Mol Breeding (2006) 18:195–208
DOI 10.1007/s11032-006-9027-0

# Characterisation of T-DNA loci and vector backbone sequences in transgenic wheat produced by *Agrobacterium*-mediated transformation

**Huixia Wu · Caroline A. Sparks · Huw D. Jones**

Received: 22 March 2006 / Accepted: 20 May 2006 / Published online: 27 September 2006


**Abstract** Detailed molecular characterisation of transgene loci is a requirement for gaining regulatory approval for environmental release of genetically modified crops. In cereals, it is generally accepted that *Agrobacterium*-mediated transformation generates cleaner transgene loci with lower copy number and fewer rearrangements than those generated by biolistics. However, in wheat there has been little detailed analysis of T-DNA insertions at genetic and molecular level. Wheat lines transformed using *Agrobacterium tumefaciens* with *bar* and *gus*A (GUS) genes were subjected to genetic and molecular analysis. Unlike previous studies of transgene loci in wheat, we used functional assays for PAT and GUS proteins, combined with PCR and Southern analysis to detect the presence, copy number, linkage and transmission of two transgenes inserted in the same T-DNA. Thirty-four independent transgenic lines were categorised into three types: type I events (38% of total) where the *gus*A and *bar* genes displayed complete genetic linkage, segregating together as a single functional locus at the expected ratio of 3:1; type II events (18%), which possessed two or more transgene loci each containing *gus*A and *bar*; and type III events (44%), containing an incomplete T-DNA in which either the *gus*A or *bar* gene was lost. Most lines in this last category had lost the *bar* gene situated near the left T-DNA border. Southern analysis indicated that 30% of all lines possessed a single T-DNA copy containing *gus*A and *bar*. However, when data on expression and molecular analysis are combined, only 23% of all lines have single copy T-DNAs in which both gene cassettes are functioning. We also report on the presence of plasmid backbone DNA sequence in transgene loci detected using primer pairs outside the left and right T-DNA borders and within the plasmid selectable marker (*Npt*I) gene. Approximately two thirds of the lines contained some vector backbone DNA, more frequently adjacent to the left border. Taken together, these data imply unstable left border function causing premature T-strand termination or read-through into vector backbone. As far as we are aware, this is the first report revealing near border T-DNA truncation and vector backbone integration in wheat transgenic lines produced by *Agrobacterium*-mediated transformation.

**Keywords** Agrobacterium · Copy number · T-DNA · Cereals · Vector backbone · Transgenic wheat

H. Wu · C. A. Sparks · H. D. Jones (✉)
CPI Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK
e-mail: huw.jones@bbsrc.ac.uk

## Introduction

The use of plant genetic transformation, whether as a research tool or as part of a commercial breeding programme, depends on producing plant lines which display a stable phenotypic change resulting from the genomic integration of recombinant DNA. Presence of DNA other than the transgene expression cassette is undesirable. Until recently, the only reliable method to generate transgenic wheat plants was to introduce recombinant DNA into immature, regenerable explants using a particle bombardment device (biolistics) (reviewed by: Barcelo et al. 2001; Christou 1992; Jones 2005; Shewry and Jones 2005; Vasil 2005). However, molecular analysis of transgenic cereal lines made by biolistic transformation has shown a tendency for high transgene copy number and insertional rearrangements (Makarevitch et al. 2003; Mehlo et al. 2000; Rooke et al. 2003; Svitashev et al. 2002). While this complexity does not necessarily hamper the use of transgenics in research, it is a major drawback in obtaining regulatory approval for field-trials and potential commercialisation. Recently, there have been several reports of *Agrobacterium*-mediated transformation of wheat (reviewed by Cheng et al. 2004; Jones et al. 2005) but there has been little detailed analysis of T-DNA insertions at genetic and molecular level to support the dogma that *Agrobacterium* generates lower copy number, cleaner transgene loci with fewer rearrangements than those generated by biolistics in this species. Of the few published reports of transgene segregation in cereal crops produced by *Agrobacterium*-mediated transformation, inheritance data has been inferred by assaying a phenotype generated by one of the inserted transgenes rather than at the level of the DNA (for example Cheng et al. 1997; Frame et al. 2002; Hiei et al. 1994; Ishida et al. 1996; Tingay et al. 1997). While this approach allows large numbers of progeny to be rapidly scored and can, in some lines, be correlated with transgene segregation, in other lines it leads to an under-estimate of transgene transmission and hence, distorted segregation ratios, because it fails to account for silenced or rearranged loci. In addition, reports of transgene segregation ratios and copy numbers have been calculated based on the presence of only one gene (or region) from a multi-gene T-DNA (for example Khanna and Daggard 2003; Tingay et al. 1997; Trifonova et al. 2001). As we report here, the relative position of Southern probes or quantitative PCR primers in the T-DNA will influence copy number determination and the segregation ratios or copy numbers of two transgenes inserted as part of the same T-DNA do not always match. Thus, the presence of a whole, intact T-DNA cannot be inferred from the presence of just one gene or component of the T-DNA. This observation has also been made recently in reference to transgenic rice lines (Afolabi et al. 2004).

In an ideal model, the T-strand to be transferred from *Agrobacterium* to plant cell is synthesised from DNA delimited by the left and right T-DNA border repeats (LB & RB) and transferred then integrated into the plant genome intact, with no extra sequences. However, it has been known for some time that partial T-DNAs can be integrated or that DNA from the Ti or binary plasmid sequence external to the T-DNA border sequences can also be incorporated into the plant genome e.g. (Cluster et al. 1996; De Buck et al. 2000; Martineau et al. 1994; Ooms et al. 1982; Sallaud et al. 2003; van der Graaff et al. 1996; Yin and Wang 2000) however this has not been previously reported for wheat.

Following our previous report on successful transformation mediated by *Agrobacterium tumefaciens* on wheat varieties Florida and Cadenza, we used biochemical assays to detect enzymatic activity of the *gus*A and *bar* gene products beta-glucuronidase (GUS) and phosphinothricin acetyltransferase (PAT) respectively. What makes this work novel is that we have combined this with PCR and Southern analysis for the *gus*A and *bar* sequences to detect the presence, linkage and transmission of both transgenes delivered within the same T-DNA. We also report on the transgene copy number and the presence of plasmid backbone DNA sequence in transgene loci and comment on the mechanisms that may have given rise to the left border T-DNA truncation and backbone sequence integration observed in our wheat lines. As far as we are aware, this is the first report revealing near border T-DNA truncation

and vector backbone integration in wheat transgenic lines produced by *Agrobacterium*-mediated transformation.

## Materials and methods

### Transgenic wheat lines

Wheat (*Triticum aestivum* L. cv. Florida) was transformed as previously described (Wu et al. 2003). All the plants were transformed with pGreen-based plasmid pAL156, containing a T-DNA incorporating the *bar* gene and a modified *gus*A (GUS) gene (Hellens et al. 2000a) each driven by the maize Ubiquitin promoter plus first intron (Christensen and Quail 1996). The *bar* gene was adjacent to the left border and *gus*A gene adjacent to the right (Fig. 1A). The primary transgenic plants ($T_0$) were grown to maturity in a temperature controlled glasshouse. All the plants grew normally, were fully fertile and produced at least 200 seeds each which is typical of non-transformed, tissue culture-derived plants, under the same conditions.

