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Use of fluorescence in situ hybridization for gross mapping of transgenes and screening for homozygous plants in transgenic barley (*Hordeum vulgare* L.)

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Abstract Introduced transgenes, *uidA*, *sgfp* (S65T) and/or *bar*, were localized using fluorescence in situ hybridization (FISH) on metaphase chromosomes of transgenic barley produced by microparticle bombardment of immature embryos. Of the 19 independent transgenic lines (eight diploid and 11 tetraploid), nine had *uidA* and ten had s*gfp* (S65T). All lines tested had three or more copies of the transgenes and 18 out of 19 lines had visibly different integration sites. At a gross level, it appeared that no preferential integration sites of foreign DNA among chromosomes were present in the lines tested; however, a distal preference for transgene integration was observed within the chromosome. In diploid T_0 plants that gave a 3:1 segregation ratio of transgene expression in the T_1, only single integration sites were detected on one of the homologous chromosomes. Homozygous diploid plants had doublet signals on a pair of homologous chromosomes. All tetraploid T_0 plants that gave a 3:1 segregation ratio in the T_1 generation had only a single integration site on one of the homologous chromosomes. In contrast, the single tetraploid T_0 plant with a 35:1 segregation ratio in the T_1 generation had doublet signals on a pair of homologous chromosomes. In the one tetraploid T_0 line, which had a homozygote-like segregation ratio (45:0), there were doublet signals at two loci on separate chromosomes. We conclude that the application of FISH for analysis of transgenic plants is useful for the gross localization of transgene(s) and for early screening of homozygous plants.

Keywords Barley · Fluorescence in situ hybridization · Transgene localization · Transgene expression · Homozygosity

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Introduction

The in situ hybridization (ISH) technique, initially developed to map the rDNA sequences on mammalian chromosomes (Gall and Pardue 1969; John et al. 1969), has become a powerful tool for detecting specific DNA sequences on genomes. Since the method for using non-radioisotopic, biotin-labeled probes was introduced to facilitate plant genome analysis (Rayburn and Gill 1985), ISH has been widely used for the study of the origin and evolution of specific genomes (reviewed by Jiang and Gill 1994).

Recently, genetic engineering technologies have been used to introduce agronomic and quality traits into major crop species (Vasil 1994). However, several studies have demonstrated that aberrant inheritance of transgenes and their expression is one of the problematic aspects of plant transformation (reviewed by Finnegan and McElroy 1994; Flavell 1994; Phillips et al. 1994; Stam et al. 1997). The physical behavior and localization of transgenes can be studied at the molecular level, using DNA hybridization analysis to investigate integration and methylation patterns, or at the cytogenetic level, using ISH analysis of chromosomes. Since the fluorescence in situ hybridization (FISH) technique was used to facilitate plant genome mapping (Schwarzacher et al. 1989), this technique has allowed detection of low-/single-copy sequences as well as rDNA and highly repetitive sequences in plants.

Detection and localization of inserted genes using ISH have been reported in *Crepis capillaris* (Ambros et al. 1986a, b), *Nicotiana tobacum* (Mouras et al. 1987; Mouras and Negrutiu 1989; Moscone et al. 1996) and *Petunia hybrida* (Wallroth et al. 1986; Wang et al. 1995), as well as other species more recently. Since the first detection of transgenes in petunia plants (Fransz et al. 1996; Hoopen et al. 1996), reports have appeared describing the use of FISH in transgenic cereal crops (Pedersen et al. 1997; Abranches et al. 2000; Leggett et al. 2000; Svitashev et al. 2000; Carlson et al. 2001; Salvo-Garrido et al. 2001).

The FISH technique can be used to provide information on the gross location of the transgenes, and to estab-

lish relationships between location and the physical stability of the transgene and its expression. The screening of transgenic lines by FISH can also be used to predict and confirm the homozygosity of lines. In the present study, we applied the FISH technique to the analysis of transgenic barley plants to: (1) locate the inserted foreign genes [*uidA*, *sgfp* (S65T) and/or *bar*] on the chromosomes, (2) attempt to correlate gross location with the physical stability of the transgene and its expression, and (3) identify homozygous plants and compare these results with segregation ratios of transgene expression.

Materials and methods

Plant materials

Transgenic barley (*Hordeum vulgare* L. cv. Golden Promise, $2n = 2x = 14$) plants were produced via microprojectile bombardment of immature embryos as described by Cho et al. (1999). Nineteen transgenic barley lines were generated; eight lines were diploid and 11 were tetraploid (see Table 1). Tetraploidy originated during the tissue-culture and transformation process. Immature embryos were bombarded with a mixture of two plasmids: (1) pAHC20 (Christensen and Quail 1996) containing *bar* under the control of the maize ubiquitin *ubi1* promoter and first exon/intron; and (2) one of the following plasmids: p16 (Sørensen et al. 1996), pD11-Hor3 (Sørensen et al. 1996), pdBhGN1-2 (M.-J. Cho, unpublished) or pdBhssGN5-6 (M.-J. Cho, unpublished), pDhsGFP-1 (M.-J. Cho, unpublished), pDhSSsGFP3-4 (M.-J. Cho, unpublished) and pAct1IsGFP-1 (Cho et al. 2000). Four plasmids (p16, pD11-Hor3, pdBhGN1-2, pdBhssGN5-6) contain *uidA* under the control of the barley endosperm B_1-(p16, pdBhGN1-2 and pdBhssGN5-6) or D-hordein promoter (pD11-Hor3). The other three plasmids (pDhsGFP-1, pDhSSsGFP3-4 and pAct1IsGFP-1) contain the synthetic green fluorescent protein gene [*sgfp* (S65T)] under the control of the D-hordein promoter (pDhsGFP-1 and pDhSSsGFP3-4) or rice actin promoter (pAct1IsGFP-1). Cultures were selected with 5 mg/l of bialaphos on DC medium (Cho et al. 1998) in the dark for about 3 months. Transgenic calli were transferred to DBC2 medium containing 5 mg/l of bialaphos for 4 to 6 weeks as an intermediate culturing step between the callus-induction (DC medium) and regeneration (FHG) steps. Regenerated shoots were transferred to Magenta boxes with BCI-DM$^-$ rooting medium containing 3 mg/l of bialaphos. When shoots reached the top of the box, plantlets were transferred to soil in the greenhouse.

