

Widely separated multiple transgene integration sites in wheat chromosomes are brought together at interphase

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Summary

We have investigated the organization of transgenes delivered by particle bombardment into the wheat genome, combining conventional molecular analysis with fluorescence *in situ* hybridization (FISH) and three-dimensional confocal microscopy. We selected a representative population of transformed wheat lines and carried out molecular and expression analysis. FISH on metaphase chromosomes showed that transgene integration sites were often separated by considerable lengths of genomic DNA (>1 Mbp), or could even be on opposite chromosome arms. Plants showing multiple integration sites on a single chromosome were selected for three-dimensional confocal analysis of interphase nuclei in root and embryo tissue sections. Confocal microscopy revealed that these sites lay in close physical proximity in the interphase nuclei. Our results clearly show that multiple transgenes physically separated by large intervening regions of endogenous DNA at metaphase can be brought together at interphase. This may reflect the original physical organization of the endogenous DNA at the moment of transformation, with DNA strand breaks introduced into several co-localized DNA loops by the intruding gold particles. Alternatively, the transgenes may be brought together after transformation, either by an ectopic homologous pairing mechanism, or by recruitment to a common transcription site.

Keywords: chromatin organization, transgenic wheat, FISH, transgene integration, confocal microscopy.

Introduction

The existence of order within the interphase nucleus was proposed over a century ago (Rabl, 1885). However, experimental evidence supporting this theory was first provided by Cremer *et al.* (1982), who showed that damage caused by UV irradiation was highly localized and affected only a small number of chromosomes. This was followed by chromosome-painting experiments that confirmed the territorial organization of chromosomes throughout the cell cycle (Cremer *et al.*, 1988; reviewed by Lamond and Earnshaw, 1998). Within each territory, chromosomes are divided into functionally independent subdomains corresponding to units of transcriptional and replicational regulation (Zink and Cremer, 1998). Within individual chromosome territories the chromatin fibre is contorted, looping back and forth between the deep and peripheral regions of each domain (Verschure *et al.*, 1999). Abranches *et al.* (1998) have shown that interphase chromosomes in wheat occupy distinct, elongated territories spanning the width of the nucleus, and that there is a highly regular Rabl

configuration with the two arms of each chromosome lying alongside each other, the centromeres clustered at one nuclear pole, and the telomeres located at the other. Chromosome territories are therefore highly organized within the interphase nucleus of both plants and animals.

There is an emerging body of evidence that chromosome organization is closely associated with the regulation of chromatin function and gene expression (Cremer *et al.*, 1993; Palladino and Gasser, 1994). In *Drosophila*, for example, genes at translocation breakpoints may be upregulated or silenced concomitant with their transfer to a different nuclear territory (Dernburg *et al.*, 1996; Sass and Henikoff, 1999). The latter authors showed that insertion of heterochromatin at the *brown* locus caused both the interrupted gene and its unaltered allele to be located at the centromeric nuclear domain, suggested to be a transcriptionally inactive region of the nucleus. The role of nuclear position as a factor influencing gene expression has been supported by recent studies of the

mammalian Ikaros proteins which are localized around centromeric heterochromatin. Brown *et al.* (1997) showed that transcriptionally inactive genes were localized to the Ikaros domains, but transcriptionally active genes were not. The effect of chromosomal position on gene expression is most noticeable in transgenic organisms, where independent transformants carrying the same transgene show different levels of transgene expression because of position effects, i.e. properties conferred by the local chromatin environment at different integration sites (Matzke and Matzke, 1998).

Variable transgene expression and transgene silencing occur frequently in transgenic plants, and there has been much interest in elucidating the factors that control transgene expression. Where transgene loci have been mapped and characterized in plants, the emphasis has been on determining the sequence context and organization of transgene sites, and not the context of the transgenic locus with respect to the architecture of the interphase nucleus. Many recent studies have focused on the molecular mechanisms of transgene integration, especially in direct DNA transfer methods that rely on endogenous properties of the plant cell, rather than the bacterial gene products involved in *Agrobacterium*-mediated transformation. Common features of such integration mechanisms include the propensity for exogenous DNA sequences to undergo complex rearrangements, and the tendency for individual transgenes to be interspersed with stretches of genomic DNA. More recent studies using fluorescence *in situ* hybridization (FISH) have been used to characterize transgene organization at the cytogenetic level. For example, such studies have revealed the random distribution of integrated T-DNA sequences on metaphase chromosomes of *Agrobacterium*-transformed dicots such as petunia (Fransz *et al.*, 1996; ten Hoopen *et al.*, 1996; Wang *et al.*, 1995) and tobacco (Iglesias *et al.*, 1997; Papp *et al.*, 1996; Park *et al.*, 1996). Although the stability of expression is likely to depend on the chromosomal position of the transgene (e.g. in centromeric or heterochromatic DNA), there have been few attempts to correlate transgene expression with cytogenetic position (Iglesias *et al.*, 1997; Papp *et al.*, 1996; Park *et al.*, 1996). Furthermore, these studies have been carried out using tobacco and petunia, which are not ideal as model species for cytogenetic analysis because there is little detailed cytogenetic information concerning specific chromosomes and hence it is difficult to discriminate between individual chromosomes. The hexaploid wheat genome has been well characterized cytologically, and hybridization probes are available for the identification of most chromosomes (Mukai *et al.*, 1993). Wheat chromosomes are large and easy to handle, and the distribution of gene-rich regions in the wheat genome has been mapped (Barakat *et al.*, 1997; Gill *et al.*, 1993; Gill *et al.*, 1996). These properties, and the

fact that it is an important crop species, make wheat an excellent model system for investigating transgene localization and integration processes.

