levels are observed in *nuc2-2* mutant

plants (Figure 1C). Wild-type profiles of VAR1 and VAR2 pre-rRNA are not restored to the wild-type level in the *nuc2-2* plants complemented with the *gNuc2* construct. By contrast, we do not detect major differences between the level of *NUC1* transcripts or protein in *nuc2-2* or *nuc2-2 gNuc2* plants compared with wild-type plants.

Thus, the results described above show that *NUC2* is a functional gene that influences flowering time and rRNA gene expression, two phenotypes that appear to be uncoupled.

***NUC2* Promoter Activity in Specific Plant Tissues and Organs**

Database analyses using Genevestigator (www.genevestigator.com/gv/) and BAR (www.bar.utoronto.ca/welcome.htm) allow the identification of major fluctuations in the levels of *NUC2* transcripts among different plant tissues, organs, developmental stages, and/or environmental conditions (Figure 2; Supplemental Figure 5). RT-PCR analyses confirm that *NUC2* transcripts are detectable 8 d after germination and then accumulate at 10 and 15 d. A peak of *NUC2* protein accumulation is also detected at 10 d. Interestingly, a concomitant decrease of the pre-rRNA VAR1 level is observed 8 d after germination. By contrast, the levels of

NUC1 transcript and protein remain similar from 4 to 15 d after germination and accumulate at similar levels in both roots and shoots (Figures 2A and 2B). In adult plants, we observed that the *NUC2* transcript level is higher in roots and flowers than in shoots and leaves (Figures 2A and 2C).

To gain insight into the developmental control of *NUC2* gene expression, we further characterized *NUC2* promoter activity using plants bearing *pNuc2:GUS* (for β -glucuronidase) transcriptional fusions. In the root apical meristem, GUS staining is detected at 4, 7, 10, and 15 d after germination (Figure 2D, top row). By contrast, staining is detected in stipules only after 7 d of germination and then at 10 and 15 d (Figure 2D, middle row), in agreement with our RT-PCR analyses. In adult plants, expression is detected specifically in leaf hydathode cells, lateral roots, flowers, and the buttress (Figure 2D, bottom row). On the other hand, *NUC2* promoter activity is induced in response to auxin treatment and during silique development, earlier in developing anthers than in developing pistils (Supplemental Figures 6 and 7).

Accordingly, these results show that *NUC2* is developmentally controlled in most plant tissues and organs analyzed. Importantly, *NUC2* expression and protein abundance increase during the establishment of rDNA VAR1 repression throughout seedling development.

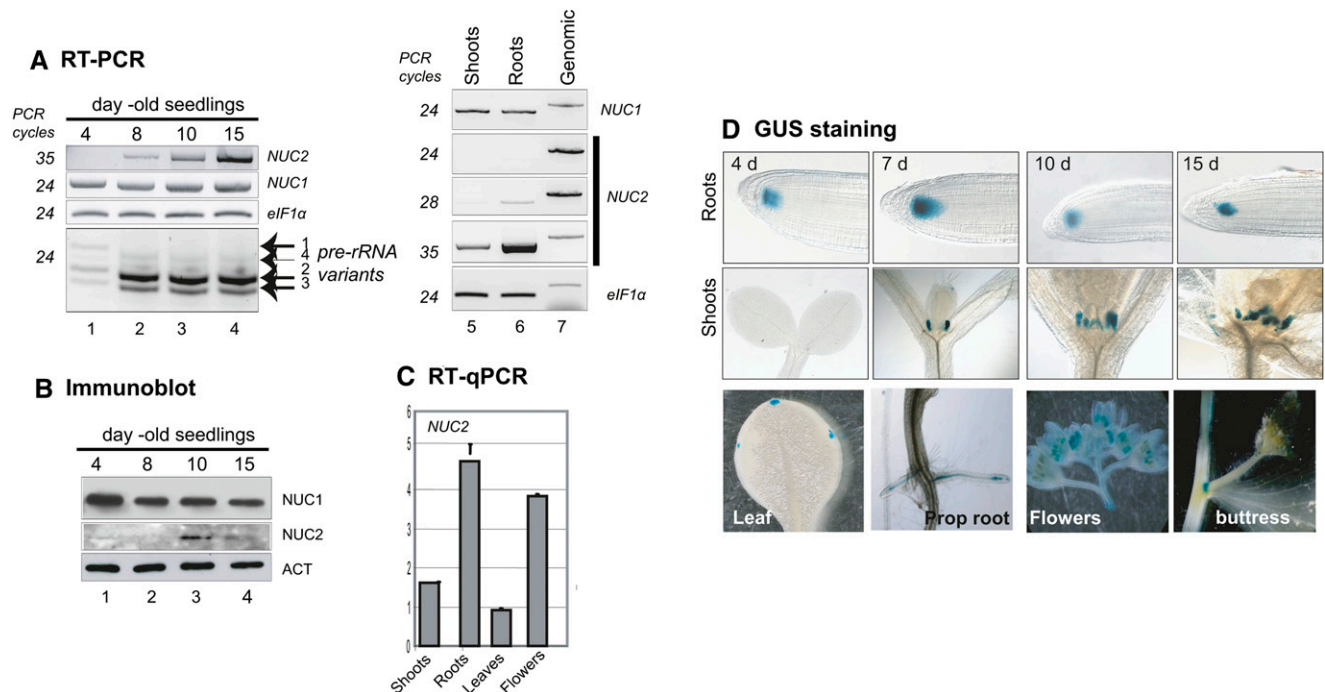


Figure 2. *NUC2* Gene Expression during Seedling Development and in Plant Organs.

(A) RT-PCR to show *NUC2* and *NUC1* transcripts during seed germination (lanes 1 to 4), in shoots (lane 5), and in roots (lane 6). Absence of genomic contamination in the cDNA samples was verified by amplification of *NUC1*, *NUC2*, and *eIF1α* genomic DNA, which generates higher molecular size bands (lane 7). The number of cycles (24, 28, and 35) of each PCR is indicated.

(B) Immunoblot experiment to determine the levels of *NUC1* and *NUC2* proteins during seed germination. α -Actin (ACT) was used to verify similar amounts of proteins.

(C) RT-qPCR to determine the relative levels of *NUC2* transcripts in shoots, roots, leaves, and flowers.

(D) Analysis of *NUC2* promoter activity in *Arabidopsis* plants transformed with the *pAtNuc2:GUS* construct. Top and middle rows, *Arabidopsis* seeds were sown on MS medium and maintained for 48 h at 4°C (day 0) and then transferred to room culture, at 22°C under continuous light conditions, for 4, 7, 10, and 15 d. Bottom row, GUS staining in leaf hydathode cells, roots, flowers, and buttress. The *NUC2* gene fused to GUS is represented in Figure 1.

NUC1 Protein Might Control NUC2 Expression

Disruption of *NUC1* induces *NUC2* overexpression (Pontvianne et al., 2007, 2010). To determine if *NUC1* might control *NUC2* expression at the transcriptional level, we transformed *nuc1* mutant plants with the *pNUC2:GUS* reporter gene construct. As shown in Figure 3A, the *nuc1* mutation considerably strengthens the *pNUC2:GUS* signal and extends *NUC2* promoter activity to the entire seedling (compare with Figure 2D, staining in the stipules and roots). In fact, the *pNUC2:GUS* signal in the *nuc1* background is similar to the *pNUC1:GUS* signal in wild-type plants (Petricka and Nelson, 2007; Pontvianne et al., 2007). Next, the possibility that *NUC1* protein physically interacts with chromatin at the *NUC2* locus was tested by chromatin immunoprecipitation (ChIP) using an anti-*NUC1* serum (Figure 3B). In wild-type chromatin, *NUC2* sequences were detected with primer pairs corresponding to the 5' region (lanes 5 and 8) but not with primers matching the upstream intergenic region (lane 2). The 5' end of *NUC2* transcripts maps to the position -87 nucleotides upstream from the ATG translation initiation codon; therefore, *NUC1* likely binds the *NUC2* promoter domain rather than its 5' untranslated region domain. No significant signals were detected in PCR using chromatin incubated with protein A alone (lanes 1, 4, and 7) or using chromatin extracted from *nuc1-2* seedlings and incubated with antibodies against *NUC1* (lanes 1, 2, 4, 5, 7, and 8). These *in vivo* analyses are corroborated by *in vitro* electrophoretic mobility shift assay (EMSA) showing that *NUC1* recombinant protein can bind different regions of *NUC2* promoter DNA indiscriminately. These observations suggest that the specificity of *NUC1* for region -307 to -38 might be conferred by the *NUC2* promoter chromatin structure or by other associated proteins in planta (Supplemental Figure 8A).