Cytological analysis

Cytological analysis of transgenic barley plants was performed as previously described (Choi et al. 2000) using healthy root tips collected from young plants grown in the greenhouse.

Chromosome preparation

Root tips, sampled from greenhouse-grown plants, were pre-treated in saturated 1-bromonaphthalene solution at 4 °C for 20–22 h, fixed in 1:3 glacial acetic acid:ethanol, and stored at 4 °C. Chromosome preparations were made by the enzymatic maceration of root tips (2% Cellulase Onozuka RS, 1.5% Macerozyme R200, 0.3% Pectolyase Y-23, 1 mM EDTA, pH 4.2) at 37 °C for 1 h. After washing with distilled water, digested meristematic parts were transferred to glass slides, spread in a drop of fresh fixative (1:3 glacial acetic acid:ethanol) with a pair of tweezers, and air-dried. The chromosomal spreads were examined by phase contrast microscopy (Axiophot, Zeiss) before the FISH analyses were conducted.

Probe preparation

The 1.9-kb *Xba*I-*Sac*I *uidA* fragment from p35SGN-3 (M.-J. Cho, unpublished), the 0.72-kb *Nco*I-*Not*I *sgfp* (S65T) fragment from pAct1IsGFP-1 (Cho et al. 2000), the 0.5-kb *Bam*HI/*Sph*I *bar* fragment from pAHC25 (Christensen and Quail 1996) and a ribosomal clone, pTa71 (Gerlach and Bedbrook 1979), were labeled with biotin-16-dUTP by nick translation (Boehringer Mannheim); pTa71 was used to map the rDNA sites of individual chromosomes as described by Leitch and Heslop-Harrison (1992). Either these fragments or the whole plasmids were used for probe labeling.

Fluorescence in situ hybridization

The FISH procedure was as described previously (Islam-Faridi and Mujeeb-Kazi 1995; Pedersen et al. 1997; Ohmido et al. 1998), with some modifications. Slides were treated with 100 µg/ml of RNase A in 2 × SSC at 37 °C for 1 h, denatured in a 70% deionized formamide solution at 70 °C for 2 min, dehydrated in an ethanol series (70%, 95% and 100% at –20 °C), and air-dried. The hybridization mixture was composed of 50% formamide, 10% dextran sulphate, 2 × SSC, 50 µg/ml of salmon sperm DNA and 5 µg/ml of probe DNA. The probe mixture was denatured at 100 °C for 5 min and quenched in ice. Approximately 25 µl of denatured probe mixture was applied per slide; the slides were covered with cover slips (22 × 30 mm), sealed with rubber cement, and then placed overnight in a humid chamber at 37 °C. After hybridization overnight, cover slips were removed carefully and washed in 2 × SSC twice (5 min each), 50% formamide/2 × SSC for 5 min, and 2 × SSC twice (5 min each) at 37 °C. Slides were washed again in 2 × SSC for 5 min and detection buffer (4 × SSC/0.2% Tween 20) for 5 min at room temperature. Slides were blocked with 5% BSA in detection buffer for 5 min at room temperature.

For detection of the probe, 5 µg/ml of FITC-avidin D (Vector Laboratories) in detection buffer containing 5% BSA was applied per slide, incubated at 37 °C for 45 min and washed three times (5 min each) in detection buffer at 37 °C. In some cases the Cy3-avidin (Jackson ImmunoResearch Laboratory) was used as an alternative to FITC-avidin. Slides were blocked with 5% goat serum in detection buffer at room temperature for 5 min. For amplification of the fluorescence signal, slides were treated with 5 µg/ml of biotinylated anti-avidin D (Vector Laboratories) in detection buffer containing 5% goat serum at 37 °C for 45 min. After incubation, the slides were washed three times in detection buffer. Slides were blocked again with 5% BSA in detection buffer. Further amplification of the FISH signal was done by applying another layer of 5 µg/ml of FITC-avidin D in detection buffer containing 5% BSA. After incubation, slides were washed three times as described above, dehydrated in an ethanol series (70%, 95% and 100%, 3 min each) and air-dried. Slides were mounted with Vectashield containing 0.7 µg/ml of propidium iodide (PI) to reduce fading of the fluorescence. In some cases slides were hybridized with a ribosomal probe to facilitate identification of specific chromosomes. Slides were examined with a Zeiss 510 confocal laser scanning microscope with filter sets 02, 10 and 15. Representative FISH images were captured and analyzed by Adobe Photoshop ver. 5.0. The nomenclature of barley chromosomes described by Linde-Laursen et al. (1997) is used in parentheses under the original chromosome numbers on the idiogram (see Fig. 2).