In the present report we have combined conventional molecular analysis with FISH to characterize a representative population of transgenic wheat lines generated by particle bombardment. Transgene integration sites were assigned to specific chromosomes, and the relationship between the stability of transgene expression and cytogenetic position was investigated. We then determined the location of transgene sites in interphase nuclei in root and embryo tissue sections. Lines were selected in which FISH analysis of metaphase chromosomes identified multiple distinct integration sites in a single chromosome for three-dimensional confocal analysis. Multiple transgenes, clearly separated along the metaphase chromosomes by intervening regions of endogenous DNA, appeared as clustered signals or a single site at interphase. This may reflect the original physical organization of the endogenous DNA at the moment of transformation, suggesting that transgene integration occurs following localized damage to DNA within a given region of the nucleus by the accelerated gold particles. Alternatively, the transgene copies may be brought together in the interphase nucleus after transformation by recruitment to a common transcription site or factory, or they may associate ectopically due to pairing of homologous DNA sequences. Such ectopic interactions have previously been suggested as a mechanism for the initiation of homology-dependent gene silencing, but have not been shown directly.

Results

Molecular and genetic characterization of transgenic lines

We analysed transgenic loci in a representative population of wheat lines produced by particle bombardment with either vector pAHC25 (containing the *gusA* and *bar* genes) or pAHC20 (containing the *bar* gene alone). We selected lines showing a diversity of integration patterns, from one to approximately 12 transgene copies, and different levels of transgene expression. Transgene copy number was determined as described earlier (Stöger *et al.*, 1999), using restriction enzymes cutting at a single site in the transformation construct (*Sst*I in pAHC25 and *Hind*III in pAHC20; Figure 1a,b). Hybridization was carried out with *gusA* and *bar* probes for plants transformed with pAHC25 and pAHC20, respectively. We observed multiple hybridization bands of different sizes but with similar intensities, suggesting random integration of plasmid fragments. Genomic DNA was then digested with *Hind*III, which releases a 4 kbp diagnostic fragment corresponding to the *gusA* expression cassette of pAHC25. Southern blot hybridization with a *gusA* probe revealed the expected

fragment in all lines, plus a number of additional fragments, some larger and some smaller than expected (data not shown), indicating the presence of rearranged transgene copies. Additional DNA analysis was carried out using genomic DNA digested with restriction enzymes known not to cut in the transforming plasmids (*BlnI* for pAHC25 and *SstI* for pAHC20; Figure 1c,d). Six of the lines we analysed showed multiple hybridizing bands. Although it is possible that minor rearrangements during integration generated new restriction sites, a more plausible explanation is that multiple transgene cassettes were interspersed with genomic DNA.

To determine whether transgenes in a given line were inherited as a unit, we performed segregation analysis in R_1 progeny of all primary transformants. Initially, segregation was determined on the basis of transgene expression using β -glucuronidase (GUS) and phosphinothricin acetyltransferase (PAT) activities, and a minimum of 30 seeds per line (with the exception of line 4 which set only six seeds). In eight lines, the data suggested 3 : 1 segregation of a single Mendelian locus. We analysed five R_1 plants from each line (except line 4 where all R_1 plants were analysed) by Southern blot hybridization. Whereas the hybridization patterns remained the same in most lines, we found two independently assorting hybridization patterns in R_1 plants of line 4, indicating two independent and unlinked transgenic loci. This was confirmed by the segregation of FISH signals in metaphase chromosome preparations in the R_1 lines 4a and 4b, derived from parental line 4.

Transgene expression and stability

In lines transformed with pAHC25, GUS activity was quantified in young leaves of R_0 and R_1 plants (Table 1). The expression levels varied widely (by about a factor of 10), with little correlation between expression level and

transgene copy number. In subsequent generations (through to R_4), segregation of GUS activity was determined by histochemical assay of endosperm tissue. PAT activity was monitored by germination assays using seeds from R_0 , R_1 , R_2 and R_3 plants. Homozygous plants could be identified in the R_2 generation of most lines, and transgene activity appeared stable in 100% of progeny. Line 2 was exceptional in that we observed silencing of both *gusA* and *bar* in two homozygous R_4 plants. This was not caused by transgene rearrangement, as Southern blot hybridization patterns remained identical in all plants analysed (data not shown). However, we were able to correlate silencing

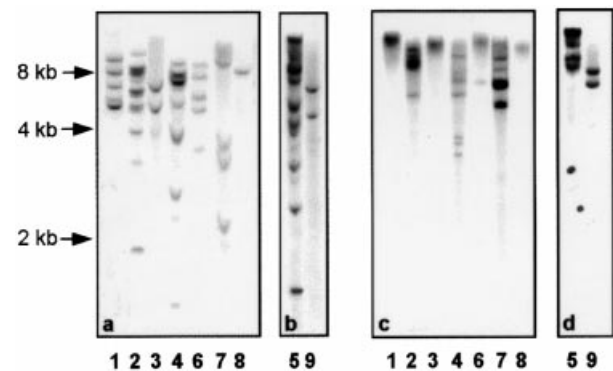


Figure 1. Molecular analysis of transgene integration. (a) Southern blot of lines 1–4 and 6–8, containing plasmid pAHC25. DNA from independent transgenic lines was digested with *SacI*, which cuts only at one site in the vector, between the *gusA* and *bar* genes. The blot was probed with the coding sequence of the *gusA* gene. (b) Southern blot of lines 5 and 9, transformed with pAHC20. DNA was digested with *HindIII*, which cuts at one site in the vector. The coding region of the *bar* gene was used as probe. (c,d) Southern blot of lines 1–9 where genomic DNA was digested with enzymes that do not cut the plasmid used for transformation (*BlnI* for pAHC25; *SacI* for pAHC20). Blot C was probed with the coding sequence of the *gusA* gene; blot D was probed with the coding sequence of the *bar* gene.