In standard growth conditions, a methylation island is detected at the 3' sequence of the *NUC2* gene (<http://signal.salk.edu/cgi-bin/methylome>). However, no changes of CG methylation are observed in this region or in the promoter sequence in *nuc1* mutants (Supplemental Figure 8B). Nevertheless, *NUC2* is also overexpressed in *met1* mutant plants that exhibit a massive loss of DNA methylation at the 3' sequence (Figure 3C). Therefore, local changes in DNA methylation levels cannot explain *NUC2* overexpression in *nuc1* plants. In addition, analyses of mutants for the RNA-directed DNA methylation pathway and histone deacetylases/acetylases do not reveal changes in the expression of *NUC2* (Supplemental Figure 8C).

To test if *NUC1* might also influence *NUC2* expression at the posttranscriptional level, we measured *NUC2* protein levels in 15-d-old wild-type and mutant plants (Figure 3D). At this stage, *NUC2* protein is detected in the two *nuc1* mutant lines tested (lanes 1 and 2) but not in wild-type (lane 6), *nuc2-2* (lane 3), *nuc2-2 gNUC2* (lane 4), or *met1* (lane 5) plants. Furthermore, overexpression of the *NUC2* gene, under the control of the *NUC1* promoter, does not lead to the accumulation of *NUC2* protein in wild-type plants (data not shown).

Together, these results indicate that *NUC1* is required to inhibit *NUC2* gene expression at the transcriptional level and may also influence the accumulation of *NUC2* protein.

NUC2 Gene Disruption Affects rDNA Chromatin Organization

Because *NUC2* gene disruption increases VAR1 rDNA expression, we further questioned whether (1) RNA pol I transcription and/or (2) rDNA chromatin states are affected in *nuc2-2* mutant plants. In *nuc1* and *histone deacetylase6 (hda6)* mutant plants, the expression of pre-rRNA VAR1 is associated with rRNA transcription initiated from an intergenic spacer and with rDNA chromatin decondensation (Earley et al., 2010; Pontvianne et al., 2010).

Primer extension analysis (Figure 4A) shows that a single transcription initiation signal (TIS) is detected in *nuc2-2* and *nuc2-2 gNUC2* samples (lanes 2 and 3), in contrast with the double TIS observed in wild-type plants (lane 1). Nevertheless, quantification of the ratio between the TIS corresponding to primary pre-rRNA and the P signal corresponding to short-lived 5' end-cleaved pre-rRNA does not reveal significant changes in the amount of primary or processed rRNA precursors in *nuc2-2* and *nuc2-2 gNUC2* compared with wild-type samples.

To assess whether *NUC2* might influence the expression and subnuclear localization of rDNA, we first tested whether *NUC2* associates with a chromatin fraction. Chromatin samples were notably purified from *nuc1* mutant plants, in which *NUC2* protein overaccumulates, and analyzed by immunoblot. *NUC2* protein was detected in *nuc1* but not in wild-type chromatin samples (Figure 4B). Similarly, the accumulation of *NUC1* protein was detected in wild-type but not in *nuc1* mutant extracts. Based on these results, *NUC2* subnuclear localization was analyzed in more detail by immunofluorescence on fixed cells. This reveals a discrete localization in the nucleolus near large heterochromatin regions corresponding to heavily 4',6-diamidino-2-phenylindole (DAPI)-stained DNA in the *nuc1* mutant background (Figure 4C; Supplemental Figure 9C). Interestingly, therefore, *NUC2* is mainly concentrated in nucleolar regions physically associated with condensed chromatin regions from which VAR1 rDNA has recently been shown to emanate (Pontvianne et al., 2013). Furthermore, the nucleolar localization of *NUC1* protein is not affected in *nuc2-2* plants (Supplemental Figure 10).

DNA fluorescence *in situ* hybridization (FISH) analysis of leaf cells shows that up to four rDNA signals are detected in interphase nuclei of wild-type plants (Figure 4D; Supplemental Table 1). These signals correspond to two each of NOR2 and NOR4 (in chromosomes 2 and 4, respectively), as expected in a diploid *Arabidopsis* cell (Pecinka et al., 2004). Interestingly, ~18% of nucleoli from *nuc2-2* and *nuc2-2 gNUC2* cells associate with three or more rDNA signals compared with one or two rDNA signals in wild-type nucleoli. By contrast, no significant differences can be detected in the number or size of the centromeres between the DAPI-labeled wild-type and *nuc2-2* and/or *nuc2-2 gNUC2* plants.

Finally, to obtain more insight into how disruption of *NUC2* induces the transcriptional activation of repressed rDNA VAR1, we purified nucleoli from *nuc2-2* mutant plants by fluorescence-assisted nucleolus sorting (FANoS) (Pontvianne et al., 2013). The four rDNA variants are detected in the nuclear fraction from wild-type and *nuc2-2* plants (Figure 4E, lanes 1 and 3). By contrast, in the nucleolar fraction, VAR1 rDNA is enriched in *nuc2-2* samples (lane 4), while only a faint band is detected in wild-type plants (lane 2).

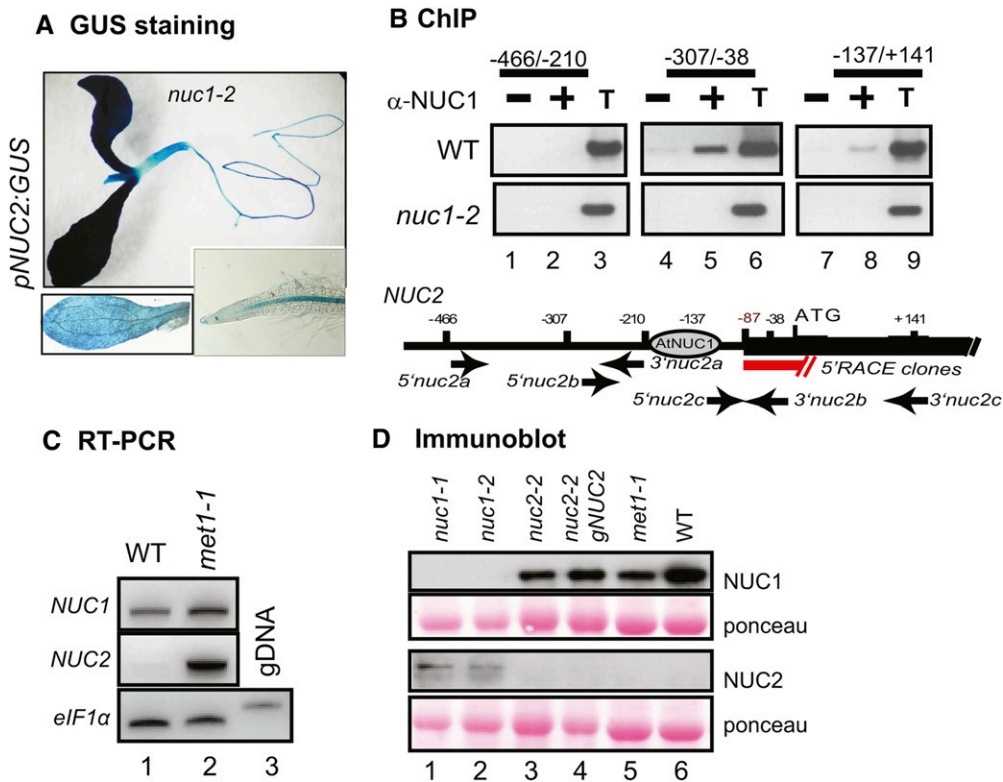


Figure 3. NUC1 Participates in the Regulation of *NUC2* Gene Expression.

(A) Analysis of *NUC2* promoter activity in *nuc1* mutant plants transformed with the *pNUC2:GUS* construct. The bottom panels show shorter staining to better visualize GUS activity in leaves and roots.

(B) Chromatin from wild-type plants was isolated and incubated either with protein A only (lanes 1, 4, and 7) or with antibodies against NUC1 (lanes 2, 5, and 8). Immunoprecipitated DNA was analyzed by PCR to detect *NUC2* sequences from -466 to -210 (lanes 1 to 3), from -307 to -38 (lanes 4 to 6), and from -137 to $+141$ (lanes 7 to 9). The positions of primers used to amplify *NUC2* sequences are indicated. The transcription initiation site is mapped by 5' rapid amplification of cDNA ends at 87 nucleotides upstream from ATG. An aliquot of total chromatin sample before the immunoprecipitation reaction was used to control PCR amplifications (lanes 3, 6, and 9). Chromatin isolated from the *nuc1* mutant was used to control the specificity of At-NUC1 antibodies in the ChIP reaction (*nuc1-2* panels).

(C) RT-PCR to show *NUC2* and *NUC1* transcripts in wild-type (lane 1) and *met1-1* (lane 2) plants. The amount of cDNA was verified in each reaction by controlling *eIF1 α* transcript levels. Absence of genomic contamination in the cDNA samples was verified by amplification of *eIF1 α* genomic DNA, which generates higher molecular size bands (lane 3).

(D) Immunoblot analysis to detect NUC1 and NUC2 proteins in *nuc1* (lanes 1 and 2), *nuc2-2* (lane 3), *nuc2-2 gNUC2* (lane 4), *met1-1* (lane 5), and wild-type (lane 6) plants. Similar amounts of protein in each membrane were verified by Ponceau S staining.

