


Plant condensin II is required for the correct spatial relationship between centromeres and rDNA arrays

Takuya Sakamoto, Tomoya Sugiyama, Tomoe Yamashita, and Sachihito Matsunaga 

Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Noda, Chiba, Japan

ABSTRACT

Plants possess the structural maintenance of chromosome (SMC) protein complexes cohesin, condensin, and SMC5/6, which function in fundamental biological processes such as sister chromatid cohesion, chromosome condensation and segregation, and damaged DNA repair. Recently, increasing evidence in several organisms has suggested that condensin is involved in chromatin organizations during interphase. In *Arabidopsis thaliana*, condensin II is localized in the nucleus throughout interphase and is suggested to be required for keeping centromeres apart and the assembly of euchromatic chromosome arms. However, it remains unclear how condensin II organizes chromatin associations. Here, we first showed the high possibility that the function of condensin II as a complex is required for the disassociation of centromeres. Analysis of the rDNA array distribution revealed that condensin II is also indispensable for the association of centromeres with rDNA arrays. Reduced axial compaction of chromosomes and impaired genome integrity in condensin II mutants are not related to the disruption of chromatin organization. In contrast, the axial compaction of chromosomes by condensin II produces the force leading to the disassociation of heterologous centromeres in *Drosophila melanogaster*. Taken together, our data imply that the condensin II function in chromatin organization differs among eukaryotes.

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Introduction


Eukaryotes possess evolutionarily conserved protein complexes composed of chromosomal ATPases that belong to the structural maintenance of chromosomes (SMC) family. There are three types of SMC protein complexes distinguished by different combinations of SMC proteins. SMC1 and SMC3 comprise the core subunits of cohesin, which is involved in sister chromatid cohesion [1,2]. Similarly, SMC2 and SMC4 are the core subunits of condensin, which is required for chromosome condensation and segregation [1–5]. The remaining two, SMC5 and SMC6, interact with each other and are crucial for damaged DNA repair and recombination [1,2].

In animals and plants that have been investigated including *Arabidopsis thaliana*, condensin is subdivided into two types, I and II, by differences in regulatory subunits. In association with SMC2 and SMC4, chromosomal associated protein (CAP)-H, CAP-G, and CAP-D3 form condensin I, while

CAP-H2, CAP-G2, and CAP-D3 form condensin II [3]. In vertebrates, repression of condensin I or II causes misregulation of both mitotic and meiotic chromosome condensation and segregation [4,5]. Likewise, defects in SMC2 or SMC4 cause missegregation of mitotic and meiotic chromosomes in *A. thaliana* [6–9]. However, *A. thaliana* condensin II is unlikely to participate in the regulation of mitotic chromosomes [10]. This is also considered to be the case in *Drosophila melanogaster* [11] and the primitive red alga *Cyanidioschyzon merolae* [12].

Unlike condensin I, which is sequestered in the cytoplasm, condensin II stays in the nucleoplasm and the nucleolus during interphase in vertebrates [13]. In plants, although condensin II subunit CAP-D3 is not expressed in the nucleolus [14], the expressions of other subunits CAP-H2 and CAP-G2 indicate that the plant condensin II during interphase shows similar localization pattern to the vertebrate condensin II [10,15]. Therefore, it is considered that condensin II plays important roles in the interphase nucleus, and conversely, that

CONTACT Sachihito Matsunaga  sachi@rs.tus.ac.jp  Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba, Japan

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condensin I has a minimal function during interphase [4]. In fact, recent studies on animal, fly and plant cells have shown that condensin II contributes to interphase chromosome functions such as damaged DNA repair [10,16,17] and local chromatin organization associated with gene transcription [18–22].

Condensin II affects a wide range of chromosome structures. In *D. melanogaster*, condensin II promotes the axial compaction of chromosomes, narrowing the distance between two distinct loci on the same chromosome arm, which drives the force that disperses heterologous centromeres and forms chromosome territories [23]. Condensin II also contributes to suppressing the association of centromeric regions in mouse neuronal stem cells and neurons [24]. A lack of CAP-D3 function in *A. thaliana* causes increased association of centromeres and disassembly of euchromatic chromosome arms [14]. Condensin I in *Schizosaccharomyces pombe* is recruited to RNA Pol III-transcribed gene loci including 5S rDNA arrays during interphase. It promotes centromere association and facilitates faithful chromosome segregation during mitosis [25,26].