### Transgene inheritance

For each $T_0$ line, between 21 and 138 $T_1$ progeny seedlings were analysed. Seeds were surface sterilized in 20% domestic bleach (Domestos, Lever Faberge, UK) for 45 min, followed by 1% Benlate (DuPont, UK) by soaking overnight with gentle agitation and finally with 10% bleach for 15 min. The seeds were rinsed at least three times with sterile distilled water between the first bleach and Benlate treatments, and again after the second bleach treatment. The washed seeds were placed in tall magenta boxes containing R medium (Wu et al. 2003) solidified with 5% agargel and incubated for 4–7 days at 4°C to synchronise germination before being transferred to a culture room at 24–25°C under fluorescent white light (250 μmol $m^{-2}$ $s^{-1}$). At the three/four-leaf stage, leaf material was harvested and subjected to simultaneous histochemical GUS and ammonium evolution assays.

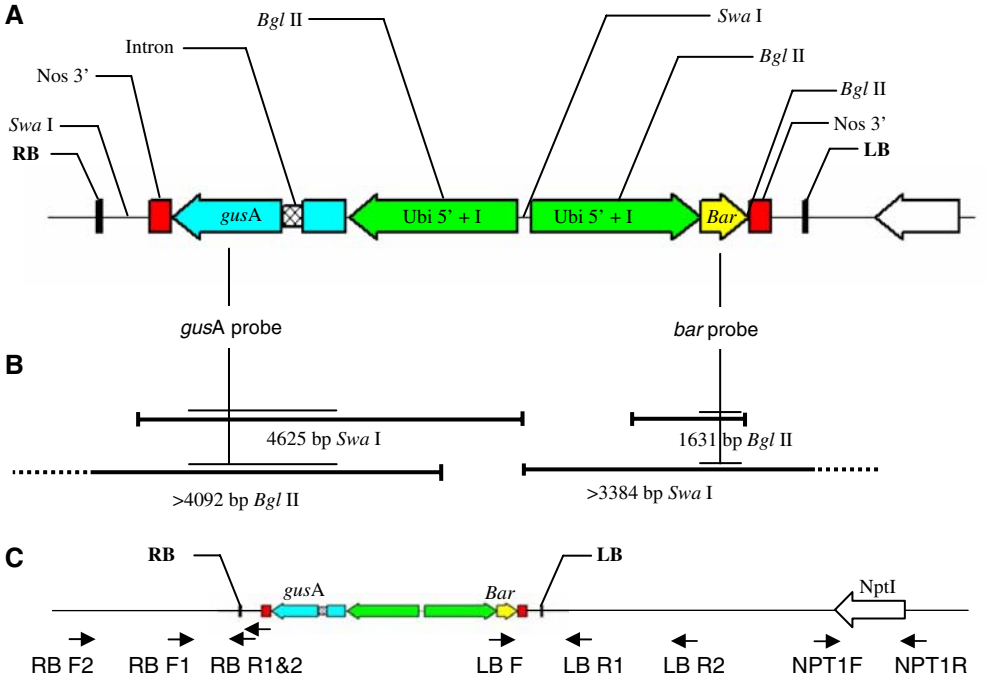


**Fig. 1** (**A**) Map of plasmid pAL156 showing *bar* and *gus*A expression cassettes relative to T-DNA borders. Restriction sites *Swa*I and *Bgl*II used in Southern analyses are also shown. (**B**) Expected size of *Swa*I and *Bgl*II restriction fragments in Southerns probed with *bar* or *gus*A. (**C**) Position of PCR primers used for the detection of plasmid backbone DNA

## Histochemical GUS assay

Leaf material from seedlings was cut into small pieces, and put into X-gluc solution in microtitre plates for GUS histochemical assay following (Jefferson et al. 1987). Explants were incubated overnight at 37°C in buffer containing 1 mM X-gluc, 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% v/v Triton X-100. The plates were incubated in darkness for 24 h at 37°C, followed by 1 day at 25°C. To remove the chlorophyll, X-gluc solution was replaced by 70–100% ethanol, and then placed under light in the culture room for a further 6 h (Fig. 2A).

## Ammonium assimilation assay for *bar* gene expression

The assay, originally developed by (Deblock et al. 1995), is based on the observation that plants expressing the *bar* gene can assimilate ammonium ions when treated with PPT whereas untransformed tissues cannot. The assay was performed according to (Rasco-Gaunt et al. 1999) with some modification. For each seedling, leaf material was cut into 6 pieces, each approximately 3 by 6 mm. They were immersed together in 1 ml incubation medium in a single well of a 24-well plate, and incubated at 24–25°C for 5–6 h under light (250 μmol $m^{-2}$ $s^{-1}$). The incubation medium contained 50 mM potassium-phosphate buffer (pH 5.8), 2% sucrose, 0.1 mg $l^{-1}$ 2,4-D, 25 mg $l^{-1}$ PPT (glufosinate ammonium, Greyhound, UK) and 0.1% Tween 20. For each sample, 200 μl of incubation medium was mixed with 1 ml Ammonium Reagent 1 (containing 34 g $l^{-1}$ sodium salicylate, 25 g $l^{-1}$ trisodium citrate, 25 g $l^{-1}$ sodium tartrate and 0.12 g $l^{-1}$ sodium nitroprusside) and 1 ml of Ammonium Reagent 2 (containing 30 g $l^{-1}$ sodium hydroxide and 0.52 g $l^{-1}$ sodium dichloroisocyanurate). After incubating the mixture at 37°C for 15 min in the dark, the colour of the solution was judged qualitatively as either positive or negative, by eye; leaf samples with no *bar* gene expression gave a dark blue colour whereas those expressing *bar* gave an obviously lighter colour ranging from pale blue to yellow (Fig. 2B).

## PCR analysis for transgene inheritance and backbone sequence detection

Mini DNA preparations were performed using 50–75 mg of leaf material according to manufacturer's instructions (Promega, USA). PCR was used to confirm the presence of *gus*A and *bar* transgenes in both $T_0$ primary transgenic lines and some of the $T_1$ segregating population (Wu et al. 2003). There were 5 primer pairs designed to check the vector backbone sequence of 34 independent $T_0$ lines. Two primers pairs were used to detect 66 bp and 700 bp fragments outside T-DNA at the left border, one primer pair was used to detect the *NptI* gene near the middle of the 2585 bp backbone sequence and another two primer pairs were used to detect sequences 54 bp and 872 bp beyond the T-DNA right border. Sequence of primer pairs, annealing temperature and expected fragment sizes are shown in Table 1. Their respective positions within the plasmid are shown in Fig. 1C. At least two replicates were carried out for each PCR analysis.

## Southern blot analysis

Genomic DNA was extracted from 100 mg to 200 mg of young leaf tissue using the CTAB method developed by (Stacey and Isaac 1994) with modifications described by (Barro et al. 1998). Genomic and plasmid DNA were each digested with two enzymes: *Swa*I and *Bgl*II (in separate reactions). After digestion, genomic DNA (5–20 μg) or pAL156 plasmid (5 pg) were separated by electrophoresis on a 0.9% (w/v) agarose gel at 20 V for ~40 h and transferred by capillary blotting onto positively charged nylon membrane (Roche Diagnostics GmbH, Lewes, East Sussex, UK) using the method of (Sambrook et al. 1989). A Digoxigenin-labelled probe was generated using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH) using *gus*A or *bar* primers as detailed above with plasmid pAL156 as template. Hybridisation and detection of probe was carried out using a non-radioactive, DIG luminescent detection kit for nucleic acids (Roche Diagnostics GmbH) according to the manufacturer's instructions.

