Spatial relationship between chromosomal domains in diploid and autotetraploid Arabidopsis thaliana nuclei

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ABSTRACT
Polyploids constitute more than 80% of angiosperm plant species. Their DNA content is often further increased by endoreplication, which occurs as a part of cell differentiation. Here, we explore the relationship between 3D chromatin architecture, number of genome copies and their origin in the model plant, Arabidopsis thaliana. Spatial proximity between pericentromeric, interstitial and subtelomeric domains of chromosomes 1 and 4 was quantified over a range of distances. The results indicate that average nuclear volume as well as chromatin density increase with the genome copy number. Similar dependence is observed when association of homologous chromosomes (in 2C/ endopolyploid nuclei) and sister chromatid separation (in endopolyploid nuclei) is studied. Moreover, clusters of chromosomal domains are detectable at the spatial scale above microscopy resolution. Subtelomeric, interstitial and pericentromeric chromosomal domains are affected to different extent by these processes, which are modulated by endopolyploidy. This factor influences fusion of heterochromatin as well. Nonetheless, local chromatin architecture of Arabidopsis thaliana depends mainly on endopolyploidy level, and to lesser extend on polyploidy.

INTRODUCTION

Nuclear architecture is influenced by various factors such as developmental stage of the organism and its environment.\textsuperscript{1-3} Those factors impact the distribution and condensation of chromatin fiber (DNA in complex with histone proteins). Consequently, chromatin may exist in relaxed (euchromatin) or compacted (heterochromatin) state,\textsuperscript{4} depending on both genetic and epigenetic regulation. Increasing data suggest that chromatin organization may be also modulated by physical features such as number of chromosomes or chromatin density.\textsuperscript{5,6}

Chromatin architecture has been extensively studied in the model plant Arabidopsis thaliana (2n = 2x = 10), which has a small genome, containing little repetitive DNA. All Arabidopsis chromosomes show similar pattern of euchromatin/heterochromatin distribution, with euchromatin covering chromosome arms and heterochromatin confined to the pericentromeric and nucleolar organizing regions (NORs).\textsuperscript{7} In the interphase nucleus chromosomes are organized into chromosome territories.\textsuperscript{8} It has been proposed that Arabidopsis chromosome territories are rosette structures, with heterochromatin constituting chromocentres, from which euchromatin loops emerge.\textsuperscript{9} The interphase chromosomes are polarized, with chromocentres on the periphery and euchromatin in the center of the nucleus\textsuperscript{10} (Fig. S1). Meristematic Arabidopsis nuclei may contain 10 (reflecting the number of centromeres) or fewer chromocentres, depending on functional state of the cells.\textsuperscript{4} However, in differentiated cells the number of chromocentres is reduced, owing to their fusion,\textsuperscript{9,10} which may be regulated by both genetic and epigenetic mechanisms.\textsuperscript{11,12,13}

Even though heterochromatin domains (ex. chromocenters, NORs) show a tendency to fuse Arabidopsis chromosome territories show random distribution. The exception are NOR-bearing chromosomes, probably due to their role in nucleolus formation.\textsuperscript{8} Interestingly, recent data suggest that some preferential interchromatin contacts may be formed by chromatin domains of 0.4 to 1 Mb size.\textsuperscript{14-16}
During cell differentiation *Arabidopsis* nuclei may undergo endoreplication, which leads to increase in the genome copy number and, in consequence, nuclear C-value (level of endopolyploidy), up to 64C. However, data on possible influence of this process on chromatin architecture remain scarce. It has been demonstrated that endoreduplicated nuclei are larger than their 2C counterparts. Moreover, with concomitant rounds of endoreplication, the number and size of chromocentres also increase. The former effect is probably caused by separation of centromeric domains of chromosomes, while their NORs remain associated. In addition, endoreplication increases the number of sister chromatids constituting a single interphase chromosome. Following replication sister chromatids remain associated, owing to the presence of cohesin complexes. In nuclei characterized by high level of endopolyploidy separation of sister chromatids may take place, which is attributed to reduction in number of these complexes. Nonetheless, it is not clear how this process affect interactions between chromatin domains and their arrangement.

It should be noted that more than 80% of angiosperm species are polyploids, which contain multiple homeologous (allopolyploids) or homologous (autopolyploids) genomes. Newer research suggest the latter are more common than previously assumed. Autopolyploidy may confer evolutional advantage, owing to the increase of the maximum number of allelic variants, which results in higher plasticity. They have also larger organ size and higher number of chloroplasts. However, genetic stability of autopolyploids may be lower than their diploid counterparts. In terms of nuclear organization increase in the genome copy number through autopolyploidy results in bigger nuclei and pronounced association of NOR domains. On the other hand, no significant changes in chromatin structure or gene expression pattern in the first generations of autopolyploids have been reported. One should note that the majority of available data pertains to behavior of meiotic chromosomes, while studies of interphase nuclei are scarce.