In this study, we established that the function of condensin II as a complex is required for the regulation of centromere and rDNA array association using condensin II mutants. We also found that defects in condensin II enhanced chromatin loosening and impaired genome integrity. However, the abnormal chromatin association between centromeres and rDNA arrays was not attributable to those impairments. Therefore, we propose a possibility that condensin II mediates the recruitment of rDNA arrays to centromeres in plants, as is the case for condensin I in *S. pombe* [25,26].

Results

Condensin II mutants show an increase in centromere association

CAP-D3, a subunit of condensin II, is required for keeping centromeres apart [14]. However, because only CAP-D3 has been analyzed among the five subunits of condensin II, it remains possible that CAP-D3 functions independently of the condensin II complex. To clarify this, we elucidated the functions of the other condensin II-specific subunits,

CAP-H2 and CAP-G2, in centromere association. The lack of CAP-D3 is known to cause an increase in centromere association into a few clusters in nuclei from rosette leaves [14]. Similarly, our FISH analysis using nuclei from flower buds not only in *cap-d3-1* but also in *cap-h2-2* and *cap-g2-1* showed a reduction in the number of centromeric signals indicated by a probe for 180 bp repeats compared with the wild-type (Figure 1(a)). Wild-type 2–8C nuclei of *A. thaliana* contain ~4 to 10 centromere clusters [14]. In our analysis, nuclei containing ~7 to 10 centromere clusters were most frequent in the wild-type, while nuclei containing ~1 to 4 centromere clusters were most frequent in *cap-h2-2* and *cap-d3-1* (Figure 1(b)). Although it did not occur frequently in *cap-g2-1* compared with *cap-h2-2* and *cap-d3-1*, centromere

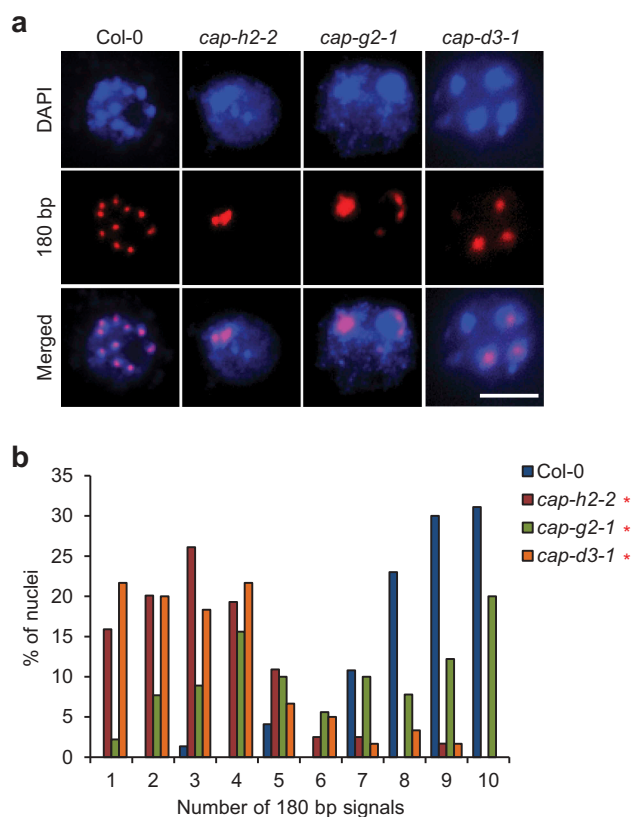


Figure 1. An increase in centromere association is observed in all mutants of condensin II-specific subunits. (a) Representative images of FISH detection of pericentromeric 180 bp signals in nuclei from flower buds of Col-0, *cap-h2-2*, *cap-g2-1*, and *cap-d3-1*. Bar = 5 μ m. (b) Number of 180 bp signals in nuclei from flower buds of Col-0, *cap-h2-2*, *cap-g2-1*, and *cap-d3-1*. The frequency distribution of the number of 180 bp signals in each mutant was statistically compared with that in Col-0 by chi-squared test (*, $p < 0.05$, $n = 74$ for Col-0, $n = 119$ for *cap-h2-2*, $n = 90$ for *cap-g2-1*, $n = 60$ for *cap-d3-1*).