Functional assays for GFP, GUS and PAT

GFP expression was observed using a Zeiss Axiophot epifluorescence microscope equipped with a Chroma filter containing a 450–490 excitation filter and an LP520 emission barrier filter (Cho et al. 2000). Histochemical staining for GUS was performed (Jefferson et al. 1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc). To determine the herbicide sensitivity of transgenic plants, a section of leaf blade at the 4- to 5-leaf stage was painted using a cotton swab with a 0.25% solution (v/v) of

Table 1 Analyses of transgene expression and FISH in transgenic barley plants

Transgenic line	Ploidy	Transgene segregation ratio in T_1 seed (+/–)	# Transgene loci	Transgene position	Transgene stability[e] (generations)
GPBhGN-7[a]	Diploid	45/18[c]	1	Chr.1(7H)S (centromeric)	Stable (T_9)
GPBhGN-12	Tetraploid	21/12[c]	1	Chr.6(6H)Sat (satellite)	Stable (T_6)
GPBhGN-14	Tetraploid	19/12[c]	1	Chr.6(6H)Sat (satellite)	Stable (T_5)
GPDhGN-6[a]	Tetraploid	43/2[d]	1	Chr.3(3H)L (centromeric)	Stable (T_5)
GPDhGN-11	Tetraploid	17/10[c]	1	Chr.5(1H)L (distal)	Stable (T_5)
GPDhGN-16	Tetraploid	7/2[c]	1	Chr.3(3H)L (distal)	Stable (T_8)
GPBhSSGN-7	Diploid	146/40[c]	1	Chr.4(4H)L (telomeric)	Stable (T_3)
GPBhSSGN-10	Diploid	136/41[c]	1	Chr.1(7H)S (telomeric)	Stable (T_3)
GPBhSSGN-23	Diploid	148/42[c]	1	Chr.4(4H)L (centromeric)	Stable (T_3)
GPGFP-44	Diploid	0/22	1	Chr.7(5H)Sat (satellite)	Unstable (T_1)
GPDhGFP-1	Tetraploid	17/3[c]	1	Chr.2(2H)S (distal)	Stable (T_4)
GPDhGFP-12[a]	Diploid	112/25[c]	1	Chr.2(2H)L (subtelomeric)	Stable (T_6)
GPDhGFP-10	Diploid	35/30	1	Chr.1(7H)L (subtelomeric)	Unstable (T_1)
GPDhSSGFP-2[a]	Tetraploid	71/2[d]	1	Chr.5(1H)S (distal)	Stable (T_5)
GPDhSSGFP-3	Tetraploid	24/6[c]	1	Chr.3(3H)L (distal)	Stable (T_2)
GPDhSSGFP-4	Tetraploid	35/7[c]	1	Chr.6(6H)L (telomeric)	Stable (T_5)
GPDhSSGFP-5[a,b]	Tetraploid	45/0[d]	2	Chr.4(4H)L (distal), Chr.7(5H)L (distal)	Stable (T_5)
GPDhSSGFP-8	Diploid	10/51	1	Chr.5(1H)S (telomeric)	Unstable (T_2)
GPDhSSATGGFP-4[a]	Tetraploid	28/1[d]	1	Chr.6(6H)S (distal)	Stable (T_5)

[a] Homozygosity in T_1 or later generations was confirmed by FISH and transgene segregation ratios
[b] $2n = 4x = 27 + 1$ telocentric
[c,d] Analyses using a χ^2-test indicate that the segregation ratios of progeny plants for transgenes were not significantly different from 3:1 and 35:1, repectively (at $\alpha = 0.05$)
[e] Expression and physical inheritance of transgenes were analyzed by transgene segregation ratios and PCR

Basta solution (starting concentration, 200 g/l of phosphinothricin) plus 0.1% Tween 20 (Cho et al. 1998). Plants were scored 1 week after herbicide application.

Genomic DNA isolation, polymerase chain reaction (PCR)

