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RESEARCH ARTICLE

Size and number of tandem repeat arrays can determine somatic homologous pairing of transgene loci mediated by epigenetic modifications in *Arabidopsis thaliana* nuclei

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Abstract The chromosomal arrangement of different transgenic repeat arrays inserted at various chromosomal positions was tested by FISH in *Arabidopsis* 2C leaf and root nuclei. Large *lacO* (~10 kb) but not *tetO* (4.8 kb) or small *lacO* (~2 kb) arrays were, in general, more often spatially associated with heterochromatic chromocenters (CC) than flanking regions (that either overlap the array insert position or are between 5 and 163 kb apart from the insert site). Allelic and ectopic pairing frequencies of *lacO* arrays were significantly increased only in nuclei of lines with two large *lacO* arrays inserted at different positions on the same chromosome arm. Within the same lines, root nuclei showed a significantly lower increase of pairing frequencies at the insert position compared to leaf nuclei but still a higher frequency than in the wild-type situation. Thus, the frequencies of homologous pairing and association with heterochromatin of transgenic repeats may differ with the construct, the chromosomal insertion position, the cell type and with the number and repetitiveness of inserts. Strong CpG methylation is correlated with a high frequency of homologous pairing at large repeat array loci in somatic cells but has no impact on their association with CCs. These results show that single low-copy arrays apparently do not alter interphase chromatin architecture and are more suitable for chromatin tagging than multiple high copy arrays.

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Introduction

The arrangement of chromosomes and of distinct chromatin domains in interphase nuclei is considered to have an impact on replication, transcription, and repair (for review see Taddei et al. 2004; Tessadori et al. 2004; Bartova and Kozubek 2006; Cremer and Cremer 2006; Kruhlak et al. 2006; Espada and Esteller 2007). The gross chromosome arrangement may nevertheless differ remarkably between phyla of higher eukaryotes (survey in Pecinka et al. 2004). Two main routes to study chromosome/chromatin arrangement in interphase nuclei use either fluorescent in situ hybridization (FISH) on fixed nuclei (Fransz et al. 2002; Pecinka et al. 2004, 2005; Berr et al. 2006) or fluorescent protein tags in living cells (Kato and Lam 2001, 2003; Thomann et al. 2002; Matzke et al. 2003, 2005). Both

approaches allow determination of the position and mobility of defined chromosomal loci (Lam et al. 2004).

Chromosomes within fixed interphase nuclei of *Arabidopsis thaliana* are mostly randomly arranged. Homologous pairing of ~100 kb regions along different chromosomes of *A. thaliana* accession Columbia occurs on average in about 5% of the somatic nuclei, corresponding with the random expectation from model simulations (Pecinka et al. 2004). However, T-DNA insertions, comprising a *lac*-operator (*lacO*)/GFP-Lac repressor tagging system, altered the local chromatin arrangement within *A. thaliana* leaf nuclei of the transgenic line EL702C. In this line, chromatin regions harboring tandem repetitive *lacO* sequences paired more frequently and were more often associated with heterochromatic chromocenters than the respective regions of wild-type nuclei (Pecinka et al. 2005). The pairing frequency was also significantly higher than the average positional pairing (~5%; Pecinka et al. 2005). The association with chromocenters was increased, while the peripherally localized centromeric chromocenters themselves fuse less frequently than expected (Berr et al. 2006) apparently because of the spatial constraints exerted by the arms on either side of the centromere. Similar results were seen for the large transgenic multicopy hygromycin phosphotransferase (HPT) locus as well (Mittelsten Scheid et al. 1991, 1998; Probst et al. 2003). Somatic pairing and association with heterochromatin of the HPT locus in *Arabidopsis* nuclei occurred significantly more often than expected (Pecinka et al. 2005). The transgenic *lacO* repeats in *A. thaliana* line EL702C are strongly methylated. This methylation is correlated with frequent homologous pairing of the transgenic loci, but not with heterochromatin association (Watanabe et al. 2005). In contrast to the observations on fixed leaf nuclei of EL702C, in vivo fluorescent chromatin tagging using similar T-DNA constructs (*lacO* or *tet*-operator sequences) did not show enhanced pairing of the transgene-bearing chromatin regions in *Arabidopsis* root nuclei (Matzke et al. 2005). In budding yeast, *tetO* repeats affect the architecture of interphase nuclei and associate frequently. This association occurred irrespective of their chromosomal context but was dependent on the presence of Tet repressor molecules (Fuchs et al. 2002).

To search for an interpretation of the contradictory results as to the homologous pairing frequency of chromatin tag sites in *Arabidopsis* and to obtain a more detailed insight regarding the possible effects of tandem repeat arrays on nuclear architecture, we performed investigations on several single and double transgenic lines harboring large or small *lacO* arrays, 9.3 or ~2 kb, respectively, or *tetO* (4.8 kb) repeat arrays. We traced by FISH homologous positional pairing of the insert sites and their association frequency with heterochromatin in flow-sorted 2C leaf and root nuclei of different transgenic lines. Furthermore, we compared the DNA methylation state of the repeat arrays that displayed either "normal" or increased pairing frequency. For four different transgenic lines (harboring short *lacO* repeat arrays) distributed in a ~100-kb region adjacent to the NOR of chromosome 2, no significant increase of homologous pairing or association with heterochromatin was observed by testing with a flanking bacterial artificial chromosome (BAC).