Here we present the analysis of chromatin organization in relation to the number of genome copies and their origin (endopolyploid, autopolyploid). Our data show that, endoreplication leads to increase of spatial clustering of chromosomal domains. However, the magnitude of these effects in diploids and autotetraploids is different and depends on the chromosome region.

**Results**

**Size of nuclei and number of heterochromatic domains**

Stem leaf nuclei with a range of C-values (2C–8C) from diploid (2x) and autotetraploid (4x) plants were analyzed in context of their morphology. In both diploid and autotetraploid plants the increase of the C-value correlated with the increase of the nuclear size (volume, estimated indirectly with nuclear area, see Materials and Methods). However, when C value doubled, size of diploid nuclei increased only by a factor of 1.82–1.9. The corresponding increase in autotetraploid plants was approximately 1.7 (Fig. 1). Thus, one may postulate that average density of nuclear chromatin increases with the DNA content. Moreover, comparison between the nuclei with the same DNA content, but different ploidy (eg. 2 £ 4C vs. 4 £ 2C) revealed that autotetraploid nuclei were smaller than their diploid counterparts (p < 0.05, Fig. 1). This result indicates that in autotetraploid plant nuclei average chromatin density might be

![Figure 1. Average area of nuclei [\(\mu m^2\)] with different C value (2C – 8C) from diploid (2x) and autotetraploid (4x) *Arabidopsis* plants. Error bars – standard deviation.](image-url)
higher than in diploid plants. Thus, to correct for this factor, further analysis steps were carried out in normalized nuclear coordinate space (see Materials and Methods).

Both *Arabidopsis* cytotypes (2x and 4x) exhibited chromocentric type of chromatin organization. Not surprisingly, mean number of chromocentres in diploid plant 2C nuclei (6 ± 1) was lower (p < 0.05) than in their autotetraploid 2C counterparts (11 ± 1) (Fig. S2). Accordingly, the number of FISH signals from centromeric repeats (main component of chromocentres) was 6 (± 1) in 2C nuclei in diploids and 11 (± 3) in autotetraploids. One may note that the numbers of chromocentres did not differ significantly in the nuclei with different endopolyploidy level. This was true for diploid and autotetraploid plants. Furthermore, in diploids and autotetraploids, the heterochromatin associated with inactive rDNA formed similar number of domains. In diploid nuclei (4 NOR chromosomes) 2 ± 1 rDNA signals were detectable. Likewise, 3 ± 1 rDNA signals were observed in autotetraploid nuclei (8 NOR chromosomes). One may thus postulate that fusion of heterochromatin domains occurs in both diploid and autotetraploid nuclei of *Arabidopsis* plants, though it is more pronounced in the latter.

**Association of pericentromeric, interstitial and subtelomeric domains**

Interphase organization of chromosome 1 and 4 (NOR-bearing) was studied using a range of pericentromeric, interstitial, and subtelomeric FISH probes (see Materials and Methods, Fig. 9). Analysis of chromosome 1 showed that in majority of 2C nuclei from diploid and autotetraploid plants mean signal number was lower (subtelomeric, Fig. 2AB and interstitial, Fig. 2CD probes) or equal (pericentromeric probe, Fig. 2E), comparing to the number of homologs (Fig. 2). This indicates that frequent association of subtelomeric and interstitial domains of homologous chromosomes occurs in both 2x and 4x plant nuclei.

In endopolyploid nuclei, the number of FISH signals (chromosome 1) was proportional to the number of endoreduplication rounds (one – 4C or two – 8C) in diploid and autotetraploid plants (Fig. 2). This suggests that separation of sister chromatids took place, though its probability was smaller than 1, as indicated by the difference between the maximum (Fig. 2) and observed signal number. The increase of FISH signal number in diploid plant nuclei was least pronounced in case of interstitial (f17l21 and t7n9, Fig. 2CD), followed by subtelomeric (f6f3 and f3f9, Fig. 2AB) and then pericentromeric (t19e23, Fig. 2E) domains. No such difference was detectable in autotetraploids, with the exception of 4 × 8C nuclei, where lower number of interstitial FISH signals detected, suggested that this domain was more cohesive (Fig. 2CD) than either subtelomeric (Fig. 2AB) or pericentromeric (Fig. 2E) domains. One may note that the reduction of interstitial signal number was more pronounced in 2 × 8C than 4 × 8C nuclei (Fig. 2CD). Therefore, it may be postulated that polyploidization correlates positively with frequency of separation of sister chromatids (chromosome 1) after endoreduplication. Nonetheless, magnitude of this effect seems to depend on the chromatin domain.