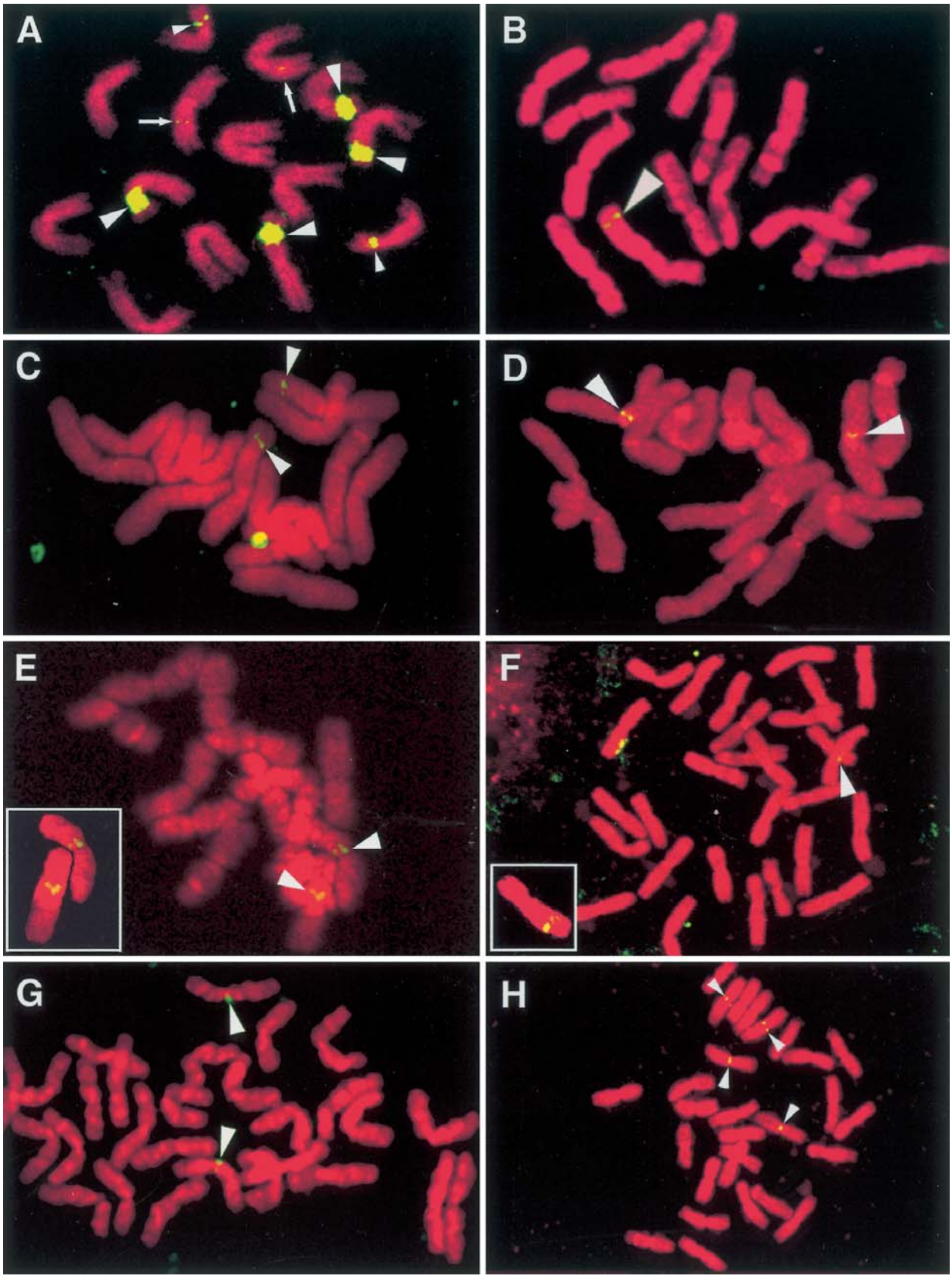
Total genomic DNA from leaf tissues was purified as described (Dellaporta 1994). To test for the presence of *sgfp* (S65T) in the progeny plants, 500 ng of genomic DNA was used in PCR amplifications using one of three primer sets, DhorsGFP1 (5′-ACGAGTCTAGACCATGGTGA-3′) plus sGFP4R (5′-agaggtaccTTACTTGTACAGCTCGTC-3′) for pDhsGFP-1 transformants (Cho et al. 2002); DhorSSsGFP5 (5′-GCTCTCACCACCGCTGTGAGCA-3′) plus sGFP4R for pDhSSsGFP3-4 transformants; or Act1int1 (5′-TCGTCAGGCTTAGATGTG-3′) plus sGFP4R (5′-agaggtaccTTACTTGTACAGCTCGTC-3′) for pAct1IsGFP-1 transformants (Cho et al. 2000). The presence of *uidA* was determined using the primer sets UIDA1 (5′-agcggccgcaTTACGTCCTGTAGAAACC-3′) and UID2R (5′-agagctcTCATTGTTTGCCTCCCTG-3′) (Cho et al. 1998). The presence of *bar* was determined using the primer set BAR5F (5′-CATCGAGACAAGCACGGTCAACTTC-3′) plus BAR1R (5′-ATATCCGAGCGCCTCGTGCATGCG-3′) (Lemaux et al. 1996). Amplifications were performed in a 25-µl reaction with *Taq* DNA polymerase (Promega, Madison, Wis.) according to a described protocol (Cho et al. 1998).

Results

Localization of transgenes

The transgenes, *uidA*, *sgfp* (S65T) and/or *bar*, were detected and localized using the FISH technique on metaphase chromosomes of transgenic barley plants produced by microprojectile bombardment of immature embryos (Table 1, Fig. 1). Of the 19 transgenic barley lines examined, eight lines were diploid [four *uidA* and four *sgfp* (S65T)] and 11 were tetraploid [five *uidA* and six *sgfp* (S65T)]. To identify individual barley chromosomes of all lines, pTa71 was used; this probe was previously employed for identification of five hybridization sites of rDNAs [two major sites on chromosomes 6 (6H) and 7 (5H), three minor sites on chromosomes 1 (7H), 2 (2H) and 5 (1H)] in barley (Leitch and Heslop-Harrison 1992). In our study, two major rDNA sites were localized easily on the NOR regions of chromosomes 6 (6H) and 7 (5H) in the diploid control plant (Fig. 1A). Two minor rDNA sites, [one (7H) and five (1H)], were weaker than the two major rDNA sites, but the signals were strong enough to be detected (Fig. 1A). The remaining one minor site [two (2H)] was not easily detected in all spreads, due to the very weak hybridization signal. Mea-

Fig. 1A–H FISH of rDNA and inserted foreign genes [*uidA* and/or *sgfp*(S65T) and *bar*] in metaphase chromosomes of transgenic barley plants. Hybridization sites are indicated with *arrows* and *arrow heads*. (**A**) Four major signals for rDNA sites on chromosomes 6 (6H) and 7 (5H) (*large arrow heads*), two minor signals on chromosome 5 (1H) (*small arrow heads*) and two minor signals on chromosome 1 (7H) (*arrows*) were found in a diploid control plant. (**B**) A *sgfp*(S65T) signal on the metaphase chromosome was found in a diploid T_0 plant (GPDhGFP-12) giving a 3:1 segregation ratio. (**C**) Two *sgfp*(S65T) signals in a homozygous T_1 diploid plant (GPDhGFP-12-7). (**D**) Two *uidA* signals in a homozygous T_1 diploid plant (GPBhGN-7-2). (**E**) Two *bar* signals in a homozygous T_1 diploid plant (GPBhGN-7-2). (**F**) A *uidA* signal in a tetraploid T_3 plant (GPDhGN-11) showing a 3:1 segregation ratio. (**G**) Two *sgfp*(S65T) signals in a tetraploid T_0 plant (GPDhGN-6) showing a 35:1 segregation ratio. (**H**) Four *sgfp*(S65T) signals in a homozygous T_2 plant (GPDhGN-6-3-4)