Material and methods

Plant material and preparation of nuclei

Plants of the homozygous lines EL702C (Kato and Lam 2001), CCP4.193; CCP4.211, CCT383, CCT431 and CCT432 (http://charting.cshl.org/; http://aesop.rutgers.edu/~lamlab/pccharting.html) or of homozygous transgenic lines 26, 125, 125/26, 25/123 and 106/112 (Matzke et al. 2005) were cultivated under long day conditions. After fixation of leaves or roots in 4% paraformaldehyde for 30 min, 2C nuclei were flow-sorted as described (Jasencakova et al. 2000).

Probe labeling and FISH

The following flanking or overlapping BAC insert clones were used as probes to trace transgenic inserts: of chromosome 1—T30E16 (GenBank accession number AC009317), of chromosome 2—F2I9 (AC005560), F3P11 (AC005917), and F11F19 (AC007017), of chromosome 3—MGL6 (AB022217) and F18C1 (AC011620), of chromosome 4—T1J1 (AF128393) and T10C21 (AL109787), of chromosome 5—F22D1 (AF296834) and K21L19 (AB024029); T15P10 containing 45S rDNA (AF167571) and plasmids containing the 180-bp centromeric repeat (pAL1, Martinez-Zapater et al. 1986) and 128× *lacO* (Kato and Lam 2001), respectively.

For FISH, slides were rinsed 3×5 min in 2× SSC (0.3 M NaCl and 0.03 M sodium citrate), treated for 2 min with pepsin (50 μg/ml 0.01 M HCl) at 38°C, rinsed 3×5 min in 2× SSC, and post-fixed for 10 min in 4% formaldehyde. Subsequently, slides were washed in 2× SSC for 3×5 min, dehydrated in 70% and 96% ethanol, for 2×5 min each, and air-dried. The BAC and plasmid DNAs were labeled with digoxigenin-dUTP, biotin-dUTP, or Cy3-dUTP using a nick translation kit (Roche) according to manufacturer's instruction. The labeled BACs were precipitated and resuspended in 25-μl hybridization buffer (50% formamide, 10% dextran sulphate, 2× SSC, 50 mM sodium phosphate, pH 7.0), dropped on the slide, covered with a cover slip, and sealed with "fixogum" before denaturation for 3 min at 80°C. Then, slides were transferred into a wet chamber and hybridized overnight at 37°C. Post-hybridization washes were 3×5 min in SF50 (50% formamide/2× SSC), 2×5 min in 2× SSC, and 5 min at 42°C in 4T (4× SSC pH 7.0 with 0.05% (*v*/*v*) Tween 20). After blocking in 3% bovine serum

albumine (BSA), 0.1% Tween 20, 4× SSC for 30 min at 37°C, digoxigenin-dUTP was detected by mouse anti-digoxigenin (1:250; Roche) and Alexa 488-conjugated goat anti-mouse antibody (1:200; Molecular Probes). Biotin-dUTP was detected by avidin conjugated with Texas Red (1:1,000; Vector Laboratories), biotin-conjugated goat anti-avidin (1:200; Vector Laboratories), and again with Texas Red-conjugated avidin, each for 30 min at 37°C. Cy3-dUTP was evaluated directly. Counterstaining of nuclei was performed with 1 μg/ml DAPI in Vectashield (Vector Laboratories).

Microscopic evaluation and image processing

The nuclei were analyzed using a fluorescence microscope "Axiophot 2" (Zeiss) and appropriate filters. Images were captured using a cooled charge-coupled device camera (Spot 2e, Diagnostic Instruments) connected with MetaVue (Universal Imaging) software. The monochromatic images were pseudocolored and merged using Adobe Photoshop 7.0 (Adobe systems). Allelic vs ectopic pairing of transgenic loci was evaluated by studying the number and arrangement of FISH signals for flanking BAC inserts and/or transgenic repeat arrays. The spatial association of the *lacO* region or the flanking BAC with heterochromatic chromocenters (CCs) was quantified as proportion of FISH signals from the insert regions that overlap with signals for the chromocenters (either DAPI or pAL + 45S rDNA FISH signals; Fig. 1).

Southern analysis

Genomic DNA was isolated from leaves or roots. Fine powder of plant tissue was suspended in 2% CTAB solution (2% cetyl trimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl pH 8.0) and incubated at 60°C for 30 min. After chloroform extraction, the aqueous phase was mixed with an equal volume of ice-cold isopropanol, and DNA was precipitated by centrifugation. The pellet was resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0) containing RNaseA.

The DNA samples digested with restriction enzymes (Fig. 2) were separated by agarose gel electrophoresis and blotted onto a nylon membrane (Hybond-N^+, GE Healthcare). Hybridization was performed in Church buffer (0.25 M Na-phosphate pH 7.2, 1% BSA, 7% SDS, 1 mM EDTA). Radio-labeled probes were generated by random priming using DecaLabelTM DNA labeling Kit (Fermentas). In the case of *lacO*, the template for random-primed DNA synthesis was prepared by excision of the insert of the clone "128× *lacO*/SK" (Kato and Lam 2001). In the case of *tetO*, a template containing multiple *tetO* units was prepared by ligation of *tetO* monomers with cohesive ends that had been obtained by annealing the oligo DNA fragments *tetO* top (5′-Pho-GATCTTTTACCACTCCC TATCAGTGATAGAGAAAAGTGAAAG-3′) and *tetO* bot (5′-Pho-GATCCTTTCACTTTTCTCTATCACTGATAGG GAGTGGTAAAA-3′). Hybridized DNA fragments were detected by autoradiography.

