Tandem repetitive transgenes and fluorescent chromatin tags alter local interphase chromosome arrangement in *Arabidopsis thaliana*

Ales Pecinka[1,‡], Naohiro Kato[2,*,‡], Armin Meister[1], Aline V. Probst[3], Ingo Schubert[1] and Eric Lam[2,§]

[1]Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstrasse 3, 06466 Gatersleben, Germany
[2]Biotech Center for Agriculture and the Environment, Rutgers, The State University of New Jersey, 59 Dudley Road, Foran Hall, New Brunswick, NJ 08901, USA
[3]Laboratory of Plant Genetics, University of Geneva, 30 Quai Ernset Ansermet, CH-1211 Geneva 4, Switzerland
*Present address: Department of Biological Sciences, Louisiana State University, 226 Life Science Buildings, Baton Rouge, LA 70808, USA
‡These authors contributed equally to this work
§Author for correspondence (e-mail: lam@aesop.rutgers.edu)

Accepted 13 May 2005
Journal of Cell Science 118, 3751-3758 Published by The Company of Biologists 2005
doi:10.1242/jcs.02498

Summary

Fluorescent protein chromatin tagging as achieved by the *lac* operator/lac repressor system is useful to trace distinct chromatin domains in living eukaryotic nuclei. To interpret the data correctly, it is important to recognize influences of the tagging system on nuclear architecture of the host cells. Within an *Arabidopsis* line that carries *lac* operator/lac repressor/GFP transgenes, the transgene loci frequently associate with each other and with heterochromatic chromocenters. Accumulation of tagged fusion protein further enhances the association frequency. Independent experiments with a transgenic plant carrying another multi-copy transgene also revealed, independent of its transcriptional state, unusually high frequencies of association with each other and with heterochromatin. From these results we conclude that the *lac* operator/lac repressor chromatin tagging system may alter the spatial chromatin organization in the host nuclei (in particular when more than one insertion locus is present) and also that loci of homologous transgenic repeats associate more often with each other and with endogenous heterochromatin than normal euchromatic regions.

Key words: *Arabidopsis thaliana,* Fluorescent chromatin tag, Homologous pairing, Interphase chromosomes, Heterochromatin

Introduction

In situ localization and direct in vivo visualization of distinct chromosome regions recently became feasible by chromatin-tagging systems. The *lac* operator/lac repressor system (Robinett et al., 1996; Straight et al., 1996) for instance uses a bacterial DNA binding protein (lac repressor) that, when fused with a green fluorescent protein (GFP) and a nuclear localization signal peptide (NLS), binds to the 256 copies of directly repeated *lac* operators (~10 kb) integrated at a specific chromosome locus. Binding at the target locus yields a fluorescent spot of higher intensity than the overall fluorescence of dispersed unbound GFP molecules in the nucleoplasm. The GFP-lac repressor protein is either transiently or stably expressed. The system was applied to various eukaryotes such as yeasts (Aragon-Alcaide and Strunnikov, 2000; Nabeshima et al., 1998; Straight et al., 1996), flies (Gunawardena and Rykowski, 2000; Vazquez et al., 2002), cultured mammalian cells (Robinett et al., 1996; Tsukamoto et al., 2000) and plants (Esch et al., 2003; Kato and Lam, 2001; Matzke et al., 2003). It revealed structural dynamics of chromosomes in interphase as well as mitotic cells by tracing the tagged loci in living cells and contributed to the understanding of chromosome functions (reviewed by Belmont et al., 1999; Gasser, 2002; Lam et al., 2004). However, tagging systems represent artificial chromosome loci and sometimes generate unusual nuclear protein localization. For instance in baby hamster kidney cells in which a *lac* operator array is amplified about ten times, a nuclear protein complex, the PML (promyelocytic leukemia) body, is formed at the integration locus (Tsukamoto et al., 2000). PML bodies are thought to play a role in regulating transcription. Because PML bodies are not formed at the transgene locus without accumulation of the *lac* operator binding fusion protein, they are thought to recognize high concentrations of induced foreign proteins (Tsukamoto et al., 2000).

Previously, we used the *lac* operator/lac repressor system to compare chromosome dynamics in *Arabidopsis* nuclei of different ploidy level (Kato and Lam, 2003), for example in 2C nuclei of guard cells (stomata) and in nuclei of pavement cells (8C on average). We found that chromosomes in interphase nuclei of *Arabidopsis* move randomly within a restricted area and that the area size correlates with the nuclear DNA content. *Lac* operator loci seemed to be stochastically associated in diploid as well as in endopolyploid nuclei according to the GFP spot numbers observed (Kato and Lam, 2003). Contrary to this, Esch and colleagues (Esch et al., 2003) reported that tagged *lac* operator loci in *Arabidopsis* are tightly, but not stochastically associated with each other because only one GFP spot was observed in most diploid as well as in polyploid cells. Therefore it was necessary to address the question of whether

lower-than-expected numbers of GFP spots are indeed due to associations of homologous loci in *Arabidopsis* nuclei and, if so, to determine why this is occurring.