A
B
C
D
E
F
G
H

surements of total chromosome length and the ratio of the short to long arms (Fukui and Kakeda 1990; Noda and Kasha 1978), when necessary, were performed to distinguish among the three chromosomes e.g., 2 (2H), 3 (3H) and 4 (4H) (data not shown)

In diploid lines showing 3:1 segregation of *sgfp* (S65T) expression from heterozygous T_0 plants, only one signal was detected on a single chromosome (GPDhGFP-12; Fig. 1B), while two signals were found on a pair of homologous chromosomes of the same locus in a T_1 homozygous plant (Fig. 1C). Another diploid homozygous line, GPBhGN-7-2, also had two signals of *uidA* in the centromeric region of a pair of the homologous chromosomes in T_1 plants (Fig. 1D). The *bar* gene was detected at the same locus on the chromosomes containing either *uidA* or *sgfp* (S65T) in this line (Fig. 1E), as well as in two other lines (GPDhGN-6 and GPDhGFP-12) tested (data not shown). One diploid plant (GPDhSSGFP-8) with a 10:51 segregation ratio of transgene expression also had one signal in the telomeric region of chromosome 5 in a T_0 plant (Table 1). Eight different loci from eight different independent diploid transgenic lines were localized: one each in a centromeric region of chromosome 1(7H)S (GPBhGN-7) (Fig. 1D and E), a telomeric region of chromosome 1(7H)S (GPBhSSGN-10), a subtelomeric region of chromosome 1(7H)L (GPDhGFP-10), a subtelomeric region of chromosome 2(2H)L (GPDhGFP-12) (Fig. 1B and C), a telomeric region of chromosome 4(4H)L (GPBhSSGN-7), a centromeric region of chromosome 4(4H)L (GPBhSSGN-23), a telomeric region of chromosome 5(1H)S (GPDhSSGFP-8) and a satellite region of chromosome 7(5H) (GPGFP-44).

Tetraploid T_0 lines were characterized by two major categories of transgene segregation ratios, 3:1 and 35:1 (Table 1). Seven tetraploid lines (GPBhGN-12, GPBhGN-14, GPDhGN-11, GPDhGN-16, GPDhGFP-1, GPDhSSGFP-3 and GPDhSSGFP-4), which had a 3:1 segregation ratio of expression in T_1 and/or later generations, had only one signal (Fig. 1F) on one of the homologous chromosomes in T_0 plants. In contrast, four tetraploid lines (GPDhGN-6, GPDhSSGFP-2, GPDhSSGFP-5 and GPDhSSATGGFP-4) with a 35:1 segregation ratio in T_1 seed had two signals on two of the four homologous chromosomes in T_0 plants (Fig. 1G). Homozygous progeny plants of tetraploid lines had four signals on all four homologous chromosomes (Fig. 1H). Various integration sites were also detected in the chromsomes of tetraploid lines: one each in a distal region of chromosome 2(2H)S (GPDhGFP-1), a centromeric region of chromosome 3(3H)L (GPDhGN-6, Fig. 1G and H), a distal region of chromosome 3(3H)L (GPDhGN-16 and GPDhSSGFP-3), a distal region of chromosome 5(1H)L (GPDhGN-11) (Fig. 1F); a distal region of chromosome 5(1H)S (GPDhSSGFP-2), a satellite region of chromosome 6(6H) (GPBhGN-12, GPBhGN-14), a telomeric region of chromosome 6(6H)L (GPDhSSGFP-4) and a distal region of chromosome 6(6H)S (GPDhSSATGGFP-4). A tetraploid line (GPDhSSGFP-5) with a 45:0 segregation ratio in T_1 had two different insertion loci for the transgenes on different chromosomes [distal positions of chromosomes 4(4H)L and 7(5H)L].

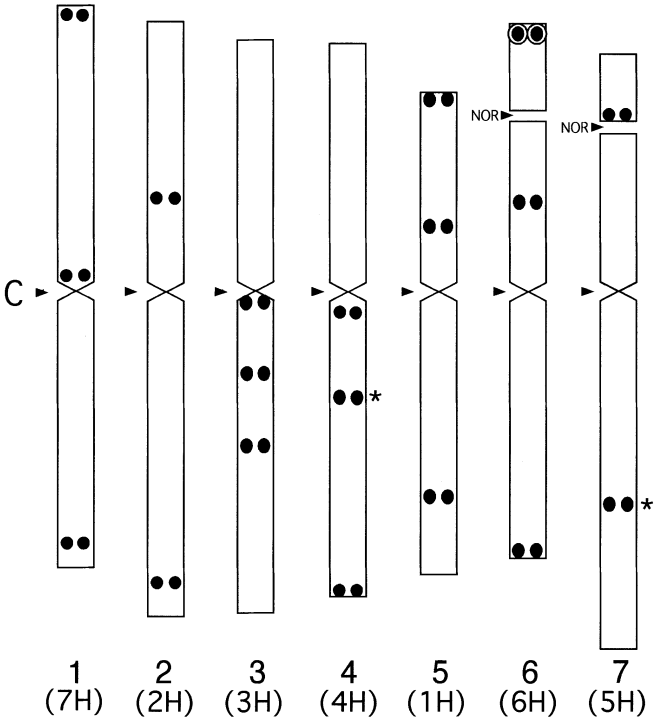


Fig. 2 An idiogram of barley chromosomes showing integrated sites (*solid circles*) of transgenes [*uidA* and *sgfp*(S65T)] in transgenic barley plants. Eighteen out of 19 lines had visibly different integration sites with apparently random gross distribution. Two independent lines with the same integration site at a gross level, detected on the satellite region of chromosome 6, are marked with *double circles*. Two *asterisk marks* (*) represent two transgenic loci from a single transgenic line (GPDhSSGFP-5). Values in parentheses are the numbering system of barley chromosomes described by Linde-Laursen et al. (1997)

