# Nucleolus-associated chromatin domains are maintained under heat stress, despite nucleolar reorganization in Arabidopsis thaliana

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**Nucleolus-associated chromatin domains are maintained under heat stress, despite nucleolar reorganization in *Arabidopsis thaliana***

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***ABSTRACT***

Several layers of mechanisms participate in plant adaptation to heat-stress. For example, the plant metabolism switches from cell growth mode to stress adaptation mode. Ribosome biogenesis is one of the most energy costly pathways. That biogenesis process occurs in the nucleolus, the largest nuclear compartment, whose structure is highly dependent on this pathway. We used a nucleolar marker to track the structure of the nucleolus, and revealed a change in its sub-nucleolar distribution under heat stress. In addition, the nucleolus is implicated in other cellular processes, such as genome organization within the nucleus. However, our analyses of nucleolus-associated chromatin domains under heat stress did not reveal significant differences compared to the control plants, suggesting a lack of connection between two of the main functions of the nucleolus: ribosome biogenesis and nuclear organization.

***KEYWORDS***

Genome organization, Heat-stress, Nucleolus, Ribosomal RNA

***INTRODUCTION***

The nucleolus is the largest nuclear compartment, implicated in ribosome biogenesis, which starts with the transcription of ribosomal RNA (rRNA) genes by the RNA polymerase I (Saez-Vasquez and Delseny 2019). rRNA genes are organized in tandem repeats in specific chromosomic locations named Nucleolus Organizer Regions or NORs. Each NOR contains several hundreds rRNA genes on the acrocentric arms of the chromosome 2 and 4 (Copenhaver and Pikaard 1996a, b). Epigenetic mechanisms, that include DNA methylation and histone modifications, regulate the number of actively transcribed rRNA genes (Tucker et al. 2010). In *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0, several rRNA gene variants co-exist and allow differentiation of active versus inactive rRNA genes (Earley et al. 2010; Pontvianne et al. 2010). Their study in vegetative tissues revealed that rRNA gene variants located in the NOR of the chromosome 4 (NOR4) associate with the nucleolus and contain actively transcribed copies. In contrast, NOR2-derived rRNA genes remain inactive and are usually excluded from the nucleolus (Pontvianne et al. 2013).

Numerous studies revealed additional roles for the nucleolus, which is also implicated in genome organization and stress response (Boulon et al. 2010; Nemeth and Grummt 2018). For example, in mammalian cells, some proteins are sequestered in the nucleolus during stress conditions (Audas and Lee 2016). The nucleolus also plays an important role in genome organization, by anchoring genomic regions with heterochromatic features at the nucleolar periphery (Nemeth and Langst 2011; Pontvianne and Liu 2019). In a previous study, we identified the nucleolus-associated chromatin domains (NADs) in *A. thaliana* (Pontvianne et al. 2016b). Excluding rRNA genes, we showed that NADs correspond to chromatin domains

depleted in actively transcribed genes and enriched in pseudogenes and transposable elements. Importantly, we demonstrated a link between rRNA gene expression and the association of the associated short arm of the chromosome with the nucleolus. Therefore, only the entire short arm of chromosome 4 associates with the nucleolus in wild-type Col-0, while in a *nucleolin 1* mutant displaying both NOR2- and NOR4-derived rRNA gene expression, the short arms of chromosome 2 and 4 associate (Pontvianne et al. 2010, 2016b).

In *A. thaliana*, prolonged heat-stress also affects global genome organization. For example, 30 h at 37°C provoke the decondensation of chromocenters and reactivate the expression of particular transposable elements (Pecinka et al. 2010; Pietzenuk et al. 2016). Genome-wide DNA and histone methylations also occur and participate in the regulation of stress-responsive genes (Kim et al. 2015b). Therefore, heat-stress both locally and globally affects chromatin organization. In that context, changes in the 3D genome organization are expected to play a role in stress response, potentially in the stress response gene activation. In this work, we aimed to identify changes in the nucleolus structure and nucleolus-associated chromatin domains in response to heat stress.

## ***Materials and methods***

### Plant materials and growth conditions

Wild-type Col-0 and *nuc1-2* plants expressing the FIB2:YFP nucleolar marker were previously reported in (Pontvianne et al. 2013, 2016b). Heat stress was performed on three week-old plants grown in a Percival® growth chamber with constant light intensity and hygrometry for stressed and unstressed plants.

*Confocal imaging*

Nuclei of *A. thaliana* plants were isolated and imaged as previously described (Picart and Pontvianne 2017). Nuclear DNA was counterstained with DAPI (4', 6-diamidino-2-phenylindole), mounted using the Vectashield medium (Vector Laboratories), and analyzed with a Zeiss LSM 700 inverted confocal microscope at the Bioenvironment microscopy platform (Perpignan University, Perpignan, France).

