Theor Appl Genet (1997) 94 : 749–757

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Localization of introduced genes on the chromosomes of transgenic barley, wheat and triticale by fluorescence in situ hybridization

Received: 28 October 1996 / Accepted: 15 November 1996

Abstract Using fluorescence in situ hybridization (FISH) we localized introduced genes on metaphase chromosomes of barley, wheat, and triticale transformed by microprojectile bombardment of microspores and scutellar tissue with the pDB1 plasmid containing the *uidA* and *bar* genes. Thirteen integration sites were detected in the nine lines analysed. Southern analysis showed that three or more copies of the plasmid were present in the lines. In a triticale line containing four copies three different integration sites were identified, indicating that the method described is sensitive enough for the detection of single-copy integrations. There was a slight tendency towards the localization of transgenes in distal chromosome regions. Using the GAA-satellite sequence for chromosome banding, the chromosomes containing the inserted genes were identified in most cases. Two barley lines derived from the same transformant showed a totally different integration pattern. Southern analysis confirmed that the inserted genes were segregating independently, resulting in different integration patterns among the progeny lines. The application of the FISH technique for the analysis of transgenic plants is discussed.

This publication is dedicated to Prof. Dr. B. Parthier on the occasion of his 65th birthday.

Communicated by G. Wenzel

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Key words Transgenic barley, wheat and triticale · Chromosomal localization · FISH · Integration pattern

Introduction

Genetic modification of plants by transformation has become of great value in basic and applied research and provides a number of promising applications in plant improvement. However, the application of transformation systems for various purposes is frequently hampered by the instability of the transgenes and their expression. It is important, therefore, to gain a better understanding of the factors affecting the stability and expression of inserted genes. One significant factor seems to be the position of the transgene in the genome, known as the "position effect". This position effect can be studied, for example, by Southern-blot analysis to examine the methylation status of the DNA, and by in situ hybridization which identifies the physical position of the transgene within the genome. However, only few reports have shown the chromosomal localization of transgenes by in situ hybridization, and this mainly using radioactive detection techniques (Ambros et al. 1986; Hoopen et al. 1996; Mouras et al. 1987; Mouras and Negrutiu 1989; Wang et al. 1995).

Here we report the localization of transgenes by fluorescence in situ hybridization (FISH) to the chromosomes of transgenic barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and triticale (× *Triticosecale* Wittmack). The integration sites of the transgene relative to heterochromatin, rDNA and other chromosomal landmarks are examined. Finally, we combine FISH and Southern hybridization to demonstrate and explain the independent segregation of inserted genes in a barley line.

Materials and methods

Plant material

The plant material studied by in situ hybridization included barley, wheat, and triticale lines which were produced by particle bombardment as described previously (Becker et al. 1994, Jähne et al. 1994, Zimny et al. 1995). The barley lines were designated I, IP, and III and were produced by particle bombardment of microspores of cv. Igri. IP is from the progeny generation R_2 and I is from the R_1 of the same transformant. Thirteen different R_1 plants of line I were in addition used for Southern analysis. The transgenic wheat lines were produced from scutellar tissue of the cultivar Florida and are designated 2/8/1/13 and 1/1/12/2. The transgenic triticale lines were derived from scutellar tissue of the line MAH 1590 and are designated 1/1, 16, 24, and 55. All lines were transformed with the pDB1 construct (Becker et al. 1994).

Root tip and chromosome preparation

Root tips were obtained from seedlings after synchronization of cell division with hydroxyurea (Dolezel et al. 1992; Pan et al. 1993). The seedlings were first germinated on moist filter paper in plastic boxes at 24°C and then transferred to filter paper containing 1.25 mM hydroxyurea when the primary root emerged. After about 18 h the seedlings were rinsed in water and incubated on filter paper with water for another 5.5 h to release the cells from the DNA block. Subsequently, the roots were pre-treated with 4 μM of APM (Amiprophos methyl, Miles Ltd) for 3 h and with icewater for 20 h before fixation in 3:1 ethanol:glacial acetic acid.

The root tips were digested with 2% cellulase (Onozuka RS) and 2% liquid pectinase (Sigma) at 37°C for 2–3 h. Chromosome preparations were made by the air-drying method (Olin-Fatih and Heneen 1992). After digestion, the enzyme solution was carefully removed and replaced with distilled water for 5 min. Then the water was replaced with fresh fixative (ethanol:glacial acetic acid 3:1). The meristematic part of a root tip was transferred to an acid-cleaned slide using a pipette, spread in a drop of fixative with a pair of tweezers, and air-dried.