Here we determined the number of *lac* operator FISH (fluorescent in situ hybridization) signals before and after expression of the GFP-lac repressor protein and the proportion of loci exhibiting a GFP spot after induced expression. Furthermore we compared the spatial organization of the tagged chromatin in hemizygous and homozygous conditions. The aim was to test whether the repeat structure and/or the expression of the GFP-lac repressor protein of the transgene have an impact on allelic/ectopic homologous pairing and on association with heterochromatic domains of the regions in question in 2C leaf nuclei. We found that the *lac* operator arrays are associated with each other more often than predicted by computer model simulation (Pecinka et al., 2004). Expression of the GFP-lac repressor protein further increases the homologous association frequency. Because the chromosome regions adjacent to the tagged loci associated with each other as well as with heterochromatin less often than *lac* operator arrays, we conclude that homologous tandem repetitive transgenes preferentially associate with each other as well as with endogenous heterochromatin in *A. thaliana.* In independent experiments homologous pairing and association with heterochromatin were studied for a homozygous multi-copy hygromycin phosphotransferase (HPT) transgene locus of ~100 kb (Probst et al., 2003) and yielded similar results to those obtained for the homozygous *lac* operator loci.

Materials and Methods

Plant material, preparation of nuclei and pachytene chromosomes

Plants of *A. thaliana* accession Columbia (Col) of hemizygous and homozygous EL702C genotypes (Kato and Lam, 2003), homozygous EL700S genotype (Kato and Lam, 2001; Kato and Lam, 2003) or homozygous line A and the mutant *mom1-1* genotype that contains the HPT locus homozygously (Probst et al., 2003) were cultivated as described. To induce expression of the GFP-lac repressor protein, young rosette leaves were detached from the plants and floated on 10 ml of 0.3 μM (homozygous EL702C) or 3 μM (wild type, homozygous EL700S) dexamethasone (Dex) solution in Petri dishes for 6-12 hours. After fixation of leaves in 4% paraformaldehyde, 2C nuclei were flow-sorted as described (Jasencakova et al., 2000). Prior to FISH, Dex-treated 2C nuclei were analyzed for the presence of GFP spots and the positions on slides of nuclei with GFP spots were recorded using a microscopic grid slide. Pachytene chromosomes of the wild type and homozygous EL702C genotypes were prepared as described (Lysak et al., 2001).

Fluorescence microscopy for GFP spot detection in living guard cell nuclei of *Arabidopsis* seedlings

The GFP signals were detected as described (Kato and Lam, 2003). Spots were defined as pixel clusters comprising more than the mean number of pixels plus 3.3-fold the value of the s.d. in the analyzed nuclei.

Probe labeling and in situ hybridization

The following DNA clones were used as probes: BAC MGL6 (GenBank accession number AB022217), BAC F18C1 (AC011620), BAC T15P10 containing 45S rDNA sequence (AF167571), 128x lacO-SK (Kato and Lam, 2001), pAL1 (Martinez-Zapater et al., 1986), the BAC contig spanning 4.2 Mb of chromosome 3 top arm from F2O10 to MSL1 (AC013454 and AB012247, respectively) and pGL2 (containing the hygromycin phosphotransferase gene under the control of the CaMV 35S promoter). BAC and plasmid DNA was labeled by nick translation (Ward, 2002) or by PCR using biotin-2'-deoxyuridine 5′-triphosphate (biotin-dUTP) (Probst et al., 2003). For chromosome painting, labeled BACs were precipitated and resuspended in 20 μl hybridization buffer (50% formamide, 10% dextran sulphate, 2× SSC, 50 mM sodium phosphate, pH 7.0) per slide. After mounting the probe, the slides were placed on a heat block at 80°C for 2 minutes and then incubated in a moist chamber at 37°C for ~12 hours. Post hybridization washes and detection steps were conducted as described (Schubert et al., 2001). Biotin-dUTP was detected by avidin conjugated with Texas Red (1:1000; Vector Laboratories), biotin-conjugated goat anti-avidin (1:200; Vector Laboratories), and again with Texas Red-conjugated avidin. Digoxigenin-dUTP was detected by mouse anti-digoxigenin (1:250; Roche) and Alexa 488-conjugated goat anti-mouse antibody (1:200; Molecular Probes). Cy3-dUTP was observed directly. Nuclei and chromosomes were counterstained with 1 μg/ml DAPI in Vectashield mounting medium (Vector Laboratories).

Microscopic evaluation and image processing

Fluorescence signals in flow-sorted 2C nuclei and on pachytene chromosomes were analyzed using an Axioplan 2 (Zeiss) epifluorescence microscope with 100×/1.4 Zeiss plan apochromat objective. Images were acquired with MetaVue (Universal Imaging) software and a cooled charge-coupled device camera (Spot 2e, Diagnostic Instruments) separately for each fluorochrome using the appropriate excitation and emission filters. The monochromatic images were pseudocolored, Gauss- or median-filtered to reduce noise and merged using Adobe Photoshop 6.0 (Adobe Systems) software. A spatial overlap of compact spheric FISH signals of homologous and/or heterologous sequences was regarded as homologous pairing and heterologous association, respectively. Allelic versus ectopic pairing of transgenic loci was distinguished on the basis of FISH signals obtained from differently labeled BACs that contain sequences flanking the respective transgene insertion loci.

Results

GFP spot numbers vary in 2C nuclei of live transgenic EL702C plants

In our previous studies, we rarely observed four GFP spots in 2C guard cell nuclei in homozygous EL702C plants with two loci on chromosome 3 containing *lac* operator insertions (Kato and Lam, 2003). Here we compare spot numbers in guard cell nuclei of cotyledons of hemizygous and homozygous EL702C plants and of homozygous transgenic EL700S plants (Fig. 1). As EL700S plants contain the same construct as EL702C plants except for the *lac* operator array, homogeneously distributed GFP signals but no GFP spots were expected in the nucleoplasm. In hemizygous EL702C plants, we found 5% of 92 nuclei without spots, 66% with one spot, 27% with two and 2% with three spots. In homozygous EL702C plants 12% of 197 nuclei had no spots, 34% showed one spot, 37% two, 11% three and 6% four spots. In homozygous EL700S plants, 55 out of 56 nuclei showed no spots and one nucleus (<2%) showed one spot. The single spot observed in an EL700S nucleus and a third spot in two hemizygous EL702C nuclei are most likely caused by spontaneous aggregation of GFP-lac repressor-NLS molecules. Taking into account our not being able to observe the maximum number of GFP spots in all nuclei