The ratio between rDNA VAR2 and VAR3 signals remains similar in *nuc2-2* (lane 4) compared with the wild-type sample (lane 2).

Altogether, these data show that in the *nuc2-2* mutant, rDNA subnuclear localization is altered; rDNA VAR1 is localized and expressed in the nucleolus. These results suggest that some modifications and/or rearrangements of rDNA chromatin occur in *nuc2-2* mutant plants as NUC2 binds chromatin.

NUC2 Is Required for the Stability of rDNA Variants Copy Number

Derepression of VAR1 rRNA genes in *nuc2-2* mutant plants could be explained by the observed reorganization of rDNA from inactive to active NOR chromatin (Jakociunas et al., 2013) but also by possible rDNA copy number variation. This phenomenon has previously been observed in *fasciata* (*fas*)

mutant plants (Figure 5A), a subunit of the H3/H4 histone chaperone Chromatin Associated Factor1 complex (Mozgová et al., 2010).

We first quantified 18S, 25S, and ITS1 rDNA sequences in wild-type, *nuc2-2*, and *nuc2-2 gNUC2* plants and observed no major variation (Figure 5A, top panel). By contrast, a global decrease of 45S rDNA copy number was detected in *fas2-4* mutant plants, as reported previously (Mozgová et al., 2010). Therefore, we further determined the amount of specific rDNA variants. In this analysis, VAR1, VAR2, and VAR3 rDNA copies represent ~46, 25, and 29%, respectively, of the rRNA genes in wild-type plants. In *nuc2-2* mutants, these numbers showed a relative increase of the VAR1 copy number (~63, 12, and 25%, respectively) (Figure 5B; Supplemental Figure 11). In *nuc2-2 gNUC2* plants, the proportions are similar to the ratio observed in *nuc2-2* mutant plants, indicating that reintroducing the *NUC2*

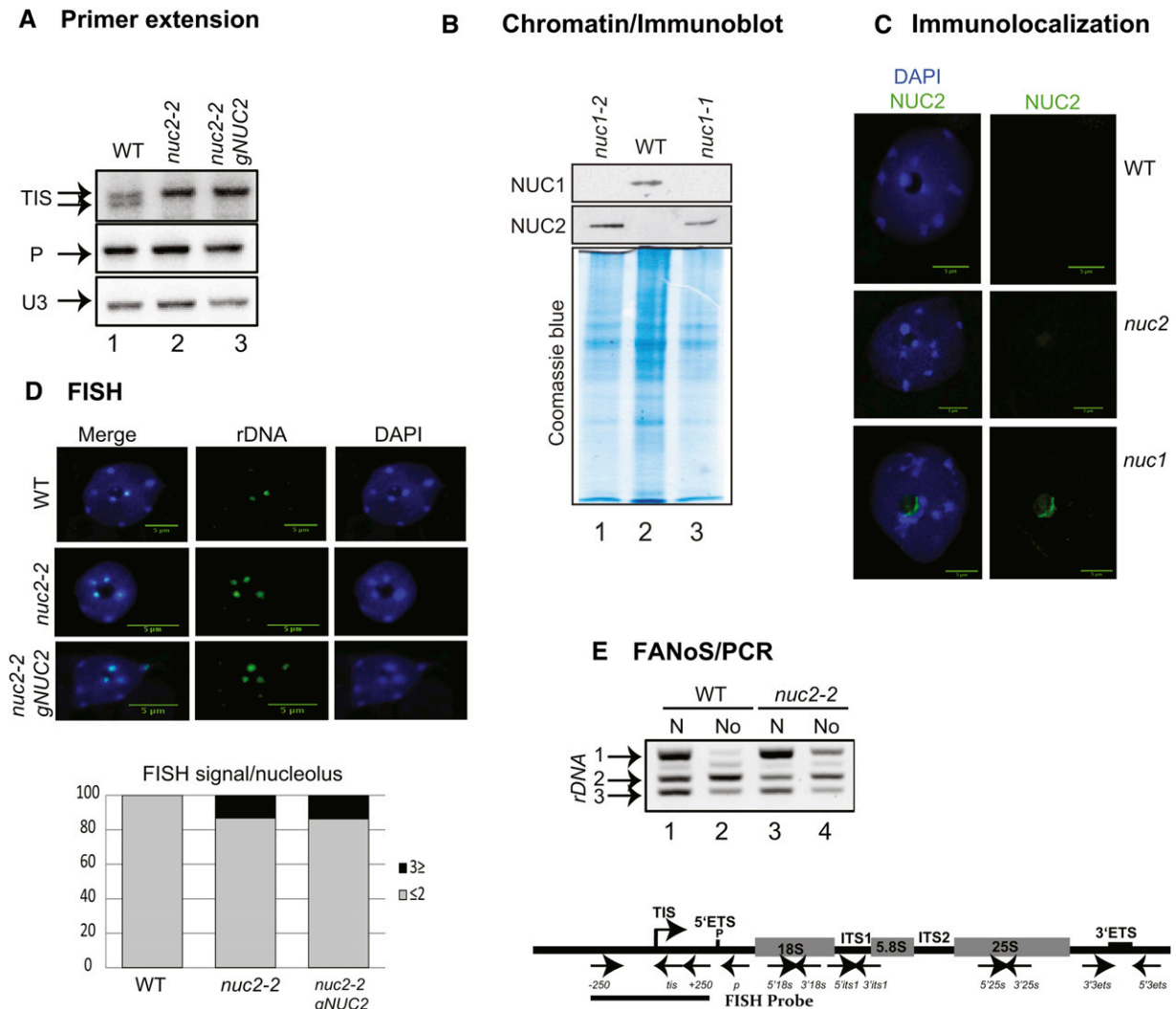


Figure 4. Transcription and rDNA Organization in *nuc2-2* Plants.

(A) Primer extension was performed using total RNA extracted from wild-type (lane 1), *nuc2-2* (lane 2), and *nuc2-2 gNUC2* (lane 3) plants. Primer *tis* detects transcription from the TIS, and primer *p* detects pre-rRNA precursors cleaved at the primary cleavage site (P) (Sáez-Vasquez et al., 2004; Pontvianne et al., 2007). Primer extension using primer *U3* that accurately maps the 5' end of U3snRNA was used to control similar amounts of total RNA in each reaction.

(B) Immunoblot analysis to detect NUC1 and NUC2 protein in *nuc1* (lanes 1 and 3) and wild-type (lane 2) chromatin extracts. The Coomassie blue gel staining reveals similar amounts of protein in each chromatin sample.

(C) Immunolocalization of NUC2 in leaves from *nuc1* plants. The NUC2 signal (green) colocalizes with chromatin situated in the periphery of the nucleolus and counterstained with DAPI (blue) (*nuc1* panels). No NUC2 signal is detected in wild-type or *nuc2* plants (used here to control the specificity of the antibodies).

(D) FISH analysis with an rDNA probe containing sequences upstream and downstream from TIS (from -250 to +250) reveals the 45S rDNA loci (green) on nuclei from wild-type, *nuc2-2*, and *nuc2-2 gNUC2* plants. Counterstaining with DAPI (blue) is shown (FISH and DAPI). The bar graphs at bottom show the percentage of rDNA loci associated with the nucleolus in wild-type, *nuc2-2*, and *nuc2-2 gNUC2* plants. Bars = 5 μ m.

(E) PCR detection of rRNA gene variants in DNA of purified nuclei (N) or nucleoli (No) of wild-type (lanes 1 and 2) and *nuc2-2* (lanes 3 and 4) plants. The positions of 5'3ets and 3'3ets primers used in PCR experiments are shown. The scheme at bottom represents the 45S rDNA unit containing the ETS (5' ETS and 3' ETS) and the structural rRNA sequences (18S, 5.8S, and 25S rRNA in gray boxes) separated by internal transcribed spacers (ITS1 and ITS2). Four repeat sequences located in the 3' ETS are represented (R1 to R4). The TIS and the primary cleavage site (P) in the 5' ETS are indicated. The positions of primers used to amplify or detect rRNA genes and/or pre-rRNA sequences are shown.

function did not restore normal rDNA variant copy numbers. In addition, analysis of subsequent *nuc2-2* and *nuc2-2 gNUC2* generations after five selfings in single-seed descent reveals no further changes in the abundance and expression of rDNA variants (data not shown). Finally, no significant changes are observed in *nuc1-2*, suggesting a specific effect of the loss of *NUC2* function on the stability of the VAR1 copy number.

rDNA Methylation State in *nuc2-2* Mutants

The expression of rRNA VAR1 was recently shown to be associated with CG hypomethylation of the 5' external transcribed spacer (ETS) region in *nuc1* and *hda6* mutant plants (Earley et al., 2010; Pontvianne et al., 2010). The methylation state of 5' ETS rDNA (from -315 to +243), therefore, was tested by bisulfite sequencing analysis in *nuc2-2*. This reveals a hypermethylated 5' ETS region in *nuc2-2* mutant lines (~35%) compared with wild-type plants (~22%), which was restored to the wild-type level in *nuc2-2 gNUC2* samples. Remarkably, the increased methylation level is restricted to the CG context in the 5' ETS region and is not observed in the upstream promoter domain. CHG and CHH methylation are not affected in *nuc2* plants (Figure 6A).