DNA probes and hybridization mixture

The pDB1 plasmid has a size of about 8.5 kb and contains the *uidA* and *bar* genes (Becker et al. 1994). The barley clone, pHvG38, contains the GAA-satellite sequence (Pedersen et al. 1996), and the ribosomal clone, pTa71, contains the entire 18s, 5.8s, 26s rDNA repeat. The plasmids were labelled with biotin-14-dATP by nick translation (Gibco) and mixed to a final concentration of 1 μg/ml in the hybridization solution containing 50% formamide, 10% dextran sulphate, 2 × SSC, 0.1% SDS and 50 μg/ml of herring sperm DNA.

Fluorescence in situ hybridization

The slides were incubated in 0.2 μg/ml proteinase K for 10 min at 37°C and washed two times with 2 × SSC and subsequently treated with 10 μg/ml of DNase-free RNase in 2 × SSC for 1 h at 37°C, washed in 2 × SSC, dehydrated in ethanol, and air-dried. Chromosomal DNA was either denatured in 0.2 N HCl at 37°C for 10 min as described previously (Pedersen and Linde-Laursen 1994), or by combined denaturation as described by Heslop-Harrison et al. (1991) using a thermal cycler equiped for slides (Biometra). The probe mixture was denatured at 95°C for 5 min and chilled on ice. About 40 μl of probe mixture was applied per slide. Slides were covered with 24 × 50 mm cover slips, sealed, and incubated in a moist chamber at 37°C overnight. Cover slips were removed and the slides were washed two times in 2 × SSC at 37°C for 10 min, once in 0.2 × SSC at 60°C for 10 min, and once in 2 × SSC at 37°C before transferring to the detection buffer (4 × SSC with 0.2% Tween 20). The slides were blocked in detection buffer containing 5% BSA for about 15 min. Biotinylated probes were detected with 5 μg/ml of FITC-avidin DCS (Vector) in detection buffer with 5% BSA followed by amplification with 5 μg/ml of biotinylated anti-avidin D (Vector) and another incubation with FITC-avidin DCS. The red fluorochrome Cy3 (Cyanine 3.18) conjugated to avidin was used as an alternative to FITC-avidin on a few occasions. Each incubation was done in a moist chamber at 37°C for 45 min and followed by washing the slides three times in detection buffer. After the final washing step the slides were dehydrated in 70% and 100% ethanol (3-min each) and air-dried. The slides were mounted in an anti-fade solution (Krenik et al. 1989) containing 0.4 μg/ml of DAPI and 0.2 μg/ml of propidium iodide.

After examination and photography of metaphases hybridized with the pDB1 probe, the preparations were in some cases hybridized with the GAA-satellite sequence or the ribosomal probe, for identifying chromosomes with positive signals as described by Pedersen and Linde-Laursen (1994).

