Theor Appl Genet (1999) 99:487–495

M. Uzé · I. Potrykus · C. Sautter

Single-stranded DNA in the genetic transformation of wheat (*Triticum aestivum* L.): transformation frequency and integration pattern

Received: 20 July 1998 / Accepted: 30 January 1999

Abstract Two non-linked marker genes (*gus* and *bar*) were co-introduced by microprojectile bombardment into wheat cells. Four different DNA structures were compared with respect to ability to integrate into the wheat genome: circular or linear (l) DNA as a single- or double-stranded plasmid (ss and ds, respectively). In eight independent experiments, linearized DNA integrated in the ds or ss form with a high efficiency of up to 14% for l-ssDNA. Molecular analyses by Southern blotting showed that all DNA forms gave a similar complicated integration pattern of the *bar* gene.

Key words Single-stranded DNA · Wheat · Transformation

Introduction

Wheat has been reproducibly transformed by bombarding the scutellum of immature embryos with various DNA-coated microprojectiles, but as shown by many reports the frequency of transformation was always low, between 0.15% and 2.34% (Weeks et al. 1993; Vasil et al. 1993; Nehra et al. 1994; Becker et al. 1994; Zhou et al. 1995; Altpeter et al. 1996, Blechl and Anderson 1996; Takumi and Shimada 1996, 1997). The question of how to increase the efficiency of wheat transformation is still open. Shillito et al. (1985) reported that tobacco protoplasts were more efficiently transformed by linear (l) double-stranded (ds) DNA than by circular (c) single-stranded (ss) DNA. Few articles have mentioned the use of ssDNA for plant transformation. Furner et al. (1989) and Rodenburg et al. (1989) obtained transgenic calli by introducing c-ssDNA into petunia and tobacco protoplasts, respectively. l-ssDNA has been more intensively studied with respect to mechanisms of targeted recombination in mammalian cells (Fujioka et al. 1993), yeast (Simon and Moore 1987) and plants (Bilang et al. 1992; De Groot et al. 1992). The common conclusion of these publications was that the ssDNA may participate efficiently in recombination with chromosomal sequences.

In the investigation reported here our specific objective was to determine if the l-ssDNA is able to integrate into the wheat genome more efficiently than the dsDNA via an illegitimate recombination. Ten-day-old precultured immature wheat embryos were bombarded with microprojectiles. DNA molecules in different configurations, c-ds, l-ds, c-ss and l-ss were bound to these particles. The role of the DNA form was monitored using both transient expression of the β-glucuronidase (*gus*) gene and stable transformation with the *bar* gene.

Communicated by F. Salamini

M. Uzé · I. Potrykus · C. Sautter (✉)
ETH Zürich, Institute of Plant Sciences, Universitätstr. 2,
CH-8092 Zürich, Switzerland
e-mail: christof.sautter@ipw.biol.ethz.ch
Fax: +41 1 6321044

Materials and methods

Plant material and tissue culture

Wheat plants (*Triticum aestivum* L. cv 'Bobwhite') were grown in the greenhouse according to Iglesias et al. (1994). At about 14 days post anthesis, immature embryos were aseptically excised and placed with the scutellum face upwards on a preculture medium (Table 1)

Plasmids

The plasmid used for wheat transformation *via* microprojectile bombardment was pBluescript II KS$^+$ (Stratagene) containing the replication origin of the phage f1. KSUG and KSAB (Fig. 1) were obtained by introducing into KS$^+$ the actin-*bar* cassette from pAB1 (rice actin 1-D promoter::*bar*::35S 3'; kindly provided by Dr. R. Bilang, ETH Zurich) in the *Eco*RI site (McElroy et al. 1990) and the ubiquitin-*gus* construct from pUbiGus (Christensen et al. 1992; Vancanneyt et al. 1990) in the *Hin*dIII site. To obtain the two constructs, we digested pUbiGus and pAB1 with *Hind*III and *Eco*RI, respectively. KSUG and KSAB were introduced by electroporation into *E. coli* strain JM109.

Table 1 Media for wheat transformation via microprojectile bombardment

Medium	Composition[a]
Preculture medium	MS, 2% maltose, 100 mg/l casein hydrolysate, 1.5 mg/l 2,4-D, 0.6% agarose, pH 5.6
Plasmolysis medium	MS, 20% maltose, 1.5 mg/l 2,4-D, 0.6% agarose, pH 5.6
Selection medium	MS, 2% maltose, 1.5 mg/l 2,4-D, 0.6% agarose, pH 5.6, 5 mg/l phosphinothricin
Regeneration medium	MS, 2% maltose, 0.5 mg/l NAA, 2 mg/l BAP, 0.6% agarose, pH 5.6, 5 mg/l phosphinothricin
Rooting medium	$^1/_2$-MS, 1% maltose, 0.2% gelrite, pH 5.6, 3 mg/l phosphinothricin

[a] The agarose was the type I Sigma and gelrite was from Scott Laboratories. MS, Murashige and Skoog medium (1962); 2,4-D, dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; NAA: 1-naphthylacetic acid