Because the 5' ETS sequence is shared by all four rDNA variants, bisulfite sequencing does not allow us to determine which rDNA variants are hypermethylated in *nuc2* mutants. Furthermore, bisulfite sequencing of the 3' ETS could not be used because of the higher number of VAR1 copies (Supplemental Figure 12). Consequently, we decided to perform modified cytosine restriction BC (McrBC) PCR restriction analysis, which consists of specifically cleaving methylated DNA and amplifying specific nonmethylated and noncleaved DNA by PCR (Figure 6B; Supplemental Figure 13). This analysis reveals that VAR1 rDNA is highly amplified in both *nuc2-2* and *nuc2-2 gNUC2* samples (lanes 6 and 7) as compared with wild-type plants (lane 5). This might either result from a global decrease of DNA methylation level on VAR1 rDNA copies or a higher number of unmethylated VAR1 rDNA copies in these plants. By contrast, similar amounts of nonmethylated rDNA VAR2 and VAR3 are detected (lanes 5 to 8). As observed before, a significant reduction of rDNA VAR2 in *nuc2-2* and *nuc2-2 gNUC2* plants is detected in undigested DNA samples (lanes 2 and 3) compared with control plants (lanes 1 and 4). Only a faint rDNA VAR1 amplification band is detected in wild-type and *nrpe1* plants upon McrBC digestion, revealing that most VAR1 rDNA is methylated in these samples (lanes 5 and 8) (Wierzbicki et al.,

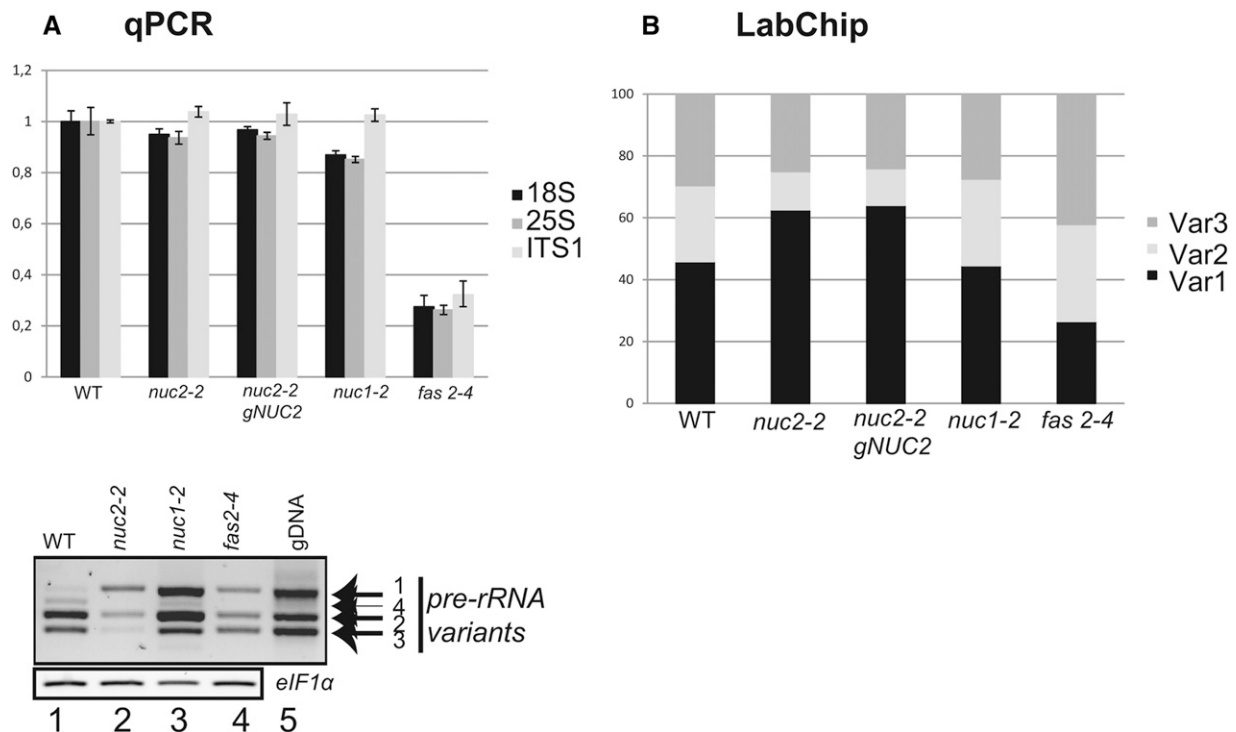
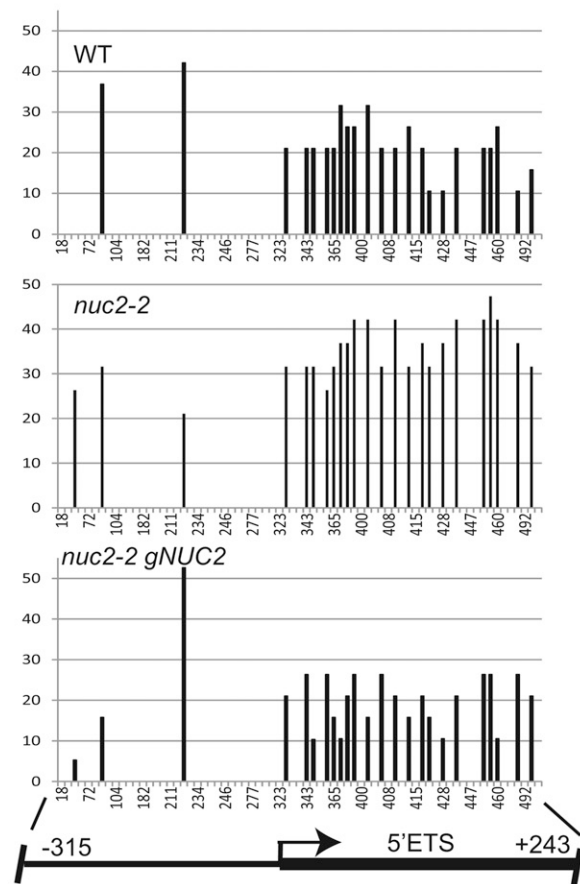
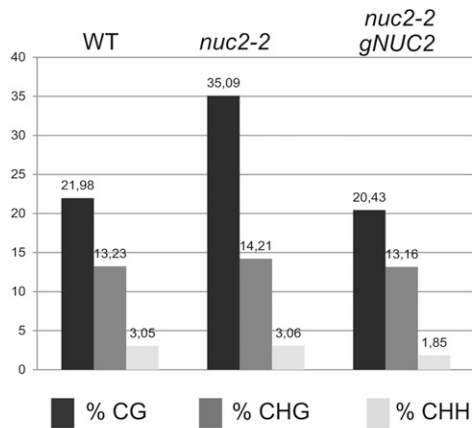
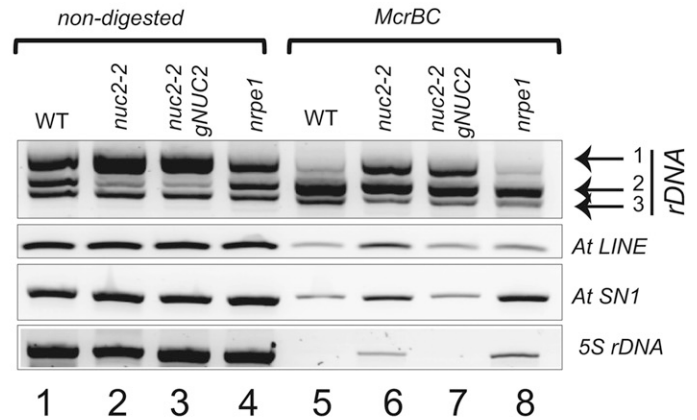


Figure 5. The Relative Abundance of rDNA Variants Is Affected in Plants with a Disrupted *NUC2*.

(A) Top, quantitative PCR analysis to amplify 18S, 25S, and ITS1 rDNA sequences from wild-type and mutant (*nuc2-2*, *nuc2-2 gNUC2*, *nuc1-2*, and *fas2-4*) plants. The bar graphs show relative amounts of 18S (black), 25S (dark gray), and ITS1 (gray) rDNA. Bottom, RT-PCR using cDNA prepared from 15-d-old wild-type (lane 1), *nuc2-2* (lane 2), *nuc1-2* (lane 3), and *fas2-4* (lane 4) seedlings to detect 3' ETS pre-rRNA transcripts. Amplification of *eIF1α* was performed to control similar amounts of cDNA in each sample. PCR on genomic DNA from wild-type plants detects the rDNA variants 1 to 4 (lane 5). **(B)** PCR amplifications of 3' ETS sequences using genomic DNA from wild-type, *nuc2-2*, *nuc2-2 gNUC2*, *nuc1-2*, and *fas2-4* plants. Relative amounts of each rDNA variant were determined using the LabChip GX system. The bar graphs show the percentage of rDNA VAR1 (black), VAR2 (gray), and VAR3 (dark gray).

