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Abiotic Stress and Induced DNA Hypomethylation Cause Interphase Chromatin Structural Changes in Rice rDNA Loci

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Key Words
Abiotic stress · 5-Azacytidine · Chromatin organization · rDNA · Rice

Abstract
Global climate change, i.e. higher and more variable temperatures, and a gain in soil salinity are increasing plant stress with direct consequences on crop yield and quality levels. Rice productivity is strongly affected by abiotic stress conditions. The regulation of chromatin structure in response to environmental stress is poorly understood. We investigated the interphase chromatin organization from rice plants in non-stress versus stress conditions. We have used a cytogenetic approach, based on fluorescence in situ hybridization (FISH) with 45S, 5S rDNA and centromeric probes on rice tissue sections. The abiotic stress conditions included cold, heat and mild salinity and were applied during seed germination. In contrast to cold, saline and heat stresses caused extensive decondensation of 45S rDNA chromatin and also an increase in the distance between the 2 homologous 5S rDNA loci. 5-Azacytidine (5-AC), a DNA hypomethylating drug, greatly increased 45S rDNA chromatin decondensation and interestingly was able to induce polarization of centromeres in rice interphase nuclei. The abiotic stresses tested did not perturb the spatial position of centromeres, typically with circular arrangement around the nucleolus. The results suggest a role for chromatin plasticity in a world of climate changes.

Genomes are constantly subjected to stress; for example, plants are constantly coping with stressful environmental conditions, e.g. cold, heat or high salinity soil, all of which seriously affect plant growth. Little is known regarding the effect of abiotic stress on chromatin organization [reviewed in Arnholdt-Schmitt, 2004; Kim et al., 2010]. McClintock [1984] predicted that stress can cause large-scale genomic changes including transposon activation and other structural modifications of chromosomes. Madlung and Comai [2004] proposed a model to explain genome regulation under normal and stress conditions, suggesting that under normal conditions, heterochromatin maintenance mechanisms repress transcription of repetitive DNA and that stress leads to relaxation of epigenetic marks and altered gene expression. Some studies have pointed out a link between environmental signals, chromatin conformation and epigenetic changes [e.g. reviewed in Chinnusamy and Zhu, 2009]. In *Arabidopsis*, a mutant analysis showed that the blue-light photoreceptor cytochrome 2 is involved in triggering chromatin decondensa-

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tion [Tessadori et al., 2007]. Also, the exposure to cold leads to chromatin changes at the *FLC* locus with reduction of acetylation at H3K9 and H3K14 sites, and increase in methylation of the H3K9 and H3K27 sites, corresponding to the formation of heterochromatin [Bastow et al., 2004]. The *FLC* locus also showed reduced H3K4 trimethylation, which has been associated with transcriptional activation after vernalization [Amasino, 2004].

Plants contain thousands of rDNA repeats which widely surpass the necessary number of genes to sustain ribosome synthesis [e.g. reviewed in Huang et al., 2006]. Only a subset of the rRNA genes is actively transcribed at any given time. For example, in pea only 5% of rDNA units is transcribed [Gonzalez-Melendi et al., 2001]. Hence, 2 contrasting types of chromatin structures may coexist: an 'open' one corresponding to transcriptionally active genes and a 'closed' one with heterochromatic configuration forming perinucleolar knobs representing the non-transcribed genes [Conconi et al., 1989]. Thus, ribosomal chromatin has been viewed as a good example to illustrate a link between chromatin states and gene activity [reviewed in McKeown and Shaw, 2009]. We have used a cytogenetic approach based on fluorescence in situ hybridization (FISH) to monitor changes in ribosomal chromatin structure and in the spatial disposition of centromeres in interphase nuclei after imposed abiotic stress or after induced DNA hypomethylation. For this study, we chose rice since its productivity and quality is seriously affected by abiotic factors (e.g. water scarcity, cold, and salinity). In contrast to cold stress, the saline and heat stresses caused extensive decondensation of 45S rDNA ribosomal chromatin and an increase in the distance between the 2 homologous 5S rDNA loci. The presence of 5-azacytidine (5-AC) during germination greatly increased ribosomal chromatin decondensation and caused polarization of centromeres in interphase nuclei. In contrast, the spatial position of centromeres in rice interphase nuclei, typically with circular arrangement around the nucleolus, was not significantly perturbed by any of the abiotic stress conditions tested. The results suggest a link between ribosomal chromatin plasticity and response to the challenges of abiotic stress.