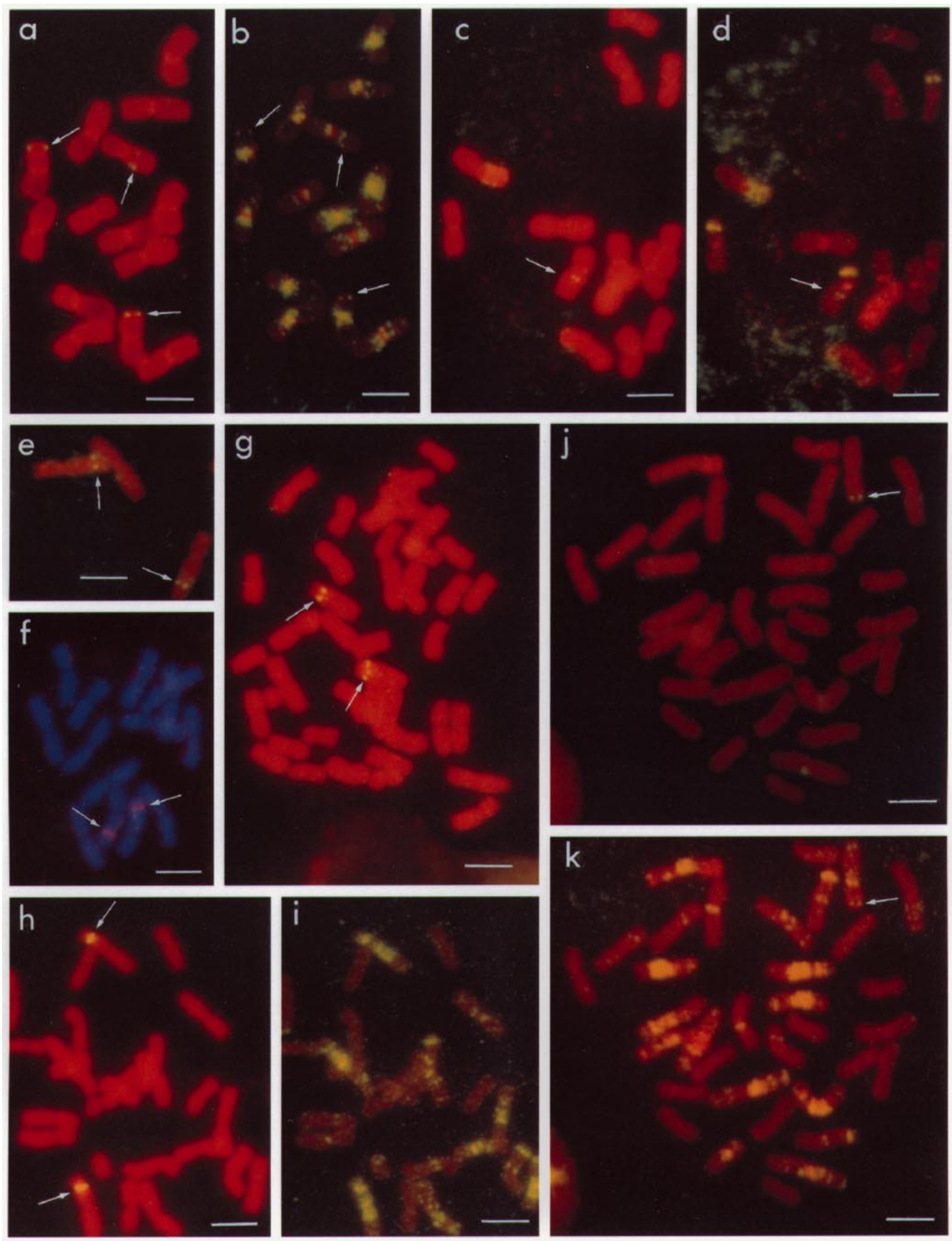
Microscopy, photography and measurements of chromosomal distances

The slides were examined with an Olympus BH-2 microscope equipped for epifluorescence and photographs were taken on Kodak Ektachrome P1600 films for colour slides. Chromosomal distances were estimated on the basis of slide projections to a magnification of about 20000 times. Only hybridization signals wich were visible as double spots were recorded. The fraction lengths (FLs) (%) of hybridizations sites were calculated as the distance from the centromere to the hybridization signal relative to the total length of the chromosome arm. Thirty to forty barley chromosomes were measured for each FL-calculation and standard deviations were estimated for all calculations. In wheat and triticale we measured only 10–20 chromosomes for each calculation. The detection frequencies express the percentage of metaphases with specific signals in the best slides analysed.

DNA isolation and Southern hybridization

The DNA isolations and the Southern hybridizations were performed as described by Becker et al. 1994. The number of integrated

Fig. 1 a–k FISH with the pDB1 probe, the GAA-satellite sequence, and rDNA probe, to metaphase chromosomes of barley and wheat. Hybridization signals of the pDB1 probe are indicated with *arrows*. **a** The hybridization signals in line I, with subtelomeric signals on both homologues of chromosome 6 and an interstitial signal on one homologue of chromosome 5. Bar = 5 μm. **b** The same metaphase as in (**a**) after hybridization with the GAA-satellite sequence (GAA-banding). Bar = 5 μm. **c** The pDB1 hybridized to line IP produced a signal on chromosome 7. Bar = 5 μm. **d** The same metaphase as in (**c**) after re-probing with the rDNA probe. Bar = 5 μm. **e** The barley line III had two clear pDB1-signals on both homologues of chromosome 7. The integration sites were detected with FITC-avidin. **f** Same as in (**e**) but detected with Cy3-avidin. Bar = 5 μm. **g** The strong signals at the secondary constriction of the satellite chromosomes of the wheat line 2/8/1/13. Bar = 5 μm. **h** Partial metaphase of the wheat line 2/8/1/13 showing the two strong signals. Bar = 5 μm. **i** The same as (**h**) after GAA-banding. Bar = 5 μm. **j** The wheat line 1/1/12/2 with a signal on one chromosome. Bar = 5 μm. **k** The same as **j** after GAA-banding. Bar = 5 μm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copies was determined by digestion with *Nco*I, *Hin*dIII and *Nde*I which have no or only a single restriction site within the transformation plasmid pDB1, followed by hybridization with the *uidA* and *bar* probes. The segregation of transgenes in transgenic barley line I was determined by digestion of genomic DNA with *Ssp*I and hybridization with the *bar* probe.