A Bisulfite sequencing**B McrBC assay****Figure 6.** rDNA Is Hypermethylated in *nuc2-2* Mutant Plants.

(A) Bisulfite sequencing analysis. Top, the bar graphs show the percentage of methylated sites in the rRNA gene sequences (from -315 to $+243$) from wild-type, *nuc2-2*, and *nuc2-2 gNUC2* plants in CG, CHG, and CHH contexts. Bottom, frequencies at which individual cytosines in a CG context are methylated between -315 and $+243$ relative to TIS. The scheme shows the positions of rDNA sequences upstream (promoter) and downstream (5' ETS) of TIS. Most of the CG sites localize in the 5' ETS region.

(B) Genomic DNA from wild-type, *nuc2-2*, *nuc2-2 gNUC2*, and *nrpe1* plants digested (lanes 5 to 8) or not (lanes 1 to 4) with McrBC. Amplifications of 3' ETS, At LINE, At SN1, and 5S were performed to detect methylated or unmethylated DNA sequences in *nuc2-2*, *nuc2-2 gNUC2*, and control (wild-type and *nrpe1*) plants.

2008). Note that *At LINE* and *At SINE* repeats, as well as 5S rRNA genes, are hypomethylated in *nuc2-2* (lane 6) as in *nrpe1* control plants (lane 8).

Altogether, *NUC2* disruption results in contrasting variations of rDNA methylation at different loci and on different variants, with a significant decrease of methylated DNA levels on 3' ETS VAR1-specific regions.

rDNA Organization and Expression in Wild-Type × *nuc2* Plants

VAR1 rDNA is derepressed in *nuc2* mutant plants, a phenomenon associated with a relative increase of VAR1 copy number in the genome of these plants. Therefore, we determined whether or not the introduction of wild-type rDNA copies could restore normal expression patterns. This was achieved by reciprocally crossing wild-type plants with *nuc2-2* plants (Figure 7). We first observed that F1 plants from backcrossed *nuc2* × wild-type (BC4 and BC3) plants and wild-type × *nuc2* (BC11 and BC9) plants display wild-type flowering timing (Figure 7A). Moreover,

VAR1 pre-rRNA is repressed, while VAR2 and VAR3 expression is restored to wild-type levels in all the backcrossed lines (Figure 7B). The abundance of rDNA VAR1, VAR2, and VAR3 is basically restored to wild-type levels in the backcrossed *nuc2/+* (45, 24, and 31%) and/or *+nuc2* (47, 23, and 30%) plants (Figure 7C, top panel; Supplemental Figure 14). Interestingly, compared with wild-type and *nuc2-2* plants, the amount of rDNA is higher in *+nuc2* (BC9) and lower in *nuc2/+* (BC4) (Figure 7C, bottom panel).

These results suggest that the expression of rDNA VAR1 in *nuc2-2* and in *nuc2-2 gNUC2* plants is intimately linked to changes in the VAR1:VAR2 copies ratio.

NUC1 but Not NUC2 Can Assist Nucleosome Remodeling in Vitro

To obtain insight into how the interaction of nucleolin proteins with chromatin might control rDNA organization and/or expression, we studied the nucleosome remodeling of NUC1 and NUC2 in vitro. Mammalian nucleolin has been reported as being

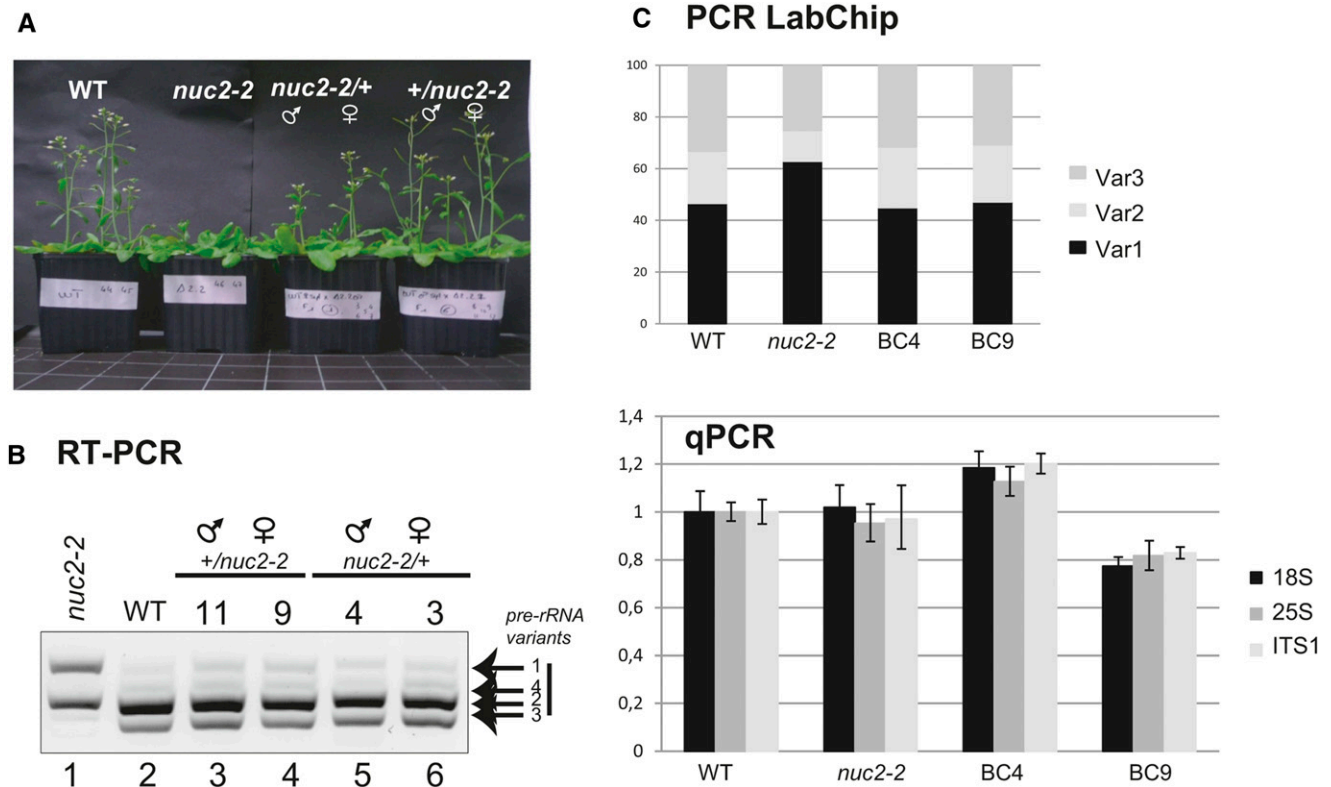


Figure 7. Reintegration of Wild-Type rDNA Restores the Abundance of rDNA Variants and the Repression of VAR1 in *nuc2-2*.

(A) Flowering time analysis of wild-type, *nuc2-2*, and backcrossed plants using pollen from *nuc2-2* (*nuc2* × wild type) or from wild-type (wild type × *nuc2*) plants.

(B) RT-PCR using cDNA prepared from 15-d-old *nuc2-2* (lane 1), wild-type (lane 2), *+nuc2* (lanes 3 and 4), and *nuc2/+* (lanes 5 and 6) seedlings to detect 3' ETS pre-rRNA transcripts.

(C) The bar graphs show relative amounts of rDNA VAR1 (black), VAR2 (gray), and VAR3 (dark gray) (top) and 18S (black), 25S (gray), and ITS1 (dark gray) (bottom) rDNA sequences in the wild type, *nuc2-2*, *nuc2/+* (BC4), and *+nuc2* (BC9).

[See online article for color version of this figure.]

able to bind nucleosomes and assist chromatin-remodeling activities *in vitro* (Angelov et al., 2006; Rickards et al., 2007). To test these potential “activities” of *Arabidopsis* NUC1 and NUC2, we used an *in vitro* nucleosome-sliding assay consisting of nucleosomes assembled *in vitro* and the remodeling activity of chromatin structure remodeling (RSC) (Angelov et al., 2006).

In vitro-reconstituted *Xenopus laevis* nucleosomes were incubated with increasing amounts of RSC, resulting in an efficient mobilization of the nucleosomes and the formation of end-positioned nucleosomes (Supplemental Figure 9A). To determine mobilization by NUC1 and NUC2, we used suboptimal RSC concentrations at which RSC was unable to remodel nucleosomes alone (Figure 8). Nucleosomes were further incubated with increasing amounts of RSC in the absence or presence of 300 ng of NUC1 (lanes 1 to 10) or NUC2 recombinant proteins (lanes 11 to 20). As expected, no detectable nucleosome sliding was observed in the absence of NUC1 (lanes 2 to 5) or NUC2 (lanes 12 to 15). By contrast, the presence of NUC1 in the remodeling reaction results in an efficient mobilization of central nucleosomes to end-positioned nucleosomes (lanes 7 to 10). No sliding was observed in the presence of NUC2 (lanes 17 to 20). Incubation of NUC1 or NUC2 proteins with nucleosomes without RSC induces a slight shift (lanes 6 and 16). Note that no free probe is detected in fractions containing nucleolin, presumably due to the strong DNA binding activity of NUC1 and NUC2 proteins (Supplemental Figure 9B). Altogether, these analyses indicate that NUC1 and NUC2 can bind nucleosomes but that only NUC1 can assist nucleosome remodeling *in vitro*.

