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Chromosome territory arrangement and homologous pairing in nuclei of *Arabidopsis thaliana* are predominantly random except for NOR-bearing chromosomes

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Abstract Differential painting of all five chromosome pairs of *Arabidopsis thaliana* revealed for the first time the interphase chromosome arrangement in a euploid plant. Side-by-side arrangement of heterologous chromosome territories and homologous association of chromosomes 1, 3 and 5 (on average in 35–50% of nuclei) are in accordance with the random frequency predicted by computer simulations. Only the nucleolus organizing region (NOR)-bearing chromosome 2 and 4 homologs associate more often than randomly, since NORs mostly attach to a single nucleolus. Somatic pairing of homologous ~100 kb segments occurs less frequently than homolog association, not significantly more often than expected at random and not simultaneously along the homologs. Thus, chromosome arrangement in *Arabidopsis* differs from that in *Drosophila* (characterized by somatic pairing of homologs), in spite of similar genome size, sequence organization and chromosome number. Nevertheless, in up to 31.5% of investigated *Arabidopsis* nuclei allelic sequences may share positions close enough for homologous recombination.

Introduction

Studies on interphase nuclei by conventional microscopy reveal chromatin regions of different density/staining

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intensity, representing (positively heteropycnotic) heterochromatin fractions of high density (Heitz 1928), euchromatin of lower density and nucleoli of lowest density. A territorial organization of interphase chromosomes was first proposed by Rabl (1885). Complete interphase chromosome territories (CTs) could only be traced one century later when chromosome painting by fluorescence in situ hybridization (FISH) became established (Lichter et al. 1988; Pinkel et al. 1988) and allowed determination of the arrangement of CTs within nuclei by three-dimensional (3D) microscopy (Cremer and Cremer 2001).

Two models considering different aspects of nuclear CT distribution have been proposed (Parada and Misteli 2002). One model, based on the radial arrangement of CTs between the center and the envelope of the nucleus, suggests that gene-dense chromosomes are located more internally than gene-poor ones. Such an arrangement was found in various types of mammalian and chicken cells (Cremer et al. 2001; Habermann et al. 2001; Kozubek et al. 2002) and appeared to be evolutionarily conserved when the positions of homeologous chromosomes were compared between human and higher primates (Tanabe et al. 2002) or human and mouse (Mahy et al. 2002a,b). However, no such arrangement was found in non-cycling cells by Bridger et al. (2000). The other model proposes specific neighborhood relationships between two or more CTs or distinct chromosome domains. Non-random side-by-side arrangement of interphase CTs is of special interest because spatial vicinity of homologs is required, at least transiently and/or punctately, for DNA repair via homologous recombination between homologs, often yielding reciprocal translocations (Rieger et al. 1973; Parada and Misteli 2002). In human cells non-random association of homologs is apparently restricted to certain chromosomes of distinct cell types, e.g., Sertoli cells (Chandley et al. 1996; Nagele et al. 1999). The relative positioning of all human heterolog combinations was proposed to be predominantly random. A weak non-random clustering was found for the gene-rich chromosomes 1, 16, 17, 19 and 22 (Cornforth et al. 2002). At least transient somatic association of homologous chromosomes

has been claimed for yeast (Burgess et al. 1999); however, no clear evidence for such an association was found by others (Fuchs et al. 2002; Lorenz et al. 2003). A development-specific and cell cycle-specific close spatial alignment of homologous chromosome segments has hitherto been observed only in *Drosophila* (Hiraoka et al. 1993; Csink and Henikoff 1998; Fung et al. 1998). For review of somatic homologous pairing see McKee (2004). Recent studies have shown by photobleaching of fluorescently labeled chromatin in vivo that the positioning of interphase chromosomes is largely inherited from mother to daughter nuclei in mammals (Gerlich et al. 2003; Walter et al. 2003; see also Bickmore and Chubb 2003; Parada et al. 2003; Williams and Fisher 2003).

In plant species with large genomes (>5000 Mb/1C), interphase chromosomes frequently show Rabl orientation with centromeres and telomeres clustered at the opposite poles of a nucleus (Dong and Jiang 1998). In *Arabidopsis* nuclei, instead of Rabl orientation, centromeres are randomly distributed in peripheral positions, while telomeres are clustered around the nucleolus (Fransz et al. 2002). Painting of plant chromosomes by chromosome in situ suppression hybridization is not yet feasible, apparently due to the high amount and complexity of interchromosomally dispersed repetitive sequences, which cannot be blocked efficiently when chromosome-derived sequences are used as probes (Fuchs et al. 1996; Schubert et al. 2001). Hitherto, individual CTs could be traced in plant interphase nuclei only for single alien chromosomes within the chromosome complements of backcross progenies from interspecific hybrids by genomic in situ hybridization (Schwarzacher et al. 1989). In the case of disomic additions, close spatial association of the added homologs barely occurs in somatic nuclei (Schwarzacher et al. 1992; Abranches et al. 1998; Schubert et al. 1998; Martínez-Pérez et al. 2001) except for tapetum cells of wheat (Aragón-Alcaide et al. 1997). However, it remains unclear whether the alien chromosomes behave in the same way as in their native background or as the host chromosomes. Experiments using FISH in diploid rice indicated homologous association of centromeres and telomeres but not of interstitial regions in root xylem and undifferentiated anther cells (Prieto et al. 2004). A significant and chromosome-specific degree of association of homologous centromeres was found in *Arabidopsis* nuclei (Fransz et al. 2002), but it remained open to what degree entire chromosome arms are involved.

So far, chromosome painting within a euploid plant is feasible only for *Arabidopsis thaliana* and its close relatives using bacterial artificial chromosome (BAC) contigs as probes (Lysak et al. 2001, 2003), apparently due to the small *Arabidopsis* genome, which has only a low amount of repetitive sequences clustered mainly within the pericentromeric heterochromatin and the nucleolus organizing regions (NORs) (The Arabidopsis Genome Initiative 2000).