Histochemical GUS assay

The GUS activity was determined histochemically as described by McCabe et al. (1988).

Results

In situ hybridization

Fluorescence in situ hybridization with the pDB1 probe produced specific signals on metaphase chromosomes of nine transgenic lines of barley, wheat and triticale. The detection frequencies varied from about 50% up to 100% depending on the line, the quality of the chromosome preparations, and the success of the FISH procedure. At least ten chromosomes with double signals, one on each chromatid, were recorded for each line and for most lines many more. The FISH results are shown in Figs. 1 and 2. The results are summarized in Fig. 3, showing the fraction-length (FL) positions of the transgenes, and in Table 1, where the detection frequencies are also given.

Barley

FISH to chromosomes of barley line III produced a clear double signal on the long arms of both homologues of chromosome 7 (Fig. 1 e–f). Chromosome 7 was distinguished from chromosome 6, the other satellited chromosome, by its uneven arm length ratio. The signals were found in more than 90% of the metaphases examined. The fraction-length position of the inserted pDB1 plasmid was estimated to be 67% (Table 1). No other specific signals were seen in this line.

In line I there was a clear signal on the satellites of chromosome 6 in more than 90% of the metaphases (Fig. 1 a). The FL position was estimated to about 88% (Fig. 3). In addition to these signals, we observed a weaker signal on chromosome 5, but always on only one of the two homologues. It was detected in about 60% of the metaphases examined at an FL of about 31% (Fig. 3). Chromosome 5 was usually identifiable because it is the only submetacentric non-satellited chromosome. In order to confirm the identities of the chromosomes with signals, we re-probed the slides with a probe containing the GAA-satellite sequence which hybridizes to the positions of the N-bands (Pedersen et al. 1996) (Fig. 1 b). These results confirmed the minor signal to be on the long arm of chromosome 5. In the line PI, which is a R_2 progeny line from the same R_0 plants as line I, we detected only a single signal and that was on chromosome 7 at a FL position of about 34% (Figs. 1 c and 3). The detection frequency was about 50% and the chromosome identification was confirmed by re-probing with the pTa71 rDNA probe (Fig. 1 d).

Wheat

In situ hybridization with the pDB1 probe produced strong signals at the secondary constriction of a pair of satellite chromosomes in the wheat line 2/8/1/13 (Figs. 1 g–h). This signal was frequently seen as two double spots on each chromatid, showing that there are two integration sites very close to each other. The chromosome could be identified as 6B since this is the only satellited chromosome in 'Florida'. It was further confirmed by re-probing with the rDNA probe, pTa71, hybridizing to the NOR, and by re-probing with the GAA-satellite sequence (Fig. 1 i). The GAA-banding patterns produced were equivalent to those obtained by Giemsa N-banding (Pedersen et al. 1996).

In another wheat line, 1/1/12/2, a weaker pDB1 hybridization signal was observed near the telomeres on the short arm of one chromosome pair (Fig. 1 j). The detection frequency was about 90%. Re-probing with the GAA-satellite produced a proximal signal on both chromosome arms and a signal near the telomere of the long arm (Fig. 1 k). Based on the GAA-bands and the arm ratio, the chromosome with the transgene was identified as 2A.

Triticale

The integrated transgenes were detected in all four Triticale lines studied (Fig. 2, Table 1). The strongest signals were seen in the lines 1/1 and 55 (Fig. 2 a–b and c). However, two other lines, 16 and 24, had several integration sites on the same chromosome arm (Figs. 2 d, f). In line 24 there were two integration sites quite close together on the same arm of a rye chromosome. Rye chromosomes could be easily identified by the stronger staining of the telomeric heterochromatin with propidium iodide and DAPI (4′,6-diamidino-2-phenylindole). In the other three lines the transformed chromosomes were of A- or B-genome origin. The transformed chromosome of line 1/1 had at least three strong proximal GAA-bands and a minor distal one on the long arm (Fig. 2 c). The arm ratio is about 1.1, indicating that the chromosome is 4B. The chromosome showing three separated integrations sites on one arm in line 16 did not have GAA-bands. Since the arm ratio is about 1, the best candidate chromosome is 6A. The transformed chromosome