Physical stability of transgenes related to their expression

Of the 19 independent transgenic lines, 18 had visibly different integration sites (Fig. 2); at a gross level it appeared that no preferential integration sites of transgenes were identified among chromosomes (Fig. 2, Table 1). However, it appeared that a preference of transgene integration for distal positions on chromosomes was observed: seven (37%) out of 19 lines examined had integration sites in distal positions of the chromosomes: four (21%) in telomeric regions, three (16%) in centromeric regions, three (16%) in satellite regions and two (10%) had subtelomeric regions. Transgene silencing was observed regardless of the gross location of transgene integration. For example, three diploid lines, i.e., GPDhSSGFP-8 (telomeric) with a 10:51 segregation ratio, GPDhGFP-10 (subtelomeric) with a 35:30 segregation ratio and GPDhGFP-44 (satellite) with a 0:22 segre-

Table 2 Analyses of expression and inheritance of transgenes in progeny plants of a transgenic barley line, GPDhGFP-12. This transgenic barley line was produced by microprojectile bombardment of immature embryos with a mixture of two plasmids pDhsGFPN-1 and pAHC20. Bombarded tissues selected on DC medium containing 5 mg/l of bialaphos and bialaphos-resistant calli were regenerated on FHG medium containing 3 mg/l of bialaphos

Transgenic barley line	Ploidy	T_0		T_1		T_2	T_3	T_4	T_5
		gfp/bar PCR) (+/–	Basta painting (+/–)	GFP (seed) (+/–)	Basta painting (+/–)	GFP (seed) (+/–)	GFP (seed) (+/–)	GFP (seed) (+/–)	GFP (seed) (+/–)
GPDhGFP-12	Diploid	+/+	+	112/25[a]	0/15				
12-1						36/20[a]			
12-1-1							13/4[a]		
12-1-2							22/7[a]		
12-1-3							5/0		
12-1-4							12/3[a]		
12-1-4-1								34/7[a]	
12-1-4-2								21/4[a]	
12-1-4-3								41/0	
12-1-4-3-1									45/0
12-1-4-3-2									33/0
12-1-4-3-3									37/0
12-1-4-4								48/0	
12-1-4-4-1									25/0
12-1-4-4-2									52/0
12-1-4-4-3									24/0
12-1-4-5								0/27	
12-1-5							10/3[a]		
12-2						0/29			
12-3						51/8			
12-4						33/7[a]			
12-5						31/0			
12-6						0/24			
12-7						48/0			
12-7-1							54/0		
12-7-2							50/0		
12-7-3							29/0		
12-8						17/9[a]			
12-9						0/16			
12-10						16/7[a]			
12-11						40/0			
12-12						14/7[a]			
12-13						31/14[a]			

[a] Analyses using a χ^2-test indicate that the segregation ratios of progeny plants for GFP were not significantly different from 3:1 (at $\alpha = 0.05$)

gation ratio, gave evidence of silencing of the transgene(s) in later generations (data not shown).

Screening of homozygous plants using FISH

To screen for homozygous plants as early as the T_1 generation, the FISH technique was employed to analyze transgenic plants. Table 2 shows an example of the expression and inheritance of transgenes in the transgenic line, GPDhGFP-12. Thirteen T_1 seeds (GPDhGFP-12-1 to GPDhGFP-12-13) originating from this line, were planted and plants were screened by FISH for homozygosity, which was confirmed by segregation ratios and PCR in subsequent generations. A T_0 plant of this line had a single fluorescing signal for the *sgfp* (S65T) gene (Fig. 1B) and T_1 seed showed a 3:1 segregation ratio of GFP expression (Table 2). Of the 13 T_1 progeny plants tested, ten plants with a 3:1 segregation ratio had one signal on their chromosomes, but the remaining three (GPDhGFP-12-5, GPDhGFP-12-7 and GPDhGFP-12-11) had two signals on a pair of homologous chromosomes (Fig. 1C). These sublines were homozygous in segregation ratios for GFP expression, 31:0 in GPDhGFP-12-5, 48:0 in GPDhGFP-12-7 and 40:0 in GPDhGFP-12-11, in the T_2 generation (Table 2). Thus, we confirmed that all three sublines identified as homozygous by FISH were true homozygotes for s*gfp* (S65T). Homozygous plants were reconfirmed by GFP segregation ratios in T_3 seed of subline GPDhGFP-12-7 and T_4, and T_5 seed of subline GPDhGFP-12-1-4, and were further confirmed by FISH and PCR analyses (data not shown). Analysis of PCR results showed that *bar* as well as s*gfp* (S65T) were present in all progeny plants of GPDhGFP-12-7 (Fig. 3A and B). Although *bar* was still present in T_2 progeny, transgene silencing of *bar* was observed (Fig. 3B and C).

Discussion

We localized transgenes [*uidA*, *sgfp* (S65T) and/or *bar*] on the metaphase chromosomes of 19 independent transgenic barley lines using the FISH technique, and all inserted genes examined were clearly visualized by this technique. DNA blot-hybridization analysis showed that transgenic lines had at least three copies of transgenes, ranging from approximately 3 to 15 copies (Cho et al. 1999, 2002). As expected, a more intense FISH signal was observed in transgenic barley lines with higher copy numbers.