DISCUSSION

Arabidopsis NUC2 Is a Functional Protein Gene

The genome of *Arabidopsis*, like most plant genomes, contains two nucleolin protein genes: *NUC1* and *NUC2* (Supplemental Figure 1). Previously, *Arabidopsis* *NUC1* was demonstrated to be

a plant functional homolog of animal and yeast nucleolins (Kojima et al., 2007; Petricka and Nelson, 2007; Pontvianne et al., 2007, 2010). These studies led us to raise the question about the physiological significance of the second nucleolin gene in plants.

Arabidopsis *NUC2* knockout plants are viable and do not exhibit a significant growth phenotype, but they have a late flowering phenotype. This suggests that *NUC2* is not an essential gene or that *NUC1* might rescue the function of *NUC2* in *nuc2* mutants. Similarly, *NUC2* at least partially rescues the loss of *NUC1* function in *nuc1* plants (Pontvianne et al., 2007). Accordingly, simultaneous disruption of *NUC1* and *NUC2* is lethal, indicating that *NUC2* can fulfill some essential functions of *NUC1*. Late flowering in *nuc2-2* mutant plants associates with the overexpression of *FLC*, a gene that plays a central role in flowering time regulation in *Arabidopsis* (Crevillén and Dean, 2011). Remarkably, *NUC2* disruption affects the expression of only a small subset of protein-coding and non-protein-coding genes besides *FLC*. No genes related to 45S pre-rRNA transcription or processing are upregulated or downregulated in *nuc2* plants, suggesting that most if not all rDNA phenotypes observed in *nuc2* plants are essentially linked to the absence of *NUC2* (Figures 1 and 4 to 7; Supplemental Figures 4, 11, 13, and 14).

Nucleolin proteins are histone chaperones (Angelov et al., 2006), and, as expected, *NUC1* and *NUC2* are able to bind nucleosomes *in vitro* and are found in chromatin fractions *in vivo* (Figures 4 and 8). The involvement of histone chaperone activities in flowering time has already been reported in plants with disrupted facilitate chromatin transcription complex (FACT) H2A-H2B histone chaperone activity genes (Lolas et al., 2010; Van Lijsebettens and Grasser, 2010). However, in contrast with the *Arabidopsis* *NUC2* gene, the absence of the SSRP1 and SPT16 FACT subunits leads to reduced expression of *FLC* and to early flowering. FACT and nucleolin might have different substrate specificities and act at different genomic positions; therefore, it remains to be determined whether their activities act directly on the *FLC* gene.

NUC2 expression is restricted to some plant tissues and organs and is developmentally controlled during early seedling establishment. *NUC2* expression is also significantly lower than *NUC1* expression. Interestingly, *NUC2* promoter activity is antagonistic to *NUC1* promoter activity (Figure 2; Supplemental Figures 5 to 7). The higher expression of *NUC2* could be a consequence of the absence or modification of *NUC1* protein. Increased expression seems to be at the transcriptional, and possibly also the posttranscriptional, level (Figure 3; Supplemental Figure 8). *NUC1* may play a direct role in regulating *NUC2* gene expression or, indirectly, by controlling the expression of an unknown repressor. In agreement with this, *NUC1* has also been implicated in the expression of *RIBOSOMAL PROTEIN* genes transcribed by RNA pol II (Kojima et al., 2007). Moreover, transcriptional and post-transcriptional regulation of pol II-transcribed genes by nucleolin proteins has been widely reported in animal cells (Mongelard and Bouvet, 2007; Tajrishi et al., 2011).

Disruption of *NUC2* Affects rDNA Genomic Organization and Gene Silencing

The expression of 45S rDNA VAR1 in *nuc2-2* mutants is a surprising observation, considering the hypermethylated state of 5'

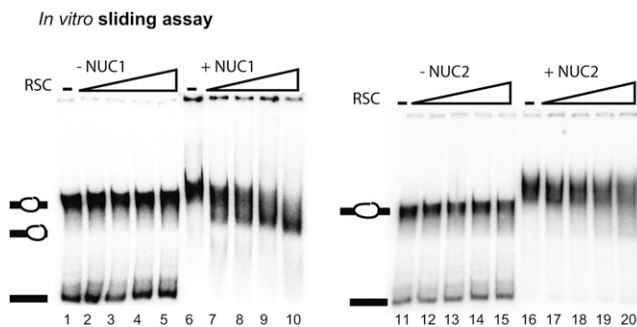


Figure 8. NUC1 and NUC2 Binding and Remodeling of Nucleosomes.

Centrally positioned nucleosomes were incubated with increasing (0.2, 0.4, 0.8, and 1.6 μ L) amounts of RSC in the absence (lanes 2 to 5 and 12 to 15) or presence (lanes 7 to 10 and 17 to 20) of 300 ng of recombinant His-NUC1 or His-NUC2. Lanes 1 and 11 show nucleosomes alone, and lanes 6 and 16 show nucleosomes with 300 ng of His-NUC1 and His-NUC2, respectively. The migrations of centrally positioned nucleosomes, mobilized nucleosomes, and the 32 P radioactively end-labeled DNA are indicated on each EMSA gel.

ETS rDNA in *nuc2-2* plants (Figure 6), which contrasts with the hypomethylation and chromatin decondensation of rDNA previously observed in *nuc1* plants (Pontvianne et al., 2007, 2010). Nevertheless, how nucleolin affects DNA methylation remains unclear. Moreover, in contrast with *nuc1* plants, in which transcription from the TIS is not greatly affected, we observed that most of the detected transcripts initiate from a single nucleotide position in *nuc2-2* plants (Figure 4). In wild-type and *nuc1* mutant plants, one signal might correspond to the transcription of rDNA VAR2 and another to VAR3. The unique TIS in *nuc2-2* could result from decreased levels of VAR3 pre-rRNA, assuming that the TIS of VAR1 pre-rRNA is similar to that of VAR2.

Quantitative analysis of 45S rDNA variants reveals no major changes in the amount of total rDNA in *nuc2-2* compared with wild-type plants. However, we observed a significant increase of VAR1 and decrease of VAR2 rDNA copy numbers in these plants (Figure 5). Similarly, plants expressing a low level of *NUC2* transcripts (*nuc2-1* mutant plants) display changes in the abundance of variants, but in these plants the amount of rRNA genes is increased (Supplemental Figure 2). This is in contrast with *fas* mutants, which display a reduction of rDNA copies and activation of VAR1, or *nuc1* mutants, where the number and abundance of rDNA variants are not affected (Figure 5) (Mozgová et al., 2010; Pontvianne et al., 2013). Disruption of the histone methyltransferases *ATXR5/6* also induces the expression of normally silent rDNA VAR1 and an increased or reduced number of silent and active rDNA copies, respectively (Pontvianne et al., 2012). However, in contrast with *atxr5/6*, we do not detect significant changes in the endoreplication of the rDNA variant copies in the *nuc2-2* mutants (data not shown). Remarkably, the relative abundance of rDNA variants is restored to the wild-type level in F1 siblings upon backcrossing the *nuc2-2* plants with wild-type plants, with repression of VAR1 and transcription of VAR2 and VAR3 (Figure 7). This shows that reintroduction of rDNA from wild-type plants, rather than the *NUC2* gene, is able to reestablish the wild-type functional organization of rDNA in *nuc2-2* plants.

In agreement with the observation that NOR2 is transcriptionally silent (Fransz et al., 2002), we have observed that inactive rRNA VAR1 genes localize mainly in NOR2, while the active VAR2 and VAR3 rRNA genes are in NOR4 (J. Sáez-Vásquez, F. Barneche, and C. Pikaard, unpublished data). Although we do not exclude alternative mechanisms, gene conversion (Schuermann et al., 2005) of rRNA could explain the variation in the abundance of rDNA variants in *nuc2-2* and *nuc2-2 gNUC2* plants. Gene conversion might provoke the translocation of VAR1 copies between different NORs, thereby relocating rDNA VAR1 copies in largely hypomethylated regions. VAR1 copies are highly methylated in a wild-type context, and their increased abundance might explain the increased global DNA methylation levels of 5' ETS rDNA in *nuc2-2* plants. However, some rDNA VAR1 copies became hypomethylated, as detected by McrBC analysis (Figure 6; Supplemental Figure 13). In the wild type, VAR1 represents 50% of all rDNA; consequently, the increased number in *nuc2-2* plants makes it difficult to determine the contribution of each rDNA variant to global rRNA gene methylation levels (Supplemental Figure 12). It is also possible that NOR2 is

not completely silenced during seedling establishment (Figure 9); consequently, some VAR1 units might be transcribed and associated with a single nucleolus in *nuc2-2* (Figure 4D). Interestingly, VAR1 rDNA copies (located either in NOR2 or NOR4) associate with the nucleolus in *nuc2-2* plants, a relocation that probably correlates with their switch to a transcriptionally active state (Figure 4E).