association was significantly increased in *cap-g2-1* compared with the wild-type (Figure 1(b)). Taken together, these data strongly suggested that CAP-H2, CAP-G2, and CAP-D3 act in a condensin II complex to maintain the correct centromere association in *A. thaliana*.

On the other hand, FISH analysis of telomere repeats revealed that condensin II mutants show nucleolar peripheral localization of telomeres without clustering as the wild-type does (Supplementary Fig. 1), suggesting that condensin II is not involved in the maintenance of telomere organization.

Condensin II mutants show disassociation of rDNA arrays from centromeres

Condensins are reported to function in axial compaction of chromosomes and recruitment of rDNA arrays to centromeres in *D. melanogaster* and *S. pombe*, respectively [23,25,26]. These facts led us to speculate that one or both functions would be conserved in *A. thaliana* condensin II. To investigate this possibility, we simultaneously visualized rDNA arrays and centromeres in nuclei of flower buds by FISH. Because in interphase nuclei of *A. thaliana*, condensin II subunits, CAP-H2 and CAP-G2, are localized in the nucleoplasm and the nucleolus [10,15] that is formed around nucleolar organizer regions (NORs) containing 45S rDNA arrays [27], we focused on the distribution of both 5S and 45S rDNA arrays. In *A. thaliana* ecotype Columbia, 5S rDNA arrays are located in close proximity to centromeres on chromosomes 3, 4 and 5; in contrast, 45S rDNA arrays are located away from centromeres on chromosomes 2 and 4 [27] (Figure 2(a)). However, similarly to 5S rDNA, the spatial location of 45S rDNA arrays basically shows an association with centromeres [28] (Figure 2(b)). Our FISH analysis revealed that more than 40% of nuclei showed the separation of 45S rDNA from centromeric regions in all condensin II mutants tested, while less than 7% of nuclei did in wild-type (Figure 2(b,c)). The differences between each mutant and wild-type were statistically significant (Figure 2(c)). We also found a reduction in the association of 5S rDNA arrays with the centromeric regions in both *cap-h2-2* and *cap-g2-1* (Figure 2(d)). The distance between the 5S rDNA signal and the nearest centromeric signal was more than twofold

longer in both condensin II mutants compared with wild-type (Figure 2(e)). These results indicate that condensin II is required for the association of rDNA arrays with centromeres during interphase in *A. thaliana*, possibly through the axial compaction of chromosomes and/or direct recruitment of rDNA arrays to centromeres (Figure 2(f)).

The abnormal arrangement of 45S rDNA association with centromeres in condensin II mutants led us to speculate the function of condensin II in controlling rRNA gene expression. Therefore, we analyzed the expression levels of 5S rRNA and 45S rRNA (5.8S, 18S and 25S rRNA) genes and found that the defects in condensin II mutant showed similar expression levels of rRNA genes to the wild-type (Supplementary Fig. 2), suggesting that condensin II is not involved in the regulation of rRNA genes.

Chromatin loosening does not cause disassociation of rDNA arrays from centromeres