Material and Methods

Plant Material and Stress Treatments

Seeds from diploid rice (*Oryza sativa* ssp. *japonica*, 2n = 24, cv. Nipponbare) were germinated for 3 days at 28°C on filter paper soaked either in water, in a solution of 150 mM sodium chloride (salinity stress) or in 80 μM 5-AC solution (Sigma) as described in Santos et al. [2002]. The imposed thermal stresses consisted of cold (4°C for 24 h) or heat (42°C for 3 h) before harvesting roots. Root-tips were fixed in 4% (w/v) formaldehyde freshly prepared from paraformaldehyde in PEM buffer (50 mM PIPES, KOH pH 6.9; 5 mM EGTA; 5 mM $MgSO_4$) for 1 h at room temperature and then washed in TBS (10 mM Tris-HCl, pH 7.4; 140 mM NaCl) for 10 min.

Root Sectioning

Root-tips were sectioned using a Vibratome Series 1000 (TAAB Laboratories Equipment Ltd., Aldermarston, UK) and allowed to dry on multi-well slides (Menzel-Glaser). The root tissue sections were approximately 20 μm thick containing about 2 cell layers and showed a good preservation and integrity of tissue structure. The slides were pre-treated by washing in 3% Decon for at least 1 h, thorough rinsing with distilled water and coating with a freshly prepared solution of 2% (v/v) 3-aminopropyltriethoxysilane (APTES, Sigma) in acetone for 10 s and activated with 2.5% (v/v) glutaraldehyde (Sigma) in PBS for 30 min, rinsed in distilled water and air-dried. The tissue sections were dehydrated in a methanol series and then digested in an enzyme mixture of cellulase 1.5% (w/v) (Onozuka R-10, Japan) and pectolyase (0.5% (w/v) Sigma) in 1× EB (0.4 mM citric acid; 0.6 mM trisodium citrate, pH4.8) for 1 h at room temperature. Finally, the sections were washed in TBS for 10 min, dehydrated in a methanol series and air-dried.

FISH on Root Tissue Sections

FISH was performed as described in Santos et al. [2002]. Briefly, the hybridization mixture contained 200 ng of DNA probe, 50% deionized formamide, 20% dextran sulfate, 10% 20× SSC, 1.25% sodium dodecyl sulfate and 1 μg of sonicated salmon sperm DNA as blocking DNA. The following plasmids were used as probes: pRY18, which carries a 3.8-kb DNA fragment containing the rice genomic 45S rDNA cluster (includes the 3′ half portion of 18S rRNA gene, the complete 5.8S rRNA gene and the 5′ half portion of 28S rRNA gene); pRTY5S carrying a 3-kb DNA fragment that contains the rice 5S rDNA (kindly provided by Prof. N. Ohmido); pRCS2 that contains rice centromeric specific *CentO* repeats [Dong et al., 1998], kindly provided by Prof. J. Jiang. Probes were labeled with digoxigenin-11-dUTP or biotin-16-dUTP (Roche) by nick translation. The hybridization mixture was denatured at 95°C for 5 min, cooled in ice for another 5 min and immediately applied to the sections. Target DNA denaturation was carried out in a modified thermocycler (Omnislide Hybaid Ltd) at 78°C for subsequent hybridization at 37°C overnight. Post-hybridization washes were performed using 20% formamide in 0.1× SSC at 42°C. Probes were labeled with digoxigenin or biotin and detected with an anti-digoxigenin antibody conjugated to FITC (Boehringer Mannheim Corp.) or to extravidin-cy3 (Sigma), respectively. Both antibodies were diluted in 3% BSA in 4× SSC, 0.2% Tween-20 (Sigma) and incubations were carried out in a humid chamber for 1 h at 37°C followed by 3 × 5 min washes in 4× SSC, 0.2% Tween-20 at room temperature. The sections were counterstained with 6 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min and mounted in Vectashield antifade solution (Vector Laboratories Inc., Burlingame, Calif., USA).