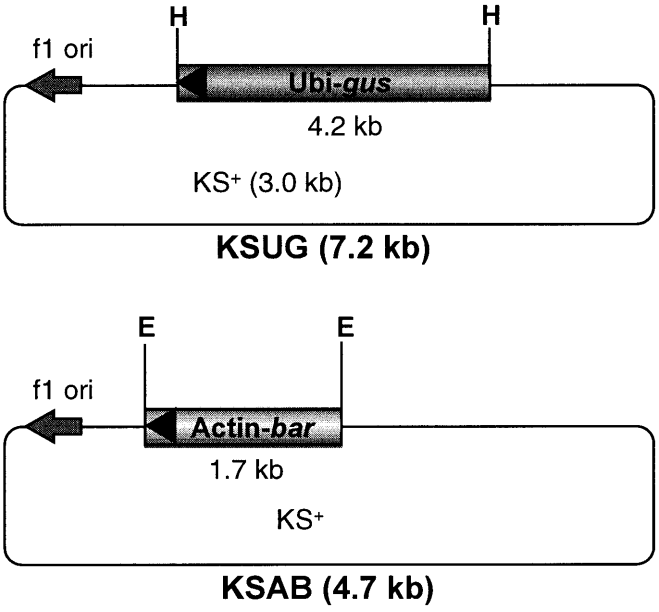

Fig. 1 Scheme of the plasmids used for wheat transformation *via* microprojectiles. *H Hin*dIII, *E Eco*RI

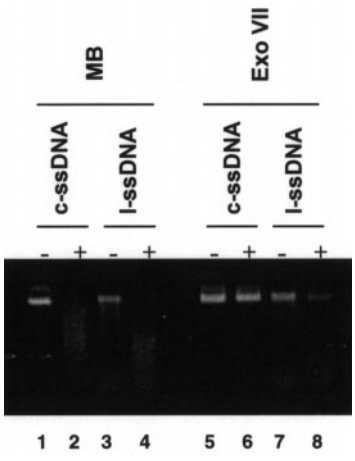

Fig. 2 Linearization of single-stranded DNA visualized by the action of specific nucleases. Circularized (*c-ss*) or linearized (*l-ss*) plasmids were mixed (+) or not (–) with the mung bean nuclease (*MB, lanes 1-4*) or the exonuclease VII (*Exo VII, lanes 5-8*)

Preparation of single-stranded DNA

c-ssDNA

KSUG and KSAB plasmids were prepared as single strands according to Sambrook et al. (1989). To determine whether the DNA was single-stranded, we mixed 1 µg of DNA with 2 U of mung bean nuclease, an enzyme which specifically digests ssDNA. As showed on Fig. 2, lanes 1-2, the c-ssDNA produced was degraded in the presence of mung bean nuclease.

l-ssDNA

Site-directed linearization of c-ssDNA molecules was performed according to Bilang et al. (1992). An oligonucleotide (5' ATCATTGGAAAACGTTCTTCGGG 3') was used for creating a ds restriction site for the *Xmn*I enzyme. After digestion, the enzyme was denaturated with phenol/chloroform (1:1) pH 8.0, and the DNA from the aqueous phase was denaturated at 80°C for 5 min to remove remaining oligonucleotides from the cut sides. The linearized ssDNA was precipitated and resuspended in 20–25 µl water; 1 µl was used to determine the DNA concentration. To confirm whether the plasmid was linearized, we mixed 1 µg of DNA in the appropriate buffer (50 m*M* TRIS pH 7.9, 50 m*M* potassium phosphate pH 7.6, 8.3 m*M* EDTA) with 2 U exonuclease VII (Exo VII) which specifically digests l-ssDNA. In Fig. 2, lanes 1-2, l-ssDNA has been degraded by mung bean nuclease. Exo VII was inactive on control c-ssDNA (lanes 5, 6) but digested the l-ss plasmids (lanes 7, 8). An efficiency of linearization of at least 75% is indicated by the remaining band on lane 8, which shows a fourfold weaker signal than that for the non-treated l-ssDNA. The Exo VII-resistant DNA is either c-ssDNA or l-ssDNA with residual oligonucleotides.

Gene transfer to wheat by particle bombardment

Immature wheat embryos were placed on preculture medium for 10 days and then subjected to osmotic pretreatment in plasmolysis medium for 4–6 h (Table 1) before bombardment. The microprojectile bombardment protocol was that of Iglesias (1994). To maintain equal amounts of ds and ss molecules, we mixed 0.5 µg of ssDNA and 1.0 µg of dsDNA of each plasmid with the gold particles. About 24 h post-bombardment the explants were placed back on the preculture medium. One week later they were transferred for 4 weeks to the dark with the addition of 5 mg/l phosphinothricin (Table 1). Calli were then transferred to regeneration medium. Regenerated shoots were subsequently transferred to selection rooting medium and to soil under greenhouse conditions (Table 1).

Enzyme assays

β-Glucuronidase (GUS) assay

The localization of GUS transient and stable expression in the transformed calli and plants was studied histochemically (Jefferson 1987) as described by Mendel et al. (1989). After a 24-h, post-

bombardment period, part of the material was incubated for another 24 h in a solution containing 5-bromo-4-chloro-3-indolylglucoronide.

Phosphinothricin acetyl transferase (PAT) assay

PAT was assayed by means of silica gel thin-layer chromatography (TLC) of leaf extracts according to Spencer et al. (1990).

