The Plant Journal (2006) **48**, 771–783 doi: 10.1111/j.1365-313X.2006.02912.x

Chromosome arrangement and nuclear architecture but not centromeric sequences are conserved between *Arabidopsis thaliana* and *Arabidopsis lyrata*

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Summary

In contrast to the situation described for mammals and *Drosophila*, chromosome territory (CT) arrangement and somatic homologous pairing in interphase nuclei of *Arabidopsis thaliana* (*n* = 5) are predominantly random except for a more frequent association of the chromosomes bearing a homologous nucleolus organizer region. To find out whether this chromosome arrangement is also characteristic for other species of the genus Arabidopsis, we investigated *Arabidopsis lyrata* ssp. *lyrata* (*n* = 8), one of the closest relatives of *A. thaliana*. First, we determined the size of each chromosome and chromosome arm, the sequence type of centromeric repeats and their distribution between individual centromeres and the position of the 5S/45S rDNA arrays in *A. lyrata*. Then we demonstrated that CT arrangement, homologous pairing and sister chromatid alignment of distinct euchromatic and/or heterochromatic regions within *A. lyrata* interphase nuclei are similar to that in *A. thaliana* nuclei. Thus, the arrangement of interphase chromosomes appears to be conserved between both taxa that diverged about 5 million years ago. Since the chromosomes of *A. lyrata* resemble those of the presumed ancestral karyotype, a similar arrangement of interphase chromosomes is also to be expected for other closely related diploid species of the Brassicaceae family.

Keywords: *Arabidopsis lyrata*, nuclear architecture, chromosome territories, karyotype evolution, chromosome painting.

Introduction

Detailed knowledge about the architecture and chromatin arrangement of the interphase nucleus will be helpful for understanding the structural basis for nuclear compartmentalization linked with the regulation of processes such as replication, chromatin remodelling, repair/recombination and transcription.

As early as 1885, Rabl (1885) proposed an interphase chromosome orientation with centromeres and telomeres clustered at opposite nuclear poles, reflecting the anaphase arrangement of chromatids. The so-called Rabl orientation has been confirmed by fluorescent *in situ* hybridization (FISH), particularly for species with large monocentric and bi-armed chromosomes, such as barley (Jasencakova *et al.*, 2001), but not for *Arabidopsis thaliana* (Fransz *et al.*, 2002; for review see Dong and Jiang, 1998). Since chromosome painting became established for mammals (Lichter *et al.*, 1988; Pinkel *et al.*, 1988), it has become obvious that individual chromosomes of euploid species occupy distinct three-dimensional chromosome territories (CTs; for review see Cremer and Cremer, 2001). A radial arrangement of CTs with gene-dense chromosomes located more internally and gene-poor ones nearer the nuclear periphery seems to be typical for many vertebrate cell types (Cremer *et al.*, 2001; Habermann *et al.*, 2001;

Kozubek *et al.*, 2002; Mahy *et al.*, 2002a,b; Tanabe *et al.*, 2002). Apart from polytene chromosomes (Metz, 1916), development- and cell cycle-specific somatic pairing of homologous chromosomes has been described for *Drosophila* (Csink and Henikoff, 1998; Fung *et al.*, 1998; Hiraoka *et al.*, 1993). A close spatial homologous association of disomic addition chromosomes was also found in tapetum nuclei but rarely in other tissues of wheat addition lines (Aragón-Alcaide *et al.*, 1997).

In *A. thaliana* nuclei, side-by-side arrangement and somatic pairing frequencies of individual CTs were found to be predominantly random. An exception was the more frequent association of chromosomes bearing a homologous nucleolus organizer region (NOR), apparently due to an early post-mitotic fusion of the four initial nucleoli (Pecinka *et al.*, 2004). Thus, in most mammalian and plant cells analysed so far, long-distance pairing of homologues was not detectable.

To ensure an equal transmission of the genetic material during mitosis, anaphase does not begin until sister chromatids are attached by their kinetochores to spindle fibres from opposite poles. In yeast, sister chromatids are aligned by ring-shaped cohesin complexes surrounding them approximately every 11 kb from early S phase until the onset of anaphase (Koshland and Guacci, 2000). However, in *A. thaliana*, sister chromatids (except at centromeres) are frequently not closely aligned along their euchromatic arms in meristematic or differentiated nuclei of 4C (1C = unreplicated haploid genome size) and higher DNA content (Schubert *et al.*, 2006).

Here, we report on karyotype analysis of *Arabidopsis lyrata* (2n = 16; 1C approximately 245 Mb), a self-incompatible diploid species separated from *A. thaliana* (2n = 10; 1C approximately 157 Mb) about 5 million years ago (Mya). By using multicolour FISH and comparative chromosome painting we determined the size of each individual chromosome, the position and the repeat type composition of each individual centromere and the position of NORs and 5S rDNA. These data were used to study CT arrangement, homologous pairing and sister chromatid alignment in *A. lyrata*. The experimental observations were compared with random chromosome/chromatin arrangement within computer-simulated nuclei as well as with corresponding data for *A. thaliana* under evolutionary aspects. The results suggest a large-scale conservation of nuclear architecture within the genus Arabidopsis.

Results

The A. lyrata *karyotype*

To study the organization of interphase chromosomes, knowledge about: (i) genome size, (ii) the size of individual chromosomes/chromosome arms and (iii) the position of the main repetitive DNA sequence clusters is required. Repetitive DNA clusters were first localized by FISH with repeat-specific probes in combination with (partial) chromosome painting. The single 5S rDNA locus was localized on the short arm of *A. lyrata* chromosome 4 (AL4). The euchromatic region between the 5S rDNA locus and the pericentromeric region did not hybridize with our bacterial artificial chromosome (BAC) probe collection (Figure 1a). Nucleolus organizer regions were found at a subterminal position on the short arm of chromosomes AL1, AL3, AL4, AL5 and AL7 (e.g. Figure 1c). The centromeres of chromosomes AL2, AL4 and AL8 were physically localized by FISH using differently labelled BAC contigs corresponding to presumed individual short and long chromosome arms. Bacterial artificial chromosomes harbouring inserts from proximal chromosome arm positions were exchanged between the arm-specific contigs until each arm flanking the strongly 4′,6-diamidino-2-phenylindole (DAPI)-stained pericentromeric regions on painted pachytene chromosomes was labelled in only one colour (e.g. Figure 1b). Centromeres of chromosomes AL1, AL3, AL5, AL6 and AL7 occupy the same positions as in *A. thaliana*.

The genome size of *A. lyrata* was estimated to be approximately 245 Mb (0.25 pg/1C; M. A. Lysak and A. Meister, unpublished data) compared to approximately 157 Mb (0.16 pg/1C; Bennett *et al.*, 2003) in *A. thaliana.* The size of individual chromosomes was estimated on the basis of comparative genetic mapping between *A. thaliana* and *A. lyrata* ssp. *lyrata* (Yogeeswaran *et al.*, 2005) and *A. lyrata.* ssp. *petraea* (Kuittinen *et al.*, 2004), respectively. The physical size (in Mb) of individual chromosome arms of *A. lyrata* was estimated according to the size of co-linear regions in *A. thaliana* expressed as distances between their most distal BACs (TAIR database, http://arabidopsis.org) without considering NORs and pericentromeres. These values were transferred proportionally to *A. lyrata* and together approached approximately 185 Mb assuming (i) a approximately 1.5-fold increase in genome size compared to *A. thaliana* and (ii) a uniform distribution of dispersed repeats for both species. The approximately 60 Mb difference between these approximately 185 Mb and the total *A. lyrata* genome (approximately 245 Mb) was distributed equally among the eight centromeres and the five NORs (i.e. approximately 4.6 Mb/heterochromatic block). These values were used for computer simulation of CTs according to the 'Spherical 1 Mb Chromatin Domain' (SCD) model (Kreth *et al.*, 2004; see Experimental procedures). Alternatively, the chromosome size was estimated by dividing the genome size of *A. lyrata* by the relative area of individually painted mitotic metaphase chromosomes. Both approaches yielded similar results (Table 1). Together, these results allowed us to define the *A. lyrata* karyotype (Figure 1h).