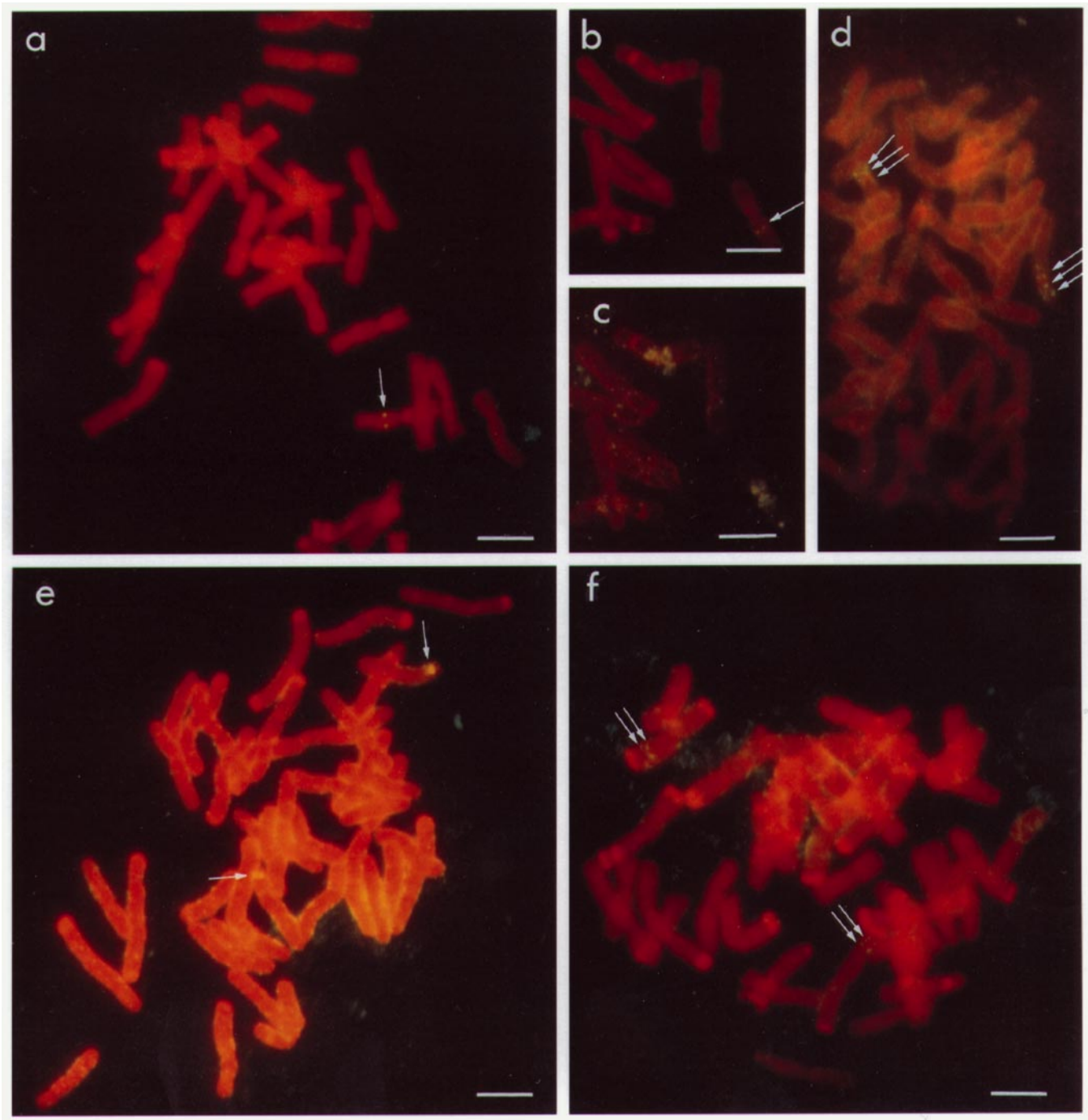

Fig. 2 FISH with the pDB1 probe to the chromosomes of the triticale lines 1 (**a–b**), 16 (**d**), 55 (**e**) and 24 (**f**). Chromosomes of line 1 are shown before (**b**) and after (**c**) GAA-banding. Hybridization signals of the pDB1 probe are indicated with *arrows*. Bars = 5 μm

in triticale line 55 had a single GAA-band on the long arm close to the centromere and an arm ratio at about 1.6. Based on this, the best candidate is chromosome 5A. Three of the four triticale lines were homozygous for the transgene signals while in line 1/1 the signal was only found on one of the homologs of chromosome 4B.

Southern and histochemical GUS analyses

Southern analysis indicate that the lines analysed by in situ hybridization contain three or more copies of the plasmid (data not shown, see Table 1).