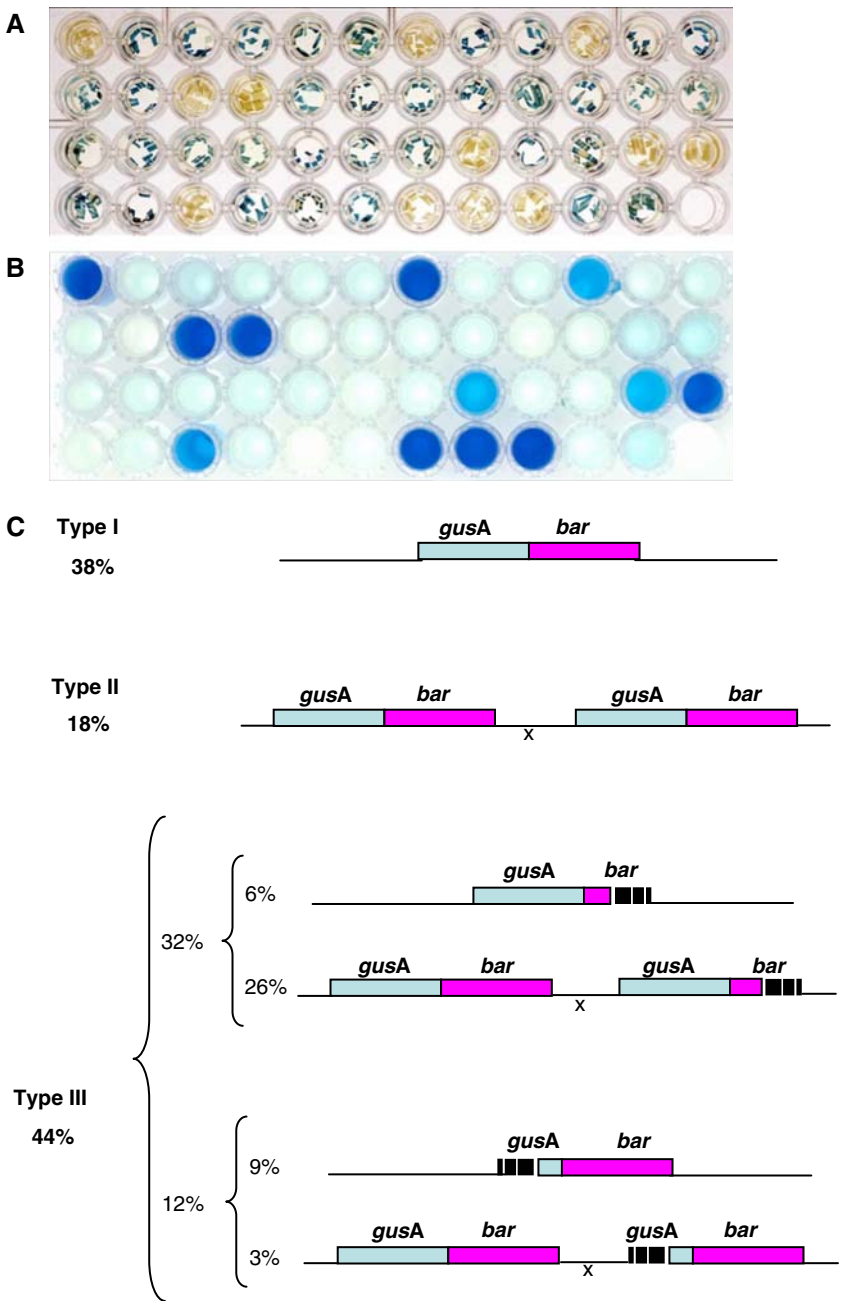


**Fig. 2** (**A**) Gus titre plate showing *gus*A expression in leaf samples of 47 $T_1$ plants of a representative line displaying a 3:1 segregation pattern. Blue colour indicates the presence of GUS. (**B**) Ammonium assay titre plate showing corresponding *bar* expression in the leaves of the same 47 $T_1$ plants. Yellow or pale blue colour indicates presence of PAT, dark blue indicates no expression. (**C**) Diagrammatic representation of transgene loci types. Type I, where *gus*A and *bar* genes display complete linkage and segregate at a ratio of 3:1. Type II, which possessed two or more transgene loci each containing at least one copy of both *gus*A and *bar*. Type III, containing a truncated T-DNA in which either the *gus*A or *bar* gene was lost

## Results

### Primary transgenic plants, transgene expression

Primary transgenic lines were originally identified from transformation experiments performed over a nine month period as previously described (Wu et al. 2003). PCR analysis on 34 confirmed independent lines showed that 29 had both *gus*A and *bar* genes present and expression analysis confirmed that all but three (in which no GUS expression could be detected) made functional GUS and PAT protein. Of the remaining five lines, three had no detectable *gus*A and two had no detectable *bar* coding sequence or expression. The latter two lines (# FL12 and 35) were selection escapes that were identified as transgenic by

**Table 1** Primer pairs for the detection of transgenes and the presence of backbone sequences

| Target | Sequence of primer pairs | Backbone sequence detected | $T_A$(°C)[a] | Fragment size (bp) |
|---|---|---|---|---|
| *gusA*F | 5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′ | N/A | 62 | 1051 |
| *gusA*R | 5′-ATCGCCGCTTTGGACATACCATCCGTA-3′ | | | |
| *bar*F | 5′-GTCTGCACCATCGTCAACC-3′ | N/A | 57 | 444 |
| *bar*R | 5′-GAAGTCCAGCTGCCAGAAAC-3′ | | | |
| LBF | 5′-CCGTCACCGAGATCTGATCC-3′ | LB66bp | 61 | 806 |
| LBR1 | 5′-ACGCGGCCTTTTTACGGTTCCT-3′ | | | |
| LBF | 5′-CCGTCACCGAGATCTGATCC-3′ | LB700bp | 61 | 1400 |
| LBR2 | 5′-TCTGCGCGTAATCTGCTGCTTGC-3′ | | | |
| NPT1F | 5′-GAGGCAGTTCCATAGGATGGCAAGATCC-3′ | NPT1 | 65 | 684 |
| NPT1R | 5′-CTTGCTCCAGGCCGCGATTAAATTCC-3′ | | | |
| RBF1 | 5′-TCCTGCAGGCTCGAGTTAAT-3′ | RB54bp | 57 | 908 |
| RBR1 | 5′-CCTCGAGGTCGACGGTATC-3′ | | | |
| RBF2 | 5′-GAGACACAACGTGGCTTTGTTG-3′ | RB872bp | 57 | 617 |
| RBR2 | 5′-CATGAAGGCCTTGACAGGAT-3′ | | | |

[a] $T_A$ (annealing temperature °C)

screening for GUS expression. All 32 lines that were positive for the *bar* gene by PCR also correctly transcribed and translated PAT protein as measured by BASTA leaf-painting (Table 2).

## Transgene segregation and linkage

To study inheritance patterns of transgene loci in the $T_1$ generation, between 21 and 138 $T_1$ plants from each of the 34 independent lines were tested for *gus*A and *bar* expression. In lines where the $T_1$ segregation ratios of *gus*A and *bar* expression did not match, PCR on genomic DNA was used to check for the presence of *gus*A and *bar* sequence.

In 13 of the 34 lines (38%), the *gus*A and *bar* genes segregated together as a single genetic locus at the expected Mendelian ratio of 3:1. Cross-referencing with Table 3 shows that in 10 of these 13 lines, both *gus*A and *bar* genes produce functional protein (lines FL42, 43 and 52 failed to synthesise active GUS enzyme). In a total of 715 $T_1$ plants tested by transgene expression assays and PCR from these 13 lines, all displayed complete linkage of the genes in the T-DNA. These were designated as type I events (Table 3, Fig. 2C). A further 6 of the 34 lines (18%) also showed complete *gus*A and *bar* linkage but possessed two or more genetic loci as indicated by segregation ratios of 15:1 or greater; these were designated as type II events (Table 3, Fig. 2C).