*NADs identification*

Nuclei and nucleoli were isolated as previously described (Pontvianne et al. 2016a) using a S3 cell sorter (Biorad®) at the Bioenvironment cytology platform (Perpignan University, Perpignan, France). Sorted nuclei or nucleoli were treated with RNase A and proteinase K prior to phenol/chloroform DNA purification, followed by two precipitation steps. DNA libraries were generated via the kit Nextera XT DNA sample preparation (Illumina®) according to manufacturer's instructions and were, then, subjected to high throughput paired-end 2X150nt sequencing on a Next-seq 550 apparatus (Illumina ®) at the Bioenvironment sequencing platform (Perpignan University, Perpignan, France). NADs identification was then performed as described in (Carpentier et al. 2018). DNA-seq data are deposited at the ENA European Nucleotide Archive under the reference PRJEB36061.

*RNA-sequencing*

Total RNAs were extracted from two pools of 3-week-old Arabidopsis plant leaf tissues of wild-type Col-0 subjected or not to a 24 h heat stress at 37°C using TRIzol reagent (MRC). Sequencing was performed by the Bioenvironment sequencing platform (Perpignan

University, Perpignan, France) using a Nextseq 550 to generate 2 × 75-bp-long reads, using the NEBNext Ultra II Directional RNA Library mRNA enrichment Prep (Biolabs, E7765S) according to manufacturer's instructions. Illumina reads were aligned to the *A. thaliana* TAIR10-annotated genome reference using HISAT 2 (Kim et al. 2015a). Count of the number of reads aligned to each genome coding sequence or TE was performed with HTseq-count (Anders et al. 2015) and differential expression profile analyzes with DESeq2 (Bioclite - R package) (Love et al. 2014). RNA-seq data are deposited at the ENA European Nucleotide Archive under the reference PRJEB36061.

## *RESULTS*

### *Changes in the nucleolar distribution of Fibrillarin*

The nucleolus structure is divided into three major compartments that are organized in a concentric manner (Medina et al. 2000). The fibrillar center (FC) is surrounded by the dense fibrillar component (DFC) which is itself enveloped by the granular component (GC). These compartments correspond to different steps of the ribosome biogenesis pathway, starting with the RNA polymerase I transcription of rRNA genes, and ending with mature ribosome subunits (Saez-Vasquez and Delseny 2019). Fibrillarin methylates rRNA precursors (pre-rRNA), is guided by small nucleolar RNA and localizes in the DFC (Medina et al. 2000; Reichow et al. 2007). In a previous work, we showed that FRIBRILLARIN 2 protein fused to the YELLOW FLUORESCENT PROTEIN (FIB2:YFP) which enables following of the subnuclear structure of the nucleolus, thanks to its localization in the DFC (Picart and Pontvianne 2017). We took advantage of this marker to follow the sub-nucleolar structures under heat stress (Fig. 1a-c). In normal growth conditions, the FIB2:YFP marker reveals the presence of rings

corresponding to the DFC. After a 24 h treatment at 37°C, the FIB2:YFP signal is much more diffuse and the entire nucleolus area becomes labelled. Such a pattern reveals a defect in the DFC maintenance under heat stress. When stressed plants return to normal growth conditions at 22°C for 3 days, ring-like shapes are once again detectable, as before stress (Fig. 1d). Nucleolin is an important nucleolar protein and is implicated in several nucleolar functions such as ribosome biogenesis (Durut and Saez-Vasquez 2015; Picart and Pontvianne 2017). Knock-out of the *nucleolin 1* (*nuc1*) gene in *A. thaliana* affects the nucleolus structure. In that case, GC and DFC are hardly detectable, even by electron microscopy (Pontvianne et al. 2007). Detection of the FIB2:YFP signal in *nuc1* mutant indeed reveals a change in the distribution of the DFC in the mutant (Picart and Pontvianne 2017). Upon heat stress, the subnuclear distribution of the FIB2:YFP marker in the *nuc1* mutant remains diffused (Fig. 1e).

We were wondering what was the dynamic of the FIB2 delocalization FIB2 from the DFC upon heat stress conditions. We, therefore, analyzed its localization at different time points during the stress, from 0 to 24 h of heat stress at 37 °C (Fig. 1b). We systematically classified the nuclei in three categories: i) when FIB2:YFP were only detected in ring-like shapes in the nucleolus, ii) when ring-like shape signals were diffused and, iii) when the FIB2:YFP distribution was completely homogeneous in the entire nucleolus. After 2 h at 37 °C, we observed an increased number of nuclei from the third category, and a decreased number of nuclei from the first category (Fig. 2). A complete shift from category 1 to 3 occurred after 8 h at 37 °C. We also tested the time needed for the FIB2:YFP to reset, and found that after a 24 h stress at 37 °C, another 24 h at 22 °C is sufficient to completely shift nuclei back from category 3 to category 1 (Figs. 1b, 2). Taken together, our data suggest a change in the nucleolus organization that occurs during a prolonged heat shock, which is resettable.