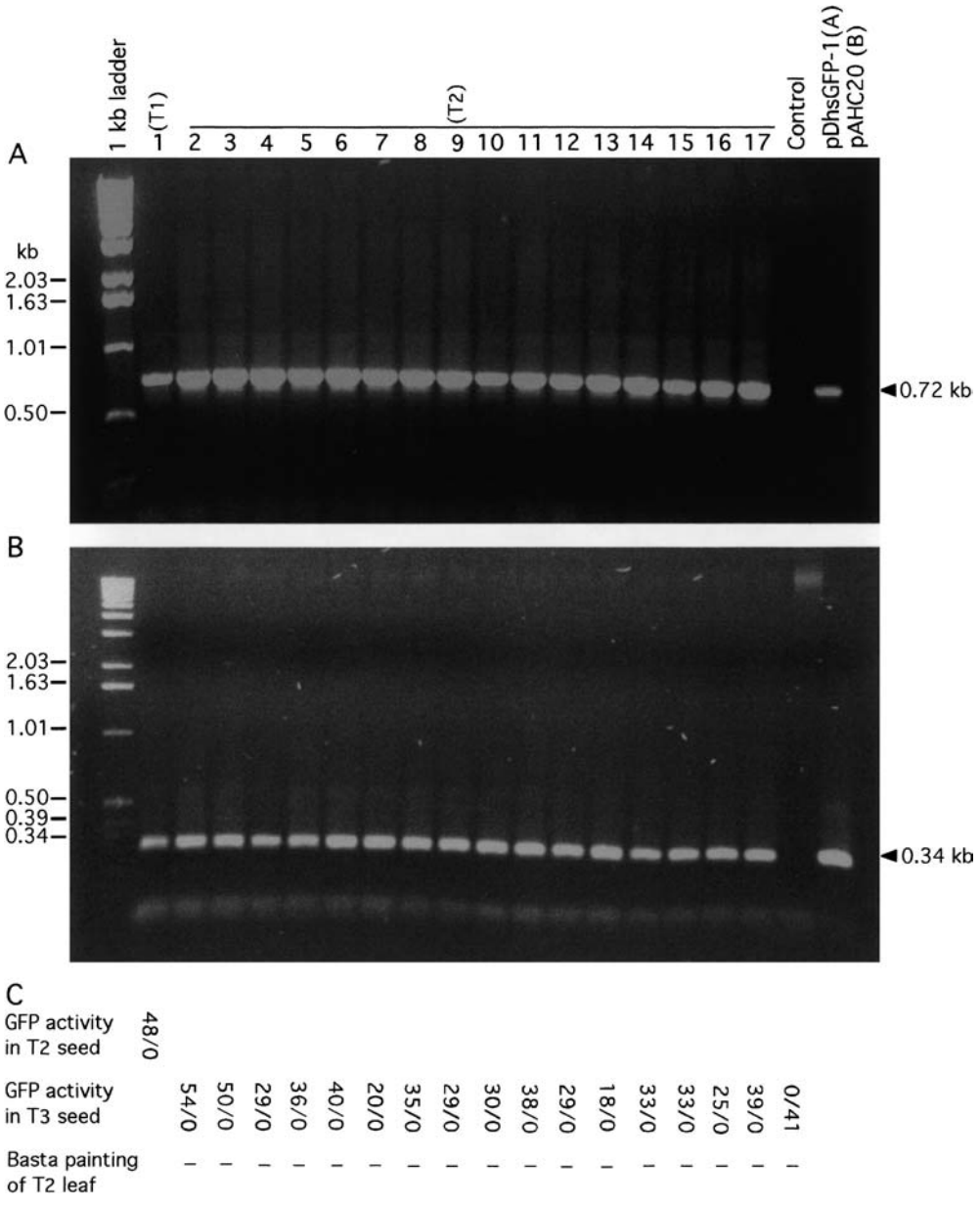


Fig. 3A–C Analyses of PCR and transgene expression of the progeny plants (T_1/T_2) of a homozygous line, derived from GPDhGFP-12. PCR products of *sgfp*(S65T) (**A**) and *bar* (**B**), amplified from T_1 and T_2 plants, were visualized by electrophoresis on a 0.8% agarose gel stained with ethidium bromide. All the T_2 plants were positive for *sgfp*(S65T) and *bar*, but only *sgfp*(S65T) yielded a functionally expressed product. *Lane 1*, marker; *lane 2*, T_1 plant; *lanes 3–17*, T_2 progeny, *lane 18*, non-transformed control plant; *lane 19*, plasmid pDhsGFP-1 (**A**) and pAHC20 (**B**). (**C**) GFP expression was observed using a Zeiss Axiophot epifluorescence microscope, and herbicide sensitivity was determined by Basta painting of a leaf blade

There were no apparent preferential gross integration sites for the foreign DNA into particular chromosomes in the 19 transgenic barley events analyzed. Transgene integration sites were randomly distributed and 18 of 19 lines had obviously different integration sites of transgenes. Svitashev et al. (2000) reported a similar result with transgenic oat lines in which there were no specific integration sites of transgenes among the chromosomes and/or genomes. However, in our study there appeared to be preferential transgene integration sites within the chromosomes in transgenic barley plants. Thirty seven percent (7/19) of transgenic barley lines had their transgenes in distal regions of the chromosomes. Other integration sites of transgenes were telomeric (21%), centromeric (16%), satellite (16%) and subtelomeric (10%). Similar results were reported in transgenic barley, wheat, triticale (Pedersen et al. 1997) and transgenic petunia (Hoopen et al. 1996). In contrast, the integration sites were in subtelomeric or telomeric regions in most transgenic oat lines examined (Svitashev et al. 2000). The tendency to insert into the telomeric region was also observed in transgenic *Crepis capillaris* (Ambros et al. 1986) and petunia (Wang et al. 1995).