Southern blot analysis

Wheat genomic DNA was isolated using the CTAB extraction method (Murray et al. 1980). Approximately 1 g of fresh leaf material was ground with liquid nitrogen and lyophilized for 12 h. Around 25 µg of total genomic DNA was digested separately with 75 U *Eco*RI and *Eco*RV at 37°C for 5 h. DNA fragments were electrophoretically separated on a 0.75% agarose gel buffered with TBE (89 m*M* TRIS borate, 89 m*M* boric acid, 2 m*M* EDTA pH 8.0), denatured, and transferred to a hybridization membrane (Hybond N, Amersham) as described by Wünn et al. (1996). The DNA was covalently crosslinked to the membrane. The blot was hybridized with a random priming dAT[^{32}P]-labelled *gus* fragment of 1900 bp or a *bar* fragment of 557 bp. The 1900-bp *gus* fragment was obtained after digesting the plasmid pBS-260gn (Hamilton et al. 1992) with *Bam*HI and *Ecl*136II. The 557-bp *bar* fragment was obtained by *Bgl*II digestion of the plasmid pCIB3064 (kindly provided by Ciba-Geigy Corporation Biotechnology Research, Research Triangle

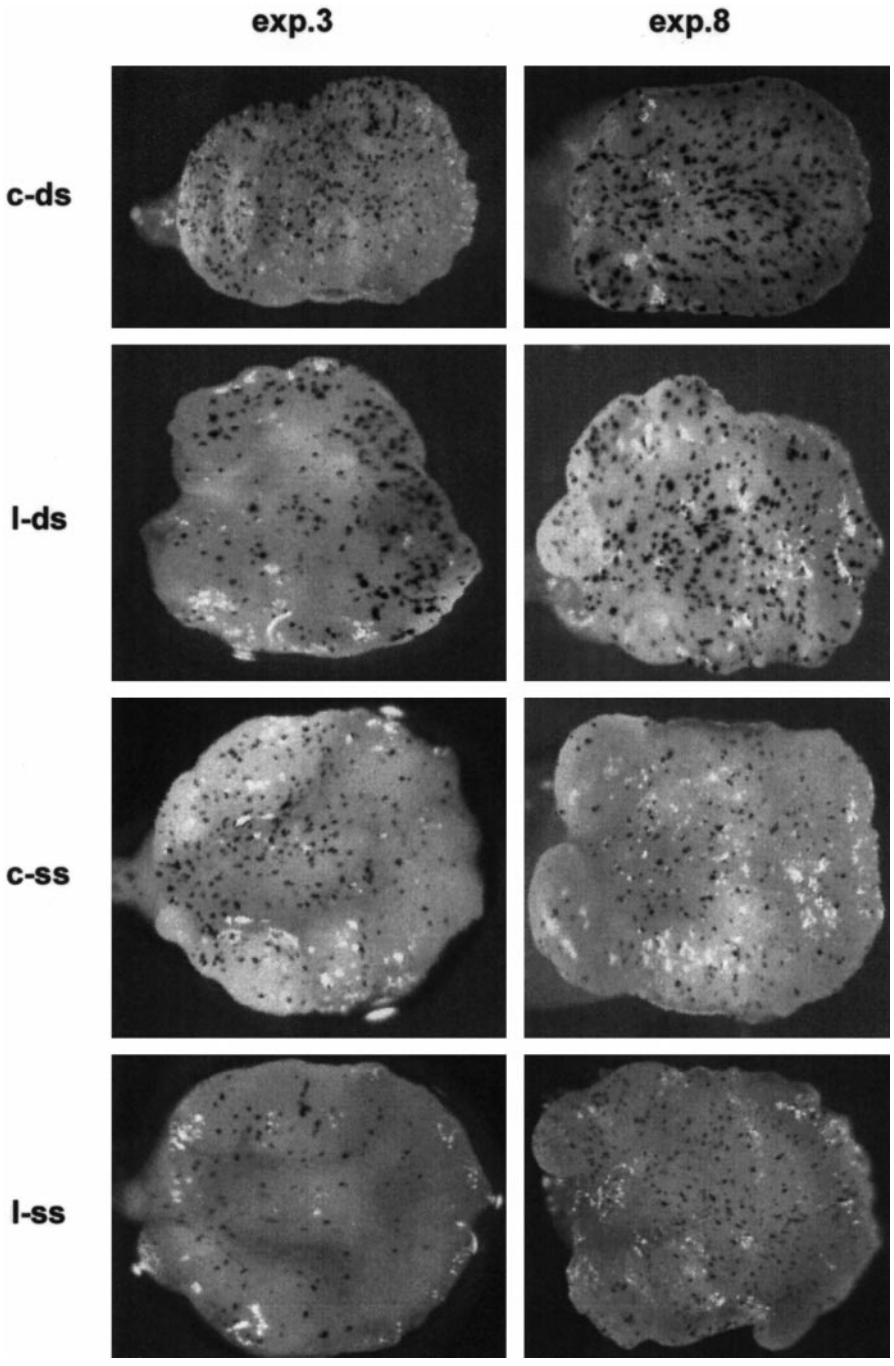

Fig. 3 GUS histochemical staining from experiments 3 and 8. Ten-day-old precultured wheat immature embryos were co-bombarded with KSUG and KSAB. GUS-expressing cells were observed 2 days later after staining. The plasmid DNA forms were double-stranded circular (*c-ds*), linear (*l-ds*), or single-stranded circular (*c-ss*) or linear (*l-ss*)

Table 2 Results of the GUS transient expression 2 days after co-bombardment of KSAB and KSUG to 10-day-old precultured immature wheat embryos (M, T mean or total)