***rRNA gene variant expression remains unchanged during heat stress***

Since FIB:YFP distribution is a read-out of the DFC, the diffusion of the FIB2:YFP signal could be interpreted as a consequence of a change in the nucleolus structure. Furthermore, the nucleolus structure is itself the consequence of ribosome biogenesis. We therefore tested whether rRNA gene transcriptional regulation was affected upon stress, taking advantage of the existence of rRNA gene variants. In *A. thaliana* Col-0, three major rRNA gene variants compose the NOR (Earley et al. 2010; Pontvianne et al. 2010). The actively transcribed NOR4 contains variants 2 and 3, while variants 1 and 3 compose the silent NOR2 (Fig. 3a) (Chandrasekhara et al. 2016). In our assay, variability is present in the 3' external transcribed region of the pre-rRNA 45S. It, therefore, allows differentiation of variants being expressed, as well as the relative accumulation of pre-rRNAs. In wild type, only variants 2 and 3 accumulate, meaning that only NOR4-derived rRNA genes are expressed (Fig. 3b). Our analyzes revealed that the same variants are expressed during and after a 24 h heat stress at 37°C. However, we observed a higher amount of pre-rRNAs from all variants expressed during the heat stress compared to the controls.

To further analyze the amount of pre-rRNAs under heat stress, we extracted total RNAs from two pools of 3-week-old *Arabidopsis* plant leaf tissues of wild-type Col-0 subjected or not to a 24 h heat stress at 37 °C. RNAs were then sequenced using the illumina® technology. Gene ontology analyses indicated that genes, up-regulated after the stress, were enriched for genes implicated in stress response (response to stimulus [GO:0050896] with a *p*-value of 6.518860e-13; Suppl. Table S1). We compared the accumulation of different species of pre-rRNAs with at least the 5'ETS or the 3'ETS regions and found a higher amount of pre-rRNAs after the heat stress (Fig. 3c). Although less pronounced than in wild-type Col-0, this bigger accumulation is also true in the *nuc1* mutant. Taken together, our data suggest that NOR2-

derived rRNA genes remain silent during the stress and that pre-rRNAs accumulate probably due to a failure of pre-rRNA processing.

***Nucleolus-Associated chromatin Domains are stable under heat stress***

Our previous experiments, and data from the literature obtained on animal cells, strongly suggest a change in the ribosome biogenesis during heat stress. However, the nucleolus is also implicated in global genome organization, anchoring genomic regions with heterochromatic features at its periphery, known as nucleolus-associated chromatin domains or NADs (Picart-Picolo et al. 2019). In *A. thaliana*, global heterochromatin seems to be affected during prolonged heat stress and chromocenter condensation can be affected (Pecinka et al. 2010; Wang et al. 2013). In our case, only mild changes in chromocenter condensation level were observed after 24 h at 37 °C (Fig. S1), but we observed that the ribosome biogenesis was affected (Figs. 1-3). We therefore analyzed whether or not NADs identity is modified during a prolonged heat stress.

To isolate nucleoli and their DNA content, we took advantage of a technique isolating either nuclei or nucleoli from entire plant tissues (Fig. 4a). The FIB2:YFP nucleolar marker is expressed to label nucleoli which are then sorted by Fluorescent Assisted Cell Sorting (FACS) (Pontvianne et al. 2013, 2016a). We therefore isolated nuclei and nucleoli from three-week-old plants subjected or not to 24 h at 37 °C. From total plant extracts, 200 000 nuclei were sorted to prepare the nuclear DNA. In parallel, two replicates of 600 000 nucleoli were isolated to prepare the nucleolar DNA. Purified genomic DNA from stressed and non-stressed samples were then sequenced by paired-end Illumina sequencing and analyzed as previously described (Carpentier et al. 2018). We found that NADs profile after a 24 h stress at 37 °C is very similar to that of control plants (Figs. 4b and S2a). Indeed, NADs mainly belong to the short arm of

chromosome 4 and to the sub-telomeric regions. We then compared the relative enrichment or depletion of NADs between the control and the stressed samples and did not find significant changes in the identity of the NAD genomic regions (Fig. S2b). As a consequence, genes present in the NADs remain largely similar with or without heat stress (Figs. 4c and S3).