The final group of lines (15 out of 34; 44%), designated as type III events, possessed an incomplete T-DNA that resulted in the loss of either the *bar* or *gus*A gene. Most lines in this

**Table 2** Summary of PCR and expression analysis of 34 independent primary $T_0$ plants (FL – variety Florida, Cd – variety Cadenza). Expression of *gus*A and *bar* was confirmed in leaf tissue using a histochemical GUS and Basta leaf-painting assay respectively.

| | $T_0$ independent plants (subtotal in brackets) | *gus*A PCR-positive | GUS expression-positive | *bar*- PCR-positive | *bar* expression-positive |
|---|---|---|---|---|---|
| Both genes fully functional | FL1; 3; 5; 6; 9; 18; 20; 26; 27; 30; 36; 37; 40; 44; 46; 53; 54; 59; 78; 80; 81; 83; 85. Cd2; 17; 22; (26) | ✓ | ✓ | ✓ | ✓ |
| *Bar* functional; *gus*A/GUS lost | FL48; 57; 98 (3) | | | ✓ | ✓ |
| *gus*A/GUS functional; *bar* lost | FL12; 35 (2) | ✓ | ✓ | | |
| *Bar* functional; *gus*A/GUS silenced | FL42; 43; 52 (3) | ✓ | | ✓ | ✓ |

**Table 3** Segregation and linkage of two transgenes (*gus*A and *bar*) located on the same T-DNA in $T_1$ progeny of 34 independent transgenic lines. The presence of *gus*A and *bar* genes was detected by simultaneous biochemical analysis of the transgene product and also confirmed by PCR if discrepancies between *gus*A and *bar* expression were found

| Type | $T_0$ line | No. of $T_1$ plants tested | No. of $T_1$ plants possessing the *gus*A and/or *bar* genes | | | | *gus*A | | | *bar* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *gus*A+ *bar*+ | *gus*A – *bar* – | *gus*A+ *bar* – | *gus*A – *bar*+ | +ve: – ve | (Ratio) | $Chi^2$ | +ve: – ve | (Ratio) | $Chi^2$ |
| | FL3 | 30 | 22 | 8 | 0 | 0 | 22:8 | (3:1) | 0.04[a] | 22:8 | (3:1) | 0.04[a] |
| I | FL6 | 65 | 47 | 8 | 0 | 0 | 47:18 | (3:1) | 0.25[a] | 47:18 | (3:1) | 0.25[a] |
| *gus*A 3:1 | Cd22 | 76 | 61 | 15 | 0 | 0 | 61:15 | (3:1) | 1.18[a] | 61:15 | (3:1) | 1.18[a] |
| *bar* 3:1 | FL26 | 40 | 33 | 7 | 0 | 0 | 33:7 | (3:1) | 1.30[a] | 33:7 | (3:1) | 1.30[a] |
| | FL27 | 47 | 35 | 12 | 0 | 0 | 35:12 | (3:1) | 0.01[a] | 35:12 | (3:1) | 0.01[a] |
| | FL42 | 56 | 41 | 15 | 0 | 0 | 41:15 | (3:1) | 0.09[a] | 41:15 | (3:1) | 0.09[a] |
| | FL43 | 21 | 17 | 4 | 0 | 0 | 17:4 | (3:1) | 0.42[a] | 17:4 | (3:1) | 0.42[a] |
| | FL52 | 35 | 26 | 9 | 0 | 0 | 26:9 | (3:1) | 0.01[a] | 26:9 | (3:1) | 0.01[a] |
| | FL53 | 38 | 27 | 11 | 0 | 0 | 27:11 | (3:1) | 0.31[a] | 27:11 | (3:1) | 0.31[a] |
| | FL54 | 24 | 18 | 6 | 0 | 0 | 18:6 | (3:1) | 0.00[a] | 18:6 | (3:1) | 0.00[a] |
| | FL78 | 73 | 47 | 26 | 0 | 0 | 47:26 | (3:1) | 4.06[b] | 47:26 | (3:1) | 4.06[b] |
| | FL83 | 138 | 111 | 27 | 0 | 0 | 111:27 | (3:1) | 2.29[a] | 111:27 | (3:1) | 2.29[a] |
| | FL85 | 72 | 52 | 20 | 0 | 0 | 52:20 | (3:1) | 0.35[a] | 52:20 | (3:1) | 0.35[a] |
| II | Cd2 | 60 | 60 | 0 | 0 | 0 | 60:0 | ND | | 60:0 | ND | |
| *gus*A ≥ 15:1 | FL5 | 31 | 30 | 1 | 0 | 0 | 30:1 | (15:1) | 0.59[a] | 30:1 | (15:1) | 0.59[a] |
| *bar* ≥ 15:1 | Cd17 | 32 | 30 | 2 | 0 | 0 | 30:2 | (15:1) | 0.00[a] | 30:2 | (15:1) | 0.00[a] |
| | FL36 | 36 | 35 | 1 | 0 | 0 | 35:1 | (15:1) | 0.92[a] | 35:1 | (15:1) | 0.92[a] |
| | FL37 | 25 | 23 | 2 | 0 | 0 | 23:2 | (15:1) | 0.12[a] | 23:2 | (15:1) | 0.12[a] |
| | FL46 | 26 | 26 | 0 | 0 | 0 | 26:0 | ND | | 26:0 | ND | |
| III | FL1 | 42 | 41 | 0 | 1 | 0 | 42:0 | ND | | 41:1 | (15:1) | 1.39[a] |
| *gus*A ≥ 15:1 | FL9 | 68 | 51 | 4 | 13 | 0 | 64:4 | (15:1) | 0.02[a] | 51:17 | (3:1) | 0.00[a] |
| *bar* 3:1 or no bar | FL12 | 48 | 0 | 12 | 36 | 0 | 36:12 | (15:1) | 0.00[a] | 0:48 | no *bar* | |
| | FL18 | 48 | 38 | 4 | 6 | 0 | 44:4 | (15:1) | 0.32[a] | 38:10 | (3:1) | 0.46[a] |
| | FL20 | 39 | 31 | 0 | 8 | 0 | 39:0 | ND | | 31:8 | (3:1) | 0.44[a] |
| | FL30 | 33 | 24 | 1 | 8 | 0 | 32:1 | (15:1) | 0.71[a] | 24:9 | (3:1) | 0.09[a] |
| | FL35 | 63 | 0 | 4 | 59 | 0 | 59:4 | (15:1) | 0.00[a] | 0:32 | no *bar* | |
| | FL40 | 38 | 30 | 1 | 7 | 0 | 37:1 | (15:1) | 1.07[a] | 30:8 | (3:1) | 0.33[a] |
| | FL59 | 46 | 30 | 3 | 13 | 0 | 43:3 | (15:1) | 0.01[a] | 30:16 | (3:1) | 2.18[a] |
| | FL80 | 34 | 27 | 1 | 6 | 0 | 33:1 | (15:1) | 0.78[a] | 27:7 | (3:1) | 0.71[a] |
| | FL81 | 32 | 26 | 0 | 6 | 0 | 32:0 | ND | | 26:6 | (3:1) | 0.37[a] |
| *bar* ≥ 15:1 | FL44 | 37 | 28 | 1 | 0 | 8 | 28:9 | (3:1) | 0.01[a] | 36:1 | (15:1) | 1.0[a] |
| *gus*A 3:1 or no *gus*A | FL48 | 36 | 0 | 12 | 0 | 24 | 0:36 | no *gus*A | | 24:12 | (3:1) | 1.25[a] |
| | FL57 | 66 | 0 | 23 | 0 | 43 | 0:32 | no *gus*A | | 20:12 | (3:1) | 6.42[b] |
| | FL98 | 37 | 0 | 10 | 0 | 27 | 0:37 | no *gus*A | | 27:10 | (3:1) | 0.08[a] |