Here we report on multicolor FISH to *Arabidopsis* interphase nuclei of different organs and ploidy levels with the aim of finding out whether there is a specific relative

positioning of homologous and heterologous CTs at the level of chromosome/chromosome arm territories and of distinct ~100 kb chromosome segments. The experimental results were compared with the predictions of computer simulations for random arrangement of *Arabidopsis* CTs and chromosome segments. This comparison suggests a largely random arrangement of CTs and random frequencies of (single-point) homologous pairing as typical features of wild-type root and leaf nuclei of *A. thaliana*. Moreover, we tested the relative position of the *FWA* gene for its presence inside or outside the territory, the bottom arm of chromosome 4, in the active versus silent state and found no significant difference.

Materials and methods

Plant material, preparation of nuclei and pachytene chromosomes

Young root tips and rosette leaves of *A. thaliana*, accessions Columbia (Col) and Landsberg (Ler) and mutant *fwa-1* in the Ler background, cultivated in a greenhouse under 16:8 h light:dark cycle, were fixed for 20 min under vacuum in 4% formaldehyde in TRIS buffer (100 mM TRIS-HCl, pH 7, 5 mM MgCl₂, 85 mM NaCl, 0.1% Triton X100) and homogenized in TRIS buffer. Suspended nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml) and flow-sorted according to their ploidy level using a FACStar^{Plus} flow cytometer (Becton Dickinson) equipped with an Argon-ion laser (INNOVA 90C-5) emitting UV light. Approximately 1000 nuclei per ploidy level were sorted on microscope slides in a drop of buffer containing 100 mM TRIS, 50 mM KCl, 2 mM MgCl₂, 0.05% Tween, 5% sucrose, air-dried and used for FISH or stored at -20°C until use.

Hybridization probes were tested on pachytene chromosomes of accessions Col and C24, which were prepared as described (Lysak et al. 2001).

Probe labeling and fluorescence in situ hybridization

The BACs used for FISH were obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio). DNA of individual clones was isolated by standard alkaline extraction without phenol-chloroform purification (Sambrook and Russell 2001). Clones that according to the sequence annotation of the Institute for Genomic Research (Rockville, Md.; <http://www.tigr.org/>) database harbor >5% of mobile elements and/or yielded strong signals in dot-blot hybridization with *A. thaliana* (Col) genomic DNA (Lysak et al. 2003) were omitted from probes designed for chromosome painting. The list of BACs used for painting of chromosomes 1–5 will be provided by the authors on request.

The BAC DNA was labeled by nick translation according to Ward (2002), either individually or, for painting, arranged into 19–38 pools (4 or 5 BACs each)

per chromosome. The nick translation mixture consisting of 1 μ g DNA, 5 μ l 10 $\times$ NT buffer (0.5 M TRIS-HCl, pH 7.5, 50 mM MgCl₂, 0.05% BSA), 5 μ l 0.1 M mercapto-ethanol, 3.75 μ l dNTP mixture [2 mM each of d(AGC) TP], 1.25 μ l dTTP (0.4 mM), 2.5–3.5 μ l DNase I (4 μ g/ml in 0.15 M NaCl/50% glycerol; Roche), 1 μ l *Escherichia coli* DNA polymerase I (Fermentas) and 2–4 μ l of labeled nucleotides (either biotin-dUTP, digoxigenin-dUTP, DNP-dUTP, Cy3-dUTP or DEAC-dUTP; prepared as described by Henegariu et al. 2000) was brought to a total volume of 50 μ l by adding distilled water and incubated at 15°C for 1.5–2 h. When the length of DNA fragments on a 1.5% agarose gel was 200–500 bp, the reaction was stopped by incubation at 65°C for 10 min or by precipitation.

Prior to FISH, slides were rinsed in 2 $\times$ SSC (2 $\times$ 5 min), treated with pepsin (100 μ g/ml in 0.01 M HCl; Roche) for 3–10 min at 38°C (only for chromosome painting), rinsed in 2 $\times$ SSC (2 $\times$ 5 min), post-fixed in 4% formaldehyde/2 $\times$ SSC (10 min), rinsed in 2 $\times$ SSC (2 $\times$ 5 min), dehydrated in 70, 90, 96% ethanol (2 min each) and air-dried.

For chromosome painting, the pools of labeled BACs were precipitated and resuspended in 20–40 μ l of hybridization mix (50% formamide, 10% dextran sulfate, 2 $\times$ SSC, 50 mM sodium phosphate, pH 7.0) per slide. The probe was denatured together with the preparation on a heating block at 80°C for 2 min before incubation in a moist chamber at 37°C for 12–36 h.

Post-hybridization washes and detection steps were as described (Schubert et al. 2001). Biotin-dUTP was detected by goat anti-avidin conjugated with biotin (1:200; Vector Laboratories) and avidin conjugated with Texas Red (1:1000; Vector Laboratories), digoxigenin-dUTP by mouse anti-digoxigenin (1:250; Roche) and goat anti-mouse conjugated with Alexa-488 (1:200; Molecular Probes), DNP-dUTP by rabbit anti-DNP (1:400; Sigma) and goat anti-rabbit conjugated with Cy5 (1:100; Jackson Laboratories). Cy3-dUTP and DEAC-dUTP were observed directly. Nuclei and chromosomes were counter-stained with DAPI (1 μ g/ml) in Vectashield (Vector Laboratories).

Evaluation by microscopy and image processing

Analysis of fluorescence signals was performed with an epifluorescence microscope (Axiophot or Axioplan 2; Carl Zeiss) using 100 $\times$ /1.4 Zeiss plan apochromat objectives and cooled CCD cameras (DXC-950P, Sony; Spot 2e, Diagnostic Instruments). Images were captured separately for each fluorochrome using the appropriate excitation and emission filters. Single plane images and stacks of optical sections through nuclei were acquired with MetaVue (Universal Imaging) or with the Digital Optical 3D Microscope system (Schwertner, Jena). The deconvolution of image stacks was performed with the point spread function algorithm. The monochromatic images were pseudocolored and merged using MetaMorph (Universal