Table 1. Location, activity and stability of transgenes

Line	Chromosome	Position	Transgene copies ^a	Expression ^b	Stability
1	2A	Subtelomeric	5	Medium	Stable
2	6B	Intercalary	>10	High	Unstable
3	6B	Intercalary	2	Medium	Stable
4a	5D?	Intercalary	≈5	High	Stable
4b	1D	Telomeric	≈2	Low	Stable
5	1D	Intercalary	>9	na	Stable
6	4A	Telomeric	5	Medium	Stable
7	2B	Telomeric	≈5	Low	Stable
8	6A	Intercalary	1	Medium	Stable

^a*gusA* gene.

^bGUS activity (nmol min⁻¹ mg⁻¹ protein): high, 1.5–3; medium, 0.5–1.5; low, <0.5. na, not applicable.

of the locus with hypermethylation of the ubiquitin-1 promoter driving the transgenes (data not shown).

Detection and mapping transgenic loci by FISH

FISH was performed on metaphase squashes from nine independent lines in order to visualize the transgenic loci and map the corresponding integration sites. Two different DNA probes (whole-plasmid DNA and the *gusA* coding region) were used for each line. In all lines, with the exception of line 9, a clear signal was visible on both chromatids. In line 5 we only used the whole plasmid probe, as this line did not contain the *gusA* gene. In lines 3 and 8 we could detect only the integrated whole plasmid DNA and not the *gusA* fragment(s). We assume that the one or two copies of the *gusA* fragment were too short a target to be detected by FISH. Complete metaphase cells (showing all 42 chromosomes) were used for chromosome counting. Only line 2 was aneuploid, containing 46 chromosomes. The loci described in this study were randomly distributed, showing no preference for the A, B or D genomes of wheat. Unlike previous reports on T-DNA loci in petunia (ten Hoopen *et al.*, 1996; Wang *et al.*, 1995), we did not observe a pronounced preference for integration at distal chromosome sites.

In three lines (2, 4a and 5) multiple signals were observed on a single chromosome arm. These signals were close together, but there was enough space to discriminate the individual signals, suggesting that transgene DNA was interspersed with extensive stretches of host genomic DNA. For example, in line 5 the distance between the two signals was equivalent to 16.7% of the long arm of 1D. The average DNA content of one wheat chromosome is 825 Mbp (Heslop-Harrison and Schwarzacher, 1993), thus the amount of interspersed genomic DNA must be in the order of several Mbp. In line 2, the four separate signals of the FISH pattern were between the nucleolar organizer region and the centromere of chromosome 6B, which is thought to have a low gene density (Gill *et al.*, 1993). Pedersen *et al.* (1997) described double hybridization signals on the same arm of chromosome 6B, albeit slightly closer to the nucleolar organizer region. Plants from this line also showed *gusA* and *bar* gene silencing associated with hypermethylation (D. Becker, personal communication).

The chromosomes carrying the transgenic DNA for each line are shown in Figure 2. In most cases the two probes identified the same sites; in line 6, however, we observed two signals with the whole plasmid probe, but only one with the *gusA* or *bar* probe. This indicated that one locus probably contained only a partial plasmid sequence which lacked the transgene coding regions. This is supported by our segregation data (Southern blot analysis with *gusA* and *bar* probes, GUS and PAT assays), which identified

only one transgenic locus in line 6. Figure 2(n) shows the chromosome (identified as 4A) with two signals when probed with pAHC25, one at the telomere of the short arm and the other intercalated in the long arm. Only the telomeric signal was detected when the *gusA* fragment was used as the probe (Figure 2m).

Following the analysis of transgene DNA, the slides were washed and reprobbed with the appropriate DNA markers for chromosome identification. This identification was based on the map of the cultivar Chinese Spring (Mukai *et al.*, 1993). The cultivar used in our study carried several polymorphisms, which altered certain regions of the physical map compared to that reported for Chinese Spring. However, by using chromosome morphology in addition to the molecular markers, we were able to identify every chromosome unambiguously except one. The only exception was in line 4a, where the identification of the chromosome carrying the *gusA* fragment was not conclusive. Although the chromosome showed the morphology and pSc119.2/dpTa1 hybridization pattern for chromosome 5D, it did not contain the 5S rDNA site present in all group 1 and 5 chromosomes.

In line 9 it was not possible to detect a FISH signal, even with antibody amplification steps and modified stringency conditions. Southern blot hybridization using an enzyme that did not cut in the transforming plasmid generated short fragments. Furthermore, very short fragments were generated using a single cutter. This suggested the integration of two partial plasmid sequences containing the *bar* gene (but very little vector sequence), separated by genomic DNA. Assuming that the two transgene sequences were not immediately adjacent to each other, they would constitute small (<6 kbp) targets. It is reasonable to assume that this is below the limit of detection of our FISH procedure. In line 8 we were able to detect a transgene region smaller than 12 kbp. As the signal was rather weak, this target size may be the approximate detection limit in complex genomes such as wheat (Menke *et al.*, 1998).

Three-dimensional analysis of root and embryonic tissue sections

FISH was performed on vibratome sections of root and embryo tissue ($\approx 30 \mu\text{m}$ thick) to localize the transgenes in three-dimensionally preserved interphase nuclei. Confocal section stacks were recorded at a spacing of 0.5–0.8 μm for about one cell layer, typically giving a stack thickness of 15–20 μm . Data sets were collected from several individual seedlings or embryos for each line, and from several different areas of each sample. Previous studies from this laboratory have shown that good preservation at the optical level can be combined with FISH labelling through-