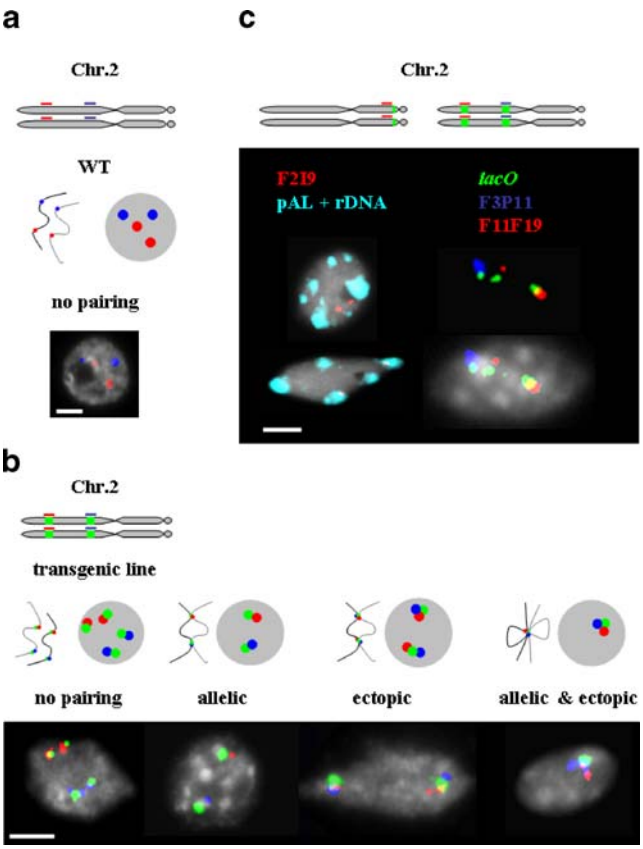

Fig. 1 Evaluation of homologous pairing and association with heterochromatin for transgenic tandem repeats and their flanking regions: **a** unpaired BAC insert regions in wild-type nuclei and **b** pairing configurations in transgenic lines (exemplified for the double transgenic line 125/26), respectively. The *lacO* arrays are in *green* and flanking BACs in *red* or *blue*. **c** Evaluation of association with heterochromatic chromocenters. *Left* BAC F2I9 flanking a 2-kb *lacO* insert on the top arm of chromosome 2 is in *red* and the heterochromatic chromocenters are represented by FISH signals for 180-bp centromeric repeats (*pAL*) and for 45S *rDNA* in *light blue*. *Above* no association of unpaired F2I9 signals. *Below* association of unpaired F2I9 signals with chromocenters (CCs). *Right* flanking BACs F3P11 and F11F19 and two *lacO* inserts (9.3 kb each) are in *blue*, *red*, and *green*, respectively. CCs are represented by strong DAPI staining in the *bottom panel*; *lacO* signals are associated with CCs; BAC signals are not associated with CCs. *Bar*=3 μm

Results and discussion

Tandem repeat arrays of different transgenic lines may vary as to their somatic pairing frequency

To investigate whether tandem repeat arrays generally mediate alterations of chromatin arrangement in 2C interphase nuclei of *A. thaliana*, we tested different