Long-term heat stress leads to reactivation of many transposons, particularly TEs of the DNA/En-Spm, DNA/MuDR, LTR/Copia, and LTR/Gypsy super-families (Pecinka et al. 2010; Tittel-Elmer et al. 2010). In our data, we can also detect a higher amount of TE transcripts which belong to these super-families (Table S2). TEs compose an important fraction of the genomic regions associating with the nucleolus, namely NAD-TEs (Pontvianne et al. 2016b). The RNA polymerase II, required for their transcriptional activation, does not localize in the nucleolus (Schubert and Weisshart 2015). Therefore, to become expressed, we expect them to leave the nucleolus area. However, we could not test this hypothesis since none of the 27 up-regulated TEs belong to the NAD-TEs which have lost their association with the nucleolus after stress (Fig. 4d).

## ***DISCUSSION***

Heat stress induces global nuclear re-organization in *A. thaliana* (Pecinka et al. 2010). This is also the case in mammal cells, where polycomb bodies composed of silent genes appear within the nucleolus (Li et al. 2015). Because the nucleolus is implicated in several stress response pathways (Boulon et al. 2010), we tested whether or not the nucleolus could be affected by a prolonged heat stress in *A. thaliana*. Using FIB2:YFP as a marker to reveal the sub-nucleolar structures, we found that 8 h at 37 °C is sufficient to change the distribution of the FIB2:YFP from the DFC (Fig. 2). We suspect that this mis-localization is a consequence of

an alteration of the DFC. Fibrillarin proteins indeed possess a strong intrinsically disordered region (IDR) required for the formation of the DFC, that behaves like a phase-separated body (Feric et al. 2016). The *A. thaliana* FIBRILLARIN 2 protein also contains an important IDR (Pontvianne and Liu 2019). These typical ring-like shapes of the DFC are also absent from the *nuc1* mutant, where the nucleolus structure is affected and where GC and DFC are hardly detectable (Pontvianne et al. 2007). As a result, we were not able to detect changes in the sub-nucleolar distribution of the FIB2:YFP marker with or without stress in the *nuc1* mutant (Fig. 1e). Ring-like shape structures also disappear after treatment with Actinomycin D, a drug that inhibits RNA transcription (Merret et al. 2017). Taken together, we are very confident that the FIB2:YFP nucleolar marker distribution is able to reveal nucleolar DFC. However, only correlative Light and Electron Microscopy experiments could fully demonstrate that the ring-like shapes labelled by the FIB2:YFP really correspond to DFC (Sartori et al. 2007).

The nucleolus structure is highly dependent on the ribosome biogenesis, and it has been known for decades that the DFC is implicated in pre-rRNA processing events (Medina et al. 2000; Stepinski 2014). Therefore, the fact that a higher amount of pre-rRNAs accumulate during the heat stress is new evidence of a default in the ribosome biogenesis process. However, since we used RNA-seq data from a mRNA enriched fraction, further work should be performed to precisely characterize the changes occurring on pre-rRNA during and after heat stress. In mammalian cells, a higher temperature provokes a shutdown of rRNA synthesis (Zhao et al. 2016). In *A. thaliana*, previous works suggest that ribosomal proteins (RPs) also assemble with pre-rRNAs in the DFC. Remarkably, RPs mRNAs are rapidly sequestered by the HEAT SHOCK PROTEIN 101 (HSP101) during heat stress, as a pause mechanism to quickly regulate the ribosome biogenesis pathway during stress (Merret et al. 2017). Taken together,

these data suggest that the ribosome biogenesis process and the internal nucleolus structure are both affected by prolonged heat stress.

The nucleolus is also implicated in global genome organization. In addition to the NORs, genomic regions with heterochromatic signatures anchor at the nucleolar periphery (Picart-Picolo et al. 2019). In *A. thaliana* cells, centromeric, pericentromeric regions, as well as repetitive sequences compose the constitutive heterochromatin (Fransz and de Jong 2011). In interphase nuclei, they form chromocenters that mostly localize at the nuclear periphery, but also at the nucleolar periphery (Pontvianne et al. 2016b; Poulet et al. 2017). Upon prolonged heat-stress, chromocenters are less condensed, suggesting a global change in nuclear distribution of the constitutive heterochromatin (Pecinka et al. 2010; Wang et al. 2013, 2015). A 30 h treatment at 37 °C is necessary to provoke a chromocenter decondensation (Pecinka et al. 2010). However, in our hands, a 24 h stress at 37 °C did not revealed a drastic change in the level of chromocenter condensation (Fig. S1). In parallel, NADs identification during heat stress did not reveal drastic changes compared to the wild-type control (Figs. 4 and S2). Although some TEs can be reactivated upon prolonged heat stress (Tittel-Elmer et al. 2010), none of them are present in the NAD-TEs identified after the prolonged heat shock (Fig. 4d). These data corroborate with previous observations in animal cells that demonstrated the stability of NADs identity during cell senescence (Dillinger et al. 2017). Taken together, those results suggest that the sub-nuclear distribution of genomic domains is globally unaffected by heat stress.