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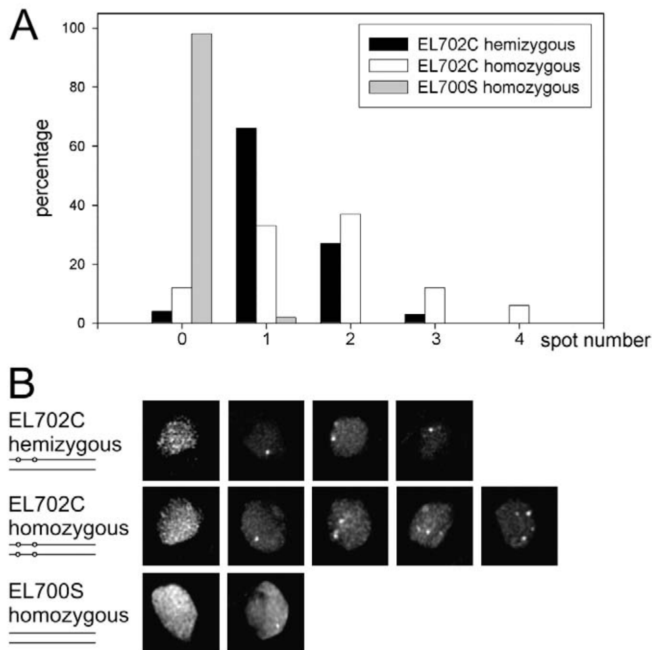

Fig. 1. GFP spot numbers in living guard cell nuclei (2C) from cotyledons of dexamethasone-treated transgenic *Arabidopsis* seedlings. (A) Percentage of nuclei with 0, 1, 2, 3 and 4 spots in hemizygous EL702C plants (n=92), homozygous EL702C plants (n=197) and homozygous EL700S plants (n=56). (B) Representative images of nuclei with 0, 1, 2, 3 and 4 spots from each of the lines and schematic view of the *lac* operator array loci on chromosome 3 in each line.

and in order to understand the nature of GFP-spot appearance patterns in the nuclei, we compared the observed data with the statistically expected percentages for nuclei with different spot numbers. We considered two statistical models.

In the first model, we assume that all *lac* operator loci in a nucleus appear as a GFP spot at random and that the two loci in the EL702C insertion line have an equal probability of interaction with GFP-lac repressor-NLS fusion proteins. In this model, spot detection should follow a binomial distribution with an average probability of spot appearance p estimated as,

$$p = (0 \times n_0 + 1 \times n_1 + \ldots + N \times n_N)/(n_0 + n_1 + \ldots + n_N)/N,$$

where N is the maximum number of spots per nucleus (N=2 for hemizygous, N=4 for homozygous nuclei) and n_i is the number of nuclei with i spots (i=0 … N). The probability of spot appearance calculated from the experimental data is significantly different for hemizygous (P=0.61) and for homozygous (P=0.41) nuclei (χ^2 test, P<0.001). Moreover, the distribution of the observed frequencies for different spot numbers per nucleus deviate significantly from the binomial distribution assuming the corresponding probability (χ^2=15.20, df=1, P=0.0001 for hemizygous nuclei; χ^2=10.46, df=3, P=0.015 for homozygous nuclei).

We previously observed that *lac* operator repeats with different lengths of the array may be detected as GFP spots with different probabilities (Kato and Lam, 2001). Therefore, in the second model we assumed that GFP spots appear with different probabilities on each tagged locus, possibly due to the different sizes of *lac* operator array in these loci (a single array in the distal and two *lac* operator arrays in the proximal insertion locus in the top arm of chromosome 3 of the EL702C line). We first estimated the probability values p_1 and p_2 for the two loci in hemizygous nuclei. On the basis of the observed percentages of hemizygous nuclei with one spot: $p_1(1-p_2) + p_2(1-p_1)$ =0.66 or with two spots: $p_1 \cdot p_2$=0.27, we calculated: p_1=0.30 and p_2=0.93. For homozygous nuclei, p_1=0.24 and p_2=0.55 were calculated similarly by numerical solution of the corresponding equations. With these estimated probabilities, the observed frequencies of hemizygous nuclei with 0, 1 or 2 spots and of homozygous nuclei with 0, 1, 2, 3 or 4 spots fitted very well with the expected values. However, our result suggests that the probability of spot appearance in hemizygous nuclei is higher than those of homozygous nuclei. Although non-homogeneous GFP accumulation in a nucleus or different levels of accumulation in hemizygous compared to homozygous nuclei might explain the lower probability of GFP spot appearance in homozygous nuclei**,** we preferred the hypothesis that the tagged loci might have intrachromosomal and/or interchromosomal interactions in the nuclei of *Arabidopsis*, thereby altering the apparent spot frequencies. To test this hypothesis, we analyzed the positional coincidence of the integrated *lac* operator loci in isolated 2C leaf nuclei by FISH analyses.