Comparison between diploids and autotetraploids with the same genome copy number (2 × 4C vs 4 × 2C and 2 × 8C vs 4 × 4C, respectively) indicated that the numbers of subtelomeric (Fig. 2AB) and pericentromeric (Fig. 2D) signals was lower in the former (2x) than in the latter (4x), which suggested that these chromatin domains are more densely packed in diploid than in autotetraploid plant nuclei.

Analysis of the NOR-bearing chromosome 4 interphase organization revealed that homologous association of subtelomeric (f6n15 and t5n17, Fig. 3AB) and pericentromeric (t1j1, Fig. 3C) regions occurred frequently in 4 × 2C but not in 2 × 2C nuclei. In case of the endopolyploid nuclei the mean number of FISH signals (chromosome 4) increased with the DNA content in diploid and autotetraploid plant nuclei (Fig. 3), suggesting disruption of homologous association. Similarly to chromosome 1, subtelomeric domains of chromosome 4 showed smallest increase in the signal number (Fig. 3AB). No significant differences between diploids (2 × 4C and 2 × 8C) and autotetraploids (4 × 4C and 4 × 8C, respectively) could be detected, regardless of the region studied (pericentromeric or subtelomeric). Furthermore, comparison of diploids and autotetraploids with the same genome copy number (2 × 4C vs 4 × 2C and 2 × 8C vs 4 × 4C) revealed the differences only in case of pericentromeric regions (Fig. 3C). Thus, organization of chromosomes 1 and 4 in diploid and autotetraploid plant nuclei is similar,
with respect to separation and fusion of subtelomeric and pericentromeric domains (occurring below resolution of optical microscopy, Table 1).

**Proximity of homologous pericentromeric, interstitial and subtelomeric domains**

The compaction of interphase chromosomes was studied with the nearest-neighbor (NN) analysis. This method quantifies proximity of homologous chromatin domains represented by FISH signals at distances above resolution of optical microscopy. Analysis of the chromosome 1 showed that the NN distances between pericentromeric signals were larger in diploid than in autotetraploid 2C nuclei (Fig. 4E). Accordingly, mean number of signals was slightly higher in the latter than in the former nuclei (Fig. 2E). Nonetheless, even though similar (albeit smaller) effect was

**Figure 2.** Numbers of chromosome 1 FISH signals of chromosome 1, corresponding to subtelomeric (A, f6f3 and B, f3f9), interstitial (C, f17f21 and D, t7f9) and pericentromeric (E, t19e23) sequences. The number distributions are shown with their 25/75 percentiles (boxes), 5/95 percentiles (whiskers) and 1/99 percentiles (circles). The distributions are plotted in shades of green (diploids, 2x) or red (autotetraploids, 4x), color saturation increasing with the DNA content. Medians of the distributions are depicted with solid (thin), whereas the means with dashed (thick) black vertical lines.
Figure 3. Numbers of FISH signals of chromosome 4, corresponding to subtelomeric (A, 6n15 and B, t5n17), and pericentromeric (C, t1j1) sequences. The number distributions are shown with their 25/75 percentiles (boxes), 5/95 percentiles (whiskers) and 1/99 percentiles (circles). The distributions are plotted in shades of green (diploids, 2x) or red (autotetraploids, 4x), color saturation increasing with the DNA content. Medians of the distributions are depicted with solid (thin), whereas the means with dashed (thick) black vertical lines.

Table 1. Organization of domains of Arabidopsis chromosome 1 and 4. The organization was studied at short distances (below microscopy resolution) by estimating reduction of the FISH signal number, ranging from non-detectable (0) to very prominent (++++.). Moderate spatial scale (directly above microscopy resolution) was probed with NN distance (short – very short, ccccc – very long) analysis. This corresponded to proximity (–closest, ccccc – farthest). Large spatial scale (up to 0.1 normalized nuclear radius) was investigated with spatial autocorrelation (ACF) functions, where chromosome clustering was estimated from moderate (#) to very prominent (+++).
observed for subtelomeric (Fig. 2AB) and interstitial (Fig. 2CD) domains, no statistically significant differences in NN distances between 2C and 4C nuclei could be detected (Fig. 4AB and 4CD, respectively). Thus, one may speculate these 2 classes of chromatin domains show similar compaction in 2C nuclei of both 2x and 4x plants.