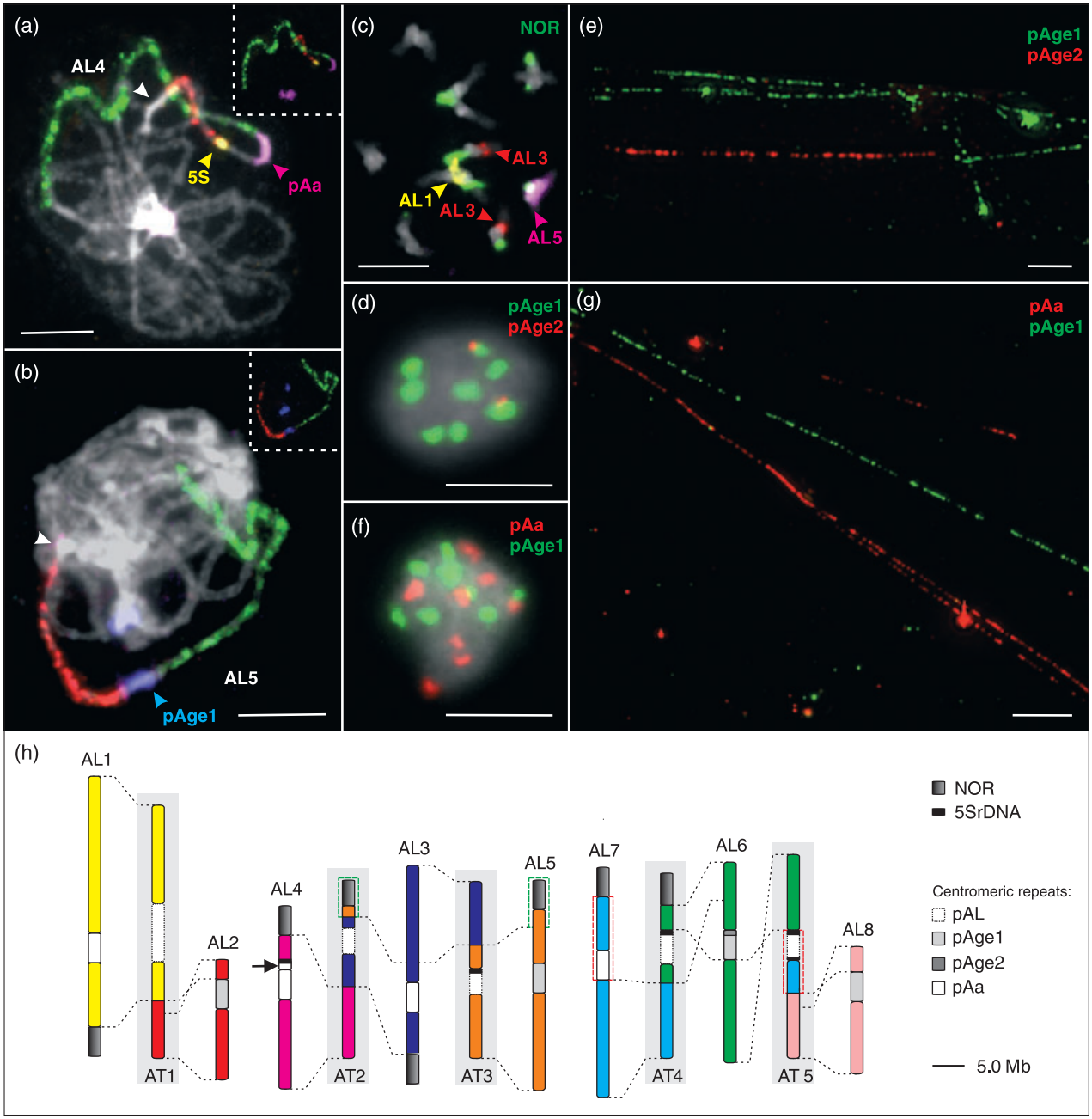

Figure 1. Examples of localization and distribution of the major heterochromatic blocks in *Arabidopsis lyrata* nuclei and the karyotype of *A. lyrata*.
(a) Localization of the 5S rDNA (yellow) close to the pAa centromere (violet) of pachytene chromosome AL4 painted in red (short arm) and in green (long arm). The NOR of AL4 is indicated by a white arrowhead.
(b) Pachytene chromosome AL5 painted in red (short arm) and in green (long arm) and the pAge1 repeat family of its centromeric region in blue. Inserts in (a) and (b) without DAPI fluorescence reveal the labelling of additional centromeric regions.
(c) Partial labelling of AL1 short arm (yellow), AL3 long arm (red) and AL5 short arm (violet) for localization of NORs (green) on mitotic metaphase chromosomes.
(d and e) Differential labelling of pAge1 (green) and pAge2 (red) on 2C interphase nuclei (d) and on DNA fibres (e).
(f and g) Differential labelling of pAa (red) and pAge1 (green) on 2C interphase nuclei (f) and on DNA fibres (g). Bars = 5 μm.
(h) Idiograms of *A. lyrata* (AL1-8) and *A. thaliana* homologues (AT1-5) based on comparative painting and co-linearity between genetic maps. The different types of centromeric repeats, NORs and 5S rDNA arrays are indicated. The eight *A. lyrata* chromosomes are highlighted in distinct colours that correspond to the homologous regions on *A. thaliana* chromosomes. The translocated short arm regions of AL5 and AL7 and the homologous parts of AT2 and AT5 are framed in green and red, respectively. The arrow indicates the region on AL4 between the 5S rDNA and the centromeric region which remained unlabelled after chromosome painting (a). The chromosome size proportions are according to Table 1 for AL1-8 and according to Supplementary Table S1 for AT1-5.

Table 1 Estimated size of individual *Arabidopsis lyrata* chromosomes (Mb)

	AL1	AL2	AL3	AL4	AL5	AL6	AL7	AL8
rDNA (NOR)[a]	4.6		4.6	4.6	4.6		4.6	
Short arm (S)	10.0	3.1	6.6	4.4	8.4	10.6	8.4	4.0
Centromeric region[a]	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
Long arm (L)	24.5	11.1	18.3	13.9	15.4	16.1	18.3	11.3
Chromosome size	43.7	18.8	34.1	27.5	33.0	31.3	35.9	19.9
	42.2[b]	20.3[b]	33.7[b]	28.9[b]	31.7[b]	29.8[b]	36.5[b]	21.9[b]
	Total genome size approximately 245.0							

Arm sizes were estimated from the physical distances between the most distal BACs of all co-linear regions comprising the *A. lyrata* linkage groups within the *A. thaliana* genome (TAIR database; Lysak *et al.*, 2006) multiplied by approximately 1.5 (see text).
[a]Estimated from the difference between the total of the arm size (approximately 185 Mb) and the *A. lyrata* genome size (approximately 245 Mb).
[b]Based on the relative area of painted mitotic metaphase chromosomes.