If we assume that axial compaction of chromosomes is required for the association of rDNA array with centromeres, defects in condensin II would cause the loosening of chromatin, at least which resides between the two loci. To verify this hypothesis, we analyzed histone acetylation, which is an epigenetic mark for loosened chromatin [29]. We found that the levels of both acetylated histone H3 and H4 in the whole nucleus were significantly higher in *cap-h2-2* (Figure 3(a-c)), suggesting that chromatin loosening is enhanced by defects in condensin II function. Based on these results, we hypothesized that chromatin loosening would cause disassociation of rDNA arrays from centromeres. This hypothesis was assessed by analyzing the spatial relationship between 45S rDNA arrays and centromeres in plants showing increased genome-wide histone acetylation caused by treatment with a histone deacetylase inhibitor, trichostatin A (TSA) [30] (Figure 3(d)). We found that TSA treatment did not affect the association between centromeres or between 45S rDNA arrays and centromeres (Figure 3(e,f)). Taken together, these results suggest that although condensin II is involved in the maintenance of the epigenetic state to prevent an increase in chromatin loosening, unlike the case in *D. melanogaster* axial

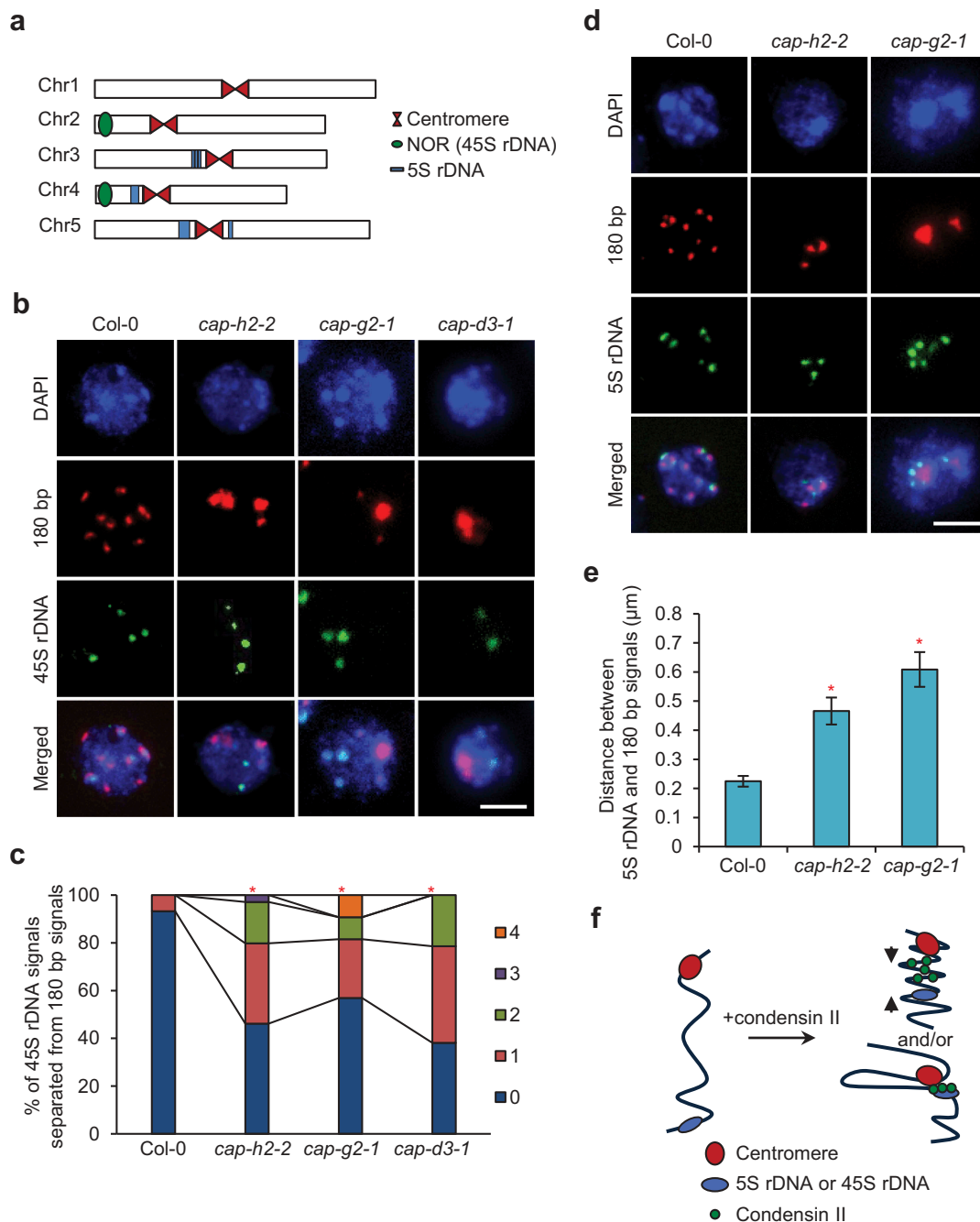


