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Molecular characterization of the fate of transgenes in transformed wheat (*Triticum aestivum* L.)

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Abstract Molecular analysis of the transgenes *bar* and *gus* was carried out over successive generations in six independent transgenic lines of wheat, until the plants attained homozygosity. Data on expression and integration of the transgenes is presented. Five of the lines were found to be stably transformed, duly transferring the transgenes to the next generation. The copy number of the transgenes varied from one to five in the different lines. One line was unstable, first losing expression of and then eliminating both the transgenes in R3 plants. Although the *gus* gene was detected in all the lines, GUS expression had been lost in R2 plants of all but one line. Rearrangement of transgene sequences was observed, but it had no effect on gene expression. All the stable lines were found to segregate for transgene activity in a Mendelian fashion.

Key words *Triticum aestivum* L. · Transformation · Transgene inheritance · Wheat

Introduction

Fertile transgenic plants have been produced from all of the major species of cereals during the past few years (Vasil 1994). Transgenic wheat plants were first obtained by biolistic bombardment of long-term embryogenic callus cultures (Vasil et al. 1992). Further improvements in transformation were realized by the bombardment of cultured immature embryos (Vasil et al. 1993; Weeks et al. 1993; Becker et al. 1994; Nehra et al. 1994). Each of the above studies demonstrated the integration and expression of various transgenes, and their Mendelian transmission to sexual progeny. In cereals, as in other higher plants, one to several copies of the transgenes are integrated at random and often at multiple sites in the host genome. It has been suggested that copy number, site of integration and the methylation of transgenes may interfere with their expression resulting in gene silencing and/or elimination (Cedar 1988; Finnegan and McElroy 1994; Flavell 1994). For the practical use of transgenic plants in agriculture, it is not only important that the transgenes be stably integrated and expressed, but also that they be transmitted to progeny in a Mendelian fashion. Earlier, we have described the production of six independent fertile transgenic lines of wheat containing the selectable marker gene *bar* and the reporter gene *gus* (Vasil et al. 1992, 1993). We now report on the Mendelian inheritance and fate of these genes up to homozygosity in the R3 generation.

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Materials and methods

Transgenic lines

We used six transgenic lines that were derived from three wheat cultivars: Bob White (line 2B-2), Pavon (lines 3P-1, 4P-1, 6P-1 and TR3), and RH770019 (line 5R-1). Bob White and Pavon are spring wheat cultivars, and RH770019 is a winter wheat cultivar (Vasil et al. 1992, 1993). Segregation analysis was carried out by determining PAT activity in R2 plants obtained by germinating up to 12 seeds derived from each R1 line. For the 5R-1 line, only four R2 seeds were available. The line had shown low seed set in R0 and R1 plants. A total of 51 R2 plants from six independent transgenic lines were assayed for PAT activity. Further, for R3 plant analysis and to obtain homozygous plants, six seeds borne on a single spike from self-fertilized R2 plants of each transgenic line were grown to maturity and assayed for PAT activity. Those showing PAT activity in all six plants were considered 'potential homozygous' and their homozygosity was confirmed by assaying six more plants for PAT activity. Transgenic lines showing PAT activity in all 12 R3 plants were considered homozygous. All plants were grown in 4-inch pots to maturity in a growth chamber at 21°/18 °C (day/night temperature) and under 300 $\mu E/m^2$ per s light. Lines 2B-2, 3P-1 and 6P-1 were transformed with pAHC25 (Vasil et al. 1993; Castillo et al. 1994) and lines TR3, 4P-1 and 5R-1 were transformed with pBARGUS (Vasil et al. 1992). Both plasmids contain *bar* and *gus* genes which are driven by different promotors (Fromm et al. 1990; Christensen et al. 1992).

Enzyme assays

PAT activity, conferred by the *bar* gene, was assayed using crude extracts of leaf tissues (Vasil et al. 1992). GUS activity, conferred by the *gus* gene, was tested histochemically as well as fluorometrically on leaf segments as described by Jefferson (1987). Specific GUS activity was calculated by measuring nmol MU/mg of total protein per h in the fluorometric assay.