Sequence organization, phylogenetic relationship and chromosomal distribution of the three centromeric repeat types of A. lyrata

Within the genus Arabidopsis, four distinct approximately 180 bp centromeric repeat types are known: (i) the pAL1 sequence of *A. thaliana* (Maluszynska and Heslop-Harrison, 1991; Martinez-Zapater *et al.*, 1986), (ii) the pAa sequence of *Arabidopsis arenosa* (Kamm *et al.*, 1995) and (iii) the pAge1 and pAge2 sequences of *Arabidopsis halleri* ssp. *gemmifera* and *A. lyrata* ssp. *kawasakiana* (Kawabe and Nasuda, 2005). We could demonstrate the presence of pAa, pAge1 and pAge2 repeat families for *A. lyrata* ssp. *lyrata* via PCR with repeat-specific primer pairs (Kawabe and Nasuda, 2005) and found a characteristic ladder pattern of approximately 180 bp intervals. Since no PCR products were obtained with single primers, the centromeric sequences are arranged as direct tandem repeats. In total six pAa (three dimers), six pAge1 (two trimers) and 13 pAge2 (two dimers and three trimers) approximately 180 bp repeats were cloned and sequenced.

The pAa family of 179 bp revealed 88.8–94.4% and 87.7–92.2% similarity within and between the three cloned dimers, respectively. The pAge1 family of 168 bp showed 91.0–94.0% and 90.4–94.0% similarity within and between the two cloned trimers, respectively. Interestingly, the pAge2 family of 176 bp revealed a particular arrangement of individual repeat units, with a sequence similarity between all first and between all last repeat units of both dimeric and trimeric clones that was higher than between the units of a single clone (86.4–98.8%, 84.6–99.4% and 77.4–88.6%, respectively; see insert to Figure 2 and Supplementary Table S2). This might indicate that dimers and trimers rather than monomers are the units of concerted evolution of the pAge2 sequence. Furthermore, phylogenetic relationships between previously reported centromeric repeats (Hall *et al.*, 2005; Kamm *et al.*, 1995; Kawabe and Nasuda, 2005; Martinez-Zapater *et al.*, 1986) and the newly isolated ones indicate that the different repeat families are with 100% probability clustered separately from each other (Figure 2).

The chromosome-specific centromere localization of the three repeat families in *A. lyrata* was determined by FISH with differently labelled pAa, pAge1 and pAge2 probes in combination with chromosome-specific painting probes. The centromeres of chromosomes AL1, AL3, AL4 and AL7 were found to possess the pAa repeat and chromosomes AL2, AL5, AL6 and AL8 the pAge1 repeat. The centromere of AL6 has an additional small cluster of pAge2 repeats. Fluorescent *in situ* hybridization on extended DNA fibres with differently labelled probes for all three centromeric repeat families confirmed a largely homogeneous repeat composition for the centromeres (Figure 1d–g). Even in the case of the AL6 centromere, the repeat families pAge1 and pAge2 were not intermingled (Figure 1e).

The determination of the centromeric repeat type for each chromosome further refined the *A. lyrata* karyotype (Figure 1h).

The major heterochromatic blocks of A. lyrata *and* A. thaliana *nuclei show a similar interphase arrangement*

Analysing the interphase nuclei of *A. lyrata*, we found that centromeric regions and NORs form intensely DAPI-stained heterochromatic chromocenters. Similar to *A. thaliana* (Fransz *et al.*, 2002), centromeric chromocenters are preferentially localized at the nuclear periphery and NORs (in most nuclei) form a single nucleolus surrounded by most of the telomeres (Figure 3).

Computer simulations were performed to test whether the centromeres of *A. thaliana* and *A. lyrata* are randomly distributed within the outermost layer (as thick as the average centromeric FISH signal diameter) of spherical nuclei. To that aim the 'Random Spatial Distribution' (RSD) model (Pecinka *et al.*, 2004) was modified (see Experimental procedures). This model considers associated NORs or centromeres as single entities corresponding to single FISH signals that may represent more than one NOR or centromere. Fluorescent *in situ* hybridization with probes for all centromeric repeats of *A. lyrata* (labelled in one colour) to interphase nuclei showed that centromeres in meristematic and in differentiated 2C and 4C nuclei are associated with a lower than expected frequency. The association tendency was similar ($P > 0.05$) for the pAa and the pAge1 repeats. The same was observed for 2C nuclei of *A. thaliana* after FISH with pAL1 repeats (Table 2). Probably due to the presence of two chromosome arms that emanate from each chromocenter, the possibility for association of centromeres is spatially restricted.

The comparison between the observed number of FISH signals obtained with a 45S rDNA probe (at maximum 10

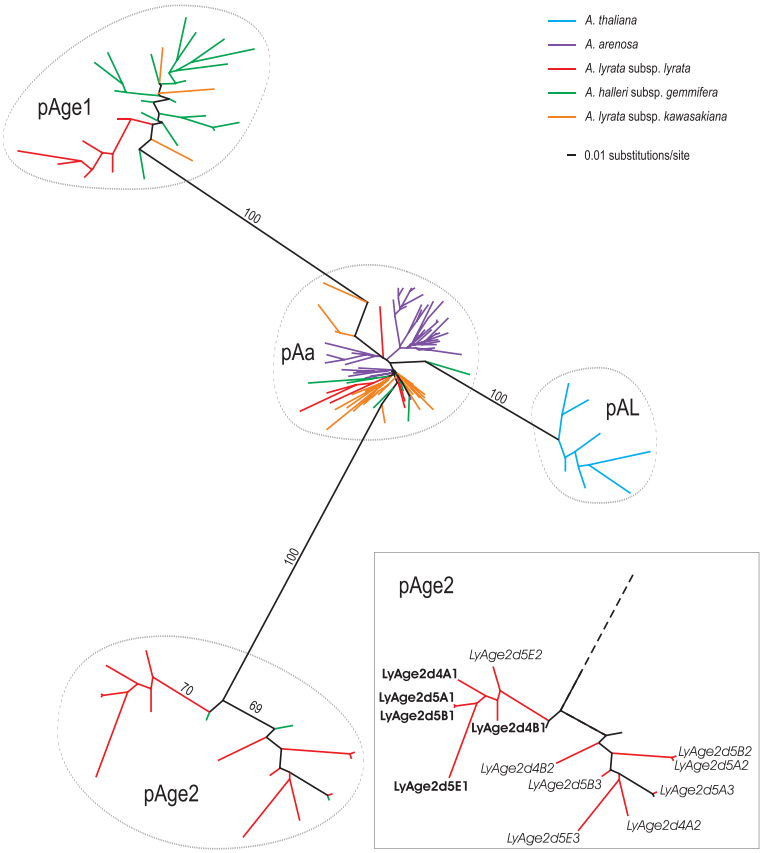

Figure 2. Neighbor-joining tree of centromeric repeats of the genus Arabidopsis.
The unrooted neighbor-joining tree was derived from the maximum-likelihood distances for different centromeric repeat types (pAL1, pAa, pAge1 and pAge2) from several Arabidopsis species. In total, 129 repeats were analysed: seven pAL1 repeats from *Arabidopsis thaliana* (Martinez-Zapater *et al.*, 1986), 45 pAa repeats from *A. arenosa* (Hall *et al.*, 2005; Kamm *et al.*, 1995), six pAa, six pAge1 and 13 pAge2 repeats from *A. lyrata* ssp. *lyrata* (present study), 21 pAa and three pAge1 repeats from *A. lyrata* ssp. *kawasakiana* and six pAa, 19 pAge1 and three pAge2 repeats from *A. halleri* ssp. *gemmifera* (Kawabe and Nasuda, 2005). Numbers along the branches indicate percentage bootstrap values supporting the major groups in the tree. Insert: clustering of the first monomeric units (in bold) as well as of the second and/or the last monomeric units (in italics) from *A. lyrata* ssp. *lyrata* pAge2 clones are shown.