[a] In good agreement with 3:1 or 15:1 at $P = 0.05$; [b] Skewed segregation ratio; ND: not determined

category possessed more *gus*A copies than *bar*. In 8 lines (Table 3: line # FL9, 18, 20, 30, 40, 59, 80 and 81, Fig. 2C) there was a single locus containing both *gus*A and *bar* which segregated 3:1 in the $T_1$ and a second locus that contained only a functional *gus*A gene. The *bar* gene was undetectable in plants that had inherited only this locus. The remaining three lines in this group were different; one had a single functional *gus*A locus but no detectable *bar* (line #12), a second had multiple *gus*A loci but no detectable *bar* (line #35) and a third had multiple loci containing both genes with an additional one (or more) loci containing only the *gus*A gene (line #1). Only four lines (12%) had the opposite configuration with more functional *bar* loci than *gus*A. Three of these lines (Table 3: line # FL48, 57 and 98) possessed a single, functional *bar* locus which segregated at 3:1 but no detectable *gus*A coding sequence or expression. The remaining line possessed two functional, independently segregating, *bar* loci but in one of these the *gus*A gene was absent (line #44).

## Transgene integration

To examine transgene integration patterns, randomly selected lines from each of the inheritance types were subjected to Southern blot analysis. Two enzymes, *Bgl*II and *Swa*I were used to cut the plasmid as shown in Fig. 1. For genomic DNA from transgenic plants, *Bgl*II cuts three times within the T-DNA and unpredictably in the genomic DNA. *Swa*I cuts twice within the T-DNA and also unpredictably in the genomic DNA. When probed with *gus*A, the *Swa*I digest gave a single diagnostic excision band at 4625 bp and the number of hybridizing bands (>4092 bp) from the *Bgl*II digest gave an indication of the insertion number of *gus*A gene. When probed with *bar*, the *Bgl*II digest gave a single diagnostic excision band at 1631 bp and the number of hybridizing bands (>3384p) in the *Swa*I digest gave an indication of the insertion number of the *bar* gene (Fig. 1B).

In general there seemed to be good correlation with the T-DNA insertion patterns predicted from Southerns and the number of loci indicated by segregation analysis (Table 4, Fig. 3). Among the nine lines randomly chosen from the Type I events (13 lines in total), seven lines (78%) displayed single insertion of both *gus*A and *bar* genes, confirming the presence of a single functional locus and indicating a single transgene copy for both genes. Of the remaining two lines, FL52 showed evidence of a rearrangement with an extra band smaller than the expected size in the *Swa*I digest when probed with *gus*A, and FL27 possessed an extra faint band in the *Bgl*II track when probed with *gus*A or *bar*. Five of the six type II lines were subjected to Southern blot analysis: multiple bands were detected with both *gus*A and *bar* probes in three of them with the number of bands ranging from two to nine, indicating multiple insertions which supported the segregation data. Lines FL36 and FL37 showed multiple bands when probed with *gus*A but only a single band was observed with *bar* even though these lines displayed a clear 15:1 segregation for both genes. We assume the *bar* Southern underestimated the true number of transgene copies in these lines, perhaps due to the loss of a restriction site or a rearrangement, which is supported by the observation of additional bands in the diagnostic *Swa*I digest. However, although *Bgl*II is not considered methylation sensitive, we cannot rule out the effect of DNA methylation. Five lines were randomly chosen from the 15 type III events. Four of them displayed multiple bands when probed with *gus*A but a single band when probed with *bar* which supported the segregation data indicating that there were at least two (segregating) copies of *gus*A with *bar* associated with only one. Another line (FL1) gave multiple hybridizing bands (5) with *gus*A probe but only two bands with the *bar* probe which again supported the segregation data showing it had at least one copy of *gus*A that was unlinked to the *bar* loci.

Rearrangements within the coding sequence were detected using the diagnostic digests of *Swa*I for *gus*A, and *Bgl*II for *bar*. As expected, most of the lines with Southern banding patterns indicative of T-DNA rearrangements were from type II and III events but there was also one line from the type I events that showed possible rearrangement of *gus*A gene only (Table 4).