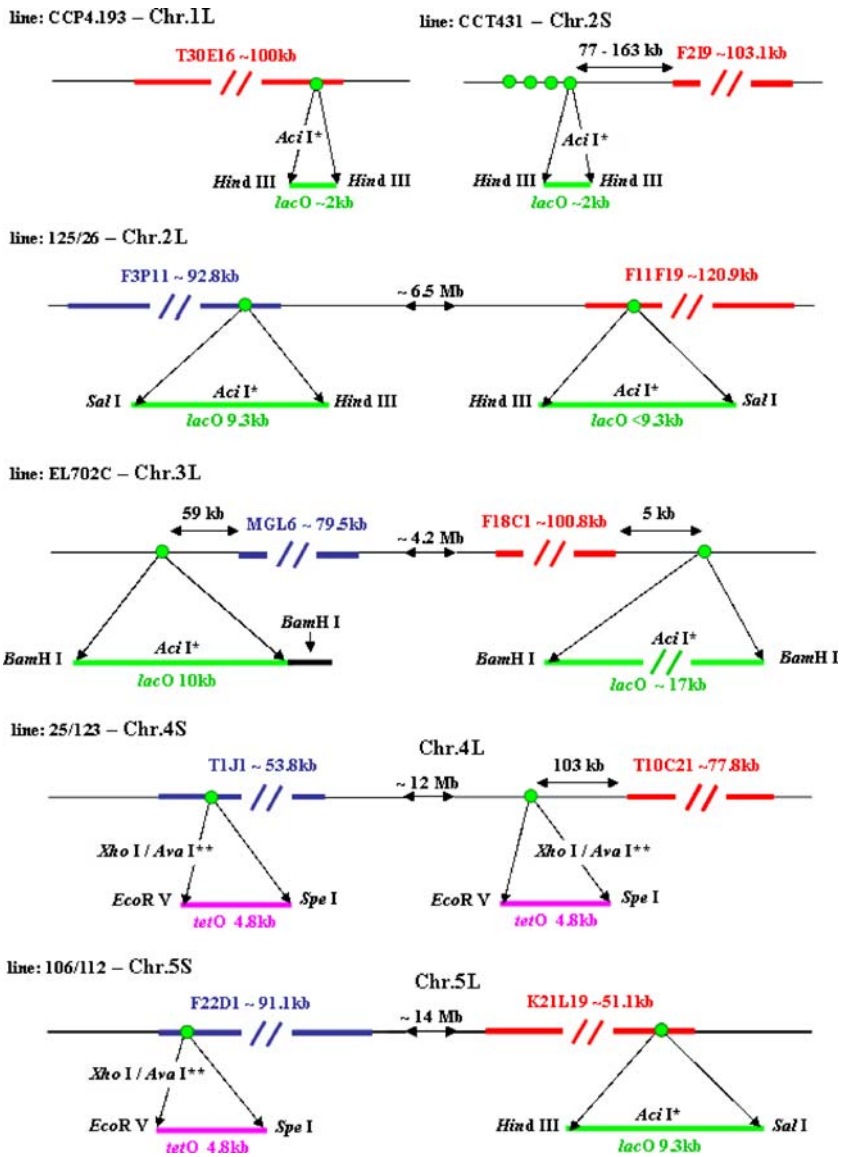

Fig. 2 Spatial arrangement of transgenic repeats and corresponding flanking BACs on different *Arabidopsis* chromosomes. The *green circles* on chromosome 2 (chr.2S) mark the insert positions within the four independent transgenic lines CCP4.193, CCT341, CCT383, and CCT432. Positions of restriction sites, sizes of flanking BACs, and their distances from transgene insertion sites are indicated. The *Eco*RV site locates 3.4-kb upstream from the 5′ end of the *tetO* array. *Asterisk* complete *Aci*I digestion of *lacO* arrays would yield 36-bp fragments. *Double asterisk* complete *Ava*I digestion of *tet*O arrays would yield 420-bp fragments

transgenic lines containing single or double *lacO* or *tetO* inserts of different size (2, 4.8, 9.3, or >10 kb) at different chromosomal positions (Fig. 3a). Transgenic *lacO* and *tetO* repeat arrays were traced by FISH for evaluation of homologous pairing (Fig. 1a,b) and association with heterochromatin (Fig. 1c). To avoid confusion with the positional sister chromatid separation which occurs at *lacO* arrays of line EL702C in ~40–60% of cases (Schubert et al. 2006), we did not extend this study to 4C nuclei. As probes, we used *lacO* repeats combined with differently labeled BAC clones carrying the sequences that flank the insertion sites of the transgene or, in case of repeat arrays of less than 9 kb, only BAC clones for the flanking sequences. Larger extension of FISH signals (because of larger probes) should have no significant impact on the estimation of pairing frequency (because of signal overlap), because for some of the 9.3 kb arrays, pairing is more frequent than for the approximately ten-fold larger flanking BACs (Fig. 3b). The pairing frequency of all transgene insertion positions was compared with that of the corresponding wild-type loci and with the "average pairing" in 2C leaf nuclei based on the pairing frequency of various chromosome positions (Pecinka et al. 2004).

For the individual transgenic lines containing a 2-kb *lacO* array, the pairing frequency of the flanking BACs (see Fig. 2) did not significantly differ from that observed in the wild-type or from average pairing frequency. The four lines, each with the 2-kb *lacO* array within a 100-kb region of chromosome 2, display different properties. CCP4.211 and CCT383 both have low transcriptional activity of a 35S: luciferase reporter gene, while CCT431 and CCT432 have higher levels of activity in leaf and root cells (F. Rosin and E. Lam, unpublished). Apparently, these differences have no impact on the pairing frequency of the 2-kb *lacO* array