Southern hybridization

Genomic DNA was isolated from leaf tissue by the CTAB method (Lassner et al. 1989). About 25 µg of DNA was digested with the appropriate restriction enzyme in a 0.5 ml volume. Electrophoresis was carried out in a 0.7% agarose gel. The gels were blotted on to a nylon membrane (Hybond, Amersham) and crosslinked by UV exposure. Hybridizations were carried out using ^{32}P-labelled *bar* or *gus* probes at 65 °C for 12–16 h. The final wash consisted of 0.25 × SSPE (pH 7.2) and 1% SDS. For determination of gene copy number by the reconstruction method, pBARGUS was digested with *Bam*HI and pAHC25 with *Eco*RI.

Results and discussion

Expression and inheritance of transgenes

The six transgenic wheat lines used in this study (TR3, 2B-2, 3P-1, 4P-1, 6P-1, and 5R-1) were obtained by bombardment of either long-term embryogenic callus tissues (Vasil et al. 1992) or of immature embryos (Vasil et al. 1993) with pBARGUS or pAHC25 DNA. Expression, integration and segregation of the *bar* gene in R0 (primary regenerants) and their progeny (R1) has been described previously (Vasil et al. 1992, 1993). In the present study, the expression (PAT and GUS activities), segregation, and inheritance of *bar* and *gus* genes were determined in R2 and R3 plants.

PAT assays of plants arising from selfed R2 plants of line TR3, derived from the bombardment of long-term callus cultures (Vasil et al. 1992), showed 3:1 segregation of PAT activity. Plants arising from cross-pollinated R0 plants of lines 5R-1, 3P-1 and 6P-1 showed 1:1 segregation and those from selfed R0 plants of line 4P-1 and 2B-2 showed 3:1 segregation of PAT activity (Vasil et al. 1993). R2 plants of all lines, except 2B-2, showed Mendelian segregation of PAT activity. R2 plants of line 2B-2 had apparently lost PAT activity and, therefore, it was considered to be an unstable line. Six plants of this line gave slightly higher PAT activity than that of the control but this was shown to be an artifact by Southern analysis (see below). Table 1 summarizes the data on PAT and GUS activity in R0, R1, R2 and R3 plants.

Strong histochemical GUS activity was seen in R0 plants of all lines, except TR3 which showed weak expression (Vasil et al. 1992, 1993). However, when R2 plants of all the lines were tested for histochemical expression of GUS, only plants of 6P-1 stained positive. Further, GUS activity was determined by fluorometric assay on up to six plants of each line (the positive control for GUS assay consisted of 48-h-old Bobwhite leaf pieces bombarded with pAHC25). In concordance with histochemical staining, plants of 6P-1 were found to contain a specific activity comparable to that of the positive control, while plants of all other lines showed no activity (Table 1).

For the determination of homozygosity, R3 seeds were collected from all R2 plants of each line. Six R3 seeds from four R2 plants of lines 3P-1, 4P-1 and 6P-1 were initially selected for PAT analysis, along with two Southern-positive R2 plants of 2B-2. Thus, 24 R3 plants each of 3P-1, 4P-1 and 6P-1; 12 R3 plants of TR3; and 10 R3 plants of 2B-2 (derived from PAT-negative but Southern-positive R2 plants) were analysed for PAT activity (a total of 94 plants). All 12 TR3 plants showed PAT activity, confirming their homozygous nature. At least one set of six plants from 3P-1, 4P-1 and 6P-1 was found to be PAT positive. Their homozygosity was confirmed by further analysis of six more plants, all of which were also positive for PAT activity (Table 1). Thus 30 R3 plants each of 3P-1, 4P-1 and 6P-1 were analysed. R2 plants of 5R-1 did not set seed, preventing any further analysis. PAT assays showed that some of the plants of each line (except 2B-2 and 5R-1) had become homozygous, while others continued to segregate (Table 1). No PAT activity could be discerned in any of the plants derived from the R3 seeds of 2B-2. This was not surprising because PAT activity was inconclusive in R2 plants of this line. Mendelian inheritance of a single