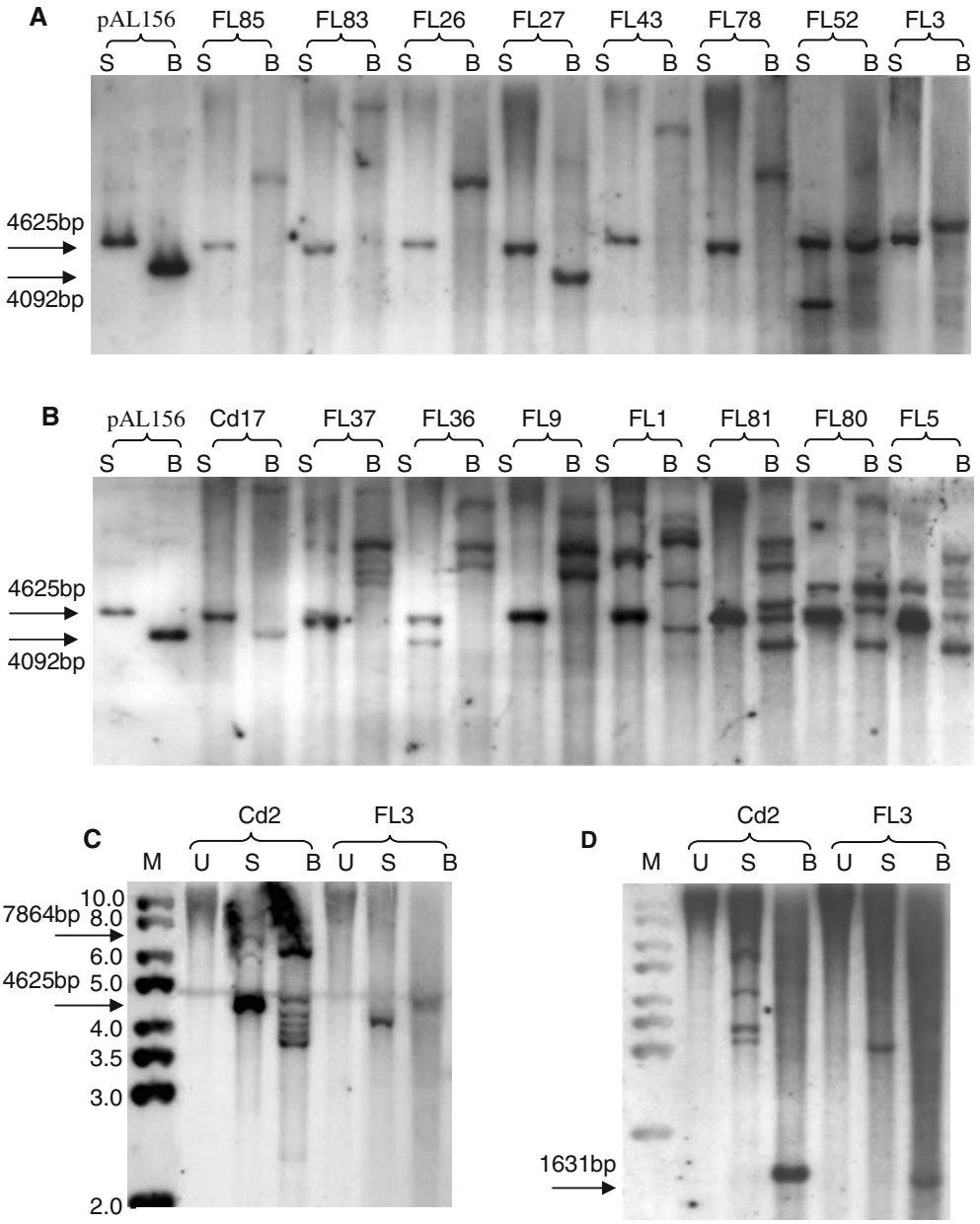


**Fig. 3** Southerns of selected lines from each group I–III. Genomic DNA was cut with *Swa*I which excises a 4625 bp fragment containing the *gus*A gene and *Bgl*II which excises a 1631 bp fragment containing the *bar* gene (see Fig. 1). When integrated into genomic DNA and probed with *bar*, *Swa*I digestion results in cuts at position 5655 in the T-DNA and unpredicted cuts in the genomic DNA beyond the left border to release a fragment of at least 3384 bp. When probed with *gus*A, *Bgl*II digestion results in cuts at position 4632 within the T-DNA and again in the genomic DNA beyond the T-DNA right border to release a fragment of at least 4092 bp. (**A**) Lines from group I (single segregating locus containing *gus*A and *bar)* probed with *gus*A. S – *Swa*I, B – *Bgl*II. (**B**) Lines from groups II and III (lines containing two or more loci or truncated T-DNAs) probed with *gus*A. S – *Swa*I, B – *Bgl*II. (**C**) and (**D**) Comparison of hybridising bands on the same filter, first probed with *gus*A (C) then stripped and re-probed with *bar* (D). M – molecular wt marker, U – uncut genomic DNA, S – *Swa*I, B – *Bgl*II

Presence of vector backbone sequence

The 34 independent lines were tested for the presence of backbone sequences. PCR primers were designed to amplify five regions of the vector backbone; two adjacent to each of the left and right T-DNA borders and one within the *Npt*I gene (Fig. 1C). Thirteen lines (38%) were free of all backbone regions tested while 21 lines (62%) contained some binary plasmid sequence outside the T-DNA. There was a clear pattern with most lines possessing backbone near to the LB and diminishing towards the RB (Table 4). Twenty lines (59% of total) contained 66 bp of backbone sequence immediately outside the LB and 17 of these (50%) also contained backbone sequence to 700 bp beyond LB. Eleven lines (32%) contained sequence reaching to the *Npt*I

**Table 4** Comparison of 34 independent ($T_0$) transgenic lines. Columns show the number of hybridising bands on Southerns, estimated functional loci for the two transgenes on the same T-DNA from segregation data, and the presence of vector backbone sequence from PCR with five primer-pairs

| Type | $T_0$ line | *gus*A | | *bar* | | Presence of backbone sequence | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Southern bands* | Loci | Southern bands** | Loci | LB – 66 bp | LB – 700 bp | *Npt1* | RB – 54 bp | RB – 872 bp |
| I | FL3 | 1 | 1 | 1 | 1 | + | + | + | + | + |
| | FL6 | ND | 1 | ND | 1 | – | – | – | – | – |
| | Cd22 | 1 | 1 | 1 | 1 | – | – | – | – | – |
| | FL26 | 1 | 1 | 1 | 1 | + | + | – | – | – |
| | FL27 | 2[a] | 1 | 2[a] | 1 | + | + | + | + | + |
| | FL42 | ND | 1 | ND | 1 | + | + | – | – | – |
| | FL43 | 1 | 1 | 1 | 1 | + | – | – | – | – |
| | FL52 | 1[b] | 1 | 1 | 1 | + | + | + | + | + |
| | FL53 | ND | 1 | ND | 1 | + | + | + | – | – |
| | FL54 | ND | 1 | ND | 1 | + | + | + | + | + |
| | FL78 | 1 | 1 | 1 | 1 | + | + | – | – | – |
| | FL83 | 1 | 1 | 1 | 1 | – | – | – | – | – |
| | FL85 | 1 | 1 | 1 | 1 | + | + | – | – | – |
| II | Cd2 | 9 | ≥2 | 6–7 | ≥2 | + | + | + | + | + |
| | FL5 | 5[b] | ≥2 | 4–5 | ≥2 | + | + | + | + | + |
| | Cd17 | 2 | 2 | 2 | 2 | – | – | – | – | – |
| | FL36 | 3[b] | ≥2 | 1 | ≥2 | – | – | – | – | – |
| | FL37 | 5[b] | 2 | 1 | 2 | – | – | – | – | – |
| | FL46 | ND | ≥2 | ND | ≥2 | + | – | – | – | – |
| III | FL1 | 5[b] | >2 | 2[b] | ≥2 | + | + | – | – | – |
| | FL9 | 3 | ≥2 | 1[b] | 1 | + | + | + | – | – |
| | FL12 | ND | 1 | 0 | 0 | – | – | + | – | – |
| | FL18 | ND | 2 | ND | 1 | – | – | – | – | – |
| | FL20 | ND | ≥2 | ND | 1 | + | + | – | – | – |
| | FL30 | ND | 2 | ND | 1 | – | – | – | – | – |
| | FL35 | 3 | 2 | 0 | 0 | + | – | – | – | – |
| | FL40 | ND | 2 | ND | 1 | – | – | – | – | – |
| | FL59 | 2 | 2 | 1 | 1 | + | + | – | – | – |
| | FL80 | 4[b] | ≥2 | 1 | 1 | + | + | + | + | + |
| | FL81 | 6–7 | ≥2 | 1 | 1 | + | + | + | + | + |
| | FL44 | ND | 1 | ND | 2 | – | – | – | – | – |
| | FL48 | 0 | 0 | N | 1 | – | – | – | – | – |
| | FL57 | ND | 0 | ND | 1 | – | – | – | – | – |
| | FL98 | ND | 0 | ND | 1 | – | – | – | – | – |

ND: not analysed; * *gus*A/*Bgl*II; ** *bar*/*Swa*I

[a] 1 + 1 faint band

[b] possibility of rearrangement

N: *bar* gene functional, but no hybridisation band detected

gene. There were 8 (24%) lines that yielded PCR fragments with all 5 primer pairs spreading across the entire backbone, which suggested that these lines probably had the whole backbone sequence integrated, as so called complete read-through. There was also one line (FL12) that consistently yielded a PCR fragment covering *Npt*I region with no PCR amplification from the other four backbone regions flanking either left or right borders. Backbone DNA less than 100 bp flanking LB was the most frequently transferred (20 out of 21; 95.2%), indicating that vector backbone DNA is much more frequently linked to the left than to the right T-DNA border which was found in only the 8 lines that showed complete read-through of the vector backbone. Significantly, there were no lines that contained backbone DNA flanking RB only. Clean and read-through lines were found in types I, II, and III segregation groups, not particularly biased toward any of these three groups. However, the four type III lines possessing more copies of *gus*A than *bar* (line # FL44, 48, 57 and 98) were interestingly, all completely backbone-free. An extra PCR fragment along with the expected size product was detected in line Cd2 when using primer pairs for detecting 66 bp backbone fragment beyond LB, indicating there could be rearrangement of the inserted backbone sequences. This type of rearrangement may also have been responsible for the presence of the *Npt*I PCR product in line FL12 mentioned above or this may be the result of truncated backbone integration independent of T-DNA as postulated by (Kononov et al. 1997). To exclude the possibility that any of the PCR products resulted from amplification of unincorporated binary vector DNA from contaminating *Agrobacterium* cells, we also used primers to the *Vir*C operon (Sawada et al. 1995) which in all cases, gave no amplification.