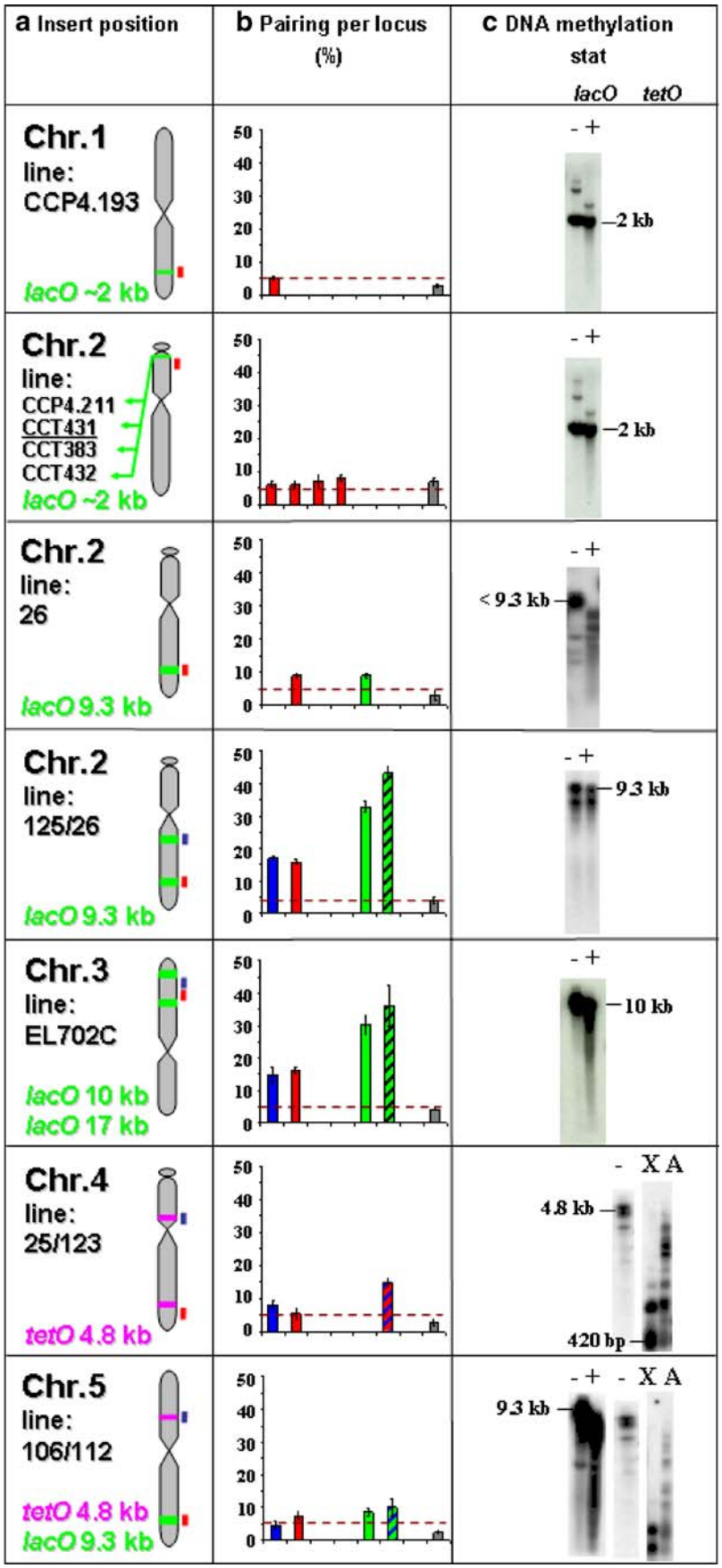

Fig. 3 Homologous pairing of the transgenic repeats and/or their flanking regions. **a** Insert positions are marked by *horizontal green* (*lacO*) or *pink* (*tetO*) *bars* and flanking BACs by *red* or *blue vertical bars* on the *right side* of the chromosomes. **b** Frequencies of somatic homologous pairing. The *color* of columns indicates the type of probes as in (**a**). The average ectopic pairing per locus is shown by *hatched columns*. In case of two *lacO* arrays, *green columns* show the average allelic pairing frequency per locus. The wild-type situation is shown in *gray* (pooled in cases of 2 BACs). The *horizontal dashed line* indicates the average pairing frequency over the genome. *Bars* indicate the standard deviation. Data for line EL702C are from Watanabe et al. (2005). **c** Southern hybridization of genomic DNA with the corresponding tandem repeats after specific digestion. The *underlined transgenic line* CCT431 was used for Southern hybridization. *Minus sign* without and *plus sign* with digestion with methylation-sensitive enzymes: Excised *lacO* arrays are digested with *Aci*I; excised *tetO* arrays were digested with a less (*Xho*I = *X*) and a more (*Ava*I = *A*) methylation-sensitive enzyme

in leaf nuclei as indicated by values for the proximally flanking region (Fig. 3). No significant difference of the pairing frequency from that observed in the wild type or from average pairing frequency was found also for the double transgenic line 25/123 with two 4.8-kb *tetO* arrays (one on the top arm near the centromere and another one on the bottom arm of chromosome 4) and for the line 106/112 with a 4.8-kb *tetO* array on the top arm and a 9.3-kb *lacO* array on the bottom arm of chromosome 5 (Fig. 3). A significant enhancement of allelic and ectopic pairing frequency was observed for line 125/26 with two 9.3 kb *lacO* arrays (both on the bottom arm of chromosome 2). Similar observations were made for the transgenic line EL702C with two insertion loci on the same arm of chromosome 3 (Pecinka et al. 2005). When the *lacO* insert positions of chromosome 2 were investigated separately in the corresponding single transgenic lines 26 (Fig. 3b) and 125 (data not shown), their pairing frequency was not significantly enhanced. It might therefore be possible that more than one (large) insert on the same chromosome arm favors an increase of pairing frequency, particularly within leaf nuclei. Whether the absence of an increased pairing frequency in case of the two *tetO* arrays on chromosome 5 is because of the sequence itself, because of the larger distance between the *tetO* arrays than between the *lacO* arrays on chromosomes 2 and 3, or rather because of the smaller insert size (4.8 kb) remains unclear. Remarkably, in all cases where the average homologous pairing per array locus was significantly higher than in wild-type situation, the flanking BACs displayed an intermediate pairing frequency between that of the array and that of the region in wild-type plants, previously discussed as a dragging effect (Pecinka et al. 2005).