Figure 2. Defects in condensin II cause dissociation of centromeres from rDNA arrays. (a) Localization of rDNA arrays in the *A. thaliana* genome. 45S rDNA arrays are contained in nucleolar organizing regions (NORs) located on the short arms of chromosomes 2 and 4. 5S rDNA arrays are located in close proximity to the centromeres of chromosomes 3, 4, and 5. (b) Representative images of simultaneous FISH detection of pericentromeric 180 bp signals and 45S rDNA signals in nuclei from flower buds of Col-0, *cap-h2-2*, *cap-g2-1*, and *cap-d3-1*. Bar = 5 μm . (c) Number of 45S rDNA signals separated from 180 bp signals in nuclei from flower buds of Col-0, *cap-h2-2*, *cap-g2-1*, and *cap-d3-1*. The frequency distribution of the number of separated 45S rDNA signals in each mutant was statistically compared with that in Col-0 by chi-squared test (*, $p < 0.05$, $n = 52$ for Col-0, $n = 104$ for *cap-h2-2*, $n = 65$ for *cap-g2-1*, $n = 42$ for *cap-d3-1*). (d) Representative images of simultaneous FISH detection of pericentromeric 180 bp signals and 5S rDNA signals in nuclei from flower buds of Col-0, *cap-h2-2*, and *cap-g2-1*. Bar = 5 μm . (e) Quantification of the distance between 5S rDNA signals and 180 bp signals in nuclei from flower buds of Col-0, *cap-h2-2*, and *cap-g2-1*. Means \pm SD are shown. The distance in each mutant was statistically compared with that in Col-0 by Student's *t*-test (*, $p < 0.01$, $n = 61$ for Col-0, $n = 51$ for *cap-h2-2*, $n = 61$ for *cap-g2-1*). (f) Two possible models for condensin II-mediated association of centromeres with rDNA arrays: i) axial compaction of chromatin located between two loci (upper), ii) direct recruitment of an rDNA array to a centromeric region (lower).