DNA structure	Experiment	Number of GUS stained calli	Total number of GUS-expressing cells	Mean blue spots per callus (+/–SEM)
c-ds	1	14	394	28.4 (+/–7.5)
	2	10	584	58.4 (+/–18.9)
	3	14	1612	115.1 (+/–28.2)
	4	20	2349	117.5 (+/–19.3)
	5	10	459	45.9 (+/–14.4)
	6	15	452	30.1 (+/–11.6)
	7	11	516	46.9 (+/–15.2)
	8	15	1849	123.3 (+/–33.2)
	M, T	**109**	**8215**	**75.4 (+/–15.5)**
l-ds	1	13	296	22.7 (+/–6.7)
	2	5	30	6.0 (+/–3.5)
	3	13	1057	81.3 (+/–28.0)
	4	23	2758	119.9 (+/– 19.0)
	5	15	1036	69.1 (+/–11.5)
	6	15	28	1.9 (+/–0.7)
	7	10	613	61.3 (+/–7.8)
	8	13	2883	221.8 (+/–28.5)
	M, T	**107**	**8701**	**81.3 (+/–22.2)**
c-ss	1	14	715	51.1 (+/–27.5)
	2	14	64	4.6 (+/– 2.0)
	3	16	299	18.7 (+/–3.1)
	4	21	1180	56.2 (+/– 14.9)
	5	15	502	33.5 (+/–13.4)
	6	10	645	64.5 (+/– 22.8)
	7	10	161	16.1 (+/–4.8)
	8	15	1836	122.4 (+/– 19.5)
	M, T	**115**	**5402**	**47.0 (+/–12.4)**
l-ss	2	20	992	49.6 (+/–10.6)
	3	14	989	70.6 (+/–13.0)
	5	15	2	0.1 (+/–0.1)
	6	14	0	0.0 (+/–0.0)
	7	9	154	17.1 (+/–8.3)
	8	15	1478	98.5 (+/–29.3)
	M, T	**87**	**3615**	**41.6 (+/–11.4)**

Park, N.C.. Preparation of the radioactive probes and hybridization were performed according to Feinberg and Vogelstein (1983). After the first hybridization, the *gus* probe was removed by soaking the membrane in boiling water with 0.1% SDS for 30 min, and the membrane was then hybridized with the *bar* probe.

Results

GUS transient expression in wheat cells after co-bombardment with KSUG and KSAB

Ten-day-old precultured wheat embryos were co-bombarded with KSUG containing the ubiquitin-*gus* cassette and KSAB containing the actin-*bar* construct. In eight experiments, two plasmids were co-bombarded for each of the four DNA forms (c-ds, l-ds, c-ss or l-ss).

Transient GUS expression varied from shot to shot for all of the independent bombardment experiments (Fig. 3). Two days after bombardment, the blue spots on scutella expressing the KSUG plasmid as a dsDNA form (c-ds and l-ds) were larger and darker than those expressing the single-stranded KSUG plasmid (c-ss and l-ss). The non-GUS-stained calli originating from bombarded embryos were kept for selection to produce stable transformants.

When the number of GUS-expressing cells was assessed 2 days after bombardment, the mean for the dsDNA structure was twice that for ssDNA, but no significant difference was found between the linearized and circularized forms (Table 2).

Stable expression of the two marker genes, *gus* and *bar*

Ten-day-old precultured immature embryos were co-bombarded with the KSAB and KSUG plasmids. A total of 90 plants were regenerated after selection on 5 mg/l PPT. Further analysis showed that 30 of these plants were escapes, i.e. contained no *bar* gene, and 5 were derived from the same transgenic line. In total, 56 independent plants containing the *bar* gene were regenerated. We carried out six independent experiments on the l-ss plasmids and eight on the three other DNA structures. Figure 4 and Table 3 summarize the results from all experiments.

PAT expression

One representative example of a PAT assay is shown in Fig. 4. The PAT activity was variable. The negative con-

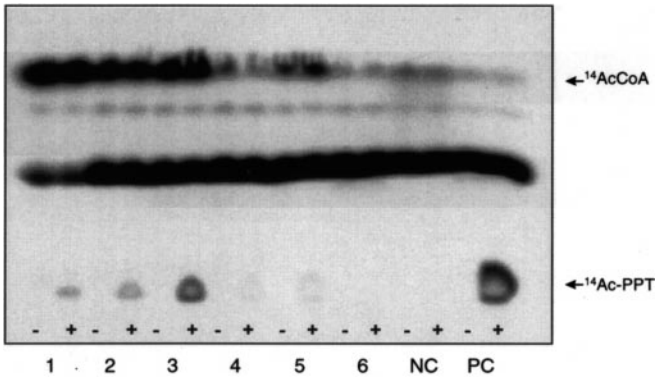

Fig. 4 PAT-TLC analysis of six regenerated plants. Each sample was reacted without PPT (–) or with PPT (+). Acetylated PPT (^{14}Ac-PPT) can be visualized by the presence of a single spot in the (+) lanes; the remaining ^{14}AcCoA is on the advancing edge. *Lanes 1-6* Regenerated plants, *NC* non-transformed wheat (negative control), *PC* transformed resistant wheat Basta (positive control)

trol (NC) showed no PAT activity whereas it was high in the positive control (PC), a *bar* gene-transformed wheat plant.

GUS expression

A T0 regenerated plant was considered to be a GUS-positive-expressing plant when cut roots or/and leaves stained blue after 24 h incubation with the GUS substrate.

Co-expression

A summary of the PAT assays and GUS-staining patterns for the different independent bombardment experiments is given in Table 3. Most of the regenerated plants co-expressing the two genes were obtained with the dsDNA, but ss plasmids were also stably expressed in wheat plantlets as were the ds plasmids usually used for wheat transformation.