GFP spots and FISH signals of *lac* operator arrays frequently colocalize in homozygous EL702C nuclei

In flow-sorted 2C nuclei of homozygous EL702C plants in which expression of the GFP-lac repressor protein was induced with Dex, GFP spots and FISH signals on the *lac* operator loci were counted (Fig. 2). Nuclei without clear GFP spots were excluded from evaluation. Out of 63 analyzed nuclei, 30% showed one, 35% two, 25% three and 10% four GFP spots. In contrast, 22% of nuclei showed four FISH signals, 35% showed two or three signals and 8% showed one FISH signal. All GFP spots coincided with a *lac* operator FISH signal (Fig. 2B) but not vice versa. In total 83% of 252 FISH signals coincided with a GFP signal. Thus, 17% of the transgene loci cannot be detected by a GFP spot in Dex-treated homozygous EL702C nuclei under the applied conditions. Either not all *lac* operator arrays were accessible to the GFP-lac repressor proteins or some GFP spots could not be discriminated owing to a high overall fluorescence intensity and/or rapid bleaching of signals within a minute of exposure. We hypothesized that less than four FISH signals per nucleus may be due to ectopic or allelic alignment of the *lac* operator arrays.

The two transgene loci of EL702C are separated by a pericentrically inverted region

The transgenic line EL702C carries three transgenes (each ~17 kb) inserted at two independent loci on the top arm of chromosome 3, ~4.2 Mb apart; the proximal locus harbors two transgenes in inverse orientation (Kato and Lam, 2003). FISH analyses with BAC probes containing insert sequences internally flanking the transgene loci were used to quantify ectopic and allelic pairing frequencies of the *lac* operator arrays in 2C nuclei. To confirm the physical position of the transgene and BAC loci on chromosome 3, we first hybridized these probes to pachytene chromosomes of EL702C. The *lac* operator probes hybridized to the predicted locations whereas

the BAC probes hybridized in reverse order suggesting an inversion of the region between the transgene loci. Also FISH with two differently labeled BAC pools (MBK21 to MSL1 corresponding to the upper region and F2O10 to F28J15 corresponding to the bottom region between the insertion loci) yielded signals of reversed orientation on pachytene chromosomes of homozygous EL702C compared to that in wild-type plants (Fig. 3). FISH signals of the BACs flanking the insertion loci externally appeared in the same order on the wild-type and EL702C bivalents (not shown). These results confirmed the inversion between the transgene loci on the top arm of chromosome 3 in EL702C plants.

Ectopic pairing of *lac* operator arrays is doubled in homozygous nuclei compared to hemizygous nuclei

Using tri-color FISH with BAC MGL6 (79.5 kb, ~54 kb downstream of the insertion, red) flanking the distal locus, BAC F18C1 (100.8 kb, ~55 kb upstream of the insertion, yellow) flanking the proximal locus and *lac* operator probe (green) (Fig. 4B-D), the ectopic and allelic pairing frequency of the *lac* operator arrays was assessed in 60 hemizygous untreated, 62 homozygous untreated and 59 homozygous Dex-treated EL702C nuclei. We classified the *lac* operator array alignments into two different pairing types. If two signals (MGL6, red and F18C1, yellow) colocalized with a *lac* operator signal (green), we identified the alignment as ectopic pairing. If all signals of either MGL6 or F18C were colocalized with a *lac* operator signal, we identified the alignment as allelic pairing. In hemizygous nuclei, ectopic pairing was detected for 13% of the *lac* operator loci without Dex treatment. In homozygous EL702C nuclei without Dex treatment, ectopic pairing was observed for 27% of the *lac* operator array loci and allelic pairing for 34% of the loci. With Dex treatment, these values increased to 35% (ectopic pairing, P=0.052) and 45% (allelic pairing, P=0.017), respectively.

Homologous pairing and ectopic association of regions flanking the transgene are more frequent in EL702C than in wild-type plants

Using the BACs F18C1 (yellow) and MGL6 (red) that flank the transgene insertions in EL702C nuclei as probes, two-color FISH was conducted to monitor the association of these regions in wild-type nuclei (n=153), hemizygous (n=60) and homozygous (n=62) EL702C nuclei without Dex treatment. We also analyzed 61 wild-type nuclei and 59 homozygous EL702C nuclei after Dex treatment (Fig. 5). Homologous pairing of both regions as well as their heterologous association occurred without significant differences (i.e. 3-6% per locus, see Fig. 5A) in wild-type nuclei and hemizygous EL702C nuclei, irrespective of Dex treatment. In homozygous EL702C nuclei, homologous pairing (10%, P<0.05 for MGL6 and 14%, P<0.001 for F18C1) and ectopic association (9%, P>0.05) occurred more often than in the wild type. A further increase