In the *nuc1* mutant, where the nucleolus structure is affected (Pontvianne et al. 2007), NAD composition was modified by rRNA gene variant 1 induction and NOR2 nucleolus association (Pontvianne et al. 2016b). NOR2 nucleolus association provoked a change in the nuclear distribution of the entire short arm of chromosome 2. In case of heat stress, NOR2-

derived rRNA genes were not reactivated, which can explain why such changes were not detected in the heat stress context.

To conclude, our data corroborate previous observations that suggest a slow-down in ribosome biogenesis under prolonged heat stress (Boulon et al. 2010; Li et al. 2015; Merret et al. 2017). However, NADs remain stable during heat stress, suggesting some independence for two of the major functions of the nucleolus: ribosome biogenesis and the heterochromatin organization within the nucleus.

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**AUTHORS CONTRIBUTION:**

FP and APC conceived and designed the analyses, APC CP and FP collected the data, APC, NP and FP performed the analyses, FP wrote the paper and acquired funding.

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**FIGURE LEGENDS**

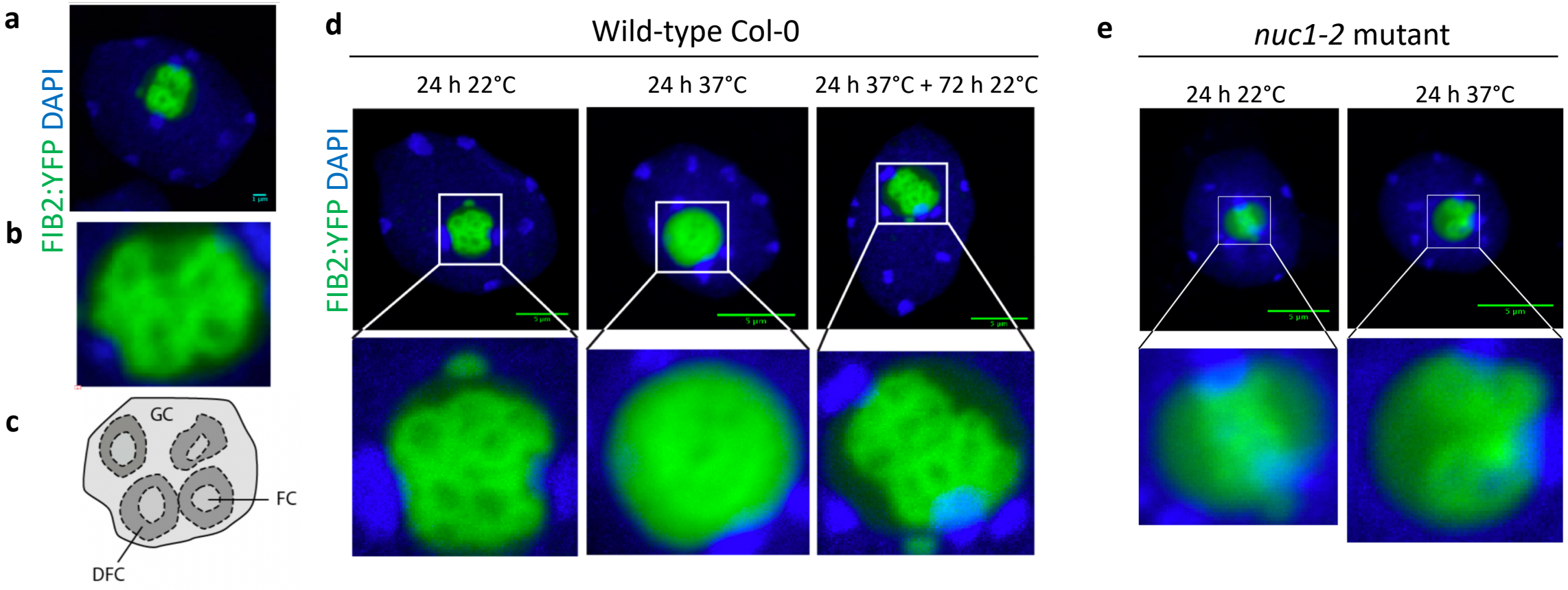
**Fig. 1: The FIB2:YFP nucleolar marker loses its DFC localization under prolonged heat stress**
**a-e.** Isolated nuclei from *A. thaliana* expressing the nucleolar marker FIB2:YFP (green). The nuclear DNA is labelled by DAPI (blue). **a**. Example of an isolated nucleus. **b**. Zoom of a nucleolus where the FIB2:YFP marker reveals ring-like shape structures that correspond to DFC. **C**. Schematic representation of the nucleolar structures from the image shown in **b**. **d-e**.

Representative isolated nuclei from three-week-old shoots (top panels) of wild-type Col-0 (**d**) or *nuc1-2* mutant (**e**) plants subjected or not to a prolonged heat stress of 24 h at 37°C. The bottom panel is a zoom of the nucleoli from the top panel.