Table 1. Number and volume (± SD) of heterochromatic knobs in distinct abiotic stress conditions

Treatment	Cells analyzed	No. of heterochromatic knobs/cell	Volume of heterochromatic knobs, μm^3
Control	75	2.08 (0.67)	1.40 (0.73)
Cold	80	2.11 (0.76)	2.19 (1.25)
Heat	37	2.03 (0.44)	1.12 (0.34)
NaCl	80	2.09 (0.90)	0.99 (0.42)

Confocal Fluorescence Microscopy, Image Acquisition and Analysis

Confocal optical section stacks were collected with a Leica TCS SP confocal microscope (Leica Heidelberg GmbH, Mannheim, Germany) equipped with a krypton and an argon laser. The microscopy data were recorded and then transferred to Image J and composite using Adobe Photoshop (Adobe Systems Inc., Mountain View, Calif., USA).

Statistical Data Analysis

Differences between treatments were evaluated with a non-parametric Analysis of Variance on Ranks (Kruskal-Wallis test) since the assumptions of the parametric ANOVA, namely normal distribution of data and homoscedasticity of treatment variances were not met. Otherwise data were analyzed with ANOVA. Post-hoc comparisons between groups were made with the non-parametric mean rank comparisons after the non-parametric ANOVA used a priori to control for type I errors. Observations greater than 1.5 times the 3rd quartile plus the interquartile range are considered as outliers. The co-localization of 5S rDNA and centromeres was evaluated with the Pearson χ^2 test of independence. Significance was accepted for $p < 0.05$.

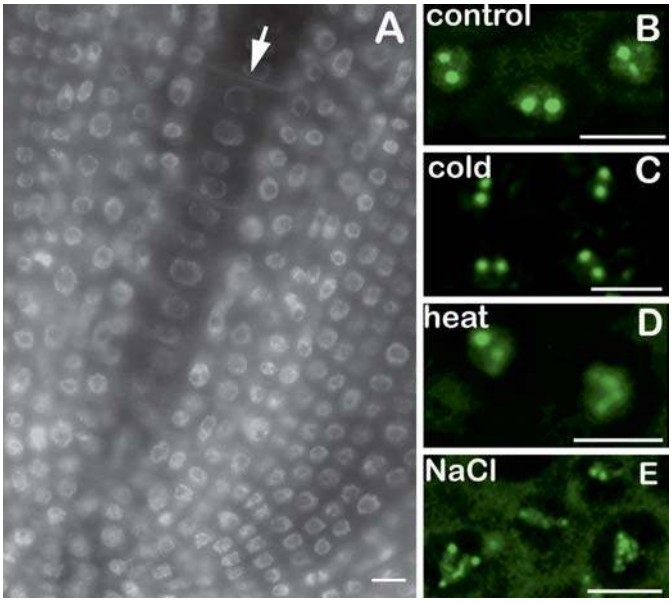

Fig. 1. Rice root tissue section labeled with DAPI, arrow indicates the xylem vessel precursor cells (**A**). 45S rDNA chromatin organization in 3D interphase nuclei of rice root sections after seed germination in water (**B**) or in the presence of abiotic stress conditions such as cold (**C**), heat (**D**), salinity (**E**). Note the 2 heterochromatic knobs at the nucleolar periphery, presumably inactive (**A**, **B**) and the threads resulting from knob unwinding (**E**). Confocal image stacks were recorded with a section spacing of 1 μm and a projection of optical confocal sections is shown. Bars: 20 μm (**A**) and 10 μm (**B**).