Contrary to our previous results on leaf nuclei of the line EL702C (Pecinka et al. 2005), the fluorescent protein tags in other *Arabidopsis* transgenic lines rarely displayed homologous pairing in living root nuclei (Matzke et al. 2005). We, therefore, examined the arrangement of repeat arrays in root and leaf nuclei of lines 125/26 and EL702C (Fig. 4). Compared to the region in wild-type nuclei, we found an increase in allelic and ectopic pairing of the large *lacO* arrays of the double transgenic lines 125/26 and EL702C in 2C root nuclei; however, the increase was significantly ($P<0.001$) lower than that observed in leaf nuclei (Fig. 4). These results suggest that somatic homologous pairing frequency of the large *lacO* arrays may be influenced by tissue specificity.

CpG methylation apparently correlates with homologous pairing at large but not at short repeat array loci

Our previous research with line EL702C indicated a correlation between DNA methylation of the *lacO* repeat array and homologous pairing frequency (Watanabe et al. 2005). To increase our understanding of the mechanism(s) controlling homologous pairing between the *lacO* repeat arrays, the DNA methylation state of the different *lacO* and *tetO* arrays in leaf nuclei and of the large *lacO* arrays of lines 125/26 and EL702C in leaf and root nuclei was analyzed. Southern hybridization results after digestion of genomic DNA prepared from leaves of all lines investigated are shown in Fig. 3. Lanes marked with (−) show the entire array excised from genomic DNA with restriction endonucleases as indicated in Fig. 2. In lanes marked with (+), the array was further digested with a methylation-sensitive enzyme as described (Fig. 2). Genomic DNA of line 25/123 was first digested with *Eco*RV and *Spe*I to excise the entire *tetO* array, followed by *Xho*I or *Ava*I to cleave the *tetO* array into 420-bp fragments. *Xho*I is impaired and *Ava*I is blocked by methylation at CpG within their recognition site (CTCGAG). Both *Xho*I and *Ava*I digested the *tetO* arrays of line 25/123 into 420-bp fragments. However, the digestion with *Ava*I was less complete than that by *Xho*I, indicating at least a partial methylation of the *tetO* arrays (Fig. 3c). The same was true for the *tetO* array on the top arm of chromosome 5 in line 106/112.

The DNA methylation state of the *lacO* repeat array was first analyzed in transgenic lines 26, 125, and 106/112, harboring a single array of *lacO* repeats (Matzke et al.

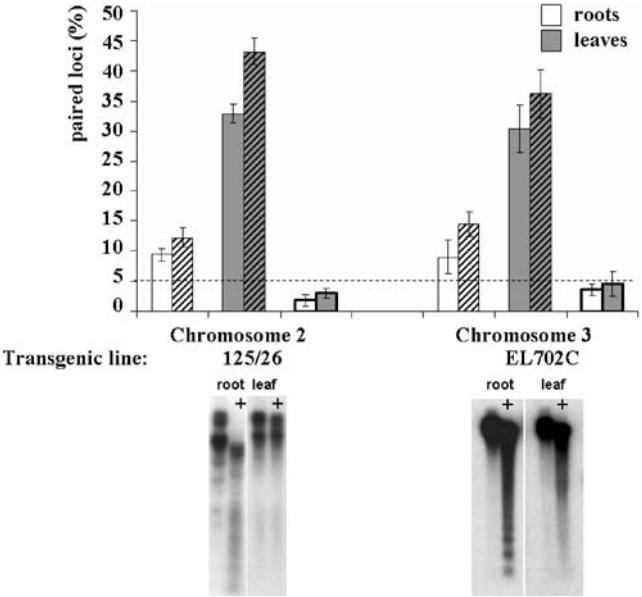

Fig. 4 Homologous pairing in 2C nuclei of ≥9.3-kb *lacO* arrays inserted at two positions of the *bottom arm* of chromosome 2 (line 125/26) or of the *top arm* of chromosome 3 (line EL702C) of *A. thaliana*. Allelic pairing is shown by *plain-colored columns*; ectopic pairing by *hatched columns*. *Strongly enframed columns* represent values for flanking BACs under wild-type condition; the *dashed horizontal line* indicates the average pairing frequency. Different methylation states in roots and leaves are shown in the *lower part* of the figure. *Plus sign* indicates digestion with the methylation-sensitive enzyme (*Aci*I)