Table 3 Results of the regenerated T0 plants after the co-bombardment of KSUG and KSAB (*M*, *T* mean or total)

DNA structure	Experiment	Number of bombarded explants in selection (A)	Number of *bar*-transformed plants (% from A)	Number of co-transformed plants (% from A)	Number of co-expressing plants (% from A)	Stable/transient[a] (×1000)
c-ds	1	58	1 (1.72)	1 (1.72)	0	35
	2	22	0	/	/	
	3	78	2 (2.56)	1 (1.28)	1 (1.28)	17
	4	66	2 (3.03)	1 (1.52)	0	17
	5	61	5 (8.20)	4 (6.56)	1 (1.64)	109
	6	78	1 (1.28)	1 (1.28)	1 (1.28)	33
	7	64	0	/	/	
	8	82	3 (3.66)	3 (3.66)	2 (2.44)	24
	M, T	**509**	**14 (2.75)**	**11 (2.16)**	**5 (0.98)**	**186**
l-ds	1	59	0	/	/	
	2	7	0	/	/	
	3	75	1 (1.33)	0	0	12
	4	87	4[b] (4.60)	1 (1.15)	0	33
	5	54	4 (7.41)	4 (7.41)	2 (3.70)	58
	6	73	2 (2.74)	1 (1.37)	1 (1.37)	105
	7	68	2 (2.94)	2 (2.94)	2 (2.94)	33
	8	82	5 (6.10)	3 (3.66)	0	23
	M, T	**505**	**18 (3.56)**	**11 (2.78)**	**5 (0.99)**	**221**
c-ss	1	108	0	/	/	
	2	51	0	/	/	
	3	71	0	/	/	
	4	74	1 (1.35)	1 (1.35)	0	18
	5	48	2 (4.17)	1 (2.08)	1 (2.08)	60
	6	55	0	/	/	
	7	60	0	/	/	
	8	87	5 (5.75)	2 (2.30)	1 (1.15)	41
	M, T	**554**	**8 (1.44)**	**4 (0.72)**	**2 (0.36)**	**170**
l-ss	2	64	0	/	/	
	3	79	11(13.92)	8 (10.13)	1 (1.27)	156
	5	108	0	/	/	
	6	62	1 (1.61)	0	0	–
	7	75	2 (2.67)	1 (1.33)	0	117
	8	94	2 (2.13)	2 (2.13)	1 (1.06)	20
	M, T	**482**	**16 (3.32)**	**11 (2.28)**	**2 (0.41)**	**385**

[a] Stable/transient is the ratio between the number of *bar*-transformed plants and the mean blue spots per callus in transient expression
[b] Eight plants were regenerated from this experiment but 5 were found to derive from a unique line after further molecular analysis

Southern blot analysis and integration pattern

To investigate the presence of non-expressing genes and for distinguishing independent plants and escapes, we performed Southern blot analysis on the 90 regenerated plants. Genomic DNA from plants analyzed for PAT and GUS activities was isolated for Southern blotting. About 25 µg of genomic DNA was digested with *Eco*RI for detecting an internal fragment of the marker genes (Fig. 5A). The membrane was first hybridized with a dAT[^{32}P]-labelled *gus* fragment and, after stripping, hybridized a second time with the *bar* probe (Fig. 5B).

When the blot was hybridized with the *gus* probe, all plants expressing the *gus* gene showed a band at the expected size of 2.9 kb (Fig. 5B, lanes 1-5, 10-13) as did some GUS-negative plants (Fig. 5B, lanes 7, 8, 14). A higher band also appeared for some GUS-positive samples (Fig 5B, lanes 3, 5, 10, 13, 14). A second hybridization with the *bar* gene showed the expected 1.7-kb fragment corresponding to the complete *bar* cassette of PAT-positive T0 plants (Fig. 5B, lanes 1, 2, 4, 6–9) and for some PAT-negative lines (Fig. 5B, lanes 10-15). A higher band appeared for 2 plants expressing the PAT protein (Fig. 5B, lanes 3, 5, 8, 9).

By comparing Southern blots hybridized with the *gus* and *bar* probes, we were able to evaluate the co-integration frequency independent of the co-expression frequency. Thus, we could detect escapes-plants which survived selection but did not contain the *bar* gene. Table 3 summarizes the results in detail for each bombardment experiment. Of the 90 regenerated lines, 60 showed a signal after Southern blotting with the *bar* probe (data not shown).

In order to test whether the transgenic lines were derived from independent transformation events, we digested genomic DNA from the 60 *bar*-transformed plants with *Eco*RV, which cuts the plasmid KSAB at a unique site after the CaMV 35S terminator (Fig. 6A). After blotting, the membrane was hybridized with a dAT[^{32}P]-labelled *bar* probe. The results are shown in Fig. 6B for 11 representative samples. As indicated by their individual band patterns, most of the detected transformation events were apparently independent. Among the 60 transgenic samples, only 5 plants showed the same integration pattern. They were regenerated from the same bombarded plate and gave the same expression pattern (data not shown). Thus, the results in Fig. 6B indicate that the integration pattern of the *bar* gene is complicated, also when l-ssDNA is used (lanes 12-15). Two