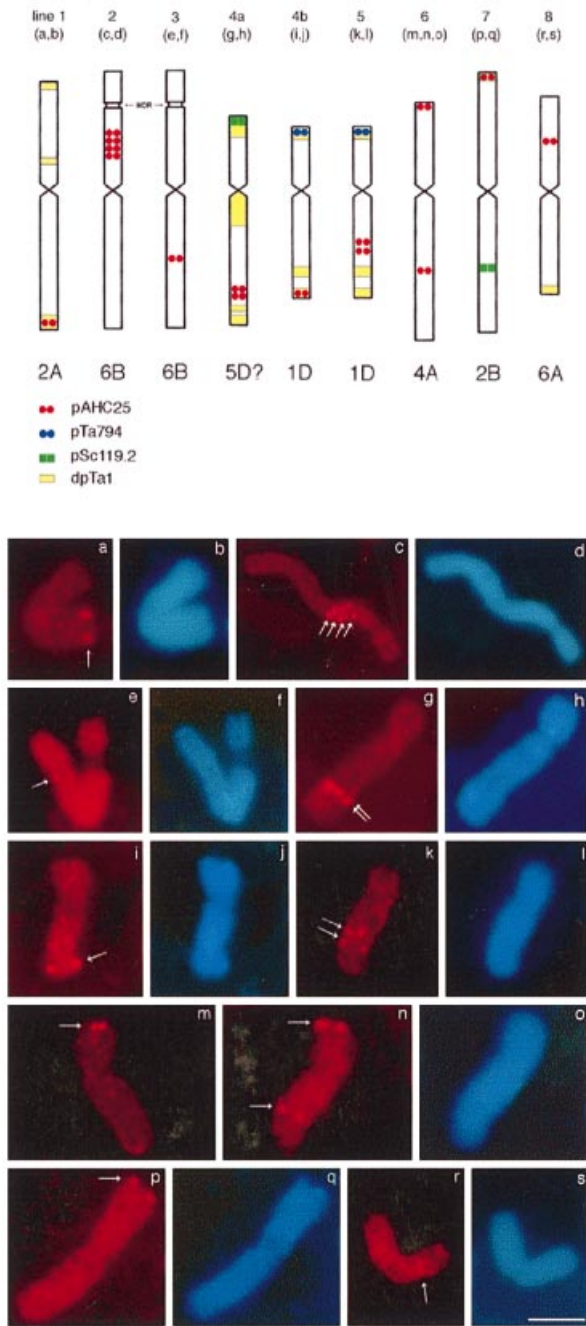


Figure 2. Summary of FISH analysis on metaphase chromosomes. Above: schematic representation of chromosomes carrying the transgene (pAHC25). For each line, the pattern obtained with FISH probes used for chromosome identification is indicated. The pTa794 probe detects the 5S rDNA, allowing identification of chromosomes from groups 1 and 5. The pSc119.2 probe hybridizes predominantly to the wheat B genome, whereas the dpTa1 probe hybridizes mainly to the D genome. Letters (a–s) below the numbers of transgenic lines correspond to letters of colour plates. Below: chromosomes showing hybridization signals with whole plasmid DNA, except (m) which shows signal with *gusA* probe, and respective DAPI counterstaining. Arrows indicate transgene sites. Bar = 1.5 μ m.

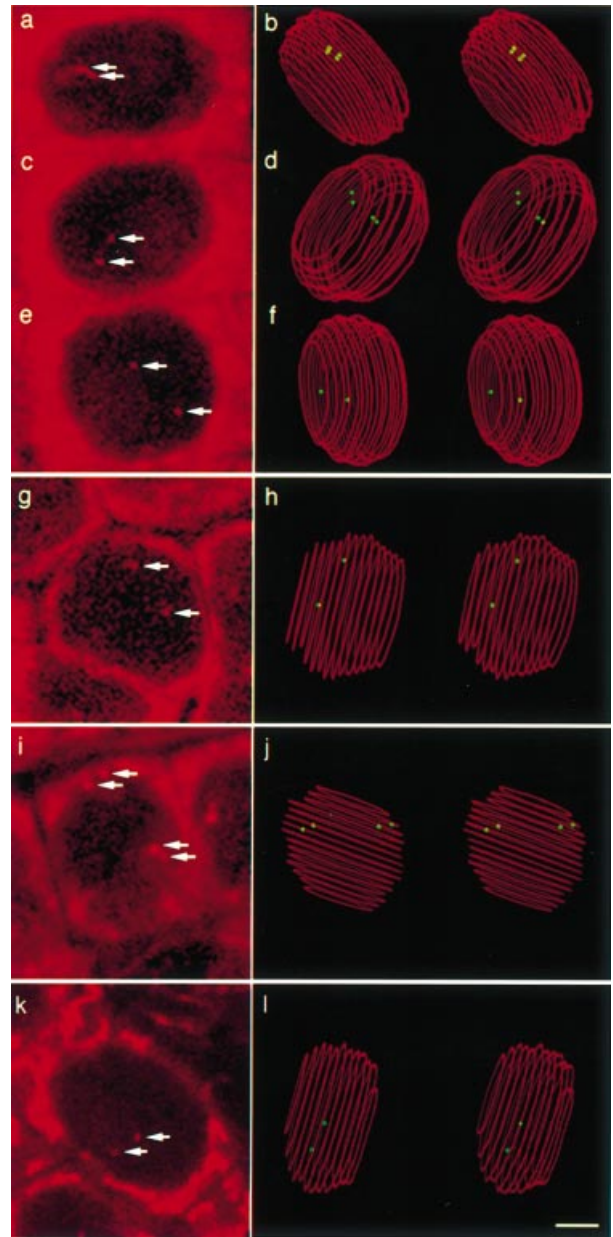


Figure 3. Visualization of transgene sites in three-dimensional interphase nuclei. (a–f) Root tissue sections of line 6 hybridized with pAHC25: (a,c,e) result from the projection of a series of confocal sections; (b,d,f,h) show stereo three-dimensional reconstruction of each nucleus (nuclear membrane in red, transgenic DNA as green dots). (a,c) Hybridization signals (indicated by arrows) appeared as pairs of dots (one pair for each homologue), whereas in (e) they appeared as single dots. (g) Nucleus from embryonic tissue section of line 2, also presenting two single dots, one for each homologue. (h) Three-dimensional stereo reconstruction. (i) Nucleus from a root tissue section of line 5, presenting two dots per homologue. (j) Three-dimensional reconstruction. (k,l) A homozygous nucleus from line 4b. Each individual signal corresponds to one homologue. Bar = 5 μ m; section spacing = 0.5 μ m.

out the depth of such vibratome sections (Abranches *et al.*, 1998; Highett *et al.*, 1993; Thompson *et al.*, 1997).