**Fig. 2: Dynamic of FIB2:YFP nucleolar marker localization under heat stress**
The nucleolar distribution of the FIB2:YFP nucleolar marker has been analyzed at different time-points during a 37°C heat stress and during a 24 h stress recovery period. Around 50 nuclei per time-point have been analyzed and classified in three categories according to the signal given by the nucleolar distribution of the FIB2:YFP signals: i) Ring-like shape signals only, ii) diffuse ring-like shape signals and iii) homogenous nucleolar localization. Our data show a complete shift from category i) to iii) after 8 h at 37°C, which completely reset 24 h after stress.

**Fig. 3: Accumulation of pre-rRNA gene variants under heat stress**
**a.** Schematic representation depicting the organization of rRNA genes at the nucleolus organizer regions. RRNA genes are organized in tandem repeats. When transcribed by the RNA polymerase I, rRNA genes produce a rRNA precursor (pre-rRNA). rRNA gene variable regions are located in the 3'ETS and allow the detection of pre-rRNA variant expression. The NOR2, inactive in leaf cells, contains variants 1 and 3, while the NOR4, active in leaf cells, is composed by variants 2 and 3. **b**. rRNA gene variant expression was analyzed by semi-quantitative RT-PCR in plant subjected or not to a 24 h heat stress at 37°C, and three days after stress (stress recovery). *ACT2* expression is used here as loading control. The bottom panel shows the no RT control. **C.** Differential accumulation of RNA-seq illumina reads corresponding to all reads or to pre-rRNAs that still have either the 5'ETS and/or the 3'ETS. The position of the sequences used to analysed pre-rRNA reads are shown in **a**.

**Fig. 4: Nucleolus-associated chromatin domains (NADs) remain unchanged under heat stress**
**a**. Schematic representation of the protocol used to identify NADs. Nuclei from *A. thaliana* expressing the nucleolar marker are isolated by FACS, or subjected to sonication to isolate nucleoli, also by FACS. Nuclear and nucleolar DNA are then purified and sequenced to identify the genomic regions enriched in the nucleolar DNA. In that case, the nuclear DNA is used as a loading control to quantify the relative enrichment in the nucleolus. **b**. Chromosome plots displaying the relative enrichment of a given genomic segment with the nucleolus. The y-axis displays the fold change nucleolus enrichment between the control plants and the plants subjected to a 37 °C heat stress for 24 h. Each dot represents a 100kb window. Nucleolus-enriched genomic regions above the threshold (red-dotted line) are colored in red. The x-axis represents the position for each chromosome in 0,1 mega-base. **c**. Venn diagram displaying the relative enrichment of NAD-regions for each chromosome in the control or heat-stressed plants. Each NAD-region corresponds to a 100kb genomic window. **d**. Venn diagram showing that none of the NAD-TEs at 22°C that lost their nucleolus association after the 37 °C-stress for 24 h become up-regulated after the 37 °C-stress for 24 h (Fold change >2).