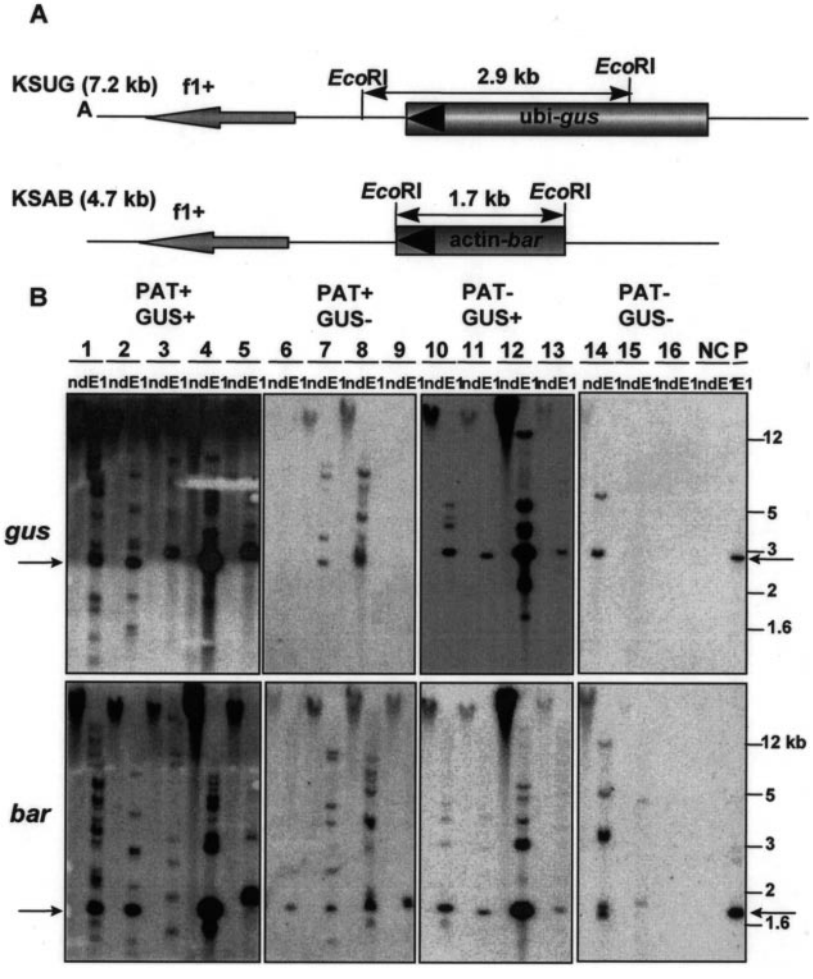

Fig. 5A, B Southern blot analysis of regenerated T0 plants after *Eco*RI restriction. **A** Maps of the two plasmids KSAB and KSUG showing the expected size of the fragment after *Eco*RI digestion **B** DNA was either digested with *Eco*RI (*E1*) or not digested (*nd*) and then probed with dAT[^{32}P]-labelled *gus* and *bar* fragments after DNA fragment separation by electrophoresis. *Lanes 1-16* Integration patterns of the marker genes (PAT+GUS+, PAT+GUS-, PAT–GUS+ and PAT–GUS–) in 16 regenerated T0 plants, *NC* negative control non-transformed wheat plant, *P* KSAB and KSUG plasmids digested with *Eco*RI. *Arrows* indicate the expected molecular size of the respective fragments. Molecular size is indicated in kb on the *right*