## Discussion

Almost 40% of the lines were what we called type I segregation events where the *gus*A and *bar* genes displayed complete genetic linkage, segregating together as a single functional locus at the expected ratio of 3:1. Furthermore, the majority of these lines produced functional transgenic protein and contained only one copy of the T-DNA (demonstrated by Southerns in 7 of the 9 tested), thus overall, 23% of all lines produced possessed a single copy of the T-DNA containing intact, functional *gus*A and *bar* genes with complete linkage. There were 18% of all lines that possessed two or more loci, each of which contained one or more copies of both transgenes (type II events). Our data broadly support the few previous reports analysing *Agrobacterium*-mediated transgene loci in wheat. For example, single transgene loci demonstrating 3:1 segregation ratios were reported in 35% of lines by Khanna and Daggard (2003) and in 46% of lines by Hu et al. (2003). Analysis of 26 plants revealed single transgene copies in 35% of lines tested, two or three copies in 50% and four to five copies in only 15% (Cheng et al. 1997), although the same author found a higher proportion (67%) of lines with single copies in a another population of transgenic wheat (Cheng et al. 2003). However, direct comparisons of transgene copy numbers in different populations must be made with caution because the choice of restriction enzymes used to cut the genomic DNA and position of the hybridising probe on the T-DNA can affect the copy number reported. In this paper we show the copy number of the whole T-DNA, calculated from Southerns using *bar* and *gus*A probes which hybridise to both ends of the T-DNA. As discussed below, we found significant numbers of lines with incomplete T-DNA copies causing a difference in the copy number of *bar* and *gus*A genes detected.

Approximately 44% of the lines made contained incomplete T-DNAs (type III events). Most of the plants in this category contained more functional *gus*A loci than *bar* gene including some selection escapes where the *bar* gene was undetectable even though a functional *gus*A gene was present. However, there were also a few lines with more *bar* loci than *gus*A including lines with no detectable *gus*A even though a functional *bar* gene was present. The *bar* gene was positioned near the LB of the T-DNA and other reports have also indicated that genes housed adjacent to the LB are more likely to be lost compared to

those near the RB. In a comprehensive survey of 171 rice lines, a higher proportion of T-DNA deletions were found at the LB (53%) compared to the RB (21%) (Kim et al. 2003). Another study in rice revealed that 70–78% of plant lines contained non-intact T-DNAs with fewer copies of the *bar* gene (2.7 copies per plant) which was situated at the LB compared with the *gus*A gene (3.8 copies per plant) situated at the RB (Afolabi et al. 2004).

Only three lines, which possessed *gus*A sequence but no observable GUS expression, displayed what could be transgene silencing or rearrangement. This relatively low frequency of gene silencing was probably due to the population being chosen for its ability to survive glufosinate selection in tissue culture. Thus the T-DNA insertions studied here are biased towards displaying good gene expression.

The presence of vector sequence outside the T-DNA borders has been reported in transgenic plants of several species but this appears to be the first analysis of this phenomenon in a population of wheat transgenics. Vector backbone sequences were detected in 21 of the 34 lines analysed (62%). This falls within the range previously reported from other species; tobacco 75% (Kononov et al. 1997), *Arabidopsis* and tobacco 20–50% (De Buck et al. 2000). In rice lines transformed with LBA4404/pGA2144, 77/171 (45%) had vector backbone integrated (Kim et al. 2003). A comprehensive study of locus composition in rice transformed using a similar pSoup/pGreen binary vector system as used in our experiments reported 53–66% of the loci vector backbone sequences had integrated with the T-DNAs (Afolabi et al. 2004). Recent work in maize has revealed 70% of transgenic lines contained various lengths of the bacterial plasmid backbone DNA sequence (Shou et al. 2004).

It has been hypothesised that the LB acts primarily to terminate the 5′ – 3′ T-strand processing initiated by VirD2 at the RB and that the LB can sometimes get skipped causing over-run of the T-strand into vector backbone DNA. An alternative explanation is that VirD2 initiates 5′ processing from the LB as well as the RB, and in this case generates a T-strand composed of vector backbone DNA and maybe T-DNA if T-strand synthesis progresses round the vector to the RB. There are data to support both models eg. (De Buck et al. 2000; Durrenberger et al. 1989; Kononov et al. 1997; Kuraya et al. 2004; Ramanathan and Veluthambi 1995; van der Graaff et al. 1996) and analysis of our wheat lines tends to support the former model.

Our study found evidence of both early termination of the T-DNA more often at the LB than the RB and of backbone sequences more often adjacent to the LB than the RB (in fact we found no vector backbone adjacent to the RB only). In addition, all but one of the 8 lines that possessed the entire backbone sequence, also possessed multiple copies of the *gus*A gene (or in one line a rearranged extra copy). This implied that the T-strand had started at the RB, failed to terminate at the LB and continued round the vector and back to the T-DNA for a second time. This is consistent with a recent study of T-DNA insertions in creeping bent grass which demonstrated that the RB termini were more intact, with a smaller range of border deletions and no backbone integration, compared to sequences adjacent to the LB (Fu et al. 2006) and fits the model that the RB more often acts to initiate 5′ T-strand synthesis and the LB to terminate (Caplan et al. 1985; De Buck et al. 2000; Miranda et al. 1992; Peralta et al. 1986; Zambryski 1988). It has been assumed that the frequency of vector backbone integration is primarily influenced by the inability of the LB to terminate T-strand synthesis. This is supported by De Buck et al. (2000) who found more than half of the *Arabidopsis* and tobacco plants containing backbone came from binary vectors with octopine borders without the additional inner border regions of the original Ti plasmid from which the vector was derived. Further support is provided by Kuraya et al. (2004) who show that additional, tandem repeat copies of the LB sequence reduce the incidence of read through in transgenic rice. For the pGreen vector used in this study, both border sequences are single copy and synthetic (Hellens et al. 2000b). They possess the RB overdrive sequence but lack the inner natural border sequence context of the octopine strains which may partly explain why a relatively large proportion (62%) of our lines possessed backbone DNA. Intriguingly, no

vector backbone was found in lines that possessed T-DNAs that were truncated at the RB.

In summary, with the vectors used here, approximately one third of the *Agrobacterium*-generated lines possessed a single, complete T-DNA which is higher than equivalent populations made by biolistics. However, approximately two thirds of these also possessed vector sequence outside the T-DNA leaving only approximately 10% with a clean, single T-DNA. It is likely that occurrence of LB read through could be significantly reduced by the inclusion of multiple LB copies which also contain additional inner border sequences.