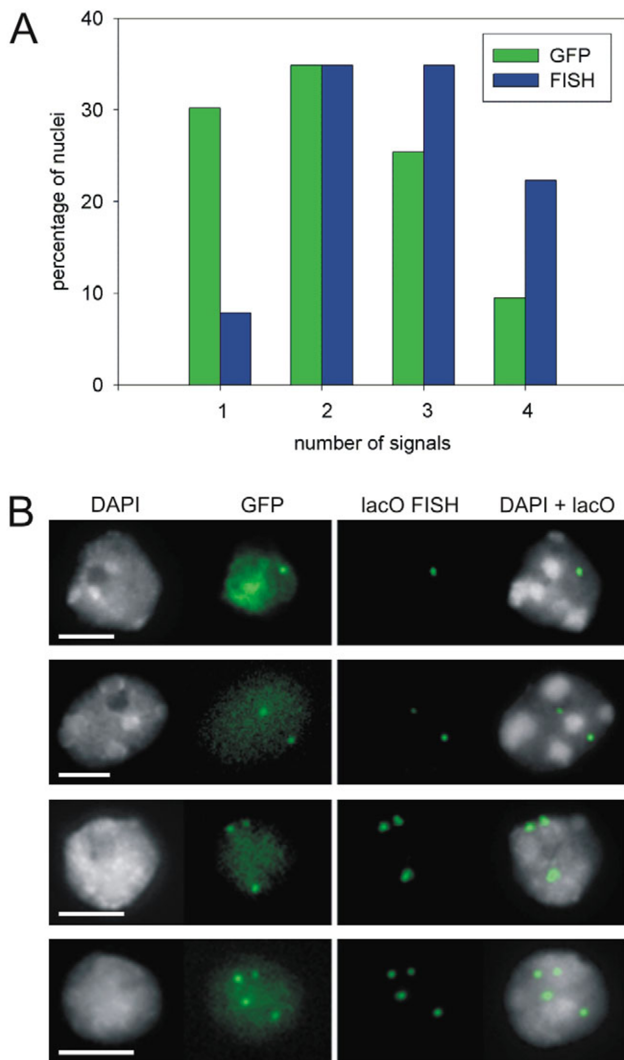

Fig. 2. Colocalization of GFP spots and *lac* operator FISH signals in Dex-treated 2C leaf nuclei of homozygous EL702L plants. (A) Percentage of nuclei with one to four GFP spots compared to FISH signals. Fewer GFP spots than FISH signals may occur in one nucleus. (B) Examples of nuclei with 1, 2, 3 or 4 GFP spots coinciding with *lac* operator FISH signals. Bars, 3 µm.

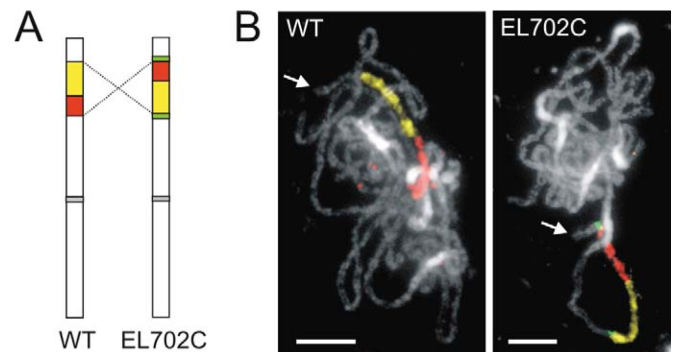

Fig. 3. The double transgene insertion in EL702C is accompanied by a paracentric inversion between the integration points. (A) Painted regions between BACs F2O10 and F28J15 (yellow) and MBK21 and MSL1 (red) schematically positioned on the top arm of chromosome 3 of the wild-type (left) and together with the transgene (green) of the EL702C genotype (right). (B) FISH of the complex probe to pachytene chromosomes of the wild type (left) and homozygous EL702C (right). Arrows indicate the top arm end of chromosome 3 bivalent. Bars, 5 µm.

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of homologous pairing (20%, *P*=0.032; and 22%, *P*=0.177, respectively) as well as of ectopic association (17%, *P*=0.013; Fig. 5A) was found after induction of GFP-lac repressor protein expression in homozygous EL702C nuclei (all at *P*<0.001 when compared to levels in the wild type).

The transgene colocalizes more often than the flanking regions with heterochromatic chromocenters

During the FISH analysis described above, we noticed a frequent spatial association of *lac* operator loci with heterochromatic chromocenters that are detected as strongly DAPI-stained regions. Therefore we determined the frequency of positional overlap (colocalization) of FISH signals of F18C1, MGL6 and *lac* operator probes with strongly DAPI-stained chromocenters in homozygous EL702C nuclei without (*n*=41) and with (*n*=31) Dex treatment. For comparison, the overlap of FISH signals of MGL6 and F18C1 probes with those of centromeric repeats and 45S rDNA, the main components of heterochromatin in *Arabidopsis* (Fransz et al., 2002), were monitored in 62 wild-type nuclei (Fig. 6). Although 8-14% of MGL6 and F18C1 FISH signals colocalized with heterochromatin in all types of nuclei tested, 37% of *lac* operator signals overlapped with chromocenters in untreated and 44% in Dex-treated homozygous EL702C nuclei (both *P*<0.001 when compared to the flanking regions). Apparently, the colocalization of *lac* operators with heterochromatin did not interfere with expression of the GFP-lac repressor protein in homozygous EL702C nuclei although the lac repressor gene is situated close to the *lac* operator array (Fig. 6). In order to test whether pairing of *lac* operator loci precedes association with heterochromatin, we counted the number of *lac* operator loci per overlap with a chromocenter. Within 31 Dex-treated homozygous EL702C nuclei (harboring 124 loci), 54 loci associated with a chromocenter, of which 14 were detected as a single locus, 24 as two, 12 as three and 4 as four paired loci suggesting that transgene pairing is not a prerequisite for association with heterochromatin.