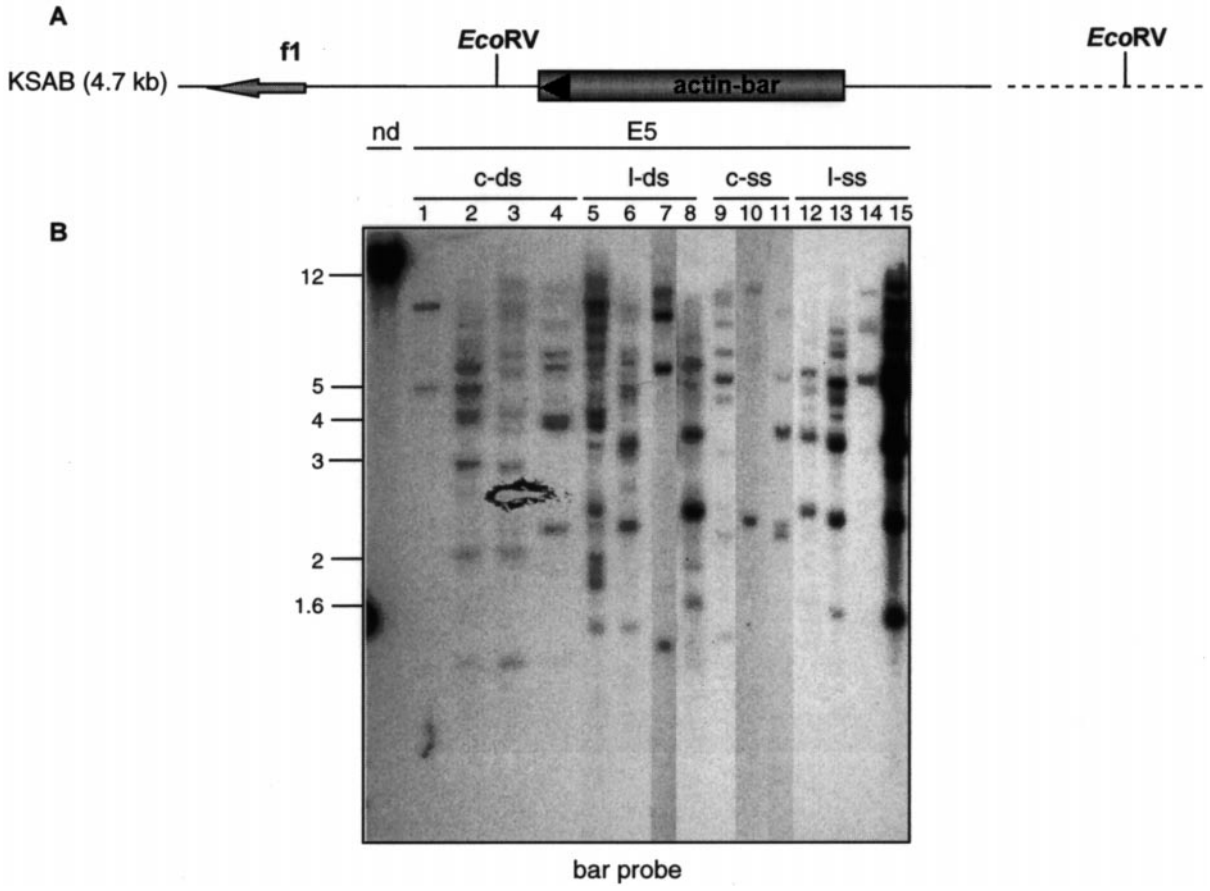

Fig. 6A, B Southern blot analysis of regenerated R0 plants after *Eco*RV restriction. **A** Map of KSAB indicating the unique *Eco*RV restriction site. **B** DNA was digested with *Eco*RV and then probed with the dAT[^{32}P]-labelled *bar* fragment after DNA fragment separation by electrophoresis. *Lanes 1-15* Regenerated R0 plants showing integration patterns of the *bar* gene (*lanes 1-4* c-dsDNA, *lanes 5-8* l-dsDNA, *lanes 9-11* c-ssDNA, *lanes 12-15* l-ssDNA), nd non-digested DNA. The molecular size is indicated in kb on the left

lines (lanes 1 and 10) showed integration of the *bar* gene in two copies, and the others showed four or more copies.

Discussion

Four different forms of DNA were introduced into wheat cells by microprojectile bombardment and their transformation efficiency and integration patterns compared. We report for the first time efficiencies of transformation of cereals with single-stranded DNA representing the same strand.

Transient expression

We observed that no correlation exists between mean number of blue spots per callus in transient expression and the number of transformed plants. Takumi and Shimada (1996) have also reported this. This is not surprising since the two marker genes, *bar* for selection and *gus* as a visible indicator, were co-bombarded on the assumption of no physical link between them. The GUS transient expression simply indicates whether the DNA-binding to particles and the bombardment were correct.

As yet there has been no report of a correlation between transient expression and stable transformation. A correlation between low transient gene expression and stable transformants is technically not possible: the nuclear genome can not be stably transformed if DNA does not enter the cell. This was also observed in our experiments when GUS transient expression was less than 20 blue spots per callus and no plants were regenerated.

The sizes of the blue spots from ds- or ssKSUG-bombarded cells were different; the blue area was always bigger with the ds plasmid. This might be due to the synthesis of the GUS protein, which may be faster with a ds strand than a ss strand or to the dsDNA, which may be more resistant to degradation. Plant cell nuclei harbor nucleases specific to ssDNA. Indeed, the plant cell is able to defeat, for example, invasive ssDNA from viruses (Citovsky et al. 1989).

Stable transformation

We showed that all four DNA structures are able to integrate into the wheat genome and that higher transformation efficiencies are obtained with linearized plas-

mids. When all of the experiments are considered, an efficiency of 3.56% for *bar*-transformed plants was observed with the l-dsDNA and 3.32% with l-ssDNA. Due to the low transient expression (Table 2), the probability of regenerating a transgenic plant with l-ssDNA was reduced compared to l-dsDNA-bombarded embryos but ultimately, their transformation efficiencies were similar.

Bilang et al. (1992) studied the efficiencies of recombination by comparing different forms of DNA introduced into tobacco protoplasts. Hygromycin-resistant colonies were produced after restoring the *hph* gene by homologous recombination. The authors observed an increase in the number of resistant calli obtained with l-ssDNA compared with c-ssDNA. Transformation with l-ssDNA has also been studied in yeast for illegitimate integration which is a rare phenomenon in such an organism (Gjuracic and Zgaga 1996). These authors demonstrated that ssDNA is more proficient at transformation via illegitimate integration than the corresponding dsDNA. Moreover, c-ds and l-ssDNA have been shown to transform with comparable efficiency. De Groot et al. (1992) supposed that ssDNA recombines before the single-strand is completely converted into dsDNA. Rodenburg et al. (1989) and Furner et al. (1989) have shown that plant protoplasts convert ssDNA into dsDNA within 48 h after gene transfer. In contrast, in mammalian cells, no conversion could be detected within 48 h (Rauth et al. 1986), and in yeast no conversion was detected within 18 h (Simon and Moore 1987). Folger et al. (1985) suggested that recombination occurs within 1 h of the introduction of the DNA. Therefore, it is possible that the introduced single-stranded DNA becomes partially double-stranded before it enters into the recombination process and that the ratio between ds and ss molecules increases, thus possibly reducing the integration frequency. Inhibition of the conversion process may improve this unfavorable situation; for example, the protection of the ssDNA with proteins similar to what *Agrobacterium* does with its T-DNA.

Molecules as l-ssDNA protected by VirD2 and VirE2 proteins might be more efficient for transformation of monocotyledonous and other crops recalcitrant to *Agrobacterium*-mediated transformation. The artificial DNA-protein complex could be delivered by bombardment into the target tissue at the correct developmental stage. An approach to ballistic delivery of the complete stochiometric complex mixture of l-ssDNA, VirD2 and VirE2 is offered by the microtargeting system developed by Sautter et al. (1991), since this system can transport water-soluble biologically active substances without binding to projectiles (Schlaman et al. 1997).

Acknowledgments We gratefully acknowledge Elke Fenner, Sabine Klarer, Katalyn Konya for caring of the plants in the greenhouse, Dr. Patrick King (FMI, Basel) for help with the English and the Swiss National Foundation for financial support. We declare that the experiments comply with the current laws of the country in which the experiments were performed.