Prompted by the surprising finding of two different in situ hybridization patterns in two barley lines

Table 1 The table summarizes the detection frequencies of the transgenes obtained by in situ hybridization with indications of the number of homologs with transgenes in each line analysed. Further, the copy numbers of the transgenes are indicated based on Southern-hybridization analyses. The GUS- and PAT-activities of each line are included

Item	Barley			Wheat		Triticale			
	III	I	IP	2/8/1	1/1/12	1/1	16	24	55
Detection freq.	90	90 + 60	50	100	90	80	80	50	80
Homo/hemi	2	2+1	1	2	2	1	2	2	2
Copy number	3	6	nd	11	3	4	4	5	5
GUS-activity	−	+	+	−/+	+	+	+	+	−
PAT-activity	+	+	+	−	+	+	+	+	+

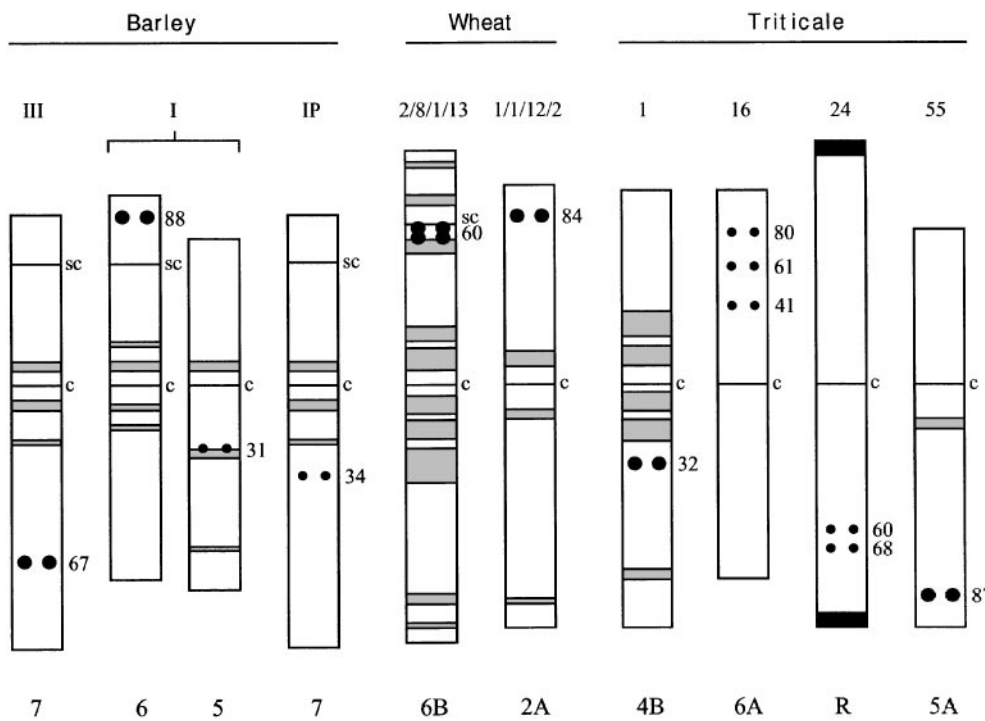

Fig. 3 Schematic illustration of the chromosomal locations of the transgenes in the nine lines analysed, with indications of the fraction- length positions of the transgenes. The *shaded bands* show the GAA-bands. The centromeres (**c**) and secondary constrictions (*sc*) are also indicated. The identity of the chromosomes is given below; the barley chromosomes are numbered according to standard barley nomenclature

derived from the same transformant, we analysed 13 F_1-plants of barley line I by Southern hybridization (Fig. 4). The parental line (lane P) showed six fragments (7.5 kb, 5.8 kb, 3.6 kb, 2.6 kb, 2.4 kb, and 0.45 kb) of various intensity. Two plants showed the same pattern as the parental line (lanes 5 and 6). In five plants (lanes 3, 9, 10, 11 and 13) only two bands (7.5 kb and 2.6 kb) were observed. A third segregation pattern (lane 8) was found in one plant (7.5 kb, 5.8 kb, 2.6 kb and 0.45 kb). Three plants (lanes 2, 4 and 7) showed five bands (5.8 kb, 3.6 kb, 2.6 kb, 2.4 kb and 0.45 kb) and one plant (lane 1) showed all fragments except two (5.8 kb and 0.45 kb). Finally, there was one plant (lane 12) which did not show any bands at all. According to the plasmid restriction map the size of the expected fragment is about 2.6 kb. This fragment was detected in the parental line and all progeny plants except for one which showed no hybridization signal at all. The larger and smaller bands in the parental line and most progeny lines indicate that fragmented copies had been integrated.