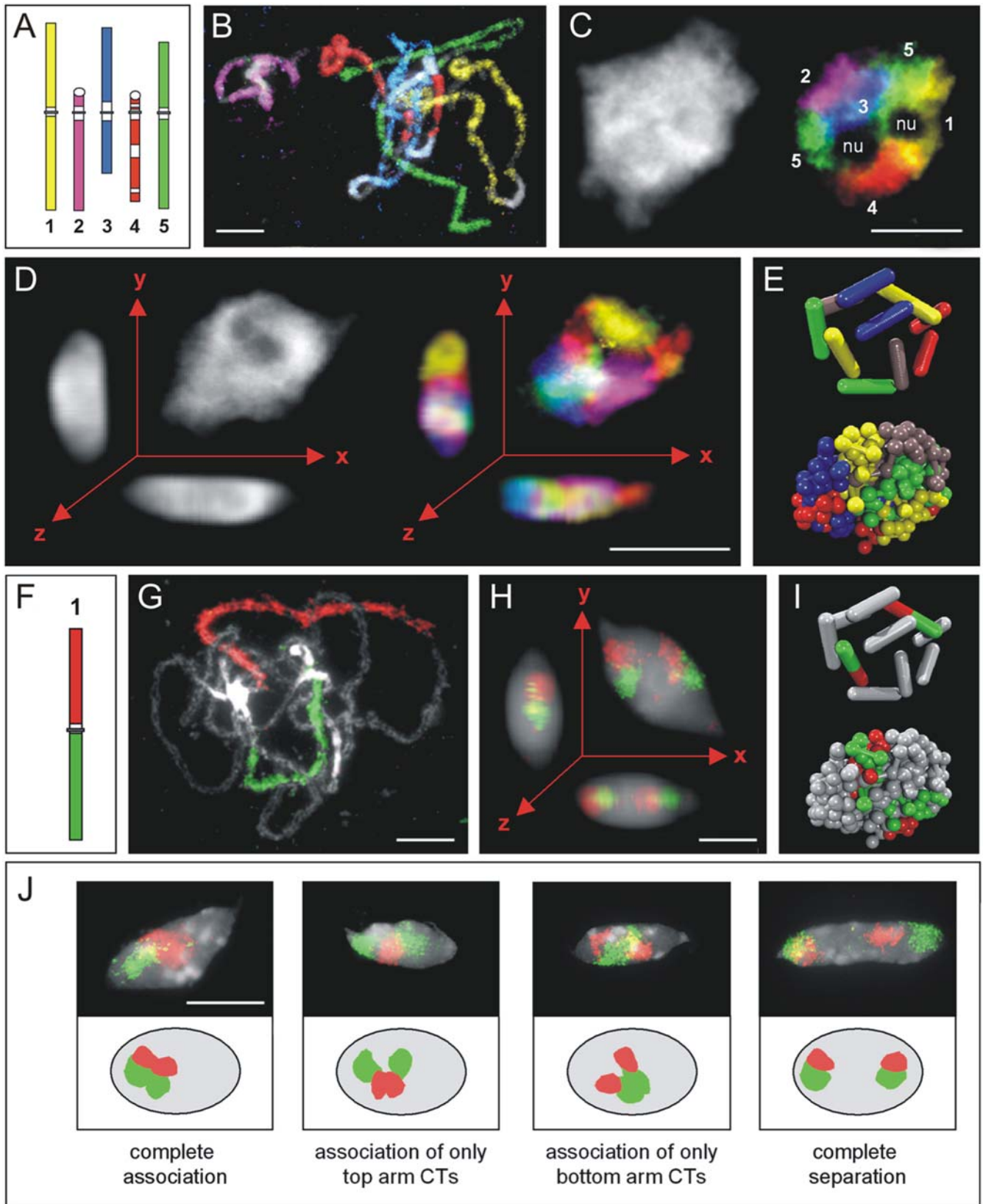
Fig. 1a–j Association of homologous and heterologous chromosome/chromosome arm territories. **a** Scheme of differential labeling of individual *Arabidopsis thaliana* chromosomes for multi-color chromosome painting. **b** Multi-color painting of the five chromosome pairs in pachytene according to the scheme in **a**. **c** Central focal plane of a 4C leaf nucleus (*left*) and of chromosome territories therein painted as in **b** (*right*); 4',6-diamidino-2-phenylindole (DAPI)-stained areas (*left*) without painting signal correspond to nucleoli (*nu*) and to the pericentromeric chromocenters containing repetitive DNA sequences that were excluded from painting probes. **d** Maximum intensity projections of a 4C leaf nucleus in three planes, *left* DAPI-stained, *right* painted chromosome territories as in **b**. **e** Spherical 1 Mb chromatin domain (SCD) model simulating random distribution of all *Arabidopsis* CTs in start configuration (*top*) and after relaxation (*bottom*). **f** Scheme showing differential labeling of the chromosome 1 *top* (red) and *bottom* (green) arm. **g** Chromosome 1 painted in pachytene, *top* arm in red, *bottom* arm in green. **h** Maximum intensity projections of an 8C root nucleus in three planes with the painted chromosome 1 arm territories. Both homologs are spatially separated as visible from *x,y* and *x,z* projections. **i** SCD model simulating random distribution of chromosome 1 arm territories; the simulated chromosomes in cylindrical start configuration (*upper part*) and after 2 $\times$ 10⁵ relaxation steps (*lower part*). **j** The four types of arrangement of homologous chromosome arm territories as images of central focal planes of 4C leaf nuclei (*upper part*) and schematically below. Bars represent 5 μ m

Imaging) and/or Adobe Photoshop 6.0 (Adobe Systems) software.

Computer simulation of interphase CTs and 100 kb segments

At first, the average volumes of 2C (root = 25.9 μ m³; leaf = 26.7 μ m³) and 4C (root = 44.9 μ m³; leaf = 39.9 μ m³) nuclei (*n*>30) and of ~100 kb segments of spherical shape therein (0.15 μ m³ and 0.22 μ m³, respectively) were determined on the basis of Digital Optical 3D Microscope image stacks for nuclei of the three predominant shapes (flattened sphere, spindle and rod).