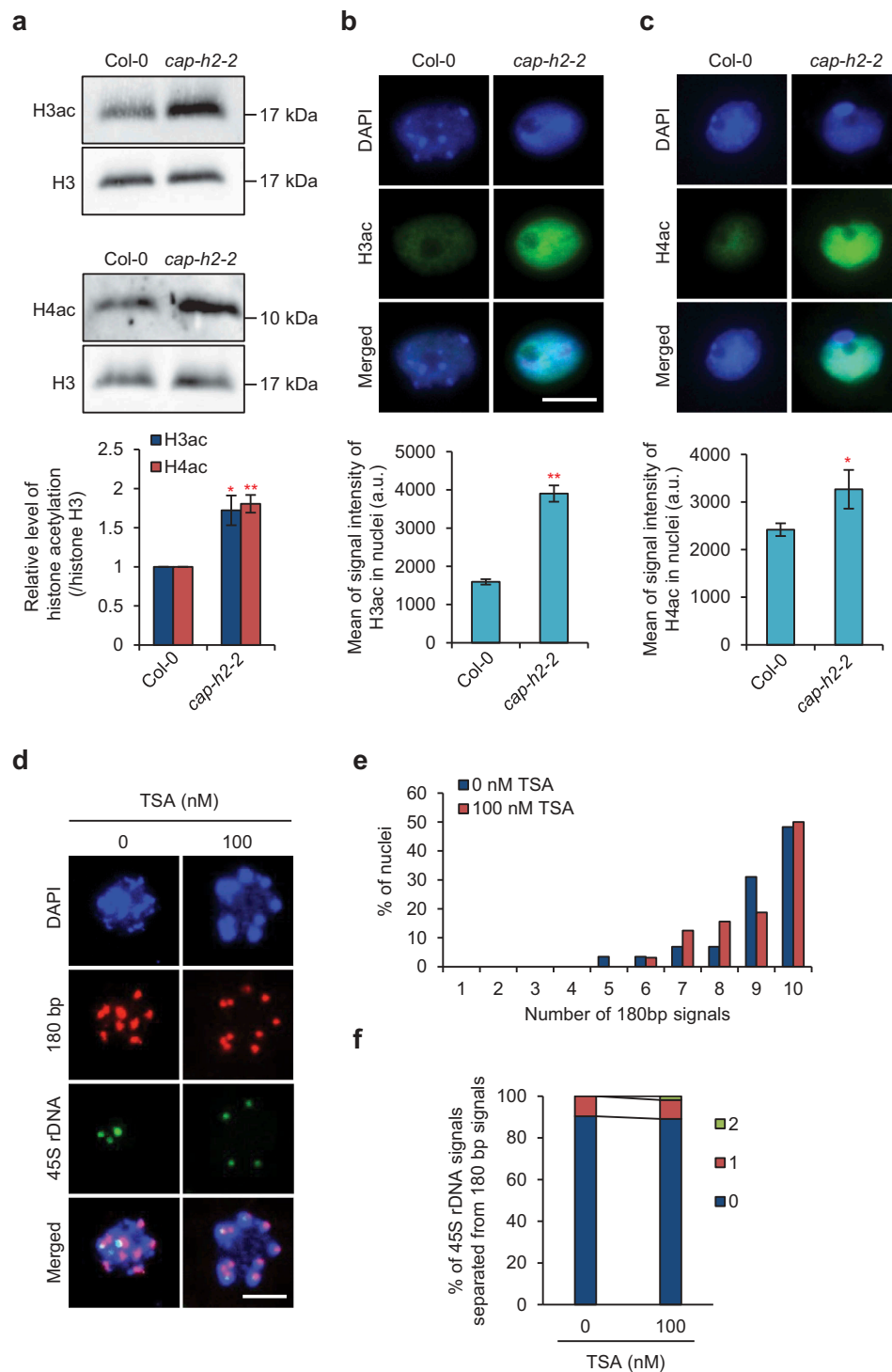


Figure 3. Defects in condensin II cause histone hyperacetylation, but this is unlikely to cause disruption of chromatin associations. (a) Immunoblotting of acetylated histone H3 and H4 in nuclei from 7-day-old seedlings of Col-0 and *cap-h2-2*. The graph shows the acetylated histone levels normalized by histone H3 levels. Error bars indicate SE. The level in *cap-h2-2* was statistically compared with that in Col-0 by Student's *t*-test (*, $p < 0.05$, **, $p < 0.01$, $n = 3$). (b,c) Immunostaining of acetylated histone H3 (b) and H4 (c) in nuclei from flower buds of Col-0 and *cap-h2-2*. Representative images of immunostained nuclei are shown. Bar = 5 μm . Each graph shows the acetylated histone levels represented by the mean intensity of immunofluorescence. Error bars indicate SE. The level in *cap-h2-2* was statistically compared with that in Col-0 by Student's *t*-test (*, $p < 0.05$, **, $p < 0.01$, $n = 53$ and 68 for H3ac, $n = 41$ and 65 for H4ac). (d) Representative images of simultaneous FISH detection of pericentromeric 180 bp signals and 45S rDNA signals in nuclei from 0 and 100 nM TSA-treated flower buds. Bar = 5 μm . (e) Number of 180 bp signals in nuclei from 0 and 100 nM TSA-treated flower buds. No significant differences in the frequency distribution of the number of 180 bp signals were detected in 100 nM TSA-treated plants compared with each control by chi-squared test ($n = 29$ for 0 nM TSA, $n = 32$ for 100 nM TSA). (f) Number of 45S rDNA signals separated from 180 bp signals in nuclei from 0 and 100 nM TSA-treated flower buds. No significant differences in the frequency distribution of the number of separated 45S rDNA signals were detected in 100 nM TSA-treated plants compared with each control by chi-squared test ($n = 46$).

compaction of chromosomes is likely not required for the association of rDNA arrays and centromeres. In other words, it is plausible that the association of rDNA arrays and centromeres results from condensin II-mediated recruitment of rDNA arrays to centromeres as is the case for condensin I in *S. pombe* [25,26].