The progeny plants were also tested for activity of the *uidA* gene and all plants showed a blue colour in stained leaf pieces including the plant which was negative for the presence of the *bar* gene in Southern analysis.

Discussion

The present study was undertaken to (1) localize transgenes on chromosomes and identify the transformed chromosomes, (2) study the integration patterns to

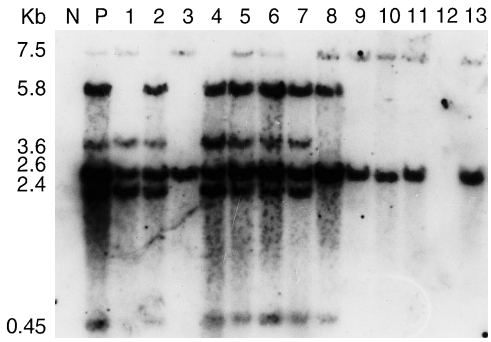

Fig. 4 Southern-blot analysis of 13 F_1-plants of barley line I (*lanes 1–13*) and the parent (*lane P*). The lane indicated with *N* contains the negative control. The genomic DNA was digested with *Ssp*I and the blot was hybridized with the *bar*-probe

reveal preferential integration sites, and (3) study the segregation of transgenes in progeny plants from a microspore-derived barley plant.

Localization of transgenes by FISH

In the recent years the FISH technique has been applied to a number of problems in the molecular cytogenetics of plants (Jiang and Gill 1994; Leitch et al. 1994). However, there have been few reports concerning the localization of transgenes on plant chromosomes and these have used radioactive or enzymatic detection techniques to localize T-DNA in petunia and tobacco plants transformed by *Agrobacterium tumefaciens* (Ambros et al. 1986; Mouras et al. 1987; Mouras and Negrutiu 1989; Wang et al. 1995). Recently, T-DNA has been located on petunia chromosomes using FISH and a cooled CCD camera for image acquisition (Fransz et al. 1996; Hoopen et al. 1996). In the present report we describe the localization of transgenes in cereal species transformed by particle bombardment. Using this delivery technique the plasmid DNA is frequently fragmented before incorporation into the genome and one to several copies of the whole plasmid, or fragments of it, are inserted in tandem at the same site (Birch and Franks 1991). Therefore, it is difficult to deduce the exact copy number from Southern analyses. In the present study we assume that nearly all lines analysed contain three or more copies of the pDB1 plasmid. The only exception might be the barley line IP which had only a single integration site. In lines containing single-copy integrations it was not possible to detect these by the method employed. However, in triticale line 16 containing four copies we detected three different integration sites, indicating that the method is sensitive enough for the detection of single-copy insertions. The clear signals and the high detection frequencies reported here show that the present technique is suitable for the physical mapping of transgenes inserted by particle bombardment. It would be desirable to improve the sensitivity to be able to detect single-copy DNA sequences of a few kb or less with a high frequency. At present it is not achievable by standard in situ hybridization with plant chromosomes (Jiang and Gill 1994). The application of a cooled CCD camera increases the sensitivity. To improve the sensitivity still further it will probably be necessary to amplify the target DNA by PCR prior to the in situ hybridization (PCR-in situ hybridization) or to do direct PCR-in situ with labelled nucleotides. Such techniques have been described for human and animal chromosomes (Gosden and Hanratty 1993; Terkelsen et al. 1993; Troyer et al. 1994). However, there has been only a single report of PCR-in situ on plant chromosomes and the results have not been of high quality (Zhu et al. 1995).

In the present study it has been possible to identify the transformed chromosomes in most cases by including DNA probes of highly repetitive sequences, such as the GAA-satellite sequence and rDNA. A previous study has shown that the GAA-banding patterns of barley, wheat, and several other *Triticeae* species are similar to the N-banding patterns (Pedersen et al. 1996). The identification of wheat and triticale chromosomes in this report has been based on the similarities between the GAA-banding patterns obtained here and the N-banding patterns reported by Endo and Gill (1984). A number of other tandemly organized highly repeated sequences have been used for identifying chromosomes in cereals and these provide tools for identifying most of the chromosomes in wheat and rye (Bedbrook et al. 1980; Mukai et al. 1993; Rayburn and Gill 1986). Another approach would be to perform C-banding or N-banding subsequent to the localization of the transgenes (Jiang and Gill 1993).