To assess the 3D topology of CTs within *Arabidopsis* nuclei, experimental data were compared with predictions derived from simulations of random association of CTs by the spherical 1 Mb chromatin domain (SCD) model (Cremer et al. 2001; Kreth et al. 2004). Based on the compartmentation of interphase CTs into subchromosomal foci of 400–800 nm in diameter (Zink et al. 1998), the SCD model considers CTs as a chain of domains of ~1 Mb (500 nm in diameter) connected by entropic spring potentials. According to their DNA content (The Arabidopsis Genome Initiative 2000), chromosomes 1–5 should correspond to 29, 20, 23, 18 and 26 Mb domains, respectively. To permit only minor overlaps, a repulsive potential between the domains was modeled and a weak energy barrier, essential for maintenance of territorial organization of simulated chromosomes, was applied around each CT. As a start configuration, the model assumes compressed cylinders corresponding to the mitotic state of the chromatin domains of the ten chromosomes to be statistically distributed within a simulated nucleus. The “start cylinders” are then allowed to relax according to the Metropolis Importance Sampling



Monte-Carlo method until thermodynamic equilibrium is reached (Fig. 1e,i). Relaxed CTs filled the nucleus uniformly after ~200,000 Monte-Carlo cycles (Metropolis

et al. 1953). Subsequently, the minimal distances between domains of interest were measured. Chromosome territories were considered as associated if boundaries were

less than 500 nm apart from each other. At this distance, CTs appear as separated at the microscopic level of resolution. Assuming a distance of 400 nm decreased the expected association frequency of heterologous CTs by no more than 1% and did not alter the significance level for comparison of experimental and simulated data for heterolog association. To test the influence of nuclear shape (flattened sphere, spindle and rod) on random arrangement of CTs, we modeled 10^3 nuclei of each shape.

Since the SCD model does not simulate domains <1 Mb, the geometrical random spatial distribution (RSD) model was established to simulate spherical chromosome segments of ~100 kb (corresponding to the average BAC insert size) within 10^6 spherical, spindle-shaped or rod-shaped nuclei according to the volumes determined for 2C and 4C nuclei and for the FISH signals therein. The coordinates of segments were calculated from random numbers. Signals that overlapped or were closer to each other than 100 nm were considered to indicate single-point homologous pairing. The random occurrence of single-point pairing was calculated using the VisualBasic 5.0 (Microsoft) software.

The differences between the experimentally obtained values and the simulated ones were compared by the χ^2 or Fisher's exact test and considered as significant at the $P < 0.001$ level.

Results

Simultaneous painting of all *Arabidopsis* chromosomes reveals random relative positioning of entire CTs

At first, we developed painting probes for differential labeling of all *Arabidopsis* chromosomes and proved the specificity of these probes by FISH on pachytene chromosomes (Fig. 1a,b). Hybridizing chromosome-specific probes (BAC contigs) to interphase nuclei revealed three-dimensional, discrete CTs (Fig. 1c,d). To test whether there is a specific side-by-side positioning between individual CTs, we performed simultaneous multi-color painting for all chromosomes on flow-sorted 4C nuclei from leaves (Fig. 1c,d). In total, 73 μ g of labeled DNA per slide were applied (~110 ng of DNA of each of 669 BACs). Association frequencies of all possible homologous and heterologous CT combinations were scored in spherical and spindle-shaped nuclei ($n=51$) and compared with the prediction for random arrangement according to the SCD model (Table 1, Fig. 1e). The random CT association frequency was calculated as a weighted average of predicted association values for spherical and spindle-shaped nuclei according to the proportion of evaluated spherical and spindle-shaped nuclei. The observed association frequency for all possible combinations ($n=15$) was very high (76.4–100%), apparently due to the low chromosome number of *A. thaliana* ($2n=10$), and not significantly different ($P > 0.05$) from the prediction (68.7–99.4%) for 10^3 simulated nuclei. The

predicted association values were lower for individual homologous (involving only the two homologs) than for individual heterologous combinations (involving the CTs of two chromosome pairs) and corresponded with the size of the chromosomes involved (Table 1).

The association frequency of homologous chromosome arm territories appears to be random for chromosomes 1, 3, 5 and higher for chromosomes 2 and 4 within all tested types of nuclei

Painting probes for differential labeling of all chromosome arms were tested for specificity by FISH on pachytene chromosomes (Fig. 1f,g). In interphase nuclei we observed either spatial association of both arms, of only the top arms or of only the bottom arms of a chromosome pair; alternatively the homologous territories were completely separated (Fig. 1j). Simulations of random CT arrangement were performed according to the SCD model (Fig. 1i, Cremer et al. 2001). Because in *Arabidopsis* roots and leaves three nuclear shapes (flattened sphere, spindle, rod) occur predominantly, independent simulations were done (10^3 nuclei per shape) to test whether the nuclear shape influences CT arrangement. Indeed, the simulations revealed an impact of nuclear shape on the random association frequency of CTs. For the symmetric chromosomes 1, 3, and 5 the computer model predicted association of entire homologs in 48.3–59.9% of spherical nuclei, in contrast to only 20.6–23.6% of rod-shaped nuclei. For the asymmetric chromosomes 2 and 4 the predicted values were 25.2–31.9% and 14.8–18.7%, respectively. Correspondingly, complete separation of homologous CTs should increase from 12.1–15.5% in

Table 1 Observed and expected frequency of pair-wise association of all *Arabidopsis* chromosome territories in 4C leaf nuclei

Chromosome combination	Association frequency (%) ^a	
	<i>A. thaliana</i> Col ($n=51$)	SCD model ($n=10^3$)
1–1	88.2	85.3
1–2	96.1	99.1
1–3	100.0	99.4
1–4	98.0	98.8
1–5	100.0	99.4
2–2	76.5	74.8
2–3	96.1	98.6
2–4	96.1	98.3
2–5	98.0	98.8
3–3	80.4	77.5
3–4	96.1	98.4
3–5	98.0	98.5
4–4	78.4	68.8
4–5	96.1	97.5
5–5	88.2	78.8