2003, 2005), and in the double transgenic lines 125/26 and 106/112 harboring either one or two *lacO* arrays. Genomic DNA was first digested with *Hin*dIII and *Sal*I to excise the entire *lacO* repeat array of 9.3 kb and then with *Aci*I, which is blocked by CpG methylation within its recognition site (CCGC) and digests unmethylated *lacO* arrays into their 36-bp repeat units. In line 26, digestion with *Hin*dIII and *Sal*I to excise the *lacO* array resulted in fragments smaller than 9.3 kb. Fragments of similar size also appeared after digestion of DNA from line 125/26 with two arrays, in addition to the expected fragments of 9.3 kb (Fig. 3c). The smaller fragments probably result from rearrangements within individual plants that reduce the size of the *lacO* arrays within the corresponding lines. Similar types of rearrangement in these lines were observed by Matzke et al. (2008). *Aci*I digestion of genomic DNA actually produced DNA fragments smaller than the entire length of the *lacO* array in all lines with a single *lacO* array of originally 9.3 kb, although these fragments were larger than 36 bp. Lack of digestion by *Aci*I indicates that the two *lacO* arrays of line 125/26 have a higher degree of methylation than the array of line 26. Next, the DNA methylation state of the ~2 kb *lacO* arrays of lines CCP4.193 and CCT431 and of the large arrays of line EL702C was tested. In this set of lines, the *lacO* array was constructed in the same way but via a different vector from that of lines 125, 26, and 112. To excise the entire *lacO* array of line EL702C, genomic DNA was digested with *Bam*HI. In case of line CCP4.193 and for line CCT431, *Hin*dIII was used for this purpose. *Aci*I was used to cleave unmethylated *lacO* arrays into their 36-bp repeat units. *Aci*I did not digest the *lacO* arrays of lines CCP4.193, CCT431, and EL702C (Figs. 3c and 4). These results indicate that the *lacO* arrays are highly methylated in the lines with a single ~2 kb *lacO* array (CCP4.193 and CCT431) as in lines with two large arrays on one chromosome arm (125/26 and EL702C). Thus, in the leaf nuclei, DNA methylation apparently correlates with homologous pairing at sites of large (~10 kb) *lacO* arrays but not at sites of smaller *tetO* or *lacO* arrays. Interestingly, high CG methylation and frequent homologous pairing was observed when two large *lacO* arrays are combined on one chromosome arm (lines 125/26 and EL702C), while the single arrays either on chromosome 5 of line 106/112 or on chromosome 2 of lines 26 and 125 revealed neither strong methylation nor frequent pairing (Fig. 3b,c). Transgenic tandem repeats of lines 125/26 and EL702C pair ~3.5-fold more often in leaf than in root nuclei (Fig. 4). Digestion with *Aci*I indicates that the methylation density of *lacO* arrays is lower in root than in leaf nuclei of line 125/26. Similar results were obtained for *lacO* arrays of line EL702C (Fig. 4). Apparently, strong methylation is necessary but not sufficient to mediate a high frequency of homologous pairing in somatic cells.

Large transgenic tandem repeats associate preferentially with heterochromatic chromocenters

To estimate the association frequency of the transgenic repeat arrays with heterochromatin in flow-sorted 2C leaf nuclei, FISH signals of *lacO* arrays or of flanking BAC insert sequences overlapping with either intensely DAPI-stained CCs or, in some cases, with FISH signals for centromeric repeats and 45S rDNA, were counted (Fig. 1c). Previous studies have shown that *lacO* arrays of line EL702C associated more often with heterochromatic CCs than the flanking BACs (Pecinka et al. 2005; Watanabe et al. 2005). In conformity with these data, we found that in all lines harboring ≥9.3 kb *lacO* inserts, association with CCs occurred significantly more often for *lacO* arrays ($P<0.001$) than for flanking BAC sequences (Fig. 5b). In the case of transgenic lines harboring a 2-kb *lacO* array or a 4.8-kb *tetO* array, we observed no increase of association frequency of the flanking BAC sequences with heterochromatin at a significance level of $P<0.01$ when compared to the wild-type situation. In contrast to the large *lacO* repeat arrays themselves, the flanking BACs of large arrays did not show a significantly higher association frequency with CCs than under wild-type conditions. However, the large *lacO* repeat arrays allow a clear differentiation between their position and those of the flanking BACs which is difficult for small arrays. For instance, in the nuclei of line CCP4.193 with a small *lacO* repeat array, the array could only have been associated more often with CCs than the BAC covering the insert site, if the array was looped out from the position of the BAC sequence. An outlooping of the 2-kb array over a larger distance from the BAC territory for association with heterochromatin is difficult to imagine. In addition, there was no correlation between transcriptional activity of a linked reporter gene and association frequency. Lines CCP4.211 and CCT383 with low luciferase activity showed results similar to those obtained for lines CCT431 and CCT432 with higher luciferase activity.

Watanabe et al. (2005) showed that a different degree of DNA methylation has no obvious effect on the association frequency of *lacO* arrays with heterochromatin in line EL702C. This observation was confirmed by lines tested in the present paper (compare *lacO* arrays of lines 125/26 and EL702C showing a high CpG methylation degree with those of lines 106/112 and 26 showing a lower CpG methylation degree; Fig. 5b,c).

The frequent association with CCs of the BAC sequence from the top arm of chromosome 2, which in four independent lines harbors 2-kb *lacO* arrays, might be because of its close vicinity to heterochromatic parts of the NOR. Thus, the association frequency of repeat arrays with CCs seems to be dependent on insert size (and possibly insert position) rather than on their methylation intensity.