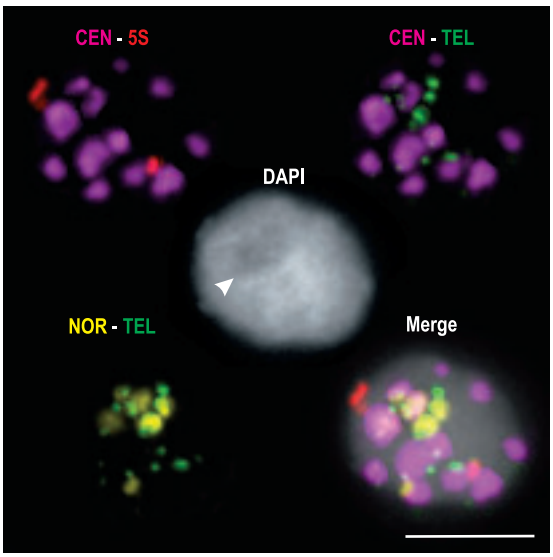

Figure 3. Arrangement of the major heterochromatic blocks in *Arabidopsis lyrata* interphase nuclei.
Centromeric chromocenters (pAa, pAge1 and pAge2 all pseudocoloured in violet) are preferentially localized at the nuclear periphery when inspected in 3D. 5S rDNA arrays (red) are associated with the centromere of AL4. The NORs (yellow) form (in most leaf nuclei) a single nucleolus (arrowhead) surrounded by most of the telomeres (green). Bar = 5 μm.

FISH signals for *A. lyrata* and four for *A. thaliana*) with the random expectation according to the RSD model revealed a strong tendency for NORs to associate in both species. This higher than random association of NORs reflects their attachment to only one nucleolus in most of the nuclei (for both species >90% of nuclei). Because of the terminal NOR positions there is less spatial restriction regarding associations than for centromeres which are flanked by two arms. As in *A. thaliana*, telomeric FISH signals usually appeared around NORs/nucleoli (Figure 3); this was to be expected at least for those 10 termini associated with 45S rDNA.

Arabidopsis lyrata *CTs show a random side-by-side positioning*

A random frequency of CT association was shown for the symmetric chromosomes AT1, AT3 and AT5 and a higher one for the NOR-bearing acrocentrics AT2 and AT4 of *A. thaliana* (Pecinka *et al.*, 2004). To test for CT arrangement in a species with a higher chromosome number and DNA content, we analysed the association frequency of 11 out of 36 possible homologous and heterologous CT combinations for six of the eight *A. lyrata* chromosomes. With this aim, differentially labelled *A. thaliana* BAC contig probes, arranged according to the corresponding linkage groups of

Table 2 Distribution of centromeric or NOR FISH signals in *Arabidopsis lyrata*[a] and *A. thaliana*[b] nuclei of different ploidy

					Number of FISH signals/nucleus (%)														
Species	Probe	Ploidy		n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A. lyrata	pAa	2C	Observed	204	0.0	1.5	1.0	2.9	6.9	21.1	40.7	26.0								
		4C	Observed	200	0.0	0.0	0.0	2.0	5.0	17.0	39.5	36.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
	pAge1	2C	Observed	204	0.0	0.5	1.9	3.4	5.9	23.0	37.7	27.4								
		4C	Observed	200	0.0	0.0	0.0	0.0	3.0	16.0	41.5	38.5	0.5	0.0	0.0	0.0	0.0	0.0	0.5	0.0
	pAa + pAge1	2C	Observed	204	0.0	0.0	0.0	0.5	0.5	0.5	2.0	0.5	2.0	2.0	5.9	14.2	16.7	20.6	21.1	13.7
			RSD model	10^6	*0.0*	*0.0*	*0.0*	*0.1*	*0.9*	*3.4*	*9.1*	*16.8*	*22.3*	*21.4*	*15.0*	*7.5*	*2.6*	*0.6*	*0.1*	*0.0*
			χ^2-test[c]		–	–	–	–	–	*	***	***	***	***	***	***	***	***	***	***
		4C	Observed	204	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	5.4	6.4	15.2	19.6	24.0	15.7	12.3
	NORs	2C	Observed	159	16.3	20.7	18.9	17.6	16.3	3.8	3.8	1.2	0.6	0.6						
			RSD model	10^6	0.8	4.9	14.0	23.7	26.1	18.7	8.8	2.5	1.4	0.0						
			χ^2-test[c]		***	***	–	*	**	**	*	–	–	–						
A. thaliana	pAL1	2C	Observed	204	0.0	0.0	0.0	4.0	5.0	18.0	32.0	18.0	17.0	6.0						
			RSD model	10^6	*0.0*	*0.1*	*1.1*	*6.0*	*17.0*	*27.9*	*27.2*	*15.3*	*4.6*	*0.6*						
			χ^2-test[c]		–	–	–	–	***	***	**	*	***	***						
	NORs	2C	Observed	221	10.9	30.8	44.8	13.6												
			RSD model	10^6	3.4	21.4	45.0	30.1												
			χ^2-test[c]		***	***	–	***												

Data obtained with the RSD model modified to simulate the centromeric repeat distribution in a peripheral nuclear layer appear in italics.

n = number of analysed nuclei.

[a]Five NORs and eight centromeres (four pAa and four pAge1) corresponding to a maximum of 10 and 16 FISH signals in 2C nuclei, respectively.

[b]Two NORs and five centromeres corresponding to a maximum of four and 10 signals in 2C nuclei, respectively.

[c]Significance level of differences between observed association frequency versus the random expectation according to the corresponding model: $-P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

A. lyrata, were applied for comparative painting to round-shaped 2C leaf nuclei of *A. lyrata* (Figure 4b). The association frequencies were scored in at least 100 nuclei and compared with those predicted by the SCD model for random arrangement (Figure 4c). The association frequencies observed for the individual CT combinations were rather high (53.4–96.6%), and not significantly different ($P > 0.05$) from that of the 10^3 simulated nuclei (52.2–98.1%; Figure 4d). Thus, in *A. lyrata* as in *A. thaliana* nuclei, the side-by-side positioning of CTs is random.

The association frequency of homologous chromosome arm territories is random for A. lyrata *chromosome 6 and higher for NOR-bearing chromosomes 3 and 5*

The association frequency of homologous chromosome arm territories was studied for *A. lyrata* chromosome 6 and for NOR-bearing chromosomes 3 and 5 in 2C leaf nuclei. Differently labelled probes for the short and the long arm of each studied chromosome were applied (Figure 5). The association of homologous chromosome arm territories was analysed in round-shaped, spindle-shaped and rod-shaped nuclei with respect to (i) association of both arms, (ii) association of only short arms, (iii) association of only long arms or (iv) complete separation (Figure 5b). For chromosome AL6, the observed association frequencies in differently shaped nuclei clearly showed a similar tendency as observed for *A. thaliana* chromosomes (Pecinka *et al.*, 2004): the highest association frequency occurred in round-shaped, an intermediate one in spindle-shaped, and the lowest one in rod-shaped nuclei (see Supplementary Table S3). The homologous arm association frequency of chromosome AL6 in round-shaped nuclei showed no deviation from random expectation according to the SCD model (Table 3). On the contrary, homologous arms of the NOR-bearing chromosomes AL3 and AL5 associated significantly more often ($P < 0.001$) than would be expected at random (Table 3).