Typical confocal images are shown in Figure 3. Although there is some cytoplasmic background (arising both from autofluorescence and from endogenous biotin) the nuclei themselves show a very low background labelling; the transgene sites can be identified very clearly and unambiguously; and both homologues can be seen in each nucleus analysed. In control experiments where probes were omitted, no labelling was seen. In other experiments, different probe sequences were used, such as centromeres, telomeres and rDNA, and the expected localization was observed (Abranches *et al.*, 1998). Although not all regions of the tissue sections were equally well labelled, we analysed several large areas of tissue (containing about 20 nuclei per confocal stack), where virtually 100% of the nuclei were labelled by the probe. For example, Figure 3(a–e) shows three adjacent nuclei, all showing labelling of both homologues. Similarly, in a homozygous plant of line 4b, both homologues could be detected in 87% of the nuclei.

We then counted the number of signals observed. In this analysis only nuclei completely and unambiguously within the three-dimensional data stack were included. In all the homozygous nuclei counted, two clearly separated sets of signals were seen, corresponding to the two homologous chromosomes. Conversely, we observed only one set of signals in the heterozygous nuclei. The number of spots seen is therefore expressed as the number of signals per chromosome. These results are summarized in Table 2. Identical results were obtained for root and embryo tissue.

We could not distinguish unambiguously between G₁ and G₂ nuclei, and therefore made no allowance for this, although very large nuclei that were clearly classifiable as G₂ were excluded. In some nuclei we saw sites arranged as sets of very close pairs, which almost certainly correspond to equivalent sites on the sister chromatids during G₂. However, we counted every distinguishable site, including these, to avoid introducing any subjective bias. Thus the number of sites counted per chromosome is likely to be overestimated if the G₁/G₂ difference is taken into account. It is not clear whether two distinguishable sites for a single locus would always be seen in G₂ or a single merged site. Highett *et al.* (1993) showed two nearly coincident sites for a locus in G₂. Similarly, in line 4b we typically observed a single signal per chromosome (homologue), also suggesting that the sister chromatids may not always be distinguishable. We have therefore made the most conservative assumptions in our statistical analysis.

In a second analysis a random sample of about 40 interphase chromosomes was chosen for lines 2, 5 and 6, and the maximum three-dimensional distance between any two signals on one chromosome was determined. These data are shown in Table 3.

Table 2. Number of FISH signals per chromosome in interphase

Number of signals	Line 2	Line 6	Line 5	Line 4b
1	37.5% (57)	70.4% (145)	11.5% (12)	91.1% (102)
2	48.6% (74)	28.6% (59)	78.9% (82)	8.9% (10)
3	7.9% (12)	1% (2)	4.8% (5)	0
4	6% (9)	0	4.8% (5)	0
	100% (152)	100% (206)	100% (104)	100% (112)

Parentheses, number of chromosomes observed.

Table 3. Maximum distance measured between any two signals of one chromosome in interphase

Maximum distance between signals (µm)	Line 2	Line 6	Line 5
1 signal (<0.3)	40% (16)	67.5% (27)	16.7% (8)
0.3–1	35% (14)	7.5% (3)	8.3% (4)
1–2	20% (8)	10% (4)	50% (24)
2–3	2.5% (1)	7.5% (3)	23.9% (11)
>3	2.5% (1)	7.5% (3)	2.1% (1)
	100% (40)	100% (40)	100% (48)

Parentheses, number of homologues measured.

Figure 3(a–f) shows a projection of optical sections and three-dimensional reconstructions of three root interphase nuclei from line 6. This line carried two separate transgenic loci, one on each arm of chromosome 4A. However, in three-dimensional interphase nuclei of a homozygous plant, we observed either four spots arranged in two pairs, each pair corresponding to one homologue (Figure 3a–d), or (in 70% of the loci) a single spot per homologue (Figure 3e,f). In line 2, which contained four integration sites on the short arm of chromosome 6B, only two spots (one corresponding to each homologue) were visible in 37.5% of the nuclei (Figure 3g,h shows an interphase nucleus from embryonic tissue). In 48.6% of the loci two signals per homologue were observed in close proximity. In 14% of the loci three or four signals were visible (Table 2). Only in 5% of the loci was the maximum distance between any two signals of one homologue greater than 2 µm (Table 3).

In line 5 we predominantly observed one pair of spots per homologue (Table 2; Figure 3i,j) rather than the single signal observed in the two other lines, but the distance between the two signals was again below 2 µm in 75% of cases (Table 3).

Line 4b, which contained only a single integration site on chromosome 1D, served as a control showing two well separated individual signals, each corresponding to one homologue (Figure 3k,l).

Discussion

Plants independently transformed with the same DNA construct show great variability in transgene expression levels, and this is often affected by the position of transgene integration. Such position effects reflect the influence of local DNA and chromatin structure, which is intimately related to the organization of DNA in the nucleus. We therefore analysed a population of transgenic wheat plants, combining molecular analysis with FISH mapping of transgene integration sites in both metaphase and interphase nuclei.

Six lines showed a single FISH signal on metaphase chromosomes, consistent with the results of genetic segregation analysis. However, three lines (2, 4a and 5) showed multiple discrete signals clustered on one chromosome arm, while one line (6) showed two signals on opposite arms of the same chromosome.