In endopolyploid nuclei (4C, 8C) the signal numbers were significantly higher than in 2C nuclei (Fig. 2), suggesting that separation of sister chromatids took place. This was coupled with the decrease in the NN distances between homologous (subtelomeric, interstitial and pericentromeric) FISH signals (chromosome 1) in both diploid and autotetraploid plant nuclei (Fig. 4). Comparison between diploids (2 × 4C and 2 × 8C) and autotetraploids (4 × 4C and 4 × 8C) revealed that higher ploidy (4x vs 2x) correlated with smaller NN distances (Fig. 4). However, this effect was not observed for

Figure 4. Normalized nearest neighbor (NN) distances between homologous chromosome 1 domains. The FISH signals, correspond to subtelomeric (A, f6f3 and B, f3f9), interstitial (C, f17121 and D, t7n9) and pericentromeric (E, t19e23) sequences. The number distributions are shown with their 25/75 percentiles (boxes), 5/95 percentiles (whiskers) and 1/99 percentiles (circles). The distributions are plotted in shades of green (diploids, 2x) or red (autotetraploids, 2x), color saturation increasing with the DNA content. Medians of the distributions are depicted with solid (thin), whereas the means with dashed (thick) black vertical lines.
interstitial domains (Fig. 4CD), where sister chromatid separation was less pronounced than in case of subtelomeric (Fig. 2AB) and pericentromeric (Fig. 2E) sequences. Thus, it might be stated that, despite sister chromatid separation, endopolyploid nuclei in both 2x and 4x plants show higher chromatin compaction than 2C nuclei, though the magnitude differs between chromatin domains. Analysis of diploids and autotetraploids with the same genome copy number (2 × 4C vs 4 × 2C and 2 × 8C vs 4 × 4C) revealed no difference in NN distances for subtelomeric (Fig. 4AB) and pericentromeric (Fig. 4E) regions. However, the NN distances calculated for interstitial domains in 4 × 4C nuclei were larger than the distances in their 2 × 8C counterparts (Fig. 4CD), even though the FISH signal numbers (Fig. 2CD) were higher in the former (4x) than the latter (2x) nuclei.

Similarly to chromosome 1, NN distances between pericentromeric signals of chromosome 4 were larger in 2C diploid (2x) than in 2C autotetraploid (4x) plant nuclei (Fig. 5C). However, this difference was not detectable for subtelomeric regions (Fig. 5AB). On the other hand a marked difference in pericentromeric (Fig. 3C) and a minor difference in subtelomeric (Fig. 3AB) signal numbers were observed. Comparison between diploid (2C) and endopolyploid (4C, 8C) nuclei indicated that the NN distances were markedly lower in the former than in the latter (Fig. 5). This effect was more pronounced in pericentromeres (Fig. 5C) than in subtelomeres (Fig. 5AB) in both 2x and 4x plants. Furthermore, when corresponding diploids (2 × 4C and 2 × 8C) and autotetraploids (4 × 4C and 4 × 8C) were compared, no significant difference in NN distances could be detected. The only exception was pericentromeric region (Fig. 5C) for which a decrease in NN distances with increasing ploidy (from 2x to 4x) was observed. Accordingly, comparison of diploids and autotetraploids with the same genome copy number (2 × 4C vs 4 × 2C and 2

Figure 5. Normalized nearest neighbor (NN) distances between homologous chromosome 4 domains. The FISH signals, correspond to subtelomeric (A, f6n15 and B, t5n17) and pericentromeric (C, t1j1) sequences. The number distributions are shown with their 25/75 percentiles (boxes), 5/95 percentiles (whiskers) and 1/99 percentiles (circles). The distributions are plotted in shades of green (diploids, 2x) or red (autotetraploids, 2x), color saturation increasing with the DNA content. Medians of the distributions are depicted with solid (thin), whereas the means with dashed (thick) black vertical lines.
$8C$ vs $4C$) showed differences in NN distances only for pericentromeres in the nuclei with $8$ genome copies ($2 \times 4C$ vs $4 \times 2C$, Fig. 5C).

Taken together, the NN analysis indicates that the sister chromatids are separated by similar distances in both diploid and autotetraploid plant nuclei (Table 1). Moreover, the organization of pericentromeric and subtelomeric domains is similar for chromosomes $1$ and $4$, as demonstrated with NN analysis. Nonetheless, association of homologous chromosomes (present in autotetraploids) seems to modulate the pattern of separation of sister chromatids (observed in diploids).