Positional pairing of homologous euchromatic and heterochromatic chromosomal segments is random in A. lyrata *somatic nuclei*

Allelic alignment or homologous pairing of approximately 100 kb segments (corresponding to the average BAC insert size) was analysed for five euchromatic regions along *A. lyrata* chromosomes 1 and 6 in 2C and 4C flow-sorted round-shaped leaf nuclei. The frequency of homologous pairing (one compact signal per BAC pair) versus separation (more than one signal per BAC pair) was scored and compared with the random pairing frequency predicted by the RSD model (Figure 6). The observed frequencies of positional homologous pairing (2.4–5.7% in 2C nuclei) were on average about 10-fold lower than that for the association of both arms of a homologous CT (Table 3), indicating that CT association does not reflect homologous alignment. Positional

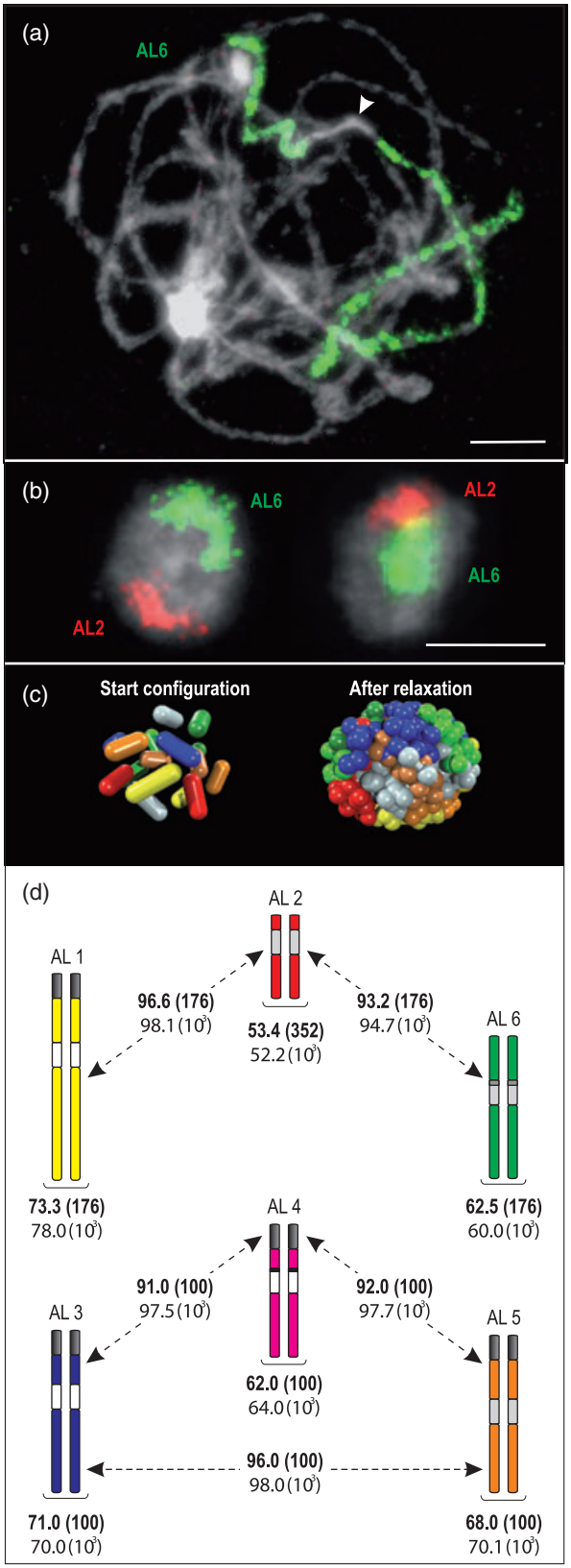

Figure 4. Association of homologous or heterologous chromosome territories in *Arabidopsis lyrata* 2C leaf nuclei.
(a) Pachytene chromosome AL6 painted in green demonstrates the specificity of probes (arrow indicates pericentromeric heterochromatin).
(b) Simultaneous painting of chromosomes AL2 (red) and AL6 (green) in interphase nuclei showing association between homologues (left and right) and additionally at least one association between heterologous (right).
(c) 'Spherical 1 Mb Chromatin Domain' (SCD) model simulating random distribution of all *A. lyrata* CTs, cylindrical start configurations representing chromosomes (left) and after 2×10^5 relaxation steps (right).
(d) Observed (bold) and expected frequencies of homologous or heterologous pairwise association of selected chromosomes in *A. lyrata* round-shaped 2C leaf nuclei (number of evaluated nuclei in parentheses). The differences between simulated and observed association values were not significant ($P > 0.05$) in Fisher's exact test. Bars = 5 μm.

homologous pairing in 2C and 4C round-shaped nuclei did not appear significantly more often than expected at random according to the RSD model prediction (4.8% in 2C and 2.6% in 4C nuclei; Figure 6a). Furthermore, the pairing frequency for the BAC pairs close to the NOR (on the AL1 short arm) was not significantly higher than at the other positions in 2C and 4C *A. lyrata* nuclei. The similar pairing frequency along chromosomes indicates that association of NORs to a single nucleolus does not reflect homologous alignment.

The frequency of homologous pairing was further analysed for two heterochromatic loci comprising tandem repetitive DNA arrays: the unique 5S rDNA locus on chromosome AL4 and the unique centromeric region of AL6 carrying a major cluster of pAge1 and a minor one of pAge2 repeats. The pairing was scored in a similar way as for BACs harbouring unique sequences. The results were compared to the random pairing frequency predicted by the corresponding model variant (Figure 6a). The observed pairing frequencies for the 5S rDNA locus (5.0% in 2C and 2.7% in 4C nuclei) and for the pAge1/pAge2 centromeric region (7.8% in 2C and 2.1% in 4C nuclei) were not significantly different ($P > 0.05$) from random expectation for 10^3 simulated nuclei (4.2% and 10.4% in 2C versus 1.7% and 2.6% in 4C nuclei, respectively).

Sister chromatids are more often aligned at centromeres and at the 5S rDNA locus than along the euchromatic chromosome arms in A. lyrata *nuclei*

It is assumed that sister chromatid alignment is mediated by cohesin complexes from replication in S phase until the onset of anaphase (reviewed in Hagstrom and Meyer, 2003). However, recent observations have shown that sister chromatid alignment is incomplete in 4C *A. thaliana* interphase nuclei except for sister centromeres which are mostly aligned (Schubert *et al.*, 2006). To elucidate the situation in *A. lyrata*, we analysed sister chromatid alignment in 4C leaf nuclei by FISH with differently labelled pairs of BACs harbouring inserts from adjacent sequences of five positions along chromosomes AL1 and AL6 (the same positions as