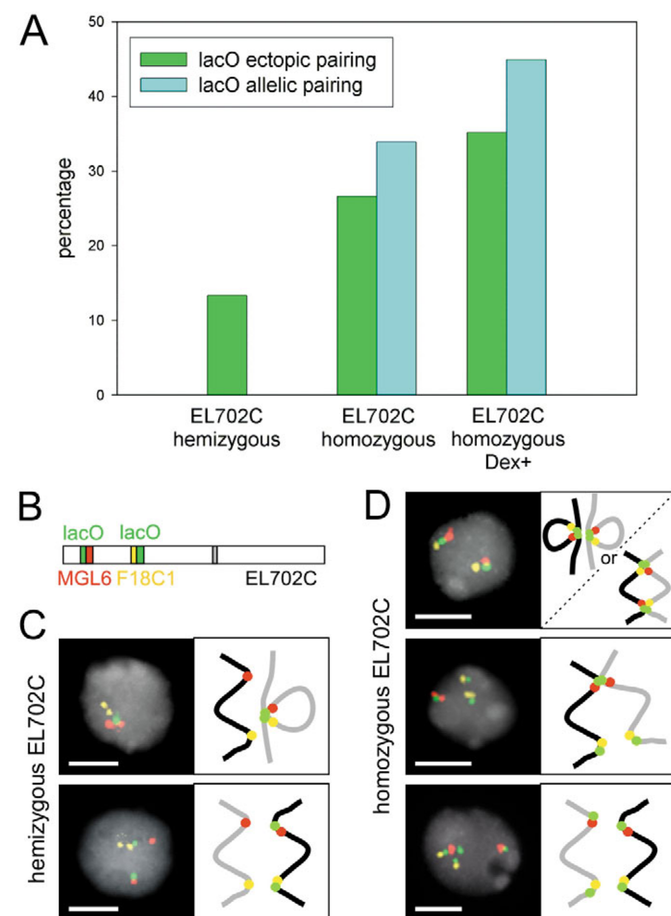

Fig. 4. Ectopic and allelic pairing of the *lac* operator array loci in EL702C plants. (A) Percentage of loci showing ectopic pairing (untreated hemizygous nuclei) or ectopic/allelic pairing (untreated and Dex-treated homozygous nuclei). (B) Scheme of chromosome 3 (EL702C) with the transgene insertion positions and the flanking regions used as markers for FISH. (C) Hemizygous nuclei showing ectopic pairing (top) or separation (bottom) of transgenic loci. (D) Homozygous nuclei with ectopic pairing of both transgenic loci (top, could be intrachromosomally or between homologues), allelic pairing of only the distal locus (middle) or separation of both loci (bottom). Bars, 3 μm.

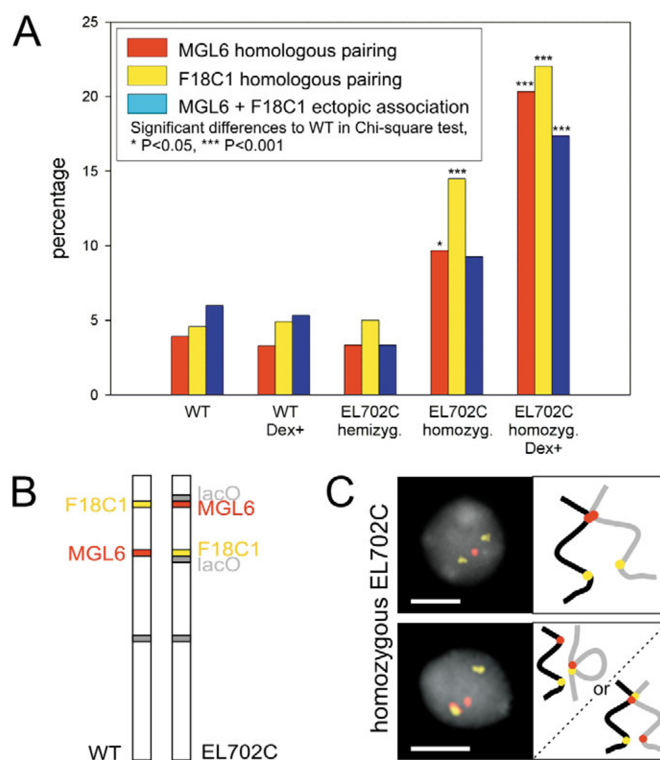

Fig. 5. Homologous pairing and ectopic association of regions flanking the *lac* operator transgene. (A) Percentage of homologously paired MGL6 loci, homologously paired F18C1 loci and association between both regions in the wild type, hemizygous and homozygous EL702C nuclei without or after Dex treatment. Significant differences *P<0.05 and ***P<0.001 were found compared to levels in the wild type determined using χ^2 test. (B) Schemes of chromosome 3 (wild type and EL702C) showing the position of BACs MGL6 and F18C1 used for FISH. (C) Homozygous EL702C nuclei showing homologous pairing of MGL6 (top) or ectopic association intrachromosomally or between two homologues (bottom). Bars, 3 μm.

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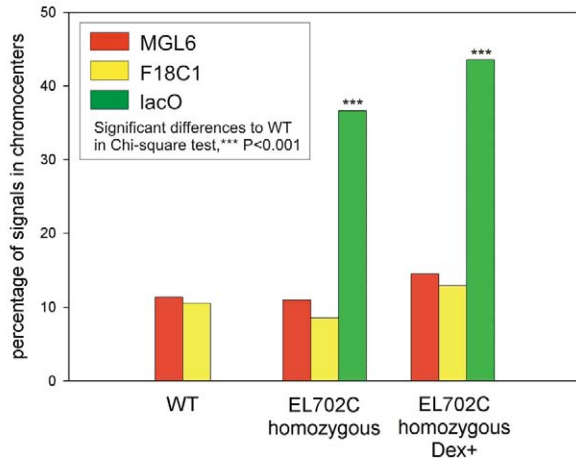
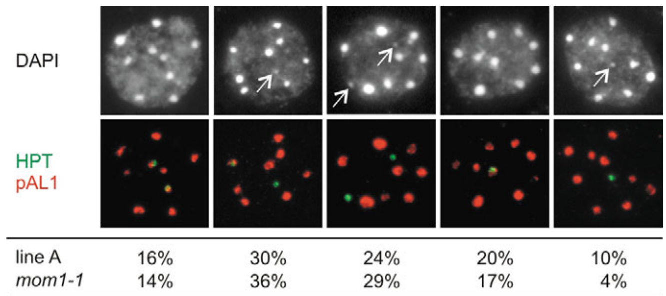


Fig. 6. Association frequency of the *lac* operator arrays with heterochromatic domains compared to that of flanking MGL6/F18C1 sequences. The percentage of FISH signals associated with heterochromatin in nuclei of wild type (MGL6, F18C1) and of homozygous EL702C plants without and after Dex treatment (MGL6, F18C1, *lac* operator) is shown. ****P*<0.001 compared to the wild-type situation determined using χ^2 test.