**Clustering of homologous pericentromeric, interstitial and subtelomeric domains**

Spatial grouping of homologous sequences was also analyzed (at a range of distances) with autocorrelation function. The value of autocorrelation function (ACF) corresponds to the average probability of encountering another signal within a certain distance from the first one, normalized to the value corresponding to the signals distributed randomly in the nuclear space (see Materials and Methods). Within the range from $0.02$ to $0.3$ (normalized nuclear radius) function values calculated for chromosome $1$ (Fig. 6) and $4$ (Fig. 7) were greater than $1$. One may thus postulate that groups (clusters) of signals occur several times more frequently (ACF value) than expected in case of random signal distribution. This was observed irrespectively of nuclear C-value (endopolyploidy level) in both diploid and autopolyploid plants when subtelomeric (Fig. 6AB and 7AB), interstitial (Fig. 6CD) and pericentromeric (Fig. 6E and 7C) domains of chromosomes $1$ and $4$ were studied. This suggests that in $2C$ and endopolyploid nuclei large-scale chromatin organization may be conserved. It may be noted that the ACF consistently decreased to $1$ (absence of clustering) at distances larger than $0.1$. This indicates that the majority of FISH signals were distributed within regions of diameter smaller than $0.1$ normalized nuclear radius, which is nonetheless larger than a typical NN distance (Figs. 4 and 5). The respective ACF values for diploids ($2x$) were equal (not significantly different) or larger than for autotetraploids ($4x$), showing that signal clusters in the former tended to be more compact than in the latter. It may be noted that for subtelomeric regions of chromosome $4$ ACF at short distances ($< 0.1$) (Fig. 7AB) had higher values than in case of pericentromeric regions of this chromosome (Fig. 7C) or subtelomeric regions of chromosome $1$ (Fig. 7AB). This may reflect the role of chromosome $4$ in nucleolus organization.

In general, these results indicate endoreplication impacts chromatin organization only at small scales, while at large scales homologous chromosome domains show similar spatial organization in $2C$ and endopolyploid nuclei (Table 1).

**Proximity of non-homologous pericentromeric, interstitial and subtelomeric domains**

Relative spatial positions of non-homologous domains of chromosomes $1$ and $4$ were studied with the NN analysis. The distance between chromosome $1$ subtelomeric (f6f3 and f3f9) domains decreased with the increase of endopolyploidy level (Fig. 8A) and were higher in autotetraploid ($4x$) than in diploid ($2x$) endoreduplicated nuclei ($4C$ and $8C$). One may note that the NN distance was inversely correlated with the FISH signal number (Fig. 2A). Similar correlation (decrease in NN distances and the increase of endoreduplication level) was also observed in case of interstitial sequences (f17l21 and t7n9) of chromosome $1$ (Fig. 8C). Interestingly, in nuclei with the corresponding ploidy and endoreduplication level the distances between interstitial sequences (Fig. 8C) were larger than the distances between subtelomeric (Fig. 8A) sequences. These data suggest that in endopolyploid nuclei non-homologous telomeric regions show tendency to form clusters. Moreover, in both diploid and endopolyploid plant nuclei subtelomeric domains seem to be more densely packed than interstitial domains.

Analysis of the spatial architecture of chromosome $4$ non-homologous domains revealed that the increase in subtelomeric signal numbers was coupled with minor decrease in NN distances between them (Fig. 8B). Thus, the NN distances in endopolyploid nuclei ($4C$ and $8C$) were higher in case of subtelomeric regions of chromosome $4$ (Fig. 8B) than in case of chromosome $1$ (Fig. 8B). This indicates that chromosome $4$ might exhibit smaller structural rigidity that chromosome $1$, which is probably due to its function in nucleolus organization.

Spatial proximity was also measured between pericentromeric regions of non-homologous chromosomes
The NN distances measured in diploid plant nuclei (2£2C and 2£4C) were larger than in autotetraploid nuclei (4£2C and 4£4C). However, in nuclei with 8C DNA level the difference was not detectable (Fig. 8D). Furthermore, the NN distances measured between pericentromeric regions of chromosomes 1 and 4 were comparable to the distances measured for other chromatin domains analyzed (both non-homologous and homologous). Thus, it seems possible that pericentromeric regions of particular non-homologous chromosomes show preferential spatial distribution in respect to each other. This putative effect may be modulated by endopolyploidization.