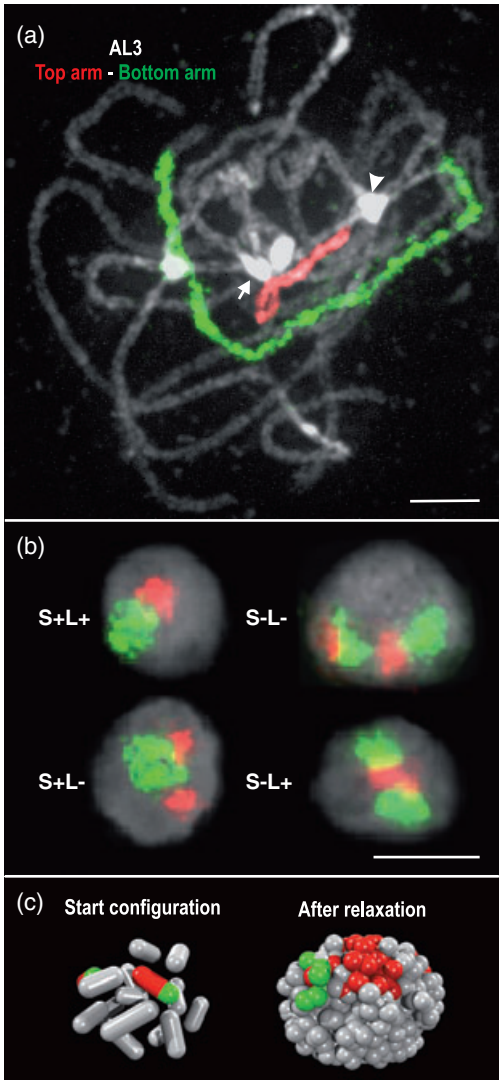

Figure 5. The association of homologous chromosome arm territories in *Arabidopsis lyrata* nuclei.
(a) Pachytene chromosome AL3 painted in red (short arm) and in green (long arm; arrow indicates NOR and arrowhead pericentromeric heterochromatin) demonstrates the specificity of probes.
(b) Possible arrangements of homologous arm territories in *A. lyrata* round-shaped 2C leaf nuclei (S, short arm; L, long arm; +, associated; –, separated).
(c) 'Spherical 1 Mb Chromatin Domain' (SCD) model simulating random distribution of AL3 arm territories, cylindrical start configurations representing chromosomes (left) and after 2×10^5 relaxation steps (right). Bars = 5 μm.

tested for positional homologous pairing; Figure 6a). Sister chromatids were considered to be aligned at both homologues if one or two FISH signals per locus appear in a nucleus. Three or four FISH signals per locus were taken as an indication of separated sister chromatids at one or both homologues, respectively. Sister chromatid alignment was not significantly different ($P > 0.05$) between the euchromatic chromosomal positions tested (73.1–78.3% of the analysed homologues; Figure 6a).

Table 3 Association frequencies of homologous arm territories in 2C round-shaped nuclei from *Arabidopsis lyrata* leaves

			Association frequency (%)			
Homologues		*n*	S+L+	S+L–	S–L+	S–L–
AL3	**Observed**	**107**	**44.8**	**10.3**	**9.3**	**35.5**
	SCD model	**10^3**	**29.0**	**2.8**	**32.9**	**35.3**
	χ^2-test[a]		*******	*******	*******	**–**
AL5	**Observed**	**112**	**51.9**	**9.8**	**13.4**	**25.0**
	SCD model	**10^3**	**35.9**	**8.1**	**22.1**	**33.9**
	χ^2-test[a]		*******	**–**	*****	*****
AL6	Observed	119	26.9	5.9	26.9	40.3
	SCD model	10^3	29.9	5.2	21.1	43.8
	χ^2-test[a]		–	–	–	–

S, short arm; L, long arm; +, associated; –, separated; *n*, number of analysed nuclei.
[a]Significance level of differences between observed association frequency and random expectation according to the SCD model: $^{-}P > 0.05$; $^{*}P < 0.05$; $^{***}P < 0.001$.
Entries for NOR-bearing chromosomes are bold.

This analysis was extended to two types of repetitive sequences, the single 5S rDNA locus on AL4 and the unique cluster of pAge1/pAge2 centromeric repeats on AL6 (Figure 6a). In these cases even 95% of pAge1/pAge2 positions and 92.7% of 5S rDNA homologous positions showed sister chromatid alignment. Furthermore, the number of centromeric FISH signals follows the same distribution in 2C and 4C leaf nuclei (Table 2), indicating that other sister centromeres should also be aligned in most cases.

Discussion

Evolution of the A. lyrata *karyotype and its tandem repeats*

Based on comparative genetic mapping (Kuittinen *et al.*, 2004; Yogeeswaran *et al.*, 2005) and previous (Lysak *et al.*, 2006) and current FISH results, we provide a detailed description of the eight chromosomes of *A. lyrata* ssp. *lyrata*. According to the currently accepted Brassicaceae phylogeny (Al-Shehbaz and O'Kane, 2002; Koch *et al.*, 2000, 2001), comparative mapping (Boivin *et al.*, 2004; Koch and Kiefer, 2005; Kuittinen *et al.*, 2004; Yogeeswaran *et al.*, 2005) and comparative painting data (Lysak *et al.*, 2006), the karyotypes of *A. lyrata* and *Capsella rubella* resemble the karyotype of a hypothetical ancestor of the Arabidopsis lineage with $n = 8$ chromosomes. Only one of the two *A. thaliana* NORs (on AT2) coincides positionally with one of the five *A. lyrata* NORs (on AL5). The single 5S rDNA locus of *A. lyrata* (on AL4) does not coincide with either of the three or four 5S rDNA loci of the *A. thaliana* karyotype (Fransz *et al.*, 1998). This is in line with the high degree of polymorphism of 5S and 45S rDNA loci reported for several groups of organisms (for higher plants see, e.g. Ali *et al.*, 2005; Fuchs *et al.*, 1998;

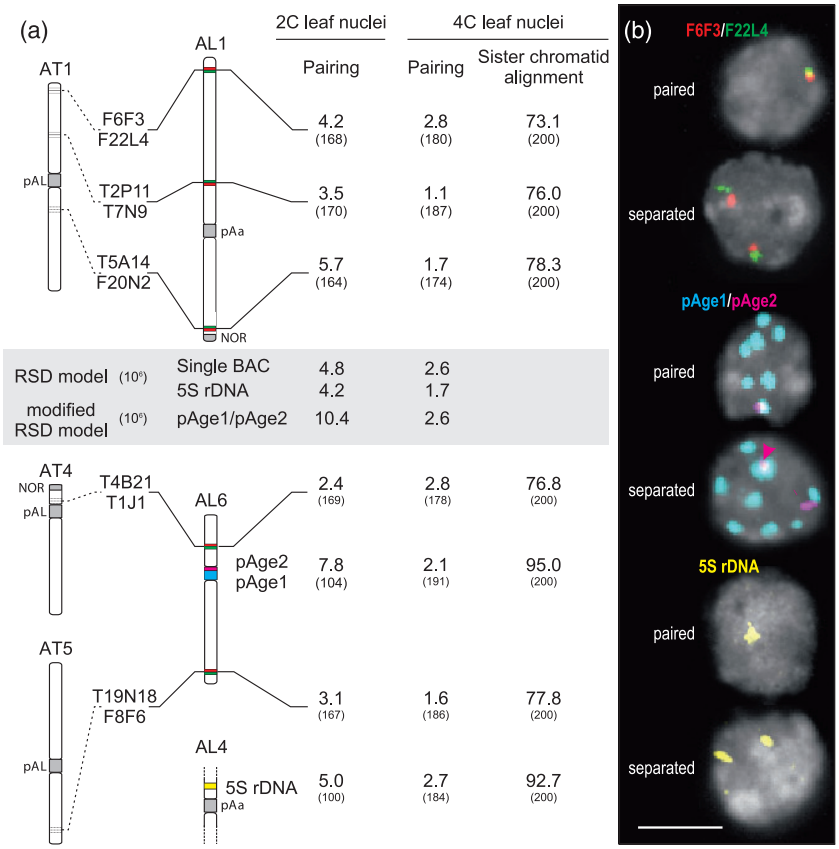

Figure 6. Positional somatic homologous pairing and sister chromatid alignment in *Arabidopsis lyrata* nuclei.
(a) The pairing frequency (% per nucleus) in 2C and 4C spherical leaf nuclei and the sister chromatid alignment (% per homologue) in 4C spherical leaf nuclei was analysed by FISH with differentially labelled BAC probes from seven positions along *A. lyrata* chromosomes AL1, AL4 and AL6 (number of evaluated nuclei in parentheses). The χ^2-test revealed no significant differences ($P > 0.05$) between simulated (grey background) and observed pairing frequencies.
(b) Homologous pairing or separation of two adjacent BAC positions (top), of centromeric (middle) and of 5S rDNA sequences (bottom) in *A. lyrata* 2C nuclei. Bar = 5 μm.