Integration patterns

In this work 13 integration sites were detected in nine lines. This sample size is too small to obtain a clear proof for preferential incorporation sites. At first sight (Fig. 3) it seems as if the transgene is integrated at random. However, there might be a slight tendency toward localization to distal regions since 9 of the 13 recorded sites are in the distal 40% of the chromosome arms. A similar tendency has been found in petunia (Wang et al. 1995; Hoopen et al. 1996), but again in limited material. Preferential incorporation into distal regions would not be surprising because these are the most decondensed parts of cereal chromosomes during interphase and contain the bulk of actively transcribed genes (Moore et al. 1993). Heterochromatin occupies a large fraction of the proximal regions of many barley and wheat chromosomes and is presumably less amenable for the incorporation of inserted genes since it is condensed during the cell cycle. It is noticeable in this

work that four out of ten chromosomes with transgenes are satellited chromosomes and in one case (chromosome 6B of wheat) a large number of copies of the transgene (about 11) is incorporated directly in the NOR. It seems that there are two adjacent incorporation sites in the rDNA region proximal to the secondary constriction. Such regions,which are highly active during the cell cycle of mitotically active cells, are probably more susceptible to the incorporation of DNA delivered into the cell nucleus.

Independent segregation of transgenes

The surprising finding of two different in situ hybridization patterns in two barley lines derived from the same transformant encouraged us to analyse 13 R_1-plants of the barley lines. The Southern analysis confirmed that inserted genes were segregating independently. The most simple model for explaining these results is to assume that the R_0-transformant was hemizygous for three different integration sites on the chromosomes 5, 6 and 7. In the first-selfing generation R_1 there will be 27 possible combinations of which some have no loci in common as demonstrated by the in situ hybridization patterns in lines I and PI. Among the 27 possible combinations in the R_1-generation, only eight different patterns can be revealed by Southern analysis. In fact, we found five different patterns by Southern hybridization while one R_1-line had completely lost the integration sites, confirming that none of the integration sites were homozygous in the R_o transformant. Therefore, the integrations must have occurred after spontaneous chromosome doubling. Though most of the transgenic plants obtained from bombarded microspores are homozygous (Jähne et al. 1994), the regeneration of hemizygous plants sometimes occurs (unpublished results). A fragment with the *uidA* gene providing GUS expression may have been integrated at a fourth locus because the plant totally lacking the *bar* gene was still GUS-positive.

Independent segregation of transgenes has previously been observed in wheat, triticale, and maize transformed by particle bombardment of immature embryos (data not shown). When several copies of the introduced gene construct are integrated at different loci in the hemizygous state, segregation of these loci may be employed to obtain lines with different integration patterns and, maybe, different expression levels. In connection with co-transformation with a selectable marker and the gene construct of interest on a different plasmid, independent segregation makes it possible to eliminate the marker gene.

General discussion

The chromosomal localization of a transgene is important for several reasons. Firstly, it is relevant to study the correlation between the chromosomal location of an inserted gene and its stable expression and inheritance. However, to determine such correlations the analysis of many lines is required. Further, it is probable that other aspects, such as the gene construct itself, the methylation status of the DNA surrounding the transgene and its copy number, play a role in the expression during subsequent generations (Finnegan and McElroy 1994; Flavell 1994).

The present study showed that the gene constucts are inserted in both A- and B-genome chromosomes as well as the R-genome chromosomes of triticale. By backcrossing the transgenic triticale lines to durum wheat and rye, depending on the chromosomal location of the transgene, it would be possible to create transgenic durum wheat and rye indirectly. This may be a useful method since rye has proved difficult to transform while triticale transformation has succeded with a relative high efficiency (one transgenic plant per 34 embryos) (Zimny et al. 1995) compared to wheat and rye (Weeks et al. 1993; Becker et al. 1994; Castillo et al. 1994; Nehra et al. 1994).

Studies in oilseed rape have shown that there is a relatively high risk of spontaneous hybridization between transgenic oilseed rape and weedy *Brassica* species, such as *B. campestris* and *B. juncea* resulting in transgene dispersal (Jorgensen and Andersen 1994; Frello et al. 1995). The transmission of the transgene is highly dependent on which *Brassica*-genome the transgene is located. Therefore, it is important to use in situ localization to select transgenic lines of oilseed rape with the transgene in a chromosomal location that minimizes the risk of transgene dispersal.

Another application is in connection with the creation of plants transformed with transposons for transposon tagging. Here, in situ localization could be a valuable method for the selection of lines with the transposon located in defined regions.

In situ hybridization provides a useful addition to the palette of tools available for the analysis of transgenic plants. It is likely to be of particular importance for subsequent genetic manipulation of transgenic lines and in the analysis of lines prior to commercialisation and release.

Acknowledgements The authors thank Dr. Peter Langridge for critical reading of the manuscript. The work was supported by a grant from the Danish agricultural and Veterinary Research Council to C. P.

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