## References


Afolabi AS, Worland B, Snape JW, Vain P (2004) A large-scale study of rice plants transformed with different T-DNAs provides new insights into locus composition and T-DNA linkage configurations. Theor Appl Genet 109:815–826

Barcelo P, Rasco-Gaunt S, Thorpe C, Lazzeri PA (2001) Transformation and gene expression. In: Shewry PR, Lazzeri PA, Edwards KJ (eds) Advances in botanical research incorporating advances in plant pathology. pp 59–126

Barro F, Cannell ME, Lazzeri PA, Barcelo P (1998) The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. Theor Appl Genet 97:684–695

Caplan AB, Vanmontagu M, Schell J (1985) Genetic-analysis of integration mediated by single T-DNA borders. J Bacteriol 161:655–664

Cheng M, Fry JE, Pang SZ, Zhou HP, Hironaka CM, Duncan DR, Conner TW, Wan YC (1997) Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiol 115:971–980

Cheng M, Hu TC, Layton J, Liu CN, Fry JE (2003) Desiccation of plant tissues post-Agrobacterium infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. In Vitro Cell Dev Biol-Plant 39:595–604

Cheng M, Lowe BA, Spencer TM, Ye XD, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol-Plant 40:31–45

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Christou P (1992) Genetic-transformation of crop plants using microprojectile bombardment. Plant J 2:275–281

Cluster PD, Odell M, Metzlaff M, Flavell RB (1996) Details of T-DNA structural organization from a transgenic Petunia population exhibiting cosuppression. Plant Mol Biol 32:1197–1203

De Buck S, De Wilde C, Van Montagu M, Depicker A (2000) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Mol Breed 6:459–468

Deblock M, Desonville A, Debrouwer D (1995) The selection mechanism of phosphinothricin is influenced by the metabolic status of the tissue. Planta 197:619–626

Durrenberger F, Crameri A, Hohn B, Koukolikovanicola Z (1989) Covalently Bound Vird2 Protein of Agrobacterium-Tumefaciens Protects the T-DNA from Exonucleolytic Degradation. Proc Natl Acad Sci U S A 86:9154–9158

Frame BR, Shou HX, Chikwamba RK, Zhang ZY, Xiang CB, Fonger TM, Pegg SEK, Li BC, Nettleton DS, Pei DQ, Wang K (2002) Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol 129:13–22

Fu D, St. Amand PC, Xiao Y, Muthukrishnan S, Lang GH (2006) Characterization of T-DNA integration in creeping bentgrass. Plant Sci 170:225–237

Hellens R, Mullineaux P, Klee H (2000a) A guide to Agrobacterium binary Ti vectors. Trends Plant Sci 5:446–451

Hellens RP, Edwards EA, Leyland NR, Bean S and Mullineaux PM (2000b) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 42:819–832

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient Transformation of Rice (*Oryza-Sativa* L.) Mediated by Agrobacterium and Sequence Analysis of the Boundaries of the T-DNA. Plant J 6:271–282

Hu T, Metz S, Chay C, Zhou HP, Biest N, Chen G, Cheng M, Feng X, Radionenko M, Lu F, Fry J (2003) *Agrobacterium*-mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. Plant Cell Reports 21:1010–1019

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by Agrobacterium tumefaciens. Nat Biotechnol 14:745–750

Jefferson RA, Kavanagh TA, Bevan MW (1987) Beta-Glucuronidase (Gus) as a Sensitive and Versatile Gene Fusion Marker in Plants. J Cell Biochem 57–57

Jones HD (2005) Wheat transformation: current technology and applications to grain development and composition. J Cereal Sci 41:137–147

Jones HD, Doherty A, Wu H (2005) Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. Plant Methods 1:5

Khanna HK, Daggard GE (2003) Agrobacterium tumefaciens-mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium. Plant Cell Reports 21:429–436

Kim SR, Lee J, Jun SH, Park S, Kang HG, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants. Plant Mol Biol 52: 761–773

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J 11:945–957

Kuraya Y, Ohta S, Fukuda M, Hiei Y, Murai N, Hamada K, Ueki J, Imaseki H and Komari T (2004) Suppression of transfer of non-T-DNA 'vector backbone' sequences by multiple left border repeats in vectors for transformation of higher plants mediated by *Agrobacterium tumefaciens*. Mol Breed 14:309–320

Makarevitch I, Svitashev SK and Somers DA (2003) Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. Plant Mol Biol 52:421–432

Martineau B, Voelker TA, Sanders RA (1994) On Defining T-DNA. Plant Cell 6:1032–1033

Mehlo L, Mazithulela G, Twyman RM, Boulton MI, Davies JW and Christou P (2000) Structural analysis of transgene rearrangements and effects on expression in transgenic maize plants generated by particle bombardment. Maydica 45:277–287

Miranda A, Janssen G, Hodges L, Peralta EG, Ream W (1992) Agrobacterium-Tumefaciens Transfers Extremely Long T-Dnas by a Unidirectional Mechanism. J Bacteriol 174:2288–2297

Ooms G, Bakker A, Molendijk L, Wullems GJ, Gordon MP, Nester EW, Schilperoort RA (1982) T-DNA Organization in Homogeneous and Heterogeneous Octopine-Type Crown Gall Tissues of Nicotiana-Tabacum. Cell 30:589–597

Peralta EG, Hellmiss R, Ream W (1986) Overdrive, a T-DNA Transmission Enhancer on the a-Tumefaciens Tumor-Inducing Plasmid. Embo J 5:1137–1142

Ramanathan V, Veluthambi K (1995) Transfer of Non-T-DNA Portions of the *Agrobacterium-Tumefaciens* Ti Plasmid pTiA6 from the Left Terminus of $T_L$-DNA. Plant Mol Biol 28:1149–1154

Rasco-Gaunt S, Riley A, Lazzeri P, Barcelo P (1999) A facile method for screening for phosphinothricin (PPT)-resistant transgenic wheats. Mol Breed 5:255–262

Rooke L, Steele SH, Barcelo P, Shewry PR, Lazzeri PA (2003) Transgene inheritance, segregation and expression in bread wheat. Euphytica 129:301–309

Sallaud C, Meynard D, van Boxtel J, Gay C, Bes M, Brizard JP, Larmande P, Ortega D, Raynal M, Portefaix M, Ouwerkerk PBF, Rueb S, Delseny M and Guiderdoni E (2003) Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. TAG Theoretical and Applied Genetics 106:1396–1408

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

Sawada H, Ieki H, Matsuda I (1995) Pcr detection of Ti and Ri plasmids from phytopathogenic agrobacterium strains. Appl Environ Microbiol 61:828–831

Shewry PR, Jones HD (2005) Transgenic wheat: where do we stand after the first 12 years? Ann Appl Biol 147:1–14

Shou HX, Frame BR, Whitham SA and Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or Agrobacteriummediated transformation. Mol Breed 13:201–208

Stacey J and Isaac P (1994) Isolation of DNA from plants. In: Isaac P (eds) Methods in molecular biology – protocols for nucleic acid analysis by nonradioactive probes. Totowa NJ, Humana Press Inc., pp 9–15

Svitashev SK, Pawlowski WP, Makarevitch I, Plank DW, Somers DA (2002) Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. Plant J 32:433–445

Tingay S, McElroy D, Kalla R, Fieg S, Wang MB, Thornton S and Brettell R (1997) Agrobacterium tumefaciens-mediated barley transformation. Plant J 11:1369–1376

Trifonova A, Madsen S, Olesen A (2001) *Agrobacterium*-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. Plant Sci 161:871–880

van der Graaff E, den Dulk-Ras A, Hooykaas PJJ (1996) Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. Plant Mol Biol 31:677–681

Vasil IK (2005) The story of transgenic cereals: The challenge, the debate, and the solution – A historical perspective. In Vitro Cell Dev Biol-Plant 41:577–583

Wu H, Sparks C, Amoah B, Jones HD (2003) Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. Plant Cell Reports 21:659–668

Yin Z, Wang GL (2000) Evidence of multiple complex patterns of T-DNA integration into the rice genome. Theor Appl Genet 100:461–470

Zambryski P (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant-cells. Annu Rev Genet 22:1–30