So far, we cannot disentangle the respective effects of rDNA variant dosage, delocalization, and activation on the derepression of VAR1 in *nuc2* mutant plants. However, it is tempting to speculate that it is due to VAR1 relocation from NOR2 to NOR4. Indeed, it has been demonstrated that rDNA can be integrated and expressed at ectopic locations and form nucleolar structures (Karpen et al., 1988; Oakes et al., 1993; Floutsakou et al., 2013). Therefore, our results fit with a model in which the switch between active and/or repressed rDNA depends on the genomic and chromatin organization of the NOR (Preuss et al., 2008).

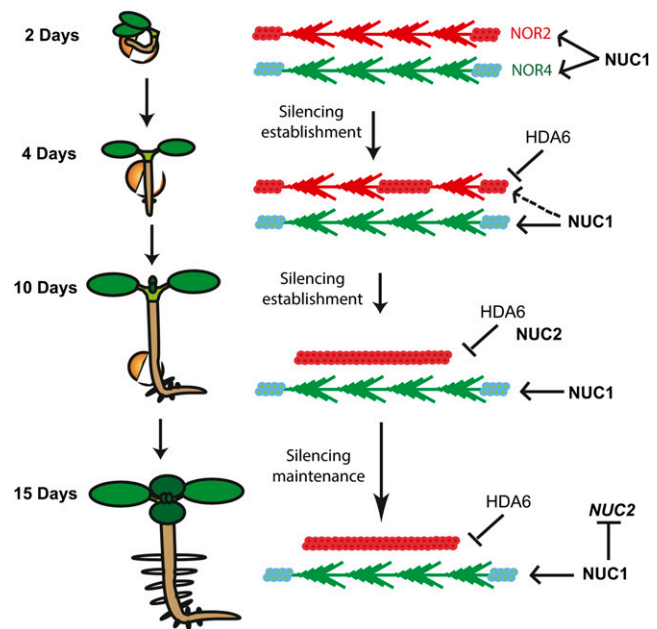


Figure 9. *NUC2* Is Required for Chromatin Dynamics during Developmental Transitions.

In this model, 45S rDNA VAR1 localizes in NOR2 (red) and VAR2 and VAR3 localize in NOR4 (green). Early in seed germination (2 d), both NOR2 and NOR4 are decondensed and transcriptionally active. NUC1 protein is associated with each NOR, as NUC1 binds to active genes (Pontvianne et al., 2010). Then, throughout germination, HDA6 participates in the chromatin silencing of rDNA VAR1 (Earley et al., 2010; Benoit et al., 2013). NOR2 becomes progressively silenced/condensed, and NUC1 dissociates. At 8 to 10 d, *NUC2* transcripts and protein accumulate and bind “silenced” chromatin to participate in the heterochromatin establishment of NOR2. At the end of seed germination transition, NUC1 might play a role in repressing *NUC2* gene expression at the transcriptional and/or posttranscriptional levels.

A Role of Nucleolin Proteins during Developmental Transition

So far, duplicated genes have been associated with gene dosage and neofunctionalization (Magadum et al., 2013). The results presented in this work, associated with previous studies, indicate that conserved and duplicated nucleolin genes have antagonistic activities in controlling rDNA organization and expression. To our knowledge, only a few duplicated genes with antagonistic roles have been described in *Arabidopsis*, including the duplicated RecQ helicases involved in homologous recombination and DNA repair (Hartung et al., 2007). How do NUC1 and NUC2 functionally interact in *Arabidopsis*? NUC2 protein accumulation during seedling development (Figure 2) temporally correlates with NOR condensation and rDNA VAR1 repression (Earley et al., 2010; van Zanten et al., 2011; Benoit et al., 2013). Accordingly, upon heterochromatin establishment of NOR2, the expression of *NUC2* could be repressed by NUC1 (Figure 3). Remarkably, both NUC1 and NUC2 proteins associate with chromatin and can bind nucleosomes, although only NUC1 is able to assist remodeling (Figure 8; Supplemental Figure 9). As reported previously, NUC1, as well as human nucleolin, can bind transcriptionally competent rDNA chromatin and might facilitate rDNA transcription (Pontvianne et al., 2010; Cong et al., 2012). Meanwhile, NUC2 might bind nucleosomes to induce and/or maintain a repressive rDNA chromatin state during seedling growth (Figure 9).

In animal and yeast cells, the maintenance of rDNA methylation and gene copy number plays an important role in genome integrity and regulation (Paredes and Maggert, 2009; Ide et al., 2010). In *Arabidopsis*, it has been demonstrated that rDNA sequences might enhance homologous recombination (Urawa et al., 2001). We also reported high variability of 45S rDNA variants in the genome of *Arabidopsis* ecotypes (Abou-Elail et al., 2011). Such a mechanism is subject to evolutionary constraints, as demonstrated by the observation that variations in genome size of *Arabidopsis* ecotypes are largely due to 45S rDNA copy number (Long et al., 2013). It would be interesting to determine how the variability of rDNA and nucleolin protein sequences, as well as other proteins involved in chromatin-based processes, can influence chromatin organization and the controlled expression of highly repeated sequences such as ribosomal genes.

METHODS

Plant Growth Conditions and Isolation of Mutants

All lines were derived from *Arabidopsis thaliana* Columbia-0 ecotype. Seeds were first sown on Murashige and Skoog (MS) medium (4.7 g/L MS medium, 0.5 g/L MES, 10 g/L Suc, and 10 g/L plant agar) or on soil and left 2 d at 4°C to synchronize. Plants were then grown either under continuous light (MS plants) or under a 16-h/8-h (light/dark) cycle (soil plants). Seeds corresponding to *nuc2-1* (SALK_542511) and *nuc2-2* (GABI_178D01) mutant plants were obtained from the Nottingham Arabidopsis Stock Centre (<http://nasc.life.nott.ac.uk>). The *nuc1* and *fas2.4* (third generation) mutant lines used in this work have been described (*nuc1*: Kojima et al., 2007; Pontvianne et al., 2007, 2010; *fas2.4*: Mozgová et al., 2010; Pontvianne et al., 2013).

Immunoblotting on Total and Chromatin Protein Fractions

Plant material (0.1 g) was homogenized and extracted in 500 μ L of 50 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mM β -mercaptoethanol, and a 1:100 dilution (v/v) of anti-protease cocktail inhibitor (Sigma). The extracts were cleared by centrifugation at 13,000g for 15 min and stored at -80°C .

To obtain protein chromatin fractions from wild-type, *nuc1-1*, and *nuc1-2* plants, chromatin samples were prepared as described previously (Pontvianne et al., 2010) and proteins were extracted in 1 \times SDS-Laemmli buffer.

Then, SDS-PAGE and immunoblot analysis were performed as described previously (Sáez-Vasquez et al., 2004). The membranes were hybridized with a 1:10,000 dilution of α -At-NUC1 or a 1:7,000 dilution of α -At-NUC2 as described previously (Pontvianne et al., 2007, 2010).

Methods Related to Nucleic Acid

Total RNA was extracted from 15-d-old *Arabidopsis* plants using Trizol reagent (Amersham Biosciences) and treated with Turbo DNase (Ambion) for 60 min to eliminate contaminant DNA. First-strand cDNA synthesis was performed on 1.5 μ g of treated RNA with the oligo(dT) and/or 3'ETS primer (for 3' ETS rDNA sequences) using the GoScript Reverse Transcription System kit following the manufacturer's instructions (Promega).

RT-PCR of 24 to 35 cycles was performed using a PTC-200 device (MJ Research). Primer pairs 5'*nuc1/3'nuc1*, 5'*nuc2/3'nuc2*, and 5'*3ets/3'3ets* were used to amplify *NUC1*, *NUC2*, and 3' ETS pre-rRNA transcripts, respectively.

Quantitative PCR was performed using a LightCycler 480 and MESA Blue qPCR Master Mix Plus for SYBR assay (Eurogentec). Primers 5'*nuc2q/3'nuc2q* and *q5'flc/q3'flc* were used to detect *NUC2* and *FLC* transcripts, respectively, and primers 5'*18S/3'18S*, 5'*25S/3'25S*, and 5'*ITS1/3'ITS1* were used to amplify 18S, 25S, and ITS1 rDNA sequences, respectively. The *Actin* gene was used as an internal standard, and results were analyzed using the comparative cycle threshold method relative to input.