FISH analysis demonstrated that in those lines with multiple clustered signals, individual transgene integration events were interspersed with substantial regions of genomic DNA at least 1 Mbp in length, and probably much greater. Previous reports have identified short DNA sequences, which could be isolated by PCR, interspersed between integrated transgenes (Kohli *et al.*, 1998). Based on these findings, the authors proposed a model for transgene integration in rice involving a two-phase mechanism where the initial integration process at a specific site rendered the site prone to further integration events, perhaps by attracting DNA-repair complexes. Based on similar observations in transgenic oat, Pawlowski and Somers (1998) proposed a model where transgene integration occurred at clustered DNA replication forks. Our Southern blot analysis, using enzymes that did not cut in the transforming plasmids, also indicated the presence of genomic DNA between the transgene copies in the transgenic wheat lines. This was apparent in several of the lines analysed, including those with single and multiple FISH signals. These data suggest that transgene copies are arranged in a hierarchical pattern, with individual copies interspersed with short stretches of genomic DNA, and clusters of transgene copies interspersed with longer regions of genomic DNA visible at the cytogenetic level.

To investigate the spatial distribution of transgene integration sites in the interphase nucleus, we analysed well preserved interphase tissue sections of lines 2, 5 and 6, which contained multiple FISH signals on metaphase chromosomes. We carried out FISH on the interphase nuclei and then determined the distribution of FISH signals by three-dimensional confocal analysis. The two integration sites in line 6, although widely separated in the metaphase chromosome, were close together in the interphase nucleus in both embryonic and root tissue. This arrangement was in agreement with the spatial

organization of wheat chromosomes known as the Rabl configuration, in which centromeres are clustered at one pole of the nucleus and telomeres at the other, bringing the two chromosome arms physically close together in a parallel arrangement (Abranches *et al.*, 1998). One integration site of line 6 is in telomeric position on the short arm of chromosome 4A. The second integration site is on the long arm of chromosome 4A, one-third of the arm length away from the telomere. In the simplest model of the Rabl configuration, where both arms of the chromosome are stretched across the diameter of the nucleus, the two transgene sites would be at a distance of 5–6 μm apart (the nucleus diameter is typically 15–20 μm). However, we observed an average distance of only 2 μm , and a single signal in more than 70% of the cases. Ten Hoopen *et al.* (1999) observed that loci adjacent to the telomere of metaphase chromosomes took more internal positions in the interphase nucleus, although the telomere itself remained associated with the nuclear periphery. This showed that adjacent loci on metaphase chromosomes tend to lie further apart, after decondensation, in interphase chromatin. On the other hand, our results clearly show that multiple transgene integration sites, physically separated by significant regions of endogenous DNA on a metaphase chromosome, lie unexpectedly close together in the interphase configuration.

In line 2 there were four metaphase FISH signals, and in interphase nuclei more than 70% of loci appeared as either a single hybridization signal, or two signals less than 1 μm apart. In the metaphase chromosome the four signals span a region that is equivalent to 29% of the short arm of 6B. Thus the expected distance between the first and last signal would be 4–6 μm in the chromosome arm stretched across the nucleus in interphase.

In line 5, we observed a pair of signals for each allele in about 80% of loci. The average distance between the signals was below 2 μm (1.6 μm), again less than the estimated distance between the sites if the arm of chromosome 1D was stretched across the nuclear diameter. However in line 5, although the sites were closer together than expected from their metaphase position, they were rarely so close together that they were visualized as a single site (i.e. closer than $\approx 0.25 \mu\text{m}$). Thus in line 5 the sites were reproducibly separated but close, whereas in the other two lines the sites were most often so close as to be indistinguishable.

Thus our results show that the multiple transgene sites in interphase are in highly reproducible configurations with respect to each other, and that they are brought close together even when widely separated on the metaphase chromosomes. This non-random localization of multiple integration sites relative to each other is reproduced over generations of interphase nuclei, probably indicating a well determined organization of the interphase chromo-

somes (Abranches *et al.*, 1998). Since interphase chromatin is highly organized, it would not be unexpected to observe the same loci lying close together in nuclei from different cells in progeny derived from each primary transformant. As our data reflect single time points within asynchronous populations of nuclei, the associations must be sustained throughout large parts of the interphase and probably in different cell types. There are several possible explanations for these results. Firstly, it is possible that DNA was delivered to a specific region of the nucleus during transformation, and that the loci were close together when transformation occurred. If nuclear organization is conserved throughout the plant, the transgenes would appear in physical proximity in all interphase nuclei. As it is rare to find multiple integration sites on different chromosomes, it is unlikely that the clustered FISH signals observed on metaphase chromosomes resulted from independent 'hits'. Furthermore, multiple FISH signals have also been described on the metaphase chromosomes of one transgenic wheat and two triticale lines produced by particle bombardment (Pedersen *et al.*, 1997), while all reports involving the use of FISH to detect T-DNA in metaphase chromosome spreads of petunia or tobacco transformed using *Agrobacterium* showed only individual signals (Fransz *et al.*, 1996; Papp *et al.*, 1996; ten Hoopen *et al.*, 1996), occasionally on two different chromosomes (Wang *et al.*, 1995). It has been reported that the gold particle is often found within the nucleus of cells transformed by particle bombardment (Yamashita *et al.*, 1991). It is therefore likely that an accelerated gold particle would cause localized damage to DNA in a particular region of the nucleus, and would affect segments of DNA not necessarily close together in the *cis*-configuration, but lying closer together in *trans* during interphase. We therefore suggest that the integration sites observed in lines 2, 5 and 6 were in close physical proximity during transformation.

Alternatively, it is possible that the transgene sites are brought together in the interphase nucleus because they are recruited to a common functional domain such as a transcription factory, perhaps reflecting the fact that they share the same promoter (Jackson *et al.*, 1998). Current models of interphase chromosome organization suggest that active genes may be located on chromatin-loop domains attached at their ends to the nuclear matrix. Our results are consistent with such a model. It may be that several loops with transgenes sharing the same promoter are brought together at specific transcription sites. Alternatively, as discussed above, an integration event may have led to transgene incorporation in one or more loops, already interacting with the same transcription site at the moment of transformation. The transgene loci we studied share extensive homology, and this may also be a

factor causing their association through ectopic homologous pairing.