^aAll differences were not significant ($P > 0.05$) in Fisher's exact test

spherical to 57.0–63.5% in rod-shaped nuclei for the symmetric chromosomes and from 21.2–25.8% (spherical nuclei) to 58.4–65.3% (rod-shaped nuclei) for asymmetric chromosomes. The values for spindle-shaped nuclei take an intermediate position as also their morphology, and thus the constraint for CT arrangement, is intermediate between spherical and rod-shaped ones. Because of the predicted differences, we merged the values simulated for different nuclear shapes by calculation of the weighted average according to the proportion of evaluated spherical, spindle and rod-shaped nuclei per experimental point. These values were compared with the sum of values for all shapes per experimental point (Table 2). The observed frequency of the types of homologous CT arrangement (Fig. 1j) did not significantly deviate from the random expectation for the differently shaped nuclei of 2C, 4C, and 8C DNA content from roots as well as from leaves in the case of chromosome 1. Corresponding data were obtained for chromosomes 3 and 5 as studied in 4C leaf nuclei (Table 2). Different observations were made for the smaller asymmetric chromosomes 2 and 4 with NORs at their top arm ends. For both these chromosomes the association of entire homologs occurred significantly more often ($P < 0.001$) and complete separation less often than expected at random in all tested types of nuclei. This increase of association also holds true when considering the values for entire homologs (T+B+) and for only top arms (T+B-) together and becomes even more pronounced with increasing ploidy level (Table 2). The significant increase in association frequency of homolo-

gous entire and top arm territories of chromosomes 2 and 4 is apparently due to the frequent attachment of the NORs to a single nucleolus (in >90% of nuclei) in a way mediating association of homologs. The pronounced increase of total top arm association (T+B+ and T+B-) in 8C leaf and root nuclei is paralleled by a decrease in the average number of FISH signals for 45S rDNA per nucleus from 3.0 in 2C to 1.6 in 8C nuclei (Z. Jasencakova and I.S. unpublished). However, in contrast to the attachment at a single nucleolus, the fusion of NORs, which progresses with increasing ploidy, does not enhance bottom arm association (T-B+).

The relative position of a gene (*FWA*) within its CT does not depend on transcriptional state

After FISH to 4C leaf nuclei ($n=359$) with differently labeled probes for the chromosome 1 top arm territory and for BAC T2P11 therein, 12.8% of FISH signals for the BAC were localized clearly outside the labeled CT. We asked whether this surprising observation might be related to an altered location due to a switch in transcriptional activity of genes of BAC inserts within different nuclei. To test whether the transcriptional activity might have an impact on CT organization, i.e., whether a transcribed gene occupies another position in relation to its CT than in the silent condition, we have chosen the flowering gene *FWA* residing in BAC M7J2 and mapped at the bottom arm of chromosome 4. In wild-type plants (*Ler*) this gene is not

Table 2 Association frequencies of homologous chromosome-arm territories in *Arabidopsis* leaf and root nuclei of different ploidy levels^a. (T top arm, B bottom arm, + associated, – separated)

Homologs	<i>A. thaliana</i> Col							SCD model (<i>n</i> =10 ³)				χ^2 test ^b
	<i>n</i>	Organ	Ploidy	Association frequency (%)				Association frequency (%)				
				T+B+	T+B–	T–B+	T–B–	T+B+	T+B–	T–B+	T–B–	
Chromosome 1	121	Leaf	2C	47.1	19.8	14.9	18.2	55.0	12.3	13.8	18.9	–
	100		4C	47.0	20.0	12.0	21.0	48.5	11.3	12.5	27.7	–
	101		8C	42.6	16.8	13.8	26.8	50.6	11.5	12.6	25.4	–
	120	Root	2C	37.5	15.8	13.4	33.3	39.0	10.4	11.4	39.2	–
	120		4C	35.0	29.2	24.2	11.6	33.3	26.7	33.3	6.7	–
	120		8C	45.8	15.0	14.2	25.0	47.6	10.9	11.9	29.6	–
Chromosome 2	120	Leaf	2C	45.8	6.7	19.2	28.3	31.1	2.5	36.2	30.2	***
	120		4C	45.0	5.8	22.5	26.7	30.6	2.4	<i>36.0</i>	31.0	***
Chromosome 3	102	Leaf	4C	47.0	26.5	6.9	19.6	43.6	20.3	6.7	29.4	–
Chromosome 4	120	Leaf	2C	42.5	3.3	25.8	<i>28.4</i>	21.3	1.2	<i>35.6</i>	<i>41.9</i>	***
	120		4C	39.2	10.0	26.7	<i>24.1</i>	23.1	1.4	<i>40.8</i>	<i>34.7</i>	***
	111		8C	42.0	21.4	10.7	25.9	21.3	1.2	35.3	42.2	***
	120	Root	2C	39.2	8.3	23.3	29.2	19.2	1.0	29.3	50.6	***
	122		4C	43.4	6.6	<i>19.7</i>	30.3	19.7	1.1	<i>31.0</i>	48.3	***
	130		8C	45.4	25.3	10.8	18.5	21.0	1.1	34.2	43.6	***
Chromosome 5	115	Leaf	4C	49.6	11.3	20.0	19.1	46.5	8.9	17.4	27.2	–

^aPer experimental point the percentage of observed values for the sum of spherical, spindle and rod-shaped nuclei is given and compared with the SCD model prediction based on the weighted average for the three nuclear shapes

^bSignificance level of differences between the entirety of observed versus expected values per experimental point in a column-wise comparison: – $P > 0.05$; *** $P < 0.001$

For individual columns (observed versus model): bold $P < 0.001$, italics $0.001 < P < 0.05$

expressed and is strongly methylated, while it is constitutively expressed and hypomethylated in leaf nuclei of the *fwa-1* mutant (Soppe et al. 2000). In 2C leaf nuclei ($n=337$) of *fwa-1*, only 4.2% of FISH signals for BAC M7J2 were found distal to the CT periphery (Fig. 2a). A similar frequency (3.8%) of signals apparently out-looped from the CT was observed also for wild-type 2C leaf

nuclei ($n=368$). In 4C nuclei, out-looping of M7J2 signals occurred in 10.7% of 121 *fwa-1* nuclei and in 6.5% of 230 wild-type nuclei. Although there is a tendency for more out-looping in *FWA*-expressing 4C nuclei, the difference is not significant at $P=0.001$ level.