Table 1 Phosphinothricin acetyltransferase (PAT) and β-glucuronidase (GUS) activity in transgenic lines and their R1, R2 and R3 progenies. All the transgenic lines are derived from GUS-positive callus tissues. GUS activity was tested by fluorometric as well as histochemical assay. Plants were self-pollinated except where indicated

Line	Plasmid	R0 PAT+	R1[a] PAT(+/−)	R2		R3 PAT(+/−)
				PAT(+/−)	GUS(+/−)	
TR3	pBARGUS	40	2/0	19/5[a]	n.d.	12/0[b]
5R-1	pBARGUS	17[c]	4/6[c]	2/2	0/4	(No seed set in R2)
2B-2	pAHC25	10	15/6	0/12	0/6	0/10
3P-1	pAHC25	14[c]	5/5	9/3	0/6	27/3[b]
4P-1	pBARGUS	25	9/4	8/3	0/6	28/2[b]
6P-1	pAHC25	29[c]	11/12	8/4	6/0	25/5[b]

n.d. – not determined
[a] Vasil et al. (1992, 1993)
[b] Homozygous plants identified
[c] Cross-pollinated

dominant locus for PAT activity was observed for lines TR3, 3P-1, 4P-1 and 6P-1 in successive generations up to homozygosity.

Integration and organization of transgenes

All the transgenic lines were shown to contain *bar* and *gus* genes in R0 and R1 plants (Vasil et al. 1992, 1993). To study the integration, organization and copy number of transgenes in R2 and R3 plants of the six lines, PAT-positive plants were used for Southern analysis. DNA of lines transformed with pAHC25 was digested with *Eco*RI and *Hin*dIII to release the 1.4-kb *bar* expression cassette and the 4.2-kb *gus* expression cassette. The same restriction enzymes were used for pBARGUS lines to release the 1.9-kb *bar* and the 3.9-kb *gus* expression cassette, respectively.

As discussed above, plants of line 2B-2 did not show distinct PAT activity. Therefore, all 12 R2 plants of this line were analyzed by Southern hybridization. Genomic DNA was restricted with *Eco*RI and hybridized with *bar* as well as *gus* probes. Two of the 12 plants were found to contain both the genes. Thus, the slightly higher PAT activity detected in 6 plants out of 12 was not correlated with the presence of transgenes and was considered to be an artifact. The hybridization patterns of both the plants were very simple, consisting of one major band corresponding with *bar* (1.4-kb) and *gus* (6.9-kb) fragments in the respective hybridizations (data not shown). The copy number of *bar* in 2B-2 plants was estimated to be about 5.

Southern analysis was carried out on four PAT-positive R2 plants each of 3P-1, 4P-1, 6P-1 and TR3, and two plants of 5R-1. All plants of each line showed hybridization with *bar* and *gus* probes (Fig. 1). Plants of 3P-1 and 6P-1 showed a simple pattern of hybridization with a low number of *bar* and *gus* copies (Fig. 1a, b); this observation was confirmed by copy number analysis using the reconstruction method (see below). However, one plant of each line did not show the expected 1.4-kb *bar* band (Fig. 1a) indicating a possible rearrangement of the *bar* gene in the R2 plants. The rearrangement in the transgene could be due to a single base change in either of the restriction-enzyme sites. Rearrangement and amplification of transgenes has also been reported in maize (Register et al. 1994).

All pBARGUS lines showed a complex hybridization pattern with *bar* (Fig. 1a). However, when the same blot was stripped and re-hybridized with the *gus* probe, a simple pattern of hybridization was observed (Fig. 1b). The *bar* hybridization pattern of TR3, 4P-1, and 5R-1 indicated that multiple copies of *bar* were inserted in the genome. Furthermore, the presence of several bands of equal intensity indicated that the various copies of the *bar* gene were not in tandem but contained unique genomic borders. Variation in *bar* hybridization pattern within all plants of 4P-1 was observed; similarly, one plant of TR3 showed variation in *bar* hybridization