Results

The 45S rDNA Chromatin Organization in Interphase Nuclei Consists of 2 Perinucleolar Heterochromatic Knobs

The 45S rDNA loci consist of tandem arrays of repeating units of the 18S, 5.8S and 28S rRNA genes and the transcribed and non-transcribed spacers, each unit being typically 10 kb long in plants. We performed FISH with the 45S rDNA probe in interphase nuclei of meristematic cells from rice root tissue sections made on a vibratome. Tissue sections were approximately 20 μm thick and showed good preservation and integrity of tissue structure. An entire rice root tip section in which the DNA is stained with DAPI is shown in figure 1A. The organization pattern of 45S rDNA consisted mainly of 2 large and well-defined heterochromatic knobs at the periphery of the nucleolus compartment which correspond to a tight cluster of non-expressing ribosomal genes (fig. 1B). Inside the nucleolus, chromatin has a diffuse appearance with no evidence for the presence of condensed chromatin foci (fig. 1B).

The 45S rDNA Chromatin Undergoes Decondensation after Exposure to Heat and Salinity Stress Factors

The organization of interphase ribosomal chromatin was evaluated after seed germination under specific abiotic stress conditions. Three-day-old seedlings were subjected either to cold (4°C for 24 h) or heat (42°C for 3 h). Another stress condition tested was germination under mild salinity (150 mM NaCl). We have analyzed the average number and the volume of heterochromatic knobs per cell (table 1). The number of knobs per cell is informative regarding any agglomeration or division of the knobs while the volume of knobs can be a better indicator for the correlation between chromatin conformation and the

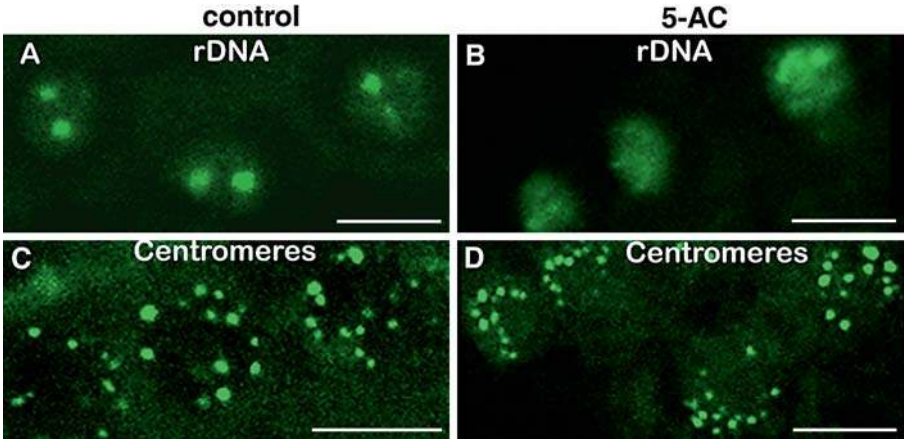

Fig. 2. 5-AC effects on rice chromatin organization: ribosomal chromatin (**A**, **B**) and centromeric chromatin (**C**, **D**). In **A**, 2 heterochromatic knobs are clearly seen. In **B**, no clear heterochromatic knobs are identified presumably due to knob unwinding inside the nucleolus, which agrees with a strong signal of diffused chromatin observed overall the nucleolus. Centromeres are dispersed circularly at the nucleus periphery (**C**). 5-AC is shown to induce centromere polarization at the nuclear periphery in one side of the nucleus (**D**). Confocal image stacks were recorded with a section spacing of 1 μm and a projection of optical confocal sections is shown. Bar: 10 μm.

fraction of genes being transcribed. The number of heterochromatic knobs around the nucleolus per cell was not statistically different between tested stress conditions and control (table 1). Regarding the volume of knobs, we observed significant differences between treatments (Kruskal-Wallis $\chi^2(4) = 90.551$; $p < 0.001$; $n = 313$). The cold stress did not result in dramatic changes either of the morphological appearance or the number of heterochromatic knobs (fig. 1C, table 1) but the knobs' volume was increased although not statistically significantly (table 1). In contrast, after heat and salinity stress conditions, the heterochromatic knobs were smaller and lost their typical circular form (fig. 1D, E). Despite the commonality of effects of saline and heat on 45S rDNA chromatin decondensation, the effect of salinity was more severe as a dispersed and complex conformation was observed (fig. 1E). After exposure to salt and heat stress factors, the volume of knobs decreased when compared to control (table 1).