Discussion

Autotetraploid (4x) plants have twice as many chromosomes as diploid (2x) plants. However, their
nuclear volume is smaller, when 2x and 4x nuclei with the same DNA content are compared. This indicates that the average chromatin density is higher in autotetraploid than in diploid plant nuclei. Moreover, the density increases with subsequent rounds of endoreplication in 2x and 4x plants. Interestingly, analysis of the number and spatial distances between FISH signals representing various chromatin domains suggests, that the differences in chromatin density are mainly due to reduction of the number of heterochromatin domains and the volume of interchromatin space. Indeed, comparison between the number of chromosomes and number of chromocentres, centromeric and rDNA repeats (FISH) supports the former notion. These results are compatible with earlier studies in Arabidopsis. Neumann et al.4,36,37 It seems that the reduction is more pronounced in 4x than in 2x plants. The fact that heterochromatin associated with inactive rDNA forms similar number of domains in both cytotypes is an illustration of this notion. The reduction of the number of heterochromatin domains was attributed to their fusion,4 which could be positively correlated with the number of chromosomes.38 One may also speculate that the positive correlation between the frequency of heterochromatin domain fusion and the genome copy number might result from macromolecular crowding (higher chromatin density, decreased volume of interchromatin space). The effect may be further modulated by genetic/epigenetic mechanisms which modulate chromatin domain organization. It is interesting to note in this context that heterochromatin architecture, studied here using fixed material, is a result of dynamic process of its reorganisation (Fig. S3).

In both diploid (2x) and autotetraploid (4x) 2C nuclei reduction of the number of chromatin domains was also observed for subtelomeric, interstitial and pericentromeric regions of chromosomes 1 and 4 (NOR-bearing), suggesting that homologous association might take place.39 Interestingly, the frequency of the association depended on the type of chromosomal domain (subtelomeric and interstitial > pericentromeric in chromosome

Figure 7. Relationship of spatial distributions of subtelomeric (A, f6n15 and B, t5n17) and pericentromeric (C, t1j1) domains of chromosome 4. The values of normalized L-functions (spatial autocorrelation) are plotted against normalized nuclear distances for diploids (2x, shades of green) and or autotetraploids (4x, shades of red), with color saturation increasing with the DNA content.
Similar data were obtained using chromosome conformation capture technique, which demonstrated that the distal chromatin domains interact more frequently than proximal domains. This suggests that distal chromatin domains might be privileged in terms of homologous association, which might be thus involved in regulating their activity.

Endoreduplication coupled with changes in chromatin organization, which are produced by the increase in sister chromatid number, contributes to differentiation of Arabidopsis cells. Our data demonstrate that sister chromatid separation occurs more often in pericentromeric, than in subtelomeric domains and is the least prominent in interstitial domains. Moreover, this process is more pronounced in 4x than in 2x plant nuclei. Thus, it might be suggested that, the higher the genome copy number the lower level of sister chromatid cohesion and thus, local chromatin density. It is tempting to speculate that sister chromatid separation might be involved in regulating chromatin accessibility, by affecting chromatin organization through disrupting/re-establishing homologous chromosome association pattern.

Polyploidization and endoreduplication affect chromatin organization at distances (quantified with NN analysis) which are immediately above those corresponding to fusion of FISH signals (probed with optical microscopy). Depending on chromosome region and the number of endoreduplication rounds, the NN proximity may correlate positively or negatively with changes in signal number due to endoreduplication and to plants polyploidy level. However, it seems likely that influence of endoreplication on chromatin organization (short and medium distance scale) is more significant than the impact of polyploidy. Furthermore, analysis of spatial distribution of ~100 kb (BAC clone size) chromatin domains (ACF function at distances < 0.1) shows that it is conserved in 2C and endopolyploid nuclei but can be affected by plants polyploidy level (2x vs 4x). Together, this indicates that process of differentiation is associated with
changes in local chromatin organization, while polyploidy modulates chromatin organization at chromosome-level. One should also note that the static picture of chromatin organization, described here, is a product of dynamic, transient interactions between various chromatin domains.

To summarize, in Arabidopsis plant nuclei, chromatin architecture is shaped by endopolyploidyization but modulated by polyploidy level. Both processes result in an increase in average chromatin density (Table 1). Main processes affecting chromatin organization are heterochromatin fusion, association of homologous chromosomes (in 2C and endopolyploidy nuclei) and sister chromatid separation (in endopolyploid nuclei). The frequency of the 2 former processes (which may be interdependent) is positively correlated with the genome copy number. Moreover, they contribute to chromatin organization at the spatial scale above resolution of optical microscopy.