It is likely that the interphase chromosome architecture, as well as specific interactions such as transcriptional recruitment or ectopic pairing, both contribute to the observed localization of FISH signals at interphase. In line 5, the two signals were reproducibly closer together than expected (about 2 μm apart), but rarely coincident, suggesting that the interphase chromatin architecture defined their relative positions but there was no physical interaction between the loci. In lines 2 and 6, however, we saw examples where the signals were close together but not coincident (as in line 5), and examples where the signals were coincident. We suggest that when the two sites are brought close together by the architecture of the chromatin, there is an opportunity for further interaction by ectopic pairing or recruitment to a common transcription site, and that this would generate a coincident signal.

Homology of transgene sequences, particularly in the promoter region, has often been proposed as a factor contributing to variable transgene expression and silencing. Silencing induced by pairing between homologous sequences has been observed in *Drosophila* through a combination of molecular and cytogenetic analysis (Sass and Henikoff, 1999). These experiments suggest that the interaction between homologous sequences located in active chromatin and heterochromatin could lead to silencing of the active copy of the gene. The most complex line we studied (line 2) showed the highest level of GUS activity. However, particularly in homozygous populations of this line, we repeatedly identified individual plants showing complete transgene silencing. This was associated with hypermethylation of the transgene promoter, which has been shown to be correlated with the transcriptional silencing of transgenes (and indeed endogenous genes) in many systems (Matzke and Matzke, 1998; Meyer, 1998). Where position effects have been abolished it is thought that other factors, such as the pairing of homologous transgene sequences in *cis* and in *trans*, may initiate silencing and induce methylation. This is conceptually similar to the effect of paramutagenic alleles, in which a repressed epigenetic state is transferred from one allele to another following a transient interaction between them (Chandler *et al.*, 1996; Jorgensen, 1993). The close physical proximity of separate transgene loci shown by our FISH analysis of interphase chromosomes confirms the possibility of such interactions between transgene sequences separated by large stretches of intervening genomic DNA, and gives direct support for such a mechanism for the induction of transcriptional silencing.

In summary, our results show that transformation by particle bombardment causes complex patterns of integration at the scale of the chromosome as well as at the level of DNA sequence, leading in some cases to multiple

transgene sites that are widely separated along the chromosome, but that these sites are very close together in *trans* in the interphase configuration, that is, the configuration in which the initial transformation event took place. Further work will be needed to distinguish the possible factors mediating this organization, but it implies a highly reproducible organization of the interphase chromosomes. As more studies show the importance of chromatin organization at all scales in the regulation of gene expression and in transcriptional silencing, a fuller understanding of organization and expression of transgenes has great relevance in practical as well as fundamental nuclear biology.

Experimental procedures

Plant material for transformation

Wheat plants (*Triticum aestivum* L. cv. Bobwhite, genomic complement AABBDD), were grown in the greenhouse and growth rooms at 15/12°C day/night temperature with a 10 h photoperiod during the first 40 days, followed by maintenance at 21/18°C day/night temperature with a 16 h photoperiod thereafter.

Target tissue and transformation

Immature embryos were removed and cultured as described (Vasil *et al.*, 1993). After 6–7 days, particle bombardment was carried out under standard conditions (Klein *et al.*, 1987) using plasmid pAHC25 or pAHC20, respectively (Christensen and Quail, 1996). Bombarded callus was selected on medium containing phosphinothricin (Altpeter *et al.*, 1996). PAT activity was assayed in leaf tissue as described, before transferring the plants to soil (Vasil *et al.*, 1992).

Southern blot analysis

DNA was prepared from leaf tissue according to Dellaporta *et al.* (1984). Aliquots of DNA (15 µg) were digested with appropriate restriction endonucleases and fractionated by 0.9% agarose gel electrophoresis. Transfer to nylon membranes and hybridization procedures were carried out according to standard protocols (Sambrook *et al.*, 1989). ³²P-labelled hybridization probes, comprising the coding region of the *gusA* or *bar* genes, respectively, were prepared using the random primer labelling kit (Gibco-BRL, Inchinnan, UK).

Copy number determination

To estimate gene copy number, genomic DNA was digested with a restriction enzyme cutting only at one site in the plasmid. Thus the number and intensity of bands reflected the copy number of integrated transgene(s). A second enzyme cutting once on the other side of the gene was chosen to confirm our determinations. A control digest releasing the full gene cassette was also carried out to distinguish between intact and truncated copies.

Preparation of plant material for FISH analysis

Seeds were germinated in a Petri dish lined with wet filter paper. For FISH analysis of metaphase chromosomes, we excised root tips approximately 1 cm long and kept them in ice-cold water for 24 h to arrest the cell cycle at metaphase before fixing in fresh ethanol : acetic acid (3 : 1). For preparation of tissue sections, root tips were excised 3 days after germination, fixed in 4% (w/v) paraformaldehyde in PEM (50 mM PIPES, 5 mM EGTA, 5 mM MgSO₄ pH 6.9) for 1 h, followed by washing for 10 min in TBS (10 mM Tris, 140 mM NaCl pH 7.4). Embryos were removed from the seed 2 weeks after pollination, and fixed in the same way.

Chromosome preparation

Chromosome spreads were prepared according to Harrison and Heslop-Harrison (1995), but using a longer enzymatic digestion (1.5 h). Samples were then treated with 100 µg ml⁻¹ RNase A (Sigma, Poole, UK) in 2 × SSC (300 mM NaCl, 30 mM sodium citrate) for 1 h at 37°C, followed by 25 µg ml⁻¹ pepsin (Sigma) in 0.01 M HCl for 10 min at 37°C. They were then fixed in 4% (w/v) paraformaldehyde for 10 min. Samples were washed in 2 × SSC between each step. Finally the samples were dehydrated for 3 min in 70% ethanol and for 3 min in absolute ethanol.