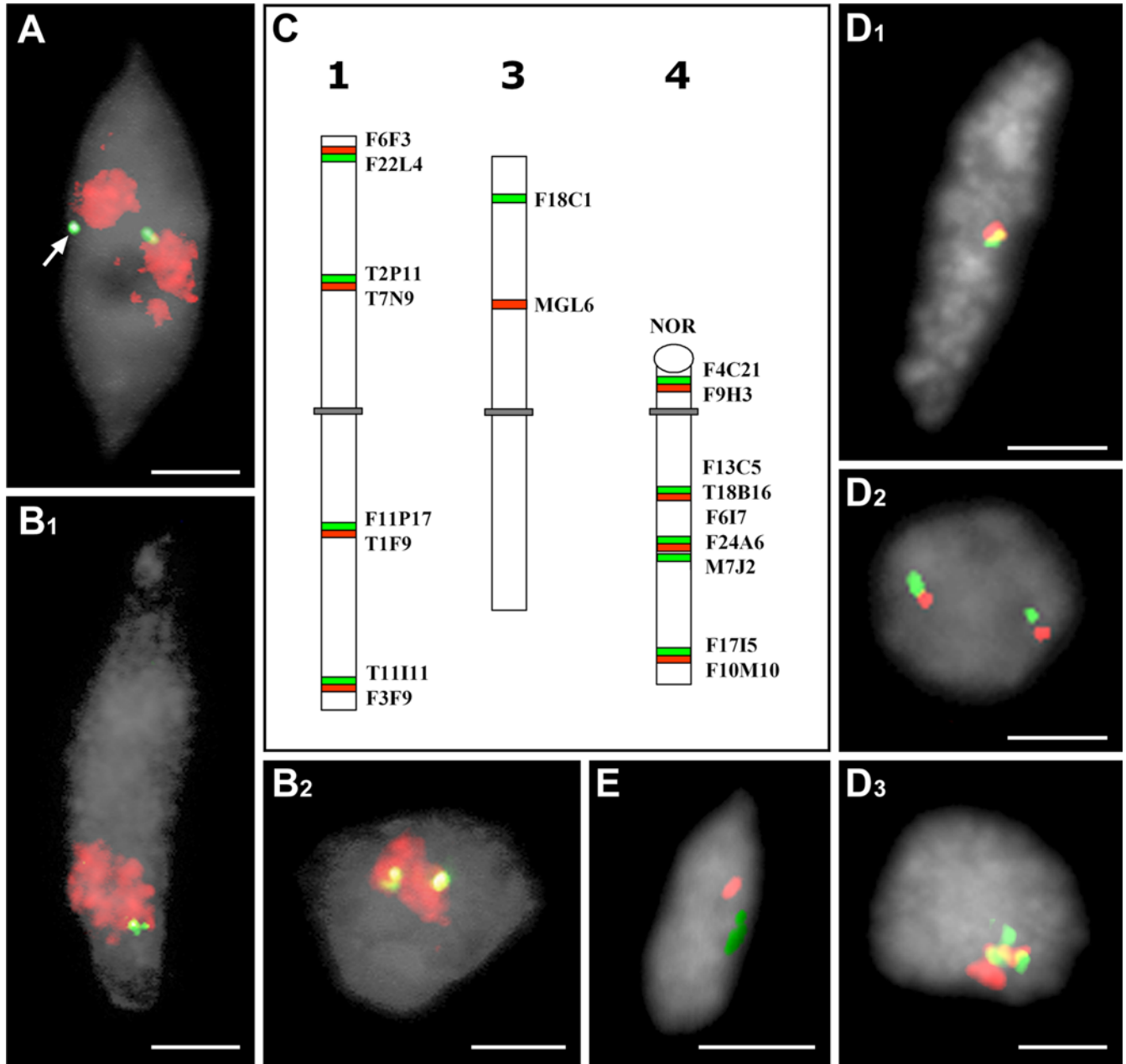


Fig. 2a–e Relative position [to each other/their chromosomal territories (CTs)] of homologous ~100 kb chromosome segments. **a** 2C leaf nucleus of the *fwa* mutant with separated chromosome 4 bottom arm territories painted in red and an ~80 kb chromosome segment of the same arm [bacterial artificial chromosome (BAC) M7J2 in green]; one segment (arrow) looped out from its territory. **b** 4C wild-type (Col) leaf nuclei with associated chromosome 1 top arm territories painted in red and therein an ~85 kb chromosome segment (BAC T2P11 in green) paired (**b1**) or separated (**b2**). **c** Scheme of chromosomes 1, 3 and 4 indicating the BAC sequence

positions used for analysis of single-point pairing by fluorescence in situ hybridization (FISH). **d** Single-point pairing of the segments T2P11/T7N9 in a 4C root nucleus (**d1**), separation of the homologous segments F11P17/T1F9 in a 2C leaf nucleus (**d2**) and loose spatial association of the segments T2P11/T7N9 in a 4C root nucleus (**d3**) based on compact (**d1** and **d2**) and dispersed signals (**d3**). **e** Rare simultaneous single-point pairing of two homologous segments (F9H3;F17I5) from opposite arms of chromosome 4 in a 2C root nucleus. All nuclei were counterstained with 4',6-diamidino-2-phenylindole. Bars represent 3 μ m

Table 3 Single-point homologous chromosome pairing and segment association analyzed by fluorescence in situ hybridization with bacterial artificial chromosome (BAC) pairs or single BACs in nuclei of different organs and ploidy levels in comparison with the random spatial distribution (RSD) model