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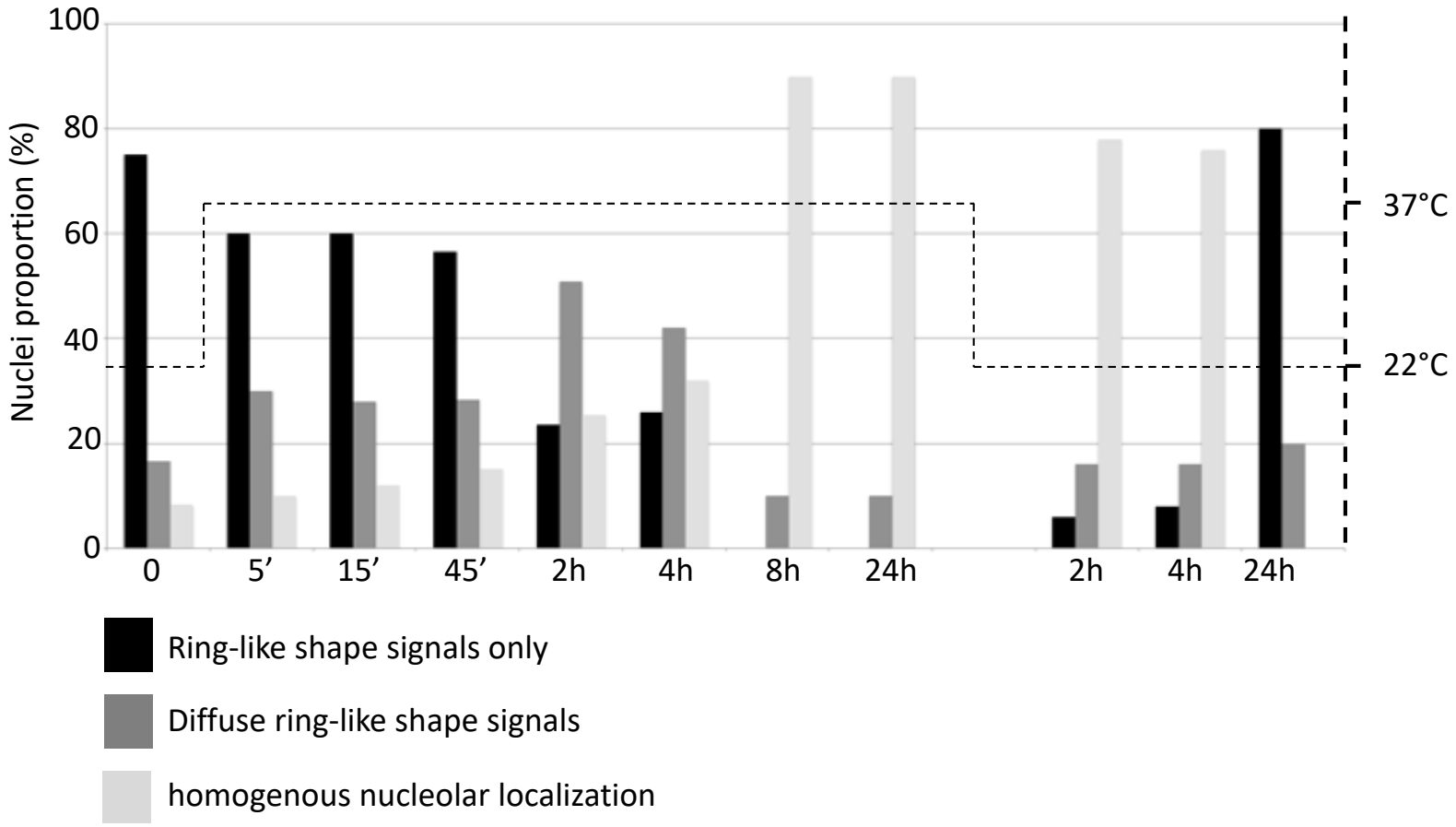


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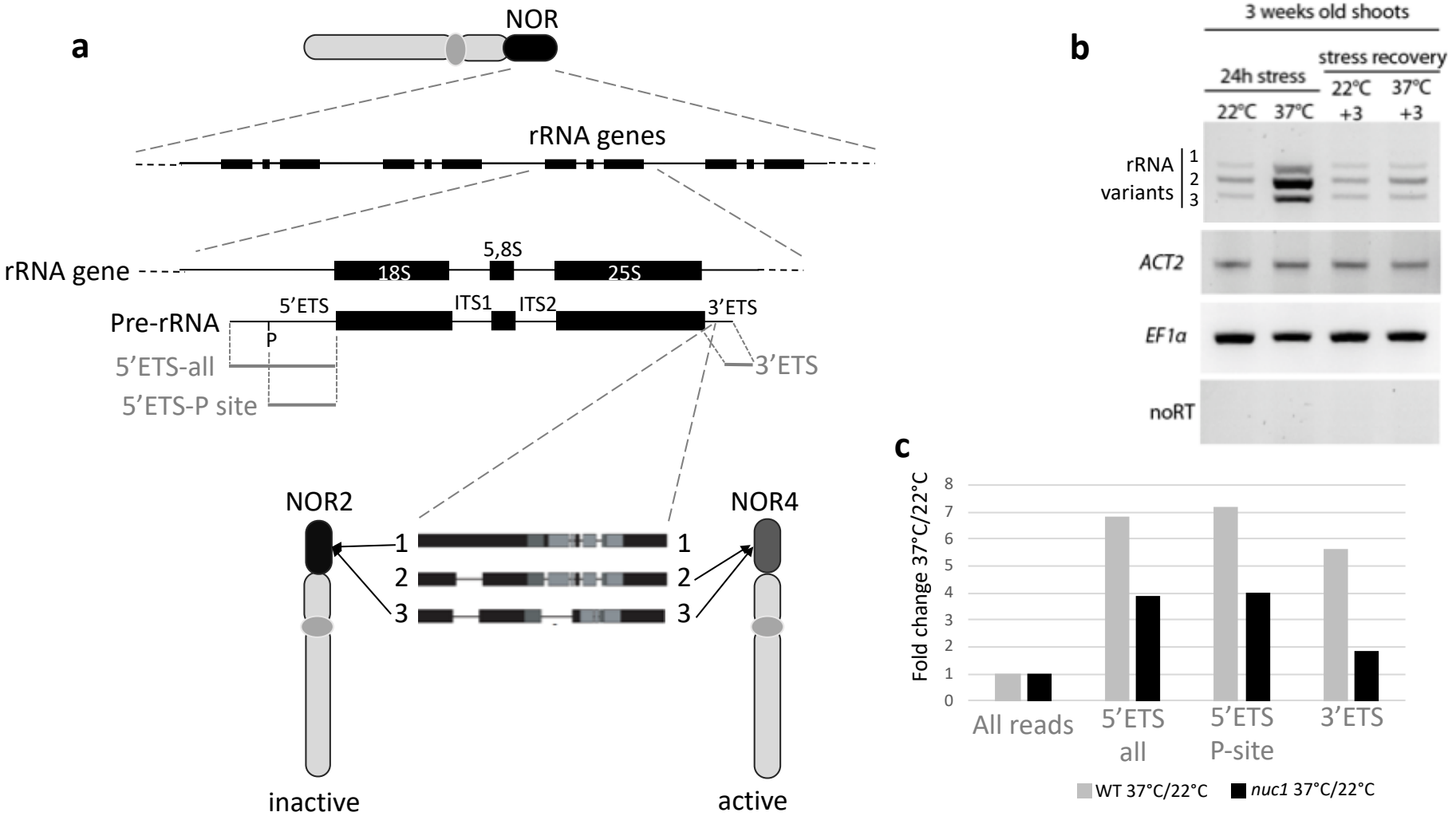