Tissue sections

Root-tip and embryonic tissue sections (30 µm) were prepared under water using a Vibratome Series 1000 (TAAB, Aldermaston, UK). Sections were immediately placed on multi-well slides (ICN Biomedicals Inc., Aurora, OH, USA) coated with glutaraldehyde-activated γ-aminopropyltriethoxysilane (APTES; Sigma) and left to air dry. Prior to *in situ* hybridization, tissue sections were treated with 2% (w/v) cellulase (Onuzuka R-10) in TBS for 1 h at room temperature and washed in TBS. Sections were then treated with RNase A (100 µg ml⁻¹ in 2 × SSC) for 1 h at 37°C, washed in 2 × SSC, dehydrated in 70% ethanol for 3 min and absolute ethanol for 3 min, and left to air dry.

FISH probes

DNA probes were prepared by nick translation of the pAHC25 plasmid or the *gusA* coding region with biotin-16-dUTP (Boehringer Mannheim, East Sussex, UK). Chromosome identification was carried out by reprobng the samples with the following probes labelled with either digoxigenin-11-dUTP (Amersham, Little Chalfont, UK) or biotin-16-dUTP (Boehringer). (1) pTa71 containing a 9 kbp *Eco*RI fragment of the 18–25S rDNA isolated from *T. aestivum* (Gerlach and Bedbrook, 1979) labelled by nick translation. (2) pSc119.2 containing a 120 bp tandem repeated sequence unit of DNA isolated from *Secale cereale* (Bedbrook *et al.*, 1980) labelled by PCR. (3) dpTa1 containing a 340 bp tandem repeated sequence unit of DNA isolated from *T. aestivum* (Vershinin *et al.*, 1994) labelled by PCR. (4) pTa794 containing a 410 bp *Bam*HI fragment of the 5S rDNA isolated from *T. aestivum* embryos (Gerlach and Dyer, 1980) labelled by PCR. (5) Rye genomic DNA labelled by nick translation.

FISH

The FISH mixture was prepared using 100 and 200 ng probe (for metaphase preparations and tissue sections, respectively) and a

50× excess salmon sperm DNA in 50% formamide, 10% dextran sulphate, 2 × SSC and 0.1% SDS. The probe was denatured in the hybridization mixture for 5 min at 95°C. Chromosomes were denatured at 78°C for 10 min in a modified thermocycler (Omnislide, Hybaid, Ashford, UK), and hybridization carried out overnight at 37°C. Post-hybridization washes were carried out using 20% formamide in 0.1 × SSC at 42°C. After collection of data, slides were washed overnight in 4 × SSC, 0.2% Tween 20 and dehydrated. Second-round FISH was carried out as above, but chromosome denaturation was performed at 70°C for 5 min.

Immunodetection

Biotinylated probes were detected with Extravidin-Cy3 (Sigma). Probes labelled with digoxigenin were detected with conjugated sheep antidigoxigenin antibody-FITC (Boehringer). Antibodies were diluted in 4 × SSC, 0.2% Tween 20 according to the manufacturer's instructions. Antibody incubation was performed in a damp chamber for 1 h at 37°C followed by 3 × 5 min washes in 4 × SSC, 0.2% Tween 20 at room temperature. Metaphase slides were counterstained with 6 µg ml⁻¹ 4', 6'-diamidino-2-phenylindole (DAPI; Sigma) for 10 min, then mounted in antifade solution (Vectashield, Vector Laboratories, Burlingame, CA, USA). Interphase nuclei were treated as above, but using 1 µg ml⁻¹ DAPI for 3 min.

Image acquisition and data analysis

Chromosome spreads were analysed with a Nikon E600 microscope, and photographs were taken on Fujicolor 400 print film and digitized with a Microtek ScanMaker 5. Tissue sections were analysed on a Leica TCS SP confocal microscope. Typical samples, comprising one or two cell layers, were scanned with an interval of 0.5–0.8 µm, generating between 18 and 40 sections per nucleus. The images were collected, transferred to NIH IMAGE (a public domain program for the Macintosh by W. Rasband, available via ftp from zippy.nimh.nih.gov), and assembled using ADOBE PHOTOSHOP 4.0. Three-dimensional reconstructions and stereo images were prepared using OBJECT-IMAGE, an extension to NIH IMAGE written by N.O. Vischer, allowing us to view reconstructed nuclei or individual layers as appropriate. Final images were printed on a Pictography P3000 printer. Counting was carried out independently by three individuals and the results averaged, producing the numbers summarized in Table 2. We then selected a random population of interphase nuclei for each line in order to measure the distances between different signals of one locus. We defined the position of each signal by measuring its coordinates within the nucleus. The vertical distance was deduced from the interval between sections. We then calculated the distance between any two of the signals belonging to a homologue. The maximum distance for each locus is given in Table 3.

Progeny analysis

The segregation of functional *bar* loci was determined by germinating excised embryos on MS agar medium containing 3 mg l⁻¹ phosphinothricin (PPT). Germination frequency was tested by placing 10 embryos on MS agar without PPT. Only data from experiments resulting in >90% germination rates were included in this study. To determine the segregation of functional *gusA* loci, endosperm tissue was tested for GUS activity by histochemical staining as described by Jefferson (1987). GUS activity was quantified in leaves of 4- to 6-week-old *R*₀ and *R*₁

plants by fluorometric assays (Jefferson *et al.*, 1987). Ten *R*₄ seedlings were tested in a mixed sample of leaves of the same stage. The level of GUS activity was stably maintained after four generations, that is, activity remained in the same category (high, medium or low) for each line.

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