Impaired genome integrity does not cause disassociation of heterochromatic loci

Condensin II is known to be crucial for the maintenance of genome integrity to alleviate DNA damage accumulation caused by genotoxic stresses, especially to high-boron stress, although the molecular mechanism is still unknown [10]. Thus, we next questioned whether impaired genome integrity would result in the disassociation of heterochromatic loci. To address this question, we focused on the mutants *lig4-4* and *rpt5a-4*. *lig4-4* is a mutant of DNA ligase IV, which functions in DNA repair [31], and *rpt5a-4* is a mutant of *RPT5a*, which encodes a subunit of the 26S proteasome that functions in protecting the genome from attack by DNA-damaging factors [30]. Both mutants are known to show the hypersensitivity to high-B stress and accumulate DNA damage even under the normal growth condition [10,30,32], which are common features to condensin II mutants [10]. The reduced genome integrity in *cap-h2-2*, *lig4-4* or *rpt5a-4* was also confirmed by the hypersensitivity of root elongation through the treatment with a DNA-damaging reagent zeocin (Supplementary Fig. 3A). We visualized both 45S rDNA arrays and centromeric regions of these two mutants by FISH (Supplementary Fig. 3B) and found that both mutants showed a normal association of centromeres and a normal spatial relationship between 45S rDNA arrays and centromeres (Supplementary Fig. 3C, D). These results suggested that the impairment of genome integrity does not cause the disassociation of heterochromatic loci.

Discussion

In this study, it is strongly suggested that the function of condensin II as a complex is required for the proper organization of centromere distribution in interphase nuclei, rather than the independent function of

individual condensin II-specific subunits, although we cannot completely show the evidence that CAP-H2, -G2, and -D3 form a complex. Considering that condensin II also prevents centromere clustering in mouse and *D. melanogaster* [23,24], its role in the separation of heterologous centromeres seems to be highly conserved among eukaryotes. However, the molecular mechanism by which the correct centromere distribution is maintained seems to differ among organisms. Our findings showed that the enforced chromatin loosening attributable to histone hyperacetylation induced by TSA did not affect the centromere distribution. Considering that histone hyperacetylation interferes with the axial compaction of meiotic chromosomes in mouse oocytes [33], unlike the case in *D. melanogaster*, *A. thaliana* condensin II acts in a different way to ensure the correct centromere distribution. Interestingly, a similar phenotype regarding the increase in centromere association is also seen in *A. thaliana* mutants of CROWDED NUCLEI (CRWN) proteins [34], which reside at the inner side of the nuclear periphery and play important roles in specifying nuclear shape and size [35,36]. This led us to speculate that condensin II and CRWNs act concertedly to ensure the correct centromere distribution during interphase in *A. thaliana*.

In yeast, as condensin II is absent, condensin I is considered to perform the job of condensin II [5]. In *S. pombe*, condensin mediates the interactions between centromeres and RNA Pol III-transcribed genes including 5S rDNA arrays and retrotransposons, which are crucial for the construction of 3D chromatin structure and contribute to gene silencing at these loci [25]. In *A. thaliana*, we found that in addition to 5S rDNA arrays, condensin II is involved in the association of 45S rDNA arrays with centromeres despite these locations being far apart on the same chromosome. The core subunit of condensin I and II, SMC4, was found to be involved in the silencing of transposons in pericentromeric heterochromatin of *A. thaliana* in conjunction with the enrichment of DNA methylation and the repressive histone modifications, H3K9me2 and H3K27me1 [37]. However, in *A. thaliana*, condensin II is not involved in repressing rRNA gene expression (Supplementary Fig. 2), unlike the case of condensin I in *S. pombe* [25].