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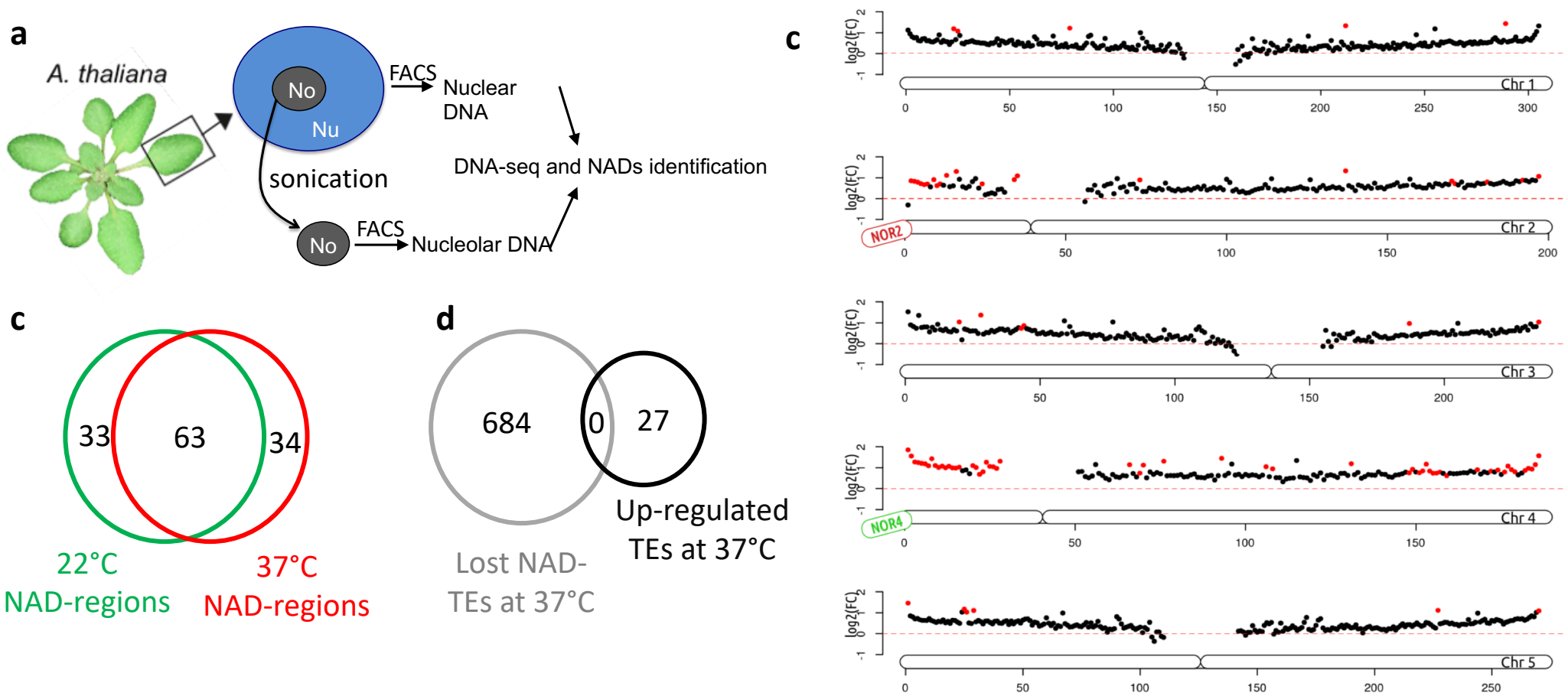


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