**Materials and Methods**

**Preparation of plant material**

Arabidopsis thaliana Columbia 0 diploid (2x = 2n =10) and autotetraploid (4x = 2n = 20) plants were grown in greenhouse in 20°C and 16/8 photoperiod. The stable autotetraploid line was obtained from diploid plants using standard colchicine treatment. The autotetraploid line has been routinely used in the laboratory for several years, with no chromosomal aberrations detected over that period. The absence of extensive genome rearrangements in synthetic Arabidopsis autotetraploids was reported also by other researchers. Stem leafs were fixed in 4% formaldehyde (PF) for 40 min in 4°C. Nuclei were isolated according to Tirichine et al.44 with minor modifications. Breathily, leafs were macerated using enzyme mixture containing 2.5% pectolyase (Sigma), 2.5% pectinase (Sigma) and 2.5% cellulase “Onozuka” (Serra) in 37°C for 40 min., washed in 1x PBS buffer (pH 7.4) and transferred to Tris buffer. Differentiated nuclei were released by chopping leaf tissue in NEB buffer (10 mM Tris HCl pH 7, 4 mM spermidine, 1 mM spermine, 5 mM MgCl2, 0.1% triton X-100) with 5mM β-mercaptoethanol added. Next, nuclei suspension was filtered through 50nm mesh and centrifuged (2500 g, 3 min, 4°C). To remove chlorophyll nuclei were washed in 1xPBS with 0.5% TritonX-100 (2500g, 3 min, 4°C). Finally, the nuclei were washed in 1xPBS (2500 g, 3 min, 4°C), supernatant was discarded and the pellet was suspended in 100 µl of 1xPBS. Nuclear suspension was added to slides and left to dry overnight in 4°C. Slides were kept in −20°C until use.

One should note that in mature stem leafs large majority of the cells are differentiated. Thus, the presence of stem cells (i.e. cells able to divide) was neglected in high-throughput analysis. Accordingly, the cells characterized by 4C DNA content were treated as endopolyploid and not diploid in G2 cycle phase.

**Probe labeling**

The following Arabidopsis sequences were used as probes for the fluorescence *in situ* hybridization (FISH): pAl1 centromeric repeat, 25S rDNA 46 and A. thaliana chromosome specific Bacterial Artificial Chromosome (BAC) clones (Fig. S5). BACs were obtained from Arabidopsis Biological Resource Center (Columbus, OH, USA) and selected to target: chromosome 1 pericentromeric region (t19e23), interstitial region (f17l21, t7n9) and subtelomeric region (f6f3, f3b9), chromosome 4 pericentromeric region (t1j1), subtelomeric region of NOR-bearing arm (f6n15) and the bottom arm (T5J17). pAl1 probe was generated by PCR with rhodoamine-dUTP. 25S rDNA and BAC clone probes were generated with nick-translation and labeled with either rhodeamine-dUTP or dig-dUTP, then detected using anti-dig FITC antibody.

**Fluorescence in situ hybridization (FISH)**

FISH procedures were carried out according to Hasterok et al.47 with minor modifications. First, slides were treated with amylase (1h 37°C), washed and incubated with RNAse (1h 37°C). Next, nuclei were fixed in 1% PF (RT, 10 min), dehydrated in ethanol series (70%, 90% and 100% ethanol) and left air-dry. All washing steps were conducted in 2xSSC (3 × 5min). Probes were added to hybridization mixture consisting of 50% deionized formamide and 10% dextran sulfate in 2x saline sodium citrate (SSC), 10 µg/µl sonicated salmon sperm DNA and 0.5% SDS. Probes were denatured in hybridization mixtures (85°C for 10 min), then applied to the slides and denatured, again, combinatorially (75°C for 4.5 min). Hybridization step was carried out for 48 h in a humid chamber at 37°C. Posthybridization washing steps
were conducted with 64% stringency. First, slides were washed at 42°C: 2xSSC, 20% formaldehyde, 3 times in 2xSSC, afterwards washed 3 times in 2xSSC at RT. For immunodetection of dig-UTP labeled probes slides and incubated in 4xSSC with 0.2% Tween20 (5 min RT) and, to block nonspecific epitopes, in 5% Milk (20 min RT). Antibodies were applied and immunodetection was carried out in 37°C for 1 h in humid chamber. Afterwards slides were washed 3 times in 4xSSC with 0.2% Tween20 in 37°C, dehydrated in ethanol series (70%, 90% and 100% ethanol) and left to air dry. Slides were mounted in Vectashield with DAPI.