In general, telomeric or centromeric regions of plant chromosomes are heterochromatic, indicating that these are highly condensed regions. Integration into these regions in barley may be related with instability of transgene expression in subsequent generations. However, in our study, all transgenic lines except one (GPDhSSGFP-8) that had transgenes in telomeric or centromeric regions showed normal expression and inheritance of transgenes. This may be because the transgene position effect on gene expression is not just dependent on the gross location of the trangene but is also dependent on

other factors, such as the DNA methylation state of the region, the copy number and the precise chromosomal position of transgenes and chromosomal rearrangements (Finnegan and McElroy 1994; Flavell 1994; Phillips et al. 1994; Stam et al. 1997).

Chromosomal aberration has been frequently observed during tissue culture and the transformation process and can also be one of the factors affecting transgene stability. Chromosomal in situ hybridization is instructive in studying the relationship between transgene expression and chromosomal status. In our previous study on regenerated transgenic barley plants (Choi et al. 2000), we observed a high frequency (46%) of cytogenetic aberrations, including numerical and structural variations such as ploidy change. Most diploid lines showed a normal Mendelian segregation ratio (3:1) in the T_1 generation, while tetraploid lines had two major categories of segregation ratios, 3:1 and 35:1 (Table 1). Tetraploid T_0 lines with a 3:1 segregation ratio had one signal on one of the homologous chromosomes, indicating that cells were already tetraploid at the time of DNA integration. However, four tetraploid lines (GPDhGN-6, GPDhSSGFP-2, GPDhSSGFP-5 and GPDhSSATGGFP-4 in Table 1) with a 35:1 segregation ratio had two signals on a pair of four homologous chromosomes in T_0 plants. This demonstrates that the cells during the callus phase were diploid and became tetraploid after foreign DNA integration.

Diploid T_0 lines with a 3:1 segregation ratio had one signal on one chromosome (Fig. 1B). As expected, the diploid homozygous T_1 plants derived from GPBhGN-7 had two signals on two pairs of homologous chromosomes, while tetraploid homozygous plants had four signals on a pair of homologous chromosomes. Based solely on analyses of PCR and segregation ratios, the homozygosity of transgenic lines can sometimes be misjudged. For example, one line (GPDhSSGFP-5) with two integration loci on two different chromosomes showed a homozygote-like segregation ratio (45:0). T_4 tetraploid plants of another line (GPDhGN-16) also showed a homozygote-like segregation ratio, but FISH results indicated that these plants were not true homozygotes. These plants had two signals on four homologous chromosomes (data not shown). Thus, the FISH technique was very useful for confirming true homozygotes.

Most transgenic lines examined in this study had more than three copies of the transgenes. The signal intensity of FISH was related to the copy number of the transgene. It was reported that microprojectile bombardment frequently produces multicopy integration at the same locus in transgenic plants (Kohli et al. 1998). Kohli et al. proposed a two-phase integration mechanism as a model of transgene integration via particle bombardment to explain transgene rearrangement and multicopy patterns. During the pre-integration phase, recombination and ligation of transgenes occur extra-chromosomally. In the second phase, rearranged transgene sequences are integrated into the plant genome at a single locus. This specific site becomes a temporary “hot spot” for the integration of foreign DNA. Subsequenty, other transgenes can also be integrated into the same locus. We detected the selectable marker gene, *bar*, and the reporter gene, either *uidA* or *sgfp* (S65T), at the same locus on the chromosomes resulting from co-bombardment, in all three transgenic lines (GPDhGN-7, GPDhGN-6 and GPDhGFP-12). Recently, two inserted genes, *bar* and *uidA*, were also found by FISH to be co-localized on the chromosome in transgenic oat (Leggett et al. 2000).

The FISH technique can be very useful in determining the number of transgene loci. Alternatively, the number of transgene integration loci can be determined by genotypic and/or phenotypic segregation ratios. However, if the transgenes are integrated into multiple loci, transgene segregation ratios will not follow a 3:1 ratio. It was previously reported that the number of transgene loci obtained from FISH and DNA blot-hybridization analyses was not in agreement with GUS segregation ratios (Svitashev et al. 2000). FISH data showed that 50% of the transgenic oat lines examined had multiple loci (two to three). This tendency of multi-locus integration was found in transgenic wheat and triticale, but was not found in barley (Pedersen et al. 1997). Our results showed that all 18 lines, except one (GPDhSSGFP-5), had transgene integration at a single locus.

Using the FISH technique, we could screen for the homozygous plants as early as the T_1 generation. For example, among the 13 T_1 plants in GPDhGFP-12, three plants (GPDhGFP-12-5, GPDhGFP-12-7 and GPDhGFP-12-11) were homozygous for both *sgfp* (S65T) and *bar* (Table 2, Fig. 1C). Analysis of PCR results showed that all T_2 progeny examined, also had both *sgfp* (S65T) and *bar* genes (Fig. 3). However, the *bar* gene was not expressed in plants of the T_1 progeny and subsequent generations. The *bar* gene in this transgenic line was localized at the same locus as the *sgfp* (S65T) gene (data not shown). Homozygous plants were reconfirmed by transgene segregation ratios in T_3 seed; and T_4 progeny plants of GPDhGFP-12-1-4-3 (41:0) and GPDhGFP-12-1-4-4 (48:0) were further confirmed as homozygous by analyses of FISH and PCR (data not shown). We also identified five other homozygous transgenic lines (two diploid and three tetraploid) with either the *uidA* or the *sgfp* (S65T) gene among 19 lines examined.

In conclusion, we have applied the FISH technique effectively to analyze transgenic barley plants. FISH was instructive in identifying the chromosomal locations of transgenes and in early screening for homozygous T_1 plants. FISH results also supported the segregation ratios of transgene expression (i.e., 3:1, 35:1 and homozygous) in most transgenic lines.

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