5-AC Causes Major Changes of Interphase Nuclear Organization: It Greatly Increases Ribosomal Chromatin Decondensation and It Induces Centromere Polarization

5-AC is widely known to induce DNA hypomethylation and here we show that it greatly affected rice ribosomal chromatin organization. Mostly, the knobs were no longer visible, and thus, not measurable in terms of volume. Instead a strong hybridization signal over the whole nucleolus indicated a dispersed and diffuse chromatin organization (fig. 2B).

FISH with a centromeric probe typically showed centromeres dispersed around the periphery of rice interphase nuclei (fig. 2C). Interestingly, seed germination on 5-AC generated polarization of centromeres in 28% of the total interphase nuclei analyzed (fig. 2D). In contrast, the spatial arrangement of centromeres in interphase was not obviously perturbed by the abiotic stress factors tested (fig. 3).

Table 2. Distance (± SD) between 5S rDNA homologous loci and co-localization analysis between 5S rDNA loci and centromeres in distinct abiotic stress conditions and in the presence of 5-AC

Treatment	Cells analyzed	Distance between 5S rDNA homologous loci, μm	Co-localization 5S rDNA/ centromeres
Control	50	2.8 (0.67)	37%
Cold	50	2.6 (0.80)	36%
Heat	50	3.6 (1.02)	44%
NaCl	50	3.5 (1.00)	42%
5-AC	50	4.08 (1.14)	55%

Spatial Disposition of 5S rDNA in Rice Interphase Nuclei Is Altered by Stress Exposure

FISH with the 5S rDNA probe revealed 2 discrete hybridization signals, relatively small, but clear, corresponding to each allele, localizing outside the nucleolus. The 5S rDNA FISH signal does not show obvious alterations with abiotic stresses or 5-AC (fig. 3). In interphase nuclei, both 5S rDNA and centromeres can co-localize and in this case there is clearly an overlapping of hybridization signals (e.g. fig. 3I). We analyzed the extent of co-localization between 5S rDNA and centromeres and the distance between 5S rDNA homologous loci after imposed abiotic stress and induced DNA hypomethylation (table 2). The frequency of co-localization between 5S rDNA and centromeres was not completely independent of the treatment ($\chi^2(4) = 4.927$; $p = 0.051$; $n = 100$). The minimum co-localization rate was observed for the cold stress and control treatments while the higher rate of co-localization was observed when germination occurred in the presence of 5-AC (table 2). Regarding the distance between 5S rDNA homologous loci, there were statistically significant differences between treatments (Kruskal-Wallis $\chi^2(4) = 92.293$; $p < 0.001$; $n = 300$). The distance between homologous loci was estimated at 2.8 ($\pm$0.67) μm and it was significantly increased to 3.6 ($\pm$1.02) μm on heat and to 3.5 ($\pm$1.00) μm on salinity stress factors. Seed germination on 5-AC also causes a notable increase in the distance between homologous 5S rDNA loci, 4.081 ($\pm$1.14) μm, which is consistent with the widely described effects of this drug on chromatin decondensation. The cold stress caused a slight decrease in the distance of homologous 5S rDNA loci although this was not statistically significant.