Pairing behavior and association frequency with heterochromatin of the *lac* operator array may not be sequence-specific

To test whether or not the higher-than-random allelic pairing frequency of the *lac* operator array is true only for this sequence, the pairing frequency of the homozygous silent transgenic HPT locus (composed of ~15 rearranged plasmid copies of ~100 kb in *A. thaliana* line A) (Mittelsten Scheid et al., 1991; Mittelsten Scheid et al., 1998) was investigated by FISH (Fig. 7). For comparison, the same locus was tested in a *mom1-1* mutant background (Amedeo et al., 2000) where HPT silencing is released without alteration of DNA methylation and histone modifications (Probst et al., 2003). In nuclei of line A, 30% of HPT FISH signals were paired. This value is significantly higher (*P*<0.001) than the ~5% of pairing observed on average for FISH signals of BACs with inserts from various endogenous euchromatic regions along the *Arabidopsis* chromosomes (Pecinka et al., 2004) but not significantly different (*P*>0.05) from the allelic pairing frequency of transgenic *lac* operator arrays (34% of loci) in homozygous EL702C nuclei. In *mom1-1* nuclei where HPT genes are expressed, association of HPT FISH signals (21%) was still significantly higher than the average pairing frequency (*P*<0.001). Colocalization with heterochromatic chromocenters was found for 50% of HPT signals in line A and for 49% in *mom1-1* nuclei. In 269 line A nuclei, 165 out of the 271 HPT loci colocalizing with chromocenters associated as a single locus and 106 as paired loci. In 355 *mom1-1* nuclei, 224 out of 346 loci colocalizing with a chromocenter associated as single and 122 as paired loci. Because 60-65% of heterochromatin-associated loci were not paired, homologous pairing seems not to be a prerequisite for spatial association of HPT loci with chromocenters. Hence the association frequency of the HPT locus with heterochromatin is even higher than that observed for the *lac* operator arrays, independent of the transcriptional status and of previous homologous pairing.



line A	16%	30%	24%	20%	10%
mom1-1	14%	36%	29%	17%	4%

Fig. 7. The association frequency of the transgenic hygromycin phosphotransferase (HPT) locus with endogenous heterochromatin is similar in line A and in the *mom1-1* background. Top row, *Arabidopsis* nuclei with intense DAPI-stained chromocenters. Middle row, the same nuclei after FISH with the 178 bp centromeric repeat (red) and pGL2 sequence labeling the HPT locus (green). From left to right: two, one or none of the separated allelic HPT signals associated with centromeric heterochromatin, and paired HPT signals associated or not associated with centromeric heterochromatin. Note the appearance of the HPT locus as small DAPI-positive chromocenter(s) in the second, third and fifth nucleus of the upper panel (arrows). Bottom row, the percentage of nuclei showing the corresponding type of association of HPT signals with centromeric heterochromatin (line A, *n*=269 nuclei; *mom1-1*, *n*=355 nuclei). Of the total HPT signals 30% were paired (see fourth and fifth nucleus) in line A and 21% in *mom1-1*. In nuclei of line A, 61% of the 271 HPT sites colocalizing with centromeric heterochromatin did so as single sites and 39% as paired sites. In *mom1-1* nuclei, 65% of the 346 HTP sites colocalized with centromeric heterochromatin as single sites and 35% as paired sites.

Discussion

FISH analyses of the transgenic line EL702C with flanking BACs revealed a previously undetected inversion between the two insertion loci of *lac* operator arrays on the top arm of chromosome 3. Without sequencing the actual breakpoints (~10-55 kb away from the insertions) we are currently not able to identify the molecular event responsible for that inversion and to decide on one of the models proposed for the generation of inversions during insertion of two transgenes in cis configuration (Laufs et al., 1999).

Homologous pairing of ~100 kb regions along different chromosomes of *A. thaliana* accession Columbia occurs on average in about 5% of somatic nuclei (Pecinka et al., 2004). In wild-type nuclei, allelic pairing and ectopic association of the regions that flank the *lac* operator loci in EL702C occur with a similar frequency (3-6% per locus). These values are within the range predicted for random appearance of 'single point' homologous pairing according to simulations based on the 'random spatial distribution' model (Pecinka et al., 2004). The homozygous presence of the *lac* operator arrays results in a four- to tenfold higher frequency of allelic as well as of ectopic pairing of these loci compared to the average values observed for endogenous sequences in wild-type nuclei (compare values for flanking sequences in Fig. 5A with those for *lac* operator arrays in Fig. 4A). The high allelic pairing frequency of the transgene may exert a 'dragging' effect on the flanking regions (Fig. 5A). In hemizygotes, a dragging effect is not obvious because pairing of the transgene is less frequent than in homozygotes and in most cases FISH signals of flanking regions are separated by those of *lac* operator loci