	BACs	Organ	Ploidy	n_1	Pairing frequency (%) ^a	n_2	Pairing + association frequency (%)
Chromosome 1	F6F3/F22L4	Leaf	2C	299	4.3	379	24.5
			4C	355	5.4	443	24.2
		Root	2C	357	4.2	435	21.4
			4C	265	8.3	343	29.1
	T2P11/T7N9	Leaf	2C	299	8.0	362	24.0
			4C	571	2.1***	670	16.6
		Root	2C	243	5.3	264	12.9
			4C	603	0.8***	659	9.3
	F11P17/T1F9	Leaf	2C	141	4.3	171	21.2
			4C	328	1.8***	382	15.6
		Root	2C	436	3.7***	503	16.5
			4C	476	4.8	530	14.5
	T11I11/F3F9	Leaf	2C	487	4.3	615	24.2
			4C	374	4.5	505	31.5
		Root	2C	528	7.0	631	22.2
			4C	544	3.9	646	19.0
Chromosome 3	F18C1	Leaf	2C	134	5.2	153	17.0
	MGL6	Leaf	2C	141	4.3	153	11.7
Chromosome 4	F4C21/F9H3	Leaf	2C	104	9.6		
			4C	114	3.5		
		Root	2C	109	10.3		
			4C	120	6.7		
	F9H3	Leaf	2C	189	7.4	222	21.2
		Root	2C	265	5.7	308	18.8
	F13C5/T18B16	Leaf	2C	107	3.7		
			4C	121	5.0		
		Root	2C	113	14.0		
			4C	116	1.7		
	F6I7/F24A6	Leaf	2C	113	6.2		
			4C	119	3.4		
		Root	2C	92	13.3		
			4C	113	5.3		
	M7J2 ^b	Leaf	2C	315	4.8	368	18.6
			4C	207	1.9	230	11.7
	F17I5/F10M10	Leaf	2C	118	7.6		
			4C	120	1.7		
		Root	2C	109	11.1		
			4C	115	5.2		
	F17I5	Leaf	2C	199	5.5	222	15.4
		Root	2C	255	9.0	308	24.7
RSD model ^c		Leaf	2C	10 ⁶	7.8		
			4C	10 ⁶	6.9		
		Root	2C	10 ⁶	7.4		
			4C	10 ⁶	5.9		

^aCompare with the simulated random values according to the RSD model in the lower part of the table; *** $P < 0.001$

^bOnly this BAC was tested in *Ler* and not in *Col* background

^cOn the basis of differences in volumes of 2C and 4C root and leaf nuclei, four expected pairing frequencies were calculated for comparison with the experimental data

n_1/n_2 : for nine positions along chromosomes 1, 3 and 4, in addition to nuclei showing either strict single-point pairing or clear separation ($\Sigma=n_1$), nuclei with stretched signals of dispersed appearance or with compact signals of a distance less than the signal diameter (together considered as "association") were scored separately and added to n_1 ($\Sigma=n_2$)

Somatic pairing of homologous chromosome segments occurs at random or less often and not simultaneously along the chromosomes

To analyze whether CT association of homologs reflects strict allelic alignment along chromosome pairs, we assessed by FISH the nuclear positions of ~100 kb chromosome segments (average BAC insert size). Simultaneous detection with differentially labeled probes of the chromosome 1 top arm territory and of BAC T2P11 therein has shown that of 94 4C leaf nuclei with associated top arm territories only seven also showed single-point pairing (one FISH signal for T2P11, Fig. 2b1). This indicates that association of homologs is not a consequence of homologous alignment.

Single-point pairing was further analyzed for different regions on chromosomes 1, 3 and 4 using either single BACs or two differently labeled adjacent BACs for FISH (Fig. 2c). A single compact signal site per nucleus is regarded as single-point pairing in contrast to clearly separated signals indicating the absence of pairing (Fig. 2d1,2). In some experiments (Table 3) we additionally scored nuclei that contained dispersed signals of spherical shape (Fig. 2d3) or separated compact signals with a distance less than the signal diameter. Such nuclei were considered to represent a loose signal association indicating spatial vicinity but not necessarily allelic alignment of homologous segments.

The RSD model simulations of 10^6 2C and 4C root and leaf nuclei, respectively, yielded a random frequency of 5.9–7.8% nuclei with homologous single-point pairing (Table 3). In contrast to the situation described for association of CTs, computer simulations revealed no significant differences as to the random expectation of single-point pairing for the three predominant nuclear shapes. The reason is presumably that FISH signals for ~100 kb segments occupy a much smaller proportion of the nuclear volume and therefore cause fewer spatial constraints than do CTs. The observed frequency of nuclei showing single-point pairing at the tested positions (0.8–14.0%; $\bar{x}$ =4.9%; Table 3) was on average 7–10 times lower than that for association of both arms of the corresponding homologous pair (35.0–49.6%; Table 1). Regardless of the chromosomal position, single-point pairing was not observed significantly more often than expected at random according to the RSD model. No significant differences for single-point pairing were found between leaf and root nuclei irrespective of the ploidy level (tested for chromosomes 1 and 4).

Compared with single-point pairing, a looser spatial signal association is up to ten times more frequent. Adding the number of nuclei showing single-point pairing to that showing loose association revealed that, depending on the chromosomal position, within 9.3–31.5% of nuclei allelic sequences occur in a close spatial proximity (Table 3). The calculation of the average volumes of ~100 kb segments for the simulations according to the RSD model was based on measurements of unpaired signals of spherical shape. For some of the larger spherical signals no clear decision

as to pairing was possible (Fig. 2d3). Therefore these signals were excluded from the pairing values and considered as associated. This might be the reason for the finding that the observed frequency of single-point pairing was significantly lower at four out of 40 experimental points ($P<0.001$) but at no point significantly higher than the values simulated on the basis of the RSD model (Table 3). For chromosome 1 the positional proximity of allelic sequences (single-point pairing and segment association together) was less pronounced at interstitial loci (15.0% on top arm; 16.1% on bottom arm) than at distal loci (24.6% on top arm; 23.8% on bottom arm) when all data from 2C and 4C, leaf and root nuclei were pooled ($P<0.001$).

Simultaneous FISH with BACs located distantly on a particular chromosome showed for four independent combinations (F6F3 and T11I11, T7N9 and F11P17 of chromosome 1; F18C1 and MGL6 of chromosome 3; and F9H3 and F17I5 of chromosome 4) that homologous pairing is indeed only single-point and does not involve entire chromosomes. Only three (0.2%) out of 1240 tested nuclei showed simultaneous pairing at two distant loci (Fig. 2e).