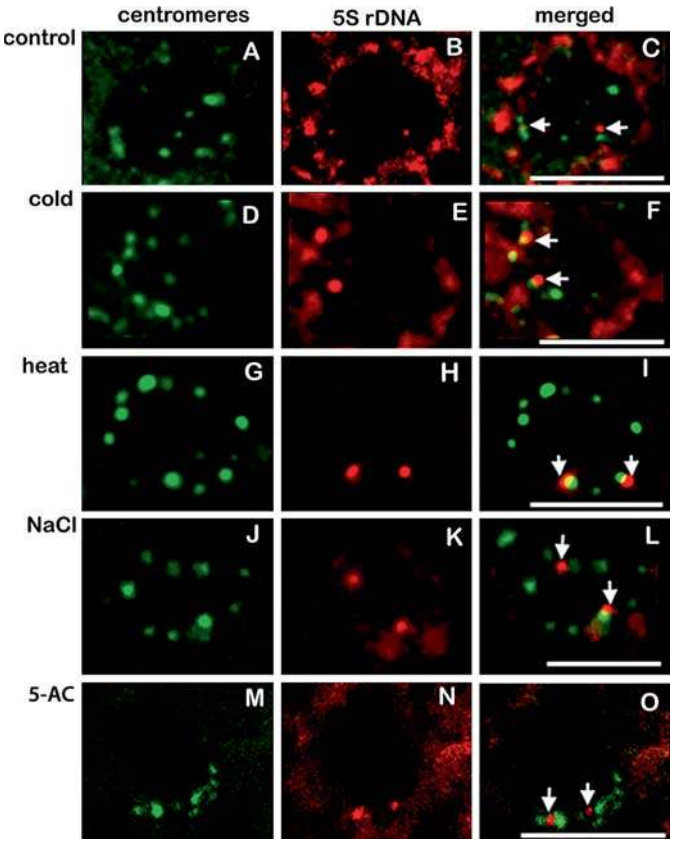

Fig. 3. Simultaneous labeling of centromeres (green) and 5S rDNA (red) in interphase nuclei of rice root sections is shown for distinct abiotic stress conditions and for 5-AC-induced DNA hypomethylation. A circular arrangement of centromeres is clearly shown on control (**A**) and in distinct abiotic stress conditions: cold (**D**), heat (**G**) and NaCl (**J**). The circular arrangement of centromeres is affected by induced DNA hypomethylation through seed germination on 5-AC and the polarization of centromeres can be seen in one side of the nucleus (**M**). Regarding 5S rDNA, 2 hybridization signals are clearly visible as small dots whose size and appearance are not altered by abiotic stresses or 5-AC (arrows in **C**, **F**, **I**, **L**, **O**). Merged images of both probes 5S rDNA and centromeres, are also shown. The overlapping of 5S rDNA alleles and centromeres can clearly be seen (e.g. **I**). Confocal image stacks were recorded with a section spacing of 1 μm and a projection of 2 confocal sections is shown. Bar: 10 μm.

Discussion

Explicit evidence of involvement of chromatin structural changes in plant response to stress is still lacking. The studies on ribosomal chromatin organization have been mainly focused on using FISH to determine the location of ribosomal genes on metaphase chromosomes. For example, in *Oryza sativa* ssp. *japonica* cv. Nipponbare (AA), the 45S rRNA genes are located at the end of the short arm of chromosome 9, while 5S rRNA genes are found on the short arm of chromosome 11, very close to the centromeric region [Shishido et al., 2000; Chung et al., 2008; Zhu et al., 2008]. We focused on the organization pattern of ribosomal chromatin in interphase nuclei from root tissue sections because transcription takes place during interphase. Firstly, we have shown that the organization pattern of 45S rDNA chromatin in rice interphase nuclei consists of 2 circular heterochromatic knobs at the periphery of the nucleolus. Inside the nucleolus, chromatin exhibits a diffuse pattern without any sign of small knobs of condensed ribosomal chromatin foci. Contrastingly, in wheat, perinucleolar knobs unwind, generating several smaller heterochromatic foci

remaining inside the nucleolus [Silva et al., 2008]. Previous unpublished experiments in wheat seedlings submitted to a heat shock of 42°C for 2 h caused drastic alterations in ribosomal chromatin organization in wheat interphase nuclei; the heterochromatic knobs are absent due to its decondensation and subsequently fibers are seen inside the nucleolus (online suppl. fig. 1, www.karger.com/doi/10.1159/000322287). In the present work, we show that heat and salinity stress exposures are both associated with a decondensation of 45S rDNA chromatin as indicated by the decrease in the volume of heterochromatin knobs. The effect of saline treatment on rDNA chromatin decondensation is considerably more severe than heat stress. This may well be due to a higher sensitivity of rice for salinity stress particularly during the germination stage. Indeed, there are studies at the ultrastructural level on rice and onion cells under salt treatment showing dramatic damages of nuclear chromatin organization [Rahman et al., 2000; Bennici and Corrado, 2009]. In yeast, environmental stress or nutritional deprivation was also shown to cause changes in the ratio of accessible vs. inaccessible rDNA chromatin [Conconi et al., 1989; Sandmeier et al., 2002]. On the other hand, the exposure to cold stress did not dramatically affect 45S rDNA chromatin organization. For instance, 2 heterochromatin perinucleolar knobs were seen with a statistically insignificant change in volume. This is not surprising, since cold treatment is often associated with slowing down of metabolism, and it is widely used as a metaphase arresting agent based on its effects on chromatin condensation [Schwarzacher and Heslop-Harrison, 2000].