Schubert and Wobus, 1985). Therefore, a direction of karyotype evolution cannot be derived from rDNA positions.

The integration of pAa, pAge1 and pAge2 centromeric repeats into the physical and the genetic map of *A. lyrata* by FISH should be helpful to interpret the events of karyotype evolution. Since the evolutionarily young karyotype of *A. thaliana* revealed only one type of centromeric repeat (pAL1), resembling more the pAa than the pAge1 or pAge2 sequences (see Supplementary Figure S1), it is of interest to know whether the ancestral karyotype of the Arabidopsis lineage possessed one (as *A. thaliana*) or more (as *A. lyrata*) types of such repeats. For four of the five *A. thaliana* centromeres, the corresponding positions in the *A. lyrata* karyotype revealed pAa sequences (Figure 1h; Lysak *et al.*, 2006). For the centromere of AL5, corresponding to the AT3 centromere of *A. thaliana*, no pAa but pAge1 sequences were detectable by FISH. The same was true for the homologous centromeric region within the ssp. *petraea* and other closely related species all with eight chromosome pairs, such as *A. halleri* and *Arabidopsis cebennensis* (A. Berr, unpublished data). According to the phylogenetic relationship between previously reported centromeric repeats (Hall *et al.*, 2005; Kamm *et al.*, 1995; Kawabe and Nasuda, 2005; Martinez-Zapater *et al.*, 1986) and the newly isolated ones, the pAa-like repeat is probably the eldest centromeric repeat within the Arabidopsis genus (Figure 2, Supplementary Figure S1). Together, these data suggest that pAL1, pAge1 and more recently pAge2 repeats evolved during the last approximately 10 Myr from an ancestral pAa-like sequence after the divergence of *A. thaliana*, *A. lyrata* and *A. halleri* from their common ancestor.

Interphase CT and chromatin arrangement are conserved between A. lyrata *and* A. thaliana

The side-by-side arrangement was random for 11 homologous and heterologous CT combinations of six *A. lyrata* chromosomes. Similar observations were made for all possible combinations in *A. thaliana* nuclei. The two NOR-bearing chromosomes investigated (AL3 and AL5) showed a significantly more frequent association of both arms than expected at random, resembling the arrangement of NOR-bearing chromosomes 2 and 4 in *A. thaliana* (Pecinka *et al.*, 2004). This non-random CT arrangement in both species might be due to frequent association of all NORs to a single nucleolus in >90% of nuclei. This is further supported by the observation that the short arm of chromosome AL6 showed a random association frequency with its homologue, while the homologous regions of the NOR-bearing chromosome AT4 are more often associated. Similar to the situation in *A. thaliana* (Pecinka *et al.*, 2004), the observed as well as the predicted association values between *A. lyrata* CTs

increased proportionally with their respective chromosome size and the observed values for homologous/heterologous CT association and for association of homologous chromosome arm territories were usually highest in round-shaped, intermediate in spindle-shaped and lowest in rod-shaped nuclei.

Apparently, a transient positional homologous pairing occurs no more often than at random along the euchromatic chromosome arms in *A. lyrata* as well as in *A. thaliana* somatic nuclei (Pecinka *et al.*, 2004). Also for two tandem repetitive heterochromatic regions, (i) the unique 5S rDNA locus and (ii) the unique pAge1/pAge2 centromeric region, a random pairing frequency was observed in *A. lyrata* nuclei. Interestingly, in *A. thaliana*, homologous transgenic tandem repeats (256 tandemly arranged *lac*O repeats = 10 kb) pair six to seven times more often than non-repetitive loci, and this higher pairing frequency seems to be correlated with strong CG methylation of those repeats (Pecinka *et al.*, 2005; Watanabe *et al.*, 2005). The reason for the different pairing frequency of the transgenic repeat arrays in *A. thaliana* and the endogenous ones in *A. lyrata* (e.g. 5S rDNA or centromeric repeats) is still unclear.

The alignment of sister chromatids was often found to be incomplete along euchromatic chromosome arm regions of *A. thaliana* (in 23.0–38.1% of 4C nuclei sister chromatids were not aligned; Schubert *et al.*, 2006). Similarly, at different euchromatic positions sister chromatids were not aligned in 21.7–26.9% of *A. lyrata* homologues in 4C nuclei. In *A. thaliana*, sister centromeres were apparently not separated, but it was not possible to analyse the heterochromatic regions individually. At the centromeric pAge1/pAge2 locus on chromosome AL6 and at the 5S rDNA locus on chromosome AL4, sister chromatids were not aligned in 5.0% and 7.3% of cases, respectively, in *A. lyrata* 4C nuclei. The high frequency of sister chromatid alignment at the 5S rDNA locus was probably due to its proximity to the centromere of AL4. For the pAge1/pAge2 locus, sister centromere separation might be overestimated when such 4C nuclei were also evaluated that resulted from mitotic division of 8C nuclei instead of representing the first endoreduplication step. On the other hand, the 100% sister centromere alignment in *A. thaliana* 4C nuclei (Schubert *et al.*, 2006) might be an overestimation because the presence of 10 FISH signals does not exclude that some of them represent associated centromeres and others separated sister centromeres. In any case, the alignment of sister centromeres is close to 100% and is required to ensure a proper segregation of sister chromatids during mitosis. An inhomogeneous cohesin distribution and/or different degrees of chromatin condensation along chromosomes might explain the variable alignment along interphase chromosomes (Blat and Kleckner, 1999).

In spite of the different chromosome size and chromosome number, the global CT/chromatin arrangement in *A. thaliana* (Fransz *et al.*, 2002; Pecinka *et al.*, 2004) and in *A. lyrata* nuclei is evolutionarily conserved and follows similar rules despite their divergence about 5 Mya (Koch *et al.*, 2000). Contrary to many other plant species (Dong and Jiang, 1998), neither taxon exposes a Rabl orientation of chromosomes. Rather, their centromeres are randomly distributed at the nuclear periphery and in most nuclei their NORs form a single nucleolus surrounded by most of the telomeres. Among mammals, CT arrangement has been highly conserved for higher primates over a period of about 30 Myr (Tanabe *et al.*, 2002) and for marsupials over a period of about 50–60 Myr (Greaves *et al.*, 2003). Since the *A. lyrata* karyotype is very similar to the proposed ancestral one (Lysak *et al.*, 2006), a similar arrangement of interphase chromosomes is also to be expected for other closely related diploid Brassicaceae species. A comparison of interphase chromosome arrangement and somatic homologous pairing between both studied Arabidopsis species and *Drosophila* (showing regular somatic pairing of homologues; Csink and Henikoff, 1998; Fung *et al.*, 1998) or vertebrates (showing a preferential radial arrangement, reviewed in Cremer and Cremer, 2001) suggests that phylogenetic relationship has a greater impact on the interphase chromosome arrangement than similarities in genome size, sequence organization and/or chromosome number.