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during ectopic transgene pairing. We also found the repeated HPT locus to be paired significantly more often (21-29%) than expected according to a random frequency that was observed for several endogenous euchromatic loci (Pecinka et al., 2004). From these data we speculate that tandem repetitive sequences promote homologous association in *Arabidopsis*. Such a tendency for homologous association of tandem repeats could also be the reason for association of multiple transgene insertion loci in wheat nuclei (Abranches et al., 2000; Santos et al., 2002). The dispersion of the wheat transgene loci observed after 5-azacytidine or trichostatin A treatment (Santos et al., 2002) might be due to chromatin modifications rather than to transcriptional activity (see below). Expression of the GFP-lac repressor protein in homozygous EL702C nuclei yielded a further increase of allelic and ectopic pairing of the transgene locus by an additional 5-10% (Fig. 4A), with an additional dragging effect on the flanking regions (Fig. 5A). Expression of HPT in the *mom1-1* background does not increase homologous pairing of the transgene locus containing the HPT repeat. We speculate that GFP-lac repressor protein binding to the *lac* operator arrays, rather than just expression of the transgene, enforces allelic and ectopic pairing of the *lac* operator arrays. Wild-type lac repressor (tetramerizing form) can bind *lac* operators on different DNA molecules, tethering together loci on different chromosomes (Straight et al., 1996; Weiss and Simpson, 1997). Because we used a dimerizing mutant form of the lac repressor (Kato and Lam, 2001), which can bind only one *lac* operator site (Robinett et al., 1996), the capability of tethering two chromosomes should be minimized in EL702C. Nevertheless, spontaneous association of GFP-lac repressor protein molecules bound to different *lac* operator loci might increase the pairing frequency. Previously, we reported that movement of tagged chromatin in *Arabidopsis* nuclei, in spite of being spatially constrained, may span up to 0.44 μm within 10 minutes (Kato and Lam, 2003). Because homologous chromosome regions of ~100 kb are either paired or separated by less than 0.2 μm in ~20% of Arabidopsis nuclei on average (Pecinka et al., 2004), it seems reasonable to assume that during the 12 hours of Dex treatment, random associations of *lac* operator sites may occur and they then become stabilized due to aggregation of GFP-lac repressor proteins.

In contrast to the FISH signals of the *lac* operator array flanking regions, of which 8-14% overlapped with heterochromatin, signals of the inserted *lac* operator arrays colocalized as single or paired loci with chromocenters in 37% of untreated and in 44% of Dex-treated homozygous EL702C nuclei. As *lac* operator arrays may associate with heterochromatin as single or as paired loci, this colocalization does not depend upon pairing of the repetitive transgene arrays. Probably, tandem repeat loci tend to associate with each other on the basis of sequence homology but also with heterochromatic chromocenters containing other repeat sequences. This would render tandem repeats better candidates for anchoring euchromatin loops to heterochromatin according to the ‘chromocenter-loop model’ (Fransz et al., 2002) than dispersed repeats such as *Emi12* elements that colocalize with chromocenters only in 1-7% of nuclei (S. Hudakova, IPK, Gatersleben, Germany and I.S., unpublished results). In total the HPT locus is clearly larger than the *lac* operator locus and often becomes visible as DAPI-intense chromocenter(s) (Fig. 7) (Probst et al., 2003). Because the HPT locus colocalized more often than the *lac* operator locus with heterochromatin, the tendency of tandem repeats to associate with heterochromatin in *Arabidopsis* interphase nuclei may correlate with the size of the entire repeat-containing locus. For the HPT locus the association with heterochromatin was independent of transcription. In the case of the *lac* operator locus, transcriptional activity of adjacent sequences does not reduce the frequency of its colocalization with endogenous heterochromatin. The mechanism by which repeat sequences are targeted to chromocenters remains to be elucidated.

Finally, our findings suggest that in many nuclei the lac repressor/*lac* operator chromatin-tagging system does not reflect the spatial organization at the integration loci under wild-type conditions and may lead to invalid conclusions as to single-point homologous pairing frequencies (Esch et al., 2003). This problem could become significant especially when multiple insertions of repetitive arrays are present either in hemizygous or in homozygous conditions. The main reason for the increase in allelic and ectopic association frequency of the *lac* operator (compared to the flanking sequences in wild-type conditions) is most likely the repetitive nature of the transgene construct. Sequence-specific but more or less location-independent somatic association of multiple inserted arrays of *tet* operator and *lac* operator has been reported for budding yeast (Aragon-Alcaide and Strunnikov, 2000), although this was not confirmed by FISH or in the absence of fusion protein. For the same organism, association of *tet* operator arrays was shown to depend on the expression of the *tet* repressor fusion protein (Fuchs et al., 2002). In *Drosophila*, *lacO* arrays apparently did not reveal a tendency for homologous pairing as it was possible to trace extensive separation of homologues and even of sister chromatids along chromosome arms during pre-meiotic mid-G2 (Vazquez et al., 2002). Our results obtained for the HPT locus further support the idea that in *A. thaliana* the tandem repetitive nature of a transgene locus might be responsible for an increased allelic and ectopic pairing frequency of homologous transgenic sequences as well as for an increased colocalization frequency with endogenous heterochromatin. GFP-lac repressor proteins tagging such loci may further enhance their tendency for homologous association. Future studies will show whether DNA methylation and histone modifications have an impact on homologous pairing and heterologous association of interstitial tandem repeats and whether such loci represent hot spots for somatic recombination, for example after genotoxin exposure.

We thank Jörg Fuchs, Rigomar Rieger, Jerzy Paszkowski and the anonymous referees for helpful suggestions and critical reading of the manuscript and Joachim Bruder for technical assistance. This work was supported in part by grants of the Deutsche Forschungsgemeinschaft (Schu 951/8-2 and 10-1) to I.S. and by a grant from the Plant Genome Research Program of NSF (#0077617) to N.K. and E.L.

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