Besides heterologous centromere associations, our results suggest that the association between

Mira cloth and then boiled for 10 min. The antibodies used in this study were anti-histone H3 (MABI0301, MBL) (1:2000 dilution), anti-acetylated histone H3 (06–599, Millipore) (1:1000 dilution), and anti-acetylated histone H4 (06–866, Millipore) (1:1000 dilution). The target proteins of each antibody were visualized with ImmunoStar (Wako, Osaka, Japan) on a Fusion Pulse system (Vilber Lourmat, Osaka, Japan). To quantify the protein levels, the signal intensities of target bands were measured using Gel Analyzer in the ImageJ software.

Immunostaining

Flower buds were fixed with 4% paraformaldehyde/1xPBS for 40 min and then washed with 1xPBS three times. The fixed flower buds were digested in solution {1% Doriselase (Sigma-Aldrich Japan, Tokyo, Japan), 0.5% Cellulase R-10 (Yakult Pharmaceutical, Tokyo, Japan), 0.025% pectolyase (Seishin Pharmaceutical, Tokyo, Japan) in water} for 30 min at 37°C. After washing with 1xPBS twice, the fixed flower buds were squashed using a coverslip on a slide coated with poly-L-lysine. The slides were immediately placed in liquid N₂ and the cover glass was removed. After the slides were dried, they were washed with 1xPBS once. Then, each sample was treated with 4% (w/v) bovine serum albumin (BSA)/1xPBS on the slide for 30 min at room temperature. After the solution was removed from the slide, 100 µl of a solution of primary antibodies diluted 100 times with 1% (w/v) BSA/1xPBS was added to the sample. Anti-H3ac rabbit IgG (Millipore, Temecula, CA, USA) and anti-H4ac rabbit IgG (Millipore) were used as the primary antibodies. The slides were incubated overnight at 4°C and then washed with 0.05% Tween20/1xPBS twice. Then, each sample was treated with 4% (w/v) bovine serum albumin (BSA)/1xPBS on the slide for 30 min at room temperature. After washing with 1xPBS twice, 50 µl of a solution of anti-rabbit IgG Alexa Fluor® 488 Fab (Invitrogen, Carlsbad, CA) diluted 200 times with 1% (w/v) BSA/1xPBS was added to the sample. The slides were incubated at 37°C for 1 h in the dark and then treated with 0.05% Tween20/1xPBS containing 1 µg/mL DAPI for 10 min. The

slides were then washed with 0.05% Tween20/1xPBS and subsequently with water three times. After drying the slides at 37°C in the dark, the hybridized nuclei were mounted with VECTASHIELD™. The nuclei were observed under the same light microscope (BX53m). The mean intensity of the fluorescence in the entire nuclei was analyzed using the ImageJ software.

rRNA gene expression

Total RNA from shoots and roots of 7-day-old seedlings was extracted as described previously [30]. Approximately 1 µg of total RNA was reverse-transcribed with a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Yokohama, Japan) following the manufacturer's protocol. Real-time PCR was performed with Thermal Cycler Dice Real Time System II (Takara Bio, Shiga, Japan) and Luna qPCR Master Mix (New England Biolabs, Tokyo, Japan). *Actin2* was used for the normalization of cDNA concentration. The primers for the detection of used were as follows: 5'-GATGCGATCATAACCAGCACT-3' (FP) and 5'-GGATGCAACACGAGGACTTC-3' (RP) for 5S rRNA, 5'-CCTGCGGCTTAATTTGACTC-3' (FP) and 5'-GACAAATCGCTCCACCAACT-3' (RP) for 18S rRNA, 5'-CGCGAGTTCTATCGGGTAAA-3' (FP) and 5'-CACTTGGAGCTCTCGATTCC-3' (RP) for 25S rRNA, and 5'-AAGTCATAACCATCGGAGCTG-3' (FP) and 5'-ACCA GATAAGACAAGACACAC-3' (RP) for *ACT2*.

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Disclosure statement

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ORCID

Sachihiro Matsunaga  <http://orcid.org/0000-0003-3024-3559>

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