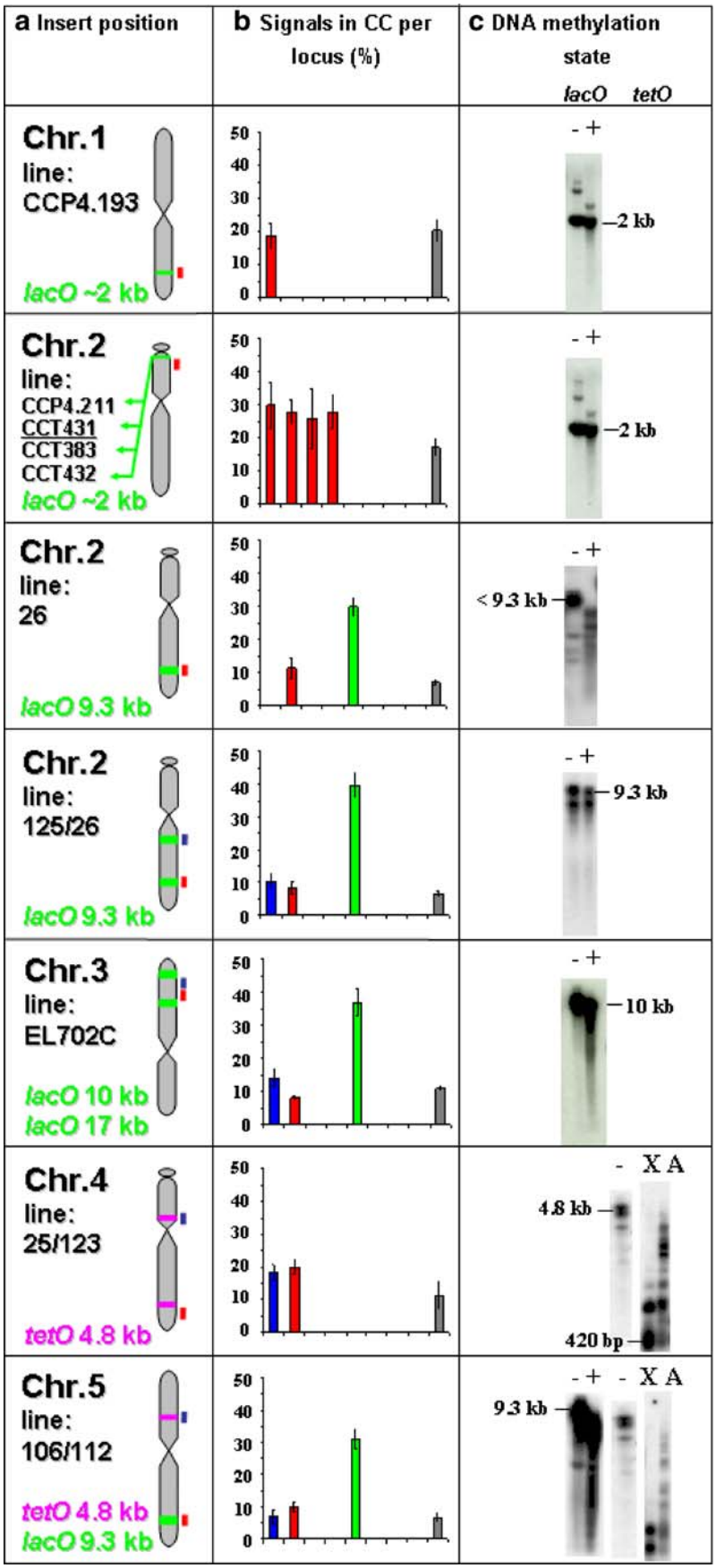

Fig. 5 Association with heterochromatin of the transgenic repeats and/or their flanking regions. **a** Insert positions are marked by *horizontal green* (*lacO*) or *pink* (*tetO*) *bars* and flanking BACs by *red* or *blue vertical bars* on the *right side* of the chromosomes. **b** Frequencies of association with heterochromatin. The *color* of columns indicates the type of probes as in (**a**). The wild-type situation is shown in *gray* (pooled in cases of 2 BACs). *Bars* indicate the standard deviation. Data for line EL702C are from Watanabe et al. (2005). **c** Southern hybridization of genomic DNA with the corresponding tandem repeats after specific digestion. The *underlined transgenic line* CCT431 was used for Southern hybridization. *Minus sign* without and *plus sign* with digestion with methylation-sensitive enzymes: Excised *lacO* arrays are digested with *Aci*I; excised *tetO* arrays were digested with a less (*Xho*I = *X*) and a more (*Ava*I = *A*) methylation-sensitive enzyme

Conclusions and outlook

Based on the present and previous data, we arrived at some preliminary conclusions regarding the impact of tandem repeats on chromatin arrangement in 2C *A. thaliana* nuclei: (a) The association frequency of repeat arrays with CCs may depend on insert size, i.e., size and number of repeat units per locus, rather than on their methylation status. (b) The frequency of somatic homologous pairing seems to be increased at insertion sites of large (≥9.3 kb) repeat arrays if these are strongly methylated and two arrays occur homozygously on one chromosome arm. Remarkably, a single (homozygous) HPT locus that is much larger (~100 kb) than the largest *lacO* arrays (~10 kb) revealed a strong increase in somatic homologous pairing frequency in 2C leaf nuclei (Pecinka et al. 2005). (c) The tested low-copy inserts do not significantly alter interphase chromatin arrangement, even if strongly methylated and independent of their transcriptional activity; therefore, they are more suitable for chromatin tagging than multiple copies of larger inserts.

Questions to be addressed in the future are: (a) Does more than one large repeat array per genome alter chromatin structure by increased homologous pairing frequency only when combined on one chromosome arm? If so, what is the reason? (b) Does more than one small repeat array per chromosome arm also increase somatic homologous pairing frequency similar to large arrays? (c) Do chromatin modifications other than CpG methylation (e.g., heterochromatin-specific histone methylation marks) have an impact on homologous pairing of large repeat arrays?

The study of chromatin arrangement at sites of transgenic repeat arrays may not only allow the prediction of intranuclear behavior of chromatin tags but may also provide insights into the mode of arrangement of endogenous tandem repeats and thus help to elucidate mechanisms for specific subnuclear organization of chromatin with distinct sequence properties.

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