References

Altpeter F, Vasil V, Srivastava V Stöger E, Vasil IK (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Rep 16:12–17

Becker D, Brettschneider R, Lörz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J 2:299–307

Bilang R, Peterhans A, Bogucki A, Paszkowski J (1992) Single-stranded DNA as a recombination substrate in plants as assessed by stable and transient recombination assays. Mol Cell Biol 12:329–336

Blechl AE, Anderson OD (1996) Expression of a novel high molecular weight glutenin subunit in transgenic wheat. Nature/Biotechnology 14:875–879

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689

Citovsky V, Wong M, Zambryski P (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single stranded DNA: implications for the T-DNA transfer process. Proc Natl Acad Sci USA 86:1193–1197

De Groot MJA, Offringa R, Does MP, Hooykaas PJJ, van den Elzen PJM (1992) Mechanisms of intermolecular homologous recombination in plants as studied with single- and double-stranded DNA molecules. Nucleic Acids Res 20:2785–2794

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Folger KR, Thomas K, Capecchi MR (1985) Nonreciprocal exchanges of information between DNA duplexes coinjected into mammalian cell nuclei. Mol Cell Biol 5:59–69

Fujioka KI, Aratani Y, Kusano K, Koyama H (1993) Targeted recombination with single-stranded DNA vectors in mammalian cells. Nucleic Acids Res 21:745–750

Furner IJ, Higgins ES, Berrington AW (1989) Single-stranded DNA transforms plant protoplast. Mol Gen Genet 220:65–68

Gjuracic V, Zgaga Z (1996) Illegitimate integration of single-stranded DNA in *Saccharomyces cerevisiae*. Mol Gen Genet 253:177–181

Hamilton DA, Roy M, Rueda J, Sindhu RK, Sanford J, Mascarenhas JP (1992) Dissection of a pollen-specific promoter from maize by transient transformation assays. Plant Mol Biol 18:211–218

Iglesias VA (1994) Genetic transformation studies in wheat using particle bombardment. PhD thesis, ETH Zurich no. 10628

Iglesias VA, Gisel A, Potrykus I, Sautter C (1994) In vitro germination of wheat proembryos to fertile plants. Plant Cell Rep 13:377–380

Jefferson RA (1987) Assaying chimeric genes in plants: the *gus* gene fusion system. Plant Mol Biol Rep 5:387–405

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163–171

Mendel RR, Muller B, Schulze J, Kolesnikov V, Zelenin A (1989) Delivery of foreign genes to intact barley cells by high velocity microprojectiles. Theor Appl Genet 78:31–34

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M, Kartha KK (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J 5:285–297

Rauth S, Song KY, Ayares D, Wallace L, Moore PD, Kucherlapati R (1986) Transfection and homologous recombination involving single-stranded DNA substrates in mammalian cells and nuclear extracts. Proc Natl Acad Sci USA 83:5587–5591

Rodenburg KW, de Groot MJA, Schilperoort RA, Hooykaas PJJ (1989) Single-stranded DNA used as an efficient new vehicle for transformation of plant protoplasts. Plant Mol Biol 13:711–719

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: laboratory manual, 2ed edn. Cold Spring Harbor Laboratory Press, New York

Sautter C, Waldner H, Neuhaus-Url G, Galli A, Neuhaus G, Potrykus I (1991) Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. Bio/Technology 9:1080–1085

Schlaman HRM, Gisel AA, Quaedvlieg EM, Bloemberg GV, Lugtenberg BJJ, Kijne JW, Potrykus I, Spaink HP, Sautter C (1997) Chitin oligosaccharides can induce cortical cell division in roots of *Vicia sativa* when delivered by ballistic microtargeting. Development 124:4887–4895

Shillito RD, Saul MW, Paszkowski J, Müller M, Potrykus I (1985) High efficiency direct gene transfer to plants. Bio/Technology 3:1099–1103

Simon JR, Moore PD (1987) Homologous recombination between single-stranded DNA and chromosomal genes in *Saccharomyces cerevisiae*. Mol Cell Biol 7:2329–2334

Spencer TM, Gordon-Kamm WJ, Daines RJ, Start WG, Lemaux PG (1990) Bialaphos selection of stable transformants from maize cell culture. Theor Appl Genet 79:625–631

Takumi S, Shimada T (1996) Production of transgenic wheat through particle bombardment of scutellar tissues: frequency is influenced by culture duration. J Plant Physiol 149:418–423

Takumi S, Shimada T (1997) Variation in transformation frequencies among six common wheat cultivars through particle bombardment of scutellar tissues. Genes Genet Syst 72:63–69

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220:245–250

Vasil V, Srivastava V, Castillo AM, Fromm ME, Vasil IK (1993) Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology 11:1553–1558

Weeks TJ, Anderson OD, Blechl AE (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L.). Plant Physiol 102:1077–1084

Wünn J, Klöti A, Burkhardt PK, Ghosh Biswas GC, Launis K, Iglesias VA, Potrykus I (1996) Transgenic indica rice breeding line IR58 expressing a synthetic *cryIA*(b) gene from *Bacillus thuringiensis* provides effective insect pest control. Bio/Technology 14:171–176

Zhou H, Arrowsmith JW, Fromm SM, Hironaka CM, Taylor ML, Rodriquez D, Pacjau ME, Brown SM, Santino CG, Fry JE (1995) Glyphosate-tolerant CP4 and GOX genes as a selectable marker in wheat transformation. Plant Cell Rep 15:159–163