There are several reports in the literature providing a link between environmental stresses, DNA methylation changes and regulation of stress-responsive gene expression. For example, in tobacco plants, aluminium, salt, cold, and oxidative stresses were shown to induce transcription and DNA demethylation of a gene encoding a glycerophosphodiesterase-like protein [Wada et al., 2004; Choi and Sano, 2007]. Moreover, studies on plant hybrids (e.g. wheat/rye) have shown that epigenetics is involved in the silencing of a set of parental rDNA clusters [Preuss and Pikaard, 2007]. Stress-induced demethylation may relax chromatin structure, thereby allowing enhanced transcription [Shilatifard, 2006]. Thus, we sought to experimentally mimic DNA hypomethylation, referred to in the literature as being associated with abiotic stress exposure. To do this, we used 5-AC, which has been shown to induce chromosome decondensation, transgene repositioning and increased gene expression during interphase [Santos et al., 2002]. Here, we showed that 5-AC causes extensive decondensation of 45S rDNA chromatin in interphase nuclei. The nucleoli acquired a 'painted appearance' resulting from decondensation of heterochromatic knobs and thus are no longer visible.

In wheat, centromeres are polarized at one side of the interphase nucleus showing a Rabl configuration [Abranches et al., 1998]. The polarized centromeric disposition in wheat interphase nuclei was not significantly disturbed by induced DNA hypomethylation through germination on a 5-AC solution [Santos et al., 2002]. In contrast, FISH with a centromeric probe clearly showed that there was no Rabl configuration in rice interphase nuclei (except in xylem vessel precursor cells), with the centromeres evenly distributed around the nuclear periphery [Prieto et al., 2004; Santos et al., 2004]. Here, we show that 5-AC induces centromere polarization in some rice interphase nuclei (28%). There is no convincing explanation for the presence or absence of a Rabl configuration in plants. Also, we cannot find a good argument for rice centromere polarization after 5-AC induced DNA hypomethylation. It may well be possible that with the genome hypomethylated and the chromatin very decondensed and presumably flexible, the normal chromosome structures and forces that move chromosomes away from the Rabl configuration at the end of telophase do not operate. Effectively, the centromeres could stay in their telophase positions and other parts of the chromosome could relocate without pulling the centromeres away. Previous studies showed that multiple homologous transgene loci located at a number of sites along a chromosome arm could associate in the interphase nuclei of wheat [Abranches et al., 2000], but the treatment with 5-AC reduced the association between transgene loci [Santos et al., 2002]. Agreeing with a higher global chromatin flexibility associated with DNA hypomethylation, we observed an increase in the distance between homologous 5S rDNA loci. Puzzlingly, it is not clear whether in plants centromeric chromatin can act as domains for gene silencing as has been shown in animals [Merkenschlager et al., 2004]. In rice, the presence of active genes in the functional domains of several centromeres has been reported [Nagaki et al., 2004]. We observed a higher percentage of co-localization between 5S rDNA and centromeres in 5-AC experiments as well as upon heat and salinity stress which we found to be associated with 45S rDNA decondensation. We cannot find a good explanation for that, but one possibility is that rice centromeres are not domains for gene silencing as has been shown to occur in

animals. In the present work, we describe inducible changes on heterochromatic domains (45S, 5S rDNA loci and centromeres) of the rice genome. However, it should be expected that other genome-wide changes associated with stress exposure, e.g. of an epigenetic nature, need further investigation since it can help to understand the huge plant plasticity responses to challenging environmental conditions.

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