**Image cytometry**

Nuclei were imaged using SCANR high content scanning station equipped with Olympus XI 81 inverted microscope, 60xPlanApo oil immersion objective (NA =1.35) and CCD camera (Orca AG, Hamamatsu). Fluorescence of DAPI (ex. 405/20, em. 460/25), FITC (ex. 490/20, em. 530/50) and Rhodamine (ex. 540/25, em. 605/55) was excited Xenon-mercury lamp (MT20 system, 150 W). The 2D fluorescence images (1024 × 1024 pixels) were registered with 0.107 μm pixel size and 12-bit precision. Nuclei were segmented from images (static thresholding) and their relative DNA content estimated with integrated fluorescence of DAPI (2 µg/µl, equilibrium staining). The histograms of DNA content (each representing minimum 1000 nuclei) were generated with Olympus ScanR Analysis software, used to identify peaks corresponding to 3 endopolloidy levels (2C, 4C and 8C). Similarly, nuclear area and number of chromocentres was measured in these populations after manual thresholding. 3D deconvolution microscopy (described below) was used to verify that nuclei, characterized by different DNA content, are similar with respect to their heights (z dimension), but differ with respect to their areas (xy dimension). Thus, nuclear area was used as proxy for volume in the 2D image cytometry measurements.

Randomly selected nuclei (50 or more) from each population (corresponding to C-value) were imaged in 3D using wide-field microscopy (0.107 × 0.107 × 0.5 μm voxel), implemented with the SCANR system. The images were processed with blind deconvolution implemented in AutoDeblur 2.0 (Media Cybernetics). The algorithm was initialized with the nominal system PSF, SNR of 25 and executed for 25 iterations. Volumes corresponding to the nuclei (DAPI) were segmented using Otsu thresholding. The method separates voxel intensity histograms into 2 classes (signal and background) by maximization of inter-class variance. The thresholding was followed by binary opening (5 × 5 × 3 structuring element). 3D ellipsoids were fitted to the segmented nuclear masks. Moreover, within these masks regions corresponding to chromocentres were isolated using triangle thresholding algorithm. The algorithm works by constructing a line between points corresponding to 5th percentile of the voxel intensity histogram (within the mask) and its 99th percentile. The intensity level corresponding to maximum distance between the histogram and the normal to the constructed line was taken as the threshold. Merged chromocentres were separated with watersheding were merging was executed for the intensity difference smaller than 250 units and volume smaller than 800 voxels. Segmentation artifacts were then removed with binary opening (3 × 3 × 3 structuring element). Images of fluorescence of FITC and Rhodamine (FISH) were denoised with median filter (spherical neighborhood, radius 1) and subjected to gray image opening (elliptical element, radius 2 × 1). Resulting (background) image was subtracted from the original (denoised) and the image containing local maxima was convolved with gaussian kernel (width corresponding to SF of 1 × 1 × 0.5). Single FISH signals were isolated by background thresholding and watersheding (15 intensity units, 25 voxels). The corresponding masks were fitted with 3D ellipsoids, with the shortest axis corresponding to the z image coordinate and the longest axis positioned within xy (optical section) plane. Lengths of the ellipsoid axes were normalized with respect to the longest one, thus providing a set of new (normalized) coordinates within nuclear mask. Positions of FISH signals (centers of mass) were calculated with respect to the ellipsoid center, using normalized ellipsoid axes as the coordinate system. The distances to other signals (starting from the nearest neighbor) were then calculated in normalized coordinates. The distributions of signals were characterized (on nucleus-by-nucleus basis) using nearest-neighbor (NN) histograms. This technique quantifies distribution of distances between the closest (microscopically resolvable) FISH signals. Furthermore, spatial correlation functions (ACF) were used to detect
grouping (clustering) of the signals at distances exceeding these captured by the NN histograms. \(^4\)

Briefly, ACF value corresponds to the probability of encountering other FISH signal within a certain distance from the first signal. The value is averaged over all pairs of signals in nuclear space and normalized to the respective value obtained when the signals are placed at random. For instance, ACF = 6 at a distance \(r = 0.1\) reflects a situation when each FISH signal is neighboring with (on average) 6 times more signals within that distance than it would if the signals were distributed randomly in nuclear space. This reflects the presence of clusters of FISH signals, characterized by radius \(r = 0.1\).

All 3D segmentation and analysis procedures were implemented under Matlab R2013b (MathWorks). Statistical significance was ascertained with Mann-Whitney U test. A significant difference between parameters corresponded to \(p < 0.05\) (95\% confidence), otherwise it was regarded as non-significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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