Experimental procedures

Plant material, preparation of pachytene chromosomes, nuclei and extended DNA fibres

Seeds of *A. lyrata* ssp. *lyrata* (2n = 16; origin: Bash Bish, Birkshire City, MA, USA) were provided by T. Mitchell-Olds (Max Planck Institute of Chemical Ecology, Jena, Germany). The plants were cultivated in a greenhouse under 16:8 h light:dark cycle. Interphase nuclei of 2C and 4C DNA content were isolated from young rosette leaves and flow-sorted as described (Pecinka *et al.*, 2004). Pachytene chromosomes were prepared from fixed immature flower buds according to Lysak *et al.* (2001). Extended chromatin fibres were prepared as described (Fransz *et al.*, 1996).

Cloning and sequencing of A. lyrata *centromeric repeats*

The three different approximately 180-bp centromeric repeat types were amplified via PCR from *A. lyrata* genomic DNA as described (Kawabe and Nasuda, 2005). The PCR products were fractionated by electrophoresis on 1% agarose gel. The amplification bands corresponding to dimers and trimers of the basic repeat units (approximately 360 bp and approximately 540 bp, respectively) were isolated using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, cloned into pCR®2.1-TOPO (Invitrogen, Karlsruhe, Germany) and sequenced with a MegaBACE® 1000 Sequencer (Amersham, Freiburg, Germany). The sequences were analysed and aligned according to the primer sequences used for PCR delineating the first and last nucleotides.

Phylogenetic analyses of centromeric repeat sequences

The sequences obtained in this study were aligned alone or together with centromeric repeat sequences of other Arabidopsis taxa (Hall *et al.*, 2005; Kamm *et al.*, 1995; Kawabe and Nasuda, 2005; Martinez-Zapater *et al.*, 1986) with CLUSTALX software. The alignment was adjusted manually and analysed with phenetic and cladistic analysis algorithms in PAUP* version 4b10 (Swofford, 2002). Pairwise genetic maximum-likelihood distances were calculated and an unrooted tree was obtained from neighbor-joining cluster analysis (NJ). A maximum parsimony analysis (MP) was conducted according to the two-step search strategy (Blattner, 2004) with the heuristic search algorithm implemented in PAUP*, restricting the number of most parsimonious trees to 20 000. Statistical support of the branches was evaluated with 500 (in NJ) and 10 000 fast-and-stepwise (MP) bootstrap re-samples of the data set.

Probe labelling, fluorescent in situ *hybridization and image processing*

The *A. thaliana* BACs used for FISH were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). Individual BACs were arranged as pools suitable for painting individual *A. lyrata* chromosomes/chromosome arms according to the corresponding linkage groups as defined by comparative genetic mapping (Kuittinen *et al.*, 2004; Yogeeswaran *et al.*, 2005). The list of BACs used for painting of *A. lyrata* chromosomes will be provided by the authors upon request.

Isolation of BAC DNA, labelling by nick translation or rolling circle amplification and FISH were performed as described (Berr and Schubert, 2006; Pecinka *et al.*, 2004). The 5S rDNA-specific probe was amplified by PCR from *A. lyrata* genomic DNA using primers designed according to the 5S sequence of *Glycine* species (Gottlob-McHugh *et al.*, 1990). Probes specific for the four centromeric repeat families (pAL1, pAa, pAge1 and pAge2) were prepared from PCR products. The telomere-specific probe was generated by PCR in absence of template using primers $(TAAACCC)_7$ and $(GGGTTTA)_7$ (Ijdo *et al.*, 1991).

Fluorescence signals were analysed using an Axioplan 2 (Zeiss, Oberkochen, Germany) epifluorescence microscope equipped with a cooled CDD camera (Spot 2e Diagnostic Instruments, Sterling Heights, MI, USA). Images were captured separately using appropriate excitation and emission filters. Single plane images and stacks of optical sections through nuclei were acquired with MetaVue (Universal Imaging, West Chester, PA, USA). Then, images were pseudo-coloured, processed and merged using ADOBE PHOTOSHOP 6.0 software (Adobe Systems, San Jose, CA, USA).

Computer model simulations of interphase CT and approximately 100 kb chromosome segment arrangement

Prior to model simulations, the average volume was determined on the basis of three-dimensional deconvolution image stacks for *A. lyrata* 2C and 4C spherical leaf nuclei, for approximately 100 kb segment FISH signals in *A. lyrata* 2C and 4C nuclei, for 5S rDNA FISH signals in *A. lyrata* 2C and 4C nuclei and for centromeric FISH signals (pAL1 in *A. thaliana* 2C leaf nuclei and both pAa and pAge1 in *A. lyrata* 2C and 4C nuclei, see Supplementary Table S4). The volume of *A. thaliana* 2C leaf nuclei (25.7 μm^3) was taken from Pecinka *et al.* (2004).

The SCD model (Cremer *et al.*, 2001; Kreth *et al.*, 2004) was used to simulate the random associations of entire chromosomes/chromosome arms in virtual *A. lyrata* 2C nuclei. Individual CTs were considered as a chain of spherical 1-Mb domains, corresponding in number to the DNA content of each chromosome (see Table 1). The RSD model (Pecinka *et al.*, 2004) was used to simulate random pairing of homologous approximately 100 kb segments (single BAC FISH signals), 5S loci and unique pAge1/pAge2 clusters of centromeric segments. The coordinates of two spheres representing homologous chromosomal regions were randomly determined in virtual 2C and 4C *A. lyrata* nuclei. Homologous spheres were considered to be paired when they either overlapped or when the distance between their edges was <100 nm (i.e. below the resolution of conventional optical microscopy). To assess the random spatial distribution of centromeric repeats in a peripheral nuclear layer as reported for *A. thaliana* by Fransz *et al.* (2002), the RSD model was modified. Chromocenters were simulated as 10 pAL1 spheres for *A. thaliana* and as eight pAge1 spheres and eight pAa spheres for *A. lyrata* nuclei within a peripheral layer with a depth corresponding to the diameter of an average centromeric FISH signal in 10^6 round-shaped virtual 2C nuclei of both species. A cluster of two or more spheres in direct contact was considered to be equal to a single centromeric FISH signal (one FISH signal/chromocenter may comprise more than one centromeric region).

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.

Acknowledgements

We thank Professor R. Rieger for critical reading of the manuscript and M. Kühne, R. Schubert and J. Bruder for technical assistance. This work was in part supported by a DFG grant to I.S. (Schu 951/10-1).

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Multiple alignments and consensus sequences of centromeric repeats of the genus Arabidopsis.

Table S1 Size of individual *A. thaliana* chromosomes as drawn in Figure 1h (Mb)

Table S2 Percentage of identity between the single ~180 bp centromeric reports cloned from *A. lyrata*

Table S3 Association frequencies of homologous arm territories in 2C nuclei of different shape from *A. lyrata* leaves

Table S4 Average dimensions and volumes of the different elements used for computer simulation

This material is available as part of the online article from http://www.blackwell-synergy.com

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All sequences newly determined in this article can be found in the EMBL Database under accession numbers AM1777597–AM177606.