For the relative abundance of each class of rDNA (variant), PCR was performed with primers 5'*3ets/3'3ets*. PCR products were run on the LabChip GX/GX II (Caliper; Life Sciences) and analyzed with the LabChip GX DNA Assay Analysis software (which calculates the size and concentration of each nucleic acid fragment) according to the manufacturer's instructions.

Primer extension analysis was performed using 15 μ g of total RNA and specific 5' end-labeled primers as described previously (Sáez-Vasquez et al., 2004). Primers used were *tis* for the detection of primary pre-rRNA precursors (+104 nucleotides from TIS) and *p* for the detection of primary pre-rRNA precursors cleaved at the P site (+1274 nucleotides from TIS). Primer 3'*U3* was used for the detection of U3snoRNA as a loading control.

Cloning of *Arabidopsis* NUC2 Promoter Sequences and Detection of GUS Activity

Approximately 1.5 kb of the *NUC2* promoter sequence was amplified from genomic DNA and fused to a GUS reporter gene in the vector pGWB433 (Nakagawa et al., 2007). The *pAtNUC1:GUS* construct in pCAMBIA 1381xa was described previously (Pontvianne et al., 2007).

Histochemical detection of GUS activity was performed using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) as a substrate. Seedlings were placed in X-Gluc buffer solution (1 mM X-Gluc, 100 mM Tris-HCl, pH 7, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, and 0.5 mM potassium ferricyanide) and incubated at 37°C for 12 h. Samples were then incubated in 70% ethanol for 2 h and cleared in Hoyer's medium. For analysis of siliques, samples were placed in 90% acetone on ice

for 1 h, followed by X-Gluc buffer as described above. All microscopic GUS images were observed using a Zeiss Axioskop 2 microscope and recorded using a Leica DC 300 FX digital camera.

ChIP and FISH

Antibodies against At-NUC1 and ChIP reactions were described previously (Pontvianne et al., 2007, 2010). PCR was performed using primers 5'*nuc2a/3'nuc2a*, 5'*nuc2b/3'nuc2b*, and 5'*nuc2c/3'nuc2c* to amplify *NUC2* sequences –466/–210, –307/–38, and –137/+141, respectively.

DNA FISH analysis was performed using nuclei from leaves of 4-week-old plants as described previously (Pontvianne et al., 2007). The biotin-labeled probe was detected using digoxigenin (1:200; Roche) followed by sheep anti-digoxigenin antibody conjugated with the fluorochrome Alexa 480 (1:200; Invitrogen). Slides were mounted using Vectashield (Vector Laboratories) mounting medium with 1 µg/mL DAPI and then observed by confocal microscopy (Axio observer.Z1 microscope with the LSM700 scanning module; Zeiss). (A more detailed description of the immunofluorescence experiment is available in Pontvianne et al., 2012).

Immunostaining

Immunofluorescence was performed using 4-week-old leaves (the wild type, *nuc1*, and *nuc2*). Treated leaves (Supplemental Methods) were incubated at 4°C overnight with α-At-NUC2 (1:500) and then with anti-rabbit antibody coupled with Alexa 488 (1:1000; Invitrogen) for 3 h at room temperature. Slides were then mounted in Vectashield containing DAPI solution. Observation and imaging were performed using a confocal microscope (LSM 700 from Zeiss).

FANoS

The *nuc2-2* plants were crossed with wild-type Columbia-0 plants expressing FIBRILLARIN2 (FIB2) protein fused to yellow fluorescent protein (YFP) and under the control of the 35S cauliflower mosaic virus promoter. Then, F1 and F2 plants were selected to isolate homozygous *nuc2-2* plants containing 35S:FIB2:YFP transgene. Three-week-old leaves from the F3 generation were then used to isolate nuclei and nucleoli by FANoS as described previously (Pontvianne et al., 2013). The cell sorter used was an ARIA II special order (BD Biosciences). Total genomic DNA was extracted from nucleoli purified from wild-type and *nuc2-2* plants expressing the FIB:YFP construct and amplified by PCR to identify the rDNA associated with the nucleolus (i.e., those that are transcriptionally active).

Bisulfite and McrBC Analyses

Genomic DNA from wild-type, *nuc2-2*, and *nuc2-2 gNUC2* mutant plants was extracted using the DNA Easy Plant Mini kit (Qiagen) following the manufacturer's instructions.

For bisulfite analysis, 500 ng of DNA was digested overnight using 20 units of *Bam*HI restriction enzyme prior to bisulfite conversion. Bisulfite treatment was performed using the Epitect Bisulfite kit (Qiagen). The primers –315 and +243 were used to amplify rRNA gene sequences (from –315 to +243); the resulting PCR products were cloned in the pGEM-T Easy vector (Promega) and sequenced. Approximately 25 clones per sample were analyzed using the CyMATE method (Hetzl et al., 2007).

For McrBC analysis, 500 ng of DNA was digested using McrBC enzyme (Qiagen), which cuts methylated DNA. Then, PCR was performed on equal amounts of digested and undigested DNA using the HotStart goTag polymerase (Promega). PCR (35 cycles) was performed with primers 5'*3ets/3'3ets*, 5'*5s/3'5s*, 5'*AtLINE/3'AtLINE*, and 5'*AtSN1/3'AtSN1* to amplify rDNA sequences (45S and 5S) and transposable elements (At *LINE* and At *SN1*). As a control, we used the demethylated *nripe1* mutant.

Nucleosome Reconstitution and Remodeling

Recombinant *Xenopus laevis* full-length histone proteins were purified from *Escherichia coli* cells as described earlier (Luger et al., 1999). The 255-bp DNA fragment containing the centrally positioned nucleosome sequence 601 at the middle or at the end of the sequence was amplified by PCR from pGEM3Z-601 (kindly provided by B. Bartholomew and J. Widom) and end labeled using T4-polymerase kinase in the presence of [γ -³²P]ATP. Nucleosome reconstitution was performed by the salt dialysis method (Angelov et al., 2006). Carrier DNA (150 to 200 bp, 2 mg) and 50 ng of γ -³²P-labeled 601 DNA was mixed with equimolar amounts of histone octamer in nucleosome reconstitution buffer containing 2 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 5 mM β-mercaptoethanol. Nucleosomes (50 ng, 0.2 pmol) were incubated with RSC in remodeling buffer containing 10 mM Tris-HCl, pH 7.4, 5% glycerol, 100 mg/mL BSA, 1 mM DTT, 0.02% Nonidet P-40, 40 mM NaCl, 2.5 mM MgCl₂, and 1 mM ATP for 45 min. The reaction was stopped by adding 1 µg of plasmid DNA. Nucleosome position was then analyzed by native PAGE.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At1g48920 (*NUC1*) and At3g18610 (*NUC2*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phylogenetic Tree of Nucleolin Proteins from Plants.

Supplemental Figure 2. Characterization of *nuc2-1* Mutant Plants.

Supplemental Figure 3. Characterization of Nucleolin Double Mutants.

Supplemental Figure 4. List of Genes Upregulated or Downregulated in *nuc2-2* Plants Identified by RNA-seq.

Supplemental Figure 5. Analysis in Silico to Compare Relative Expression Levels of *NUC1* and *NUC2*.

Supplemental Figure 6. *NUC1* and *NUC2* Expression in Response to Auxin.

Supplemental Figure 7. *NUC2* and *NUC1* Promoter Activities during Silique Development.

Supplemental Figure 8. Mechanisms Controlling *NUC2* Gene Expression.

Supplemental Figure 9. Titration of RSC, EMSA with *NUC1* and *NUC2*, and Immunolocalization of *NUC2*.

Supplemental Figure 10. Immunolocalization of *NUC1* in Wild-Type and *nuc2* Mutant Plants.

Supplemental Figure 11. LabChip Data of the Abundance of rDNA Variants in Wild-Type, *nuc2*, *nuc2 gNUC2*, *nuc1*, and *fas2* Plants.

Supplemental Figure 12. Bisulfite Analysis of 3' ETS rDNA Sequences.

Supplemental Figure 13. PCR and LabChip Data with Different PCR Cycles for McrBC Experiments.

Supplemental Figure 14. PCR and LabChip Data with Different PCR Cycles for the Abundance of rDNA Variants in Wild-Type, *nuc2*, and Wild-Type × *nuc2* Backcrossed Plants.

Supplemental Table 1. FISH Data in Wild-Type, *nuc2-2*, and *nuc2-2 gNUC2* Plants.

Supplemental Table 2. Primer List.

Supplemental Methods. Immunostaining, RNA-seq, Bisulfite, EMSA, and Bioinformatics Analyses.

Supplemental Data Set 1. Alignment of Nucleolin Proteins.**ACKNOWLEDGMENTS**

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AUTHOR CONTRIBUTIONS

J.S.-V. conceived and directed the project and wrote the article. K.N., P.B., and M.E. designed research. N.D. and M.A.-E. conducted the majority of experiments. F.P. and S.D. performed FANoS and nucleosome assays. S.N. and S.R. performed RNA sequence analysis. H.K., S.U., A.d.B., P.C., and R.M. contributed to the research.

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