Discussion

In general, *Arabidopsis* CTs are arranged randomly in relation to each other

After establishing chromosome painting for all *A. thaliana* chromosomes, we could address questions as to the arrangement of entire CTs during interphase in this euploid model plant. As observed for other eukaryotes, all *Arabidopsis* chromosomes occupy discrete, three-dimensional CTs in 2C, 4C and 8C interphase nuclei of roots and leaves. A significant deviation from a random radial arrangement was not to be expected because of the low DNA content, the small proportion of repetitive sequences (clustered mainly at the pericentromeric chromocenters and at the NORs), the low number and similar size of *Arabidopsis* chromosomes and the predominantly peripheral location of pericentromeric chromocenters (The Arabidopsis Genome Initiative 2000; Fransz et al. 2002). Indeed no obvious preference as to the radial arrangement of specific CTs was observed during evaluation of nuclei after multicolor painting of all chromosomes and a large proportion of all CTs touch the nuclear periphery. Thus, our main focus was directed on somatic pairing of homologs as a special case of side-by-side arrangement of CTs. The remarkably high frequency of spatial association of entire homologous CTs (T+B+) of symmetric chromosomes 1, 3 and 5 (on average in 35–50% of nuclei and up to 70% in spherical nuclei) is in agreement with the model predictions of a random arrangement for these chromosomes, and is obviously due to the small number of chromosome pairs. The low chromosome number is apparently also responsible for the high frequency of the side-by-side arrangement (76.4–100%) for all possible

homologous and heterologous chromosome combinations, which again was not deviating from model predictions for random arrangement.

The higher-than-expected frequency of homologous CT association for the asymmetric chromosomes 2 and 4 with a bias toward association of the homologous short, NOR-bearing top arms of these chromosomes is likely mediated by the attachment of NORs to only a single nucleolus in >90% of nuclei. A “strong tendency” for association of homologs (in 53–70% of nuclei) was found (exceptionally) in human Sertoli cells for six chromosomes tested by chromosome painting (Chandley et al. 1996). However, among the tested chromosomes only the acrocentric NOR-bearing chromosomes 13 and 21 showed a high frequency of homologous association (50%) also in dividing lymphocytes, apparently due to attachment of NORs at one nucleolus (Chandley et al. 1996). Investigation of meristematic tissues will show whether nuclear CT arrangement in mitotically active cells is similar to that observed in differentiated tissues.

Comparative chromosome painting (with probes consisting of chromosome-specific BAC contigs of *A. thaliana*, arranged according to comparative genetic maps) to related species that differ with respect to the number and shape of chromosomes and to the number of NORs is now feasible (Lysak et al. 2003). A comparative chromosome painting approach will allow testing of whether a chromosomal constitution different from that of *A. thaliana* will result in a different CT arrangement and is in accordance with the corresponding prediction of the SCD model.

In 8C nuclei, in general no more than two CTs were found per homolog. Also the number of chromocenters (at maximum 14, i.e., 10 pericentromeres and 4 NORs, but usually not more than 10, Fransz et al. 2002) did not significantly increase in >4C nuclei. Both observations suggest that CTs of endoreduplicated chromatids are usually not separated but remain associated, at least within the pericentromeric regions.

The activity of an individual gene does not necessarily modify its relative position in a CT

As shown for BAC M7J2 harboring the *FWA* gene, the relative position of a sequence to the CT (inside, at the edge or outside) to which the sequence belongs does not obviously depend on the transcriptional state of that sequence. This agrees with the observations made on mammalian cells where active genes were found to be located on the surface as well as in the interior of a CT and were not relocated when switching the expression status (Mahy et al. 2002a). Thus, transcriptional activity of a gene is not necessarily a reasonable explanation for the relatively high frequency (12.8%) at which the FISH signal for BAC T2P11 was found “outside” the chromosome 1 top arm territory. However, regions of “high gene density and transcription” may frequently extend from their territory (Mahy et al. 2002b) as already described for

the MHC region, which may locate outside its CT depending on cell type and gene activity (Volpi et al. 2000). The gene density along *Arabidopsis* chromosome arms is rather uniform, but we cannot exclude that most of the 21 presumed genes of BAC T2P11 are simultaneously expressed in nuclei showing this region out-looped from its CT. Although we did not test the position of specific regions after simultaneous painting of all chromosomes, the results obtained with BACs T2P11 and M7J2 imply that CTs do not always have a smooth surface, i.e., out-looping of chromatin into interchromosomal spaces (mimicking intermingling of CTs) might occur to some extent.

Somatic homologous pairing is the exception rather than the rule in *A. thaliana*

Fluorescence in situ hybridization experiments with ~100 kb segments (BAC insert sequences) from distinct positions along chromosomes 1, 3 and 4 showed that association of homologous CTs does not reflect contiguous allelic alignment since single-point pairing (on average 4.9%) occurs 7–10 times less frequently than homolog association and not simultaneously along the chromosomes. Thus, in *A. thaliana* root and leaf nuclei somatic pairing of entire homologs is the exception rather than the rule. The opposite has been shown for *Drosophila melanogaster* with homologous pairing in 60–90% of somatic nuclei from the 13th embryonic cell cycle on (Csink and Henikoff 1998; Fung et al. 1998). The comparison of *Arabidopsis* and *Drosophila* shows that similarity in genome size, sequence organization and chromosome number does not necessarily cause an identical CT arrangement.

In spite of the constraints as to the chromatin dynamics within interphase nuclei of all tested organisms a certain flexibility of chromatin positions has been found (for review see Lam et al. 2004). The average movement of GFP-tagged chromatin loci is ~0.085 $\mu\text{m}/\text{min}$ (Kato and Lam 2003). Therefore, at least in the 9.3–31.5% of nuclei that show either single-point pairing or close association of allelic sequences, these allelic sequences might occupy nuclear positions sufficiently close for homologous recombination (for instance in the course of double-strand break repair), in spite of the lack of regular and contiguous alignment of homologs.

Consideration of single-point pairing and loose spatial association together, as exemplified for chromosome 1, suggests that interstitial chromosome arm regions are less often in close vicinity than distal loci. This agrees with the clustering of telomeric regions around the nucleolus and a high frequency of homologous chromocenter association (Fransz et al. 2002). Therefore, interstitial chromosome regions should associate—and consequently be involved in spontaneous and induced homologous exchange aberrations—less frequently than chromocenters and chromosome termini. During the first post-treatment mitosis after mutagen exposure, multicolor chromosome painting

should allow identification of the chromosomes involved in exchange aberrations. If *Arabidopsis* chromosome regions containing tandemly repeated sequences are preferentially involved in mutagen-induced structural chromosome aberrations as observed for other organisms (Schubert et al. 1994, 2004), the breakpoints of exchange aberrations in the first post-treatment mitoses should predominantly occur within pericentromeric or NOR regions detectable by size and composition of anaphase bridges and/or acentric fragments after multicolor chromosome painting.

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