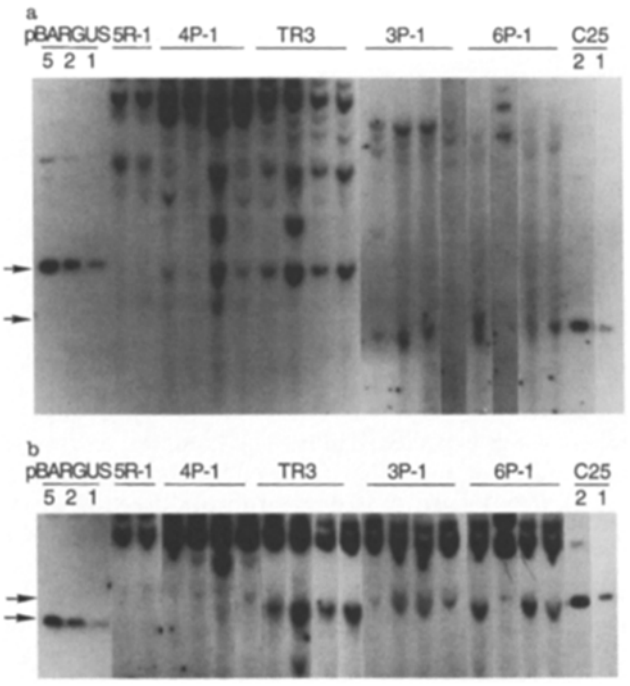


Fig. 1a, b Southern hybridization of transgenic lines. Genomic DNA of all the lines were restricted with *Eco*RI and *Hin*dIII, resolved in a 0.7% gel, blotted on a nylon membrane and then hybridized with *bar* (**a**) and *gus* (**b**) probes. Copy number reconstruction of pBARGUS and pAHC25 digested with same restriction enzymes is shown. Four plants of each line were analysed. *Arrows* in **a** indicate 1.9-kb and 1.4-kb bands for *bar* from pBARGUS and pAHC25, respectively. *Arrows* in **b** indicate 3.9-kb and 4.2-kb bands for *gus* from pBARGUS and pAHC25, respectively

pattern (Fig. 1a). The variation of hybridization patterns within the plants of a line suggests a rearrangement of the transgene after sexual transmission. The comparison of *bar* and *gus* hybridizations of pBARGUS lines indicated that the *gus* gene is not physically linked with every *bar* gene. Amongst the pBARGUS lines, 5R-1 plants did not show the 1.9-kb *bar* as well as the 3.9-kb *gus* expression cassettes, whereas all plants of 4P-1 showed the 1.9-kb *bar* band but did not show the 3.9-kb *gus* band while TR3 plants showed both the expected bands in the respective hybridizations. (Fig. 1 a, b). The presence of multiple gene copies and insertion sites in TR3 and 4P-1 was confirmed by further Southern analysis (see below). When additional plants (2–3) of all the transgenic lines (except 5R-1) were studied for transgene integration and variation in transgene organization (data not shown), it was found that two plants of 3P-1 and one plant of 4P-1 do not contain a *gus* gene (Table 2). In order to further study the complex hybridization patterns of pBARGUS lines, their DNAs were digested with *Bam*HI and hybridized with *bar* and *gus* probes (data not shown). The *Bam*HI hybridization patterns of TR3 and 4P-1 with both *bar* and *gus* probes were simple, showing only one prominent band with both the probes, corresponding to the 1.4-kb *bar* fragment and the 7.8-kb *gus* fragment; but the 5R-1 plants again did not show

Table 2 Southern analysis of R2 and R3 plants of transgenic lines

Line	R2 plants			R3 plants		
	No. plants tested	*bar* (+)	*Gus* (+)	Copies		Integration sites
				bar	*Gus*	
TR3	7	7	7	~5	2–3	2
5R-1	2	2	2	n.d.	n.d.	n.d.
2B-2	12	2	2	n.d.	n.d.	n.d.
3P-1	6	6	4	1	1	1
4P-1	6	6	5	2–3	2–3	3
6P-1	6	6	6	1–2	1–2	1

n.d. = not determined

either of the expected bands. The complex rearrangement in the 5R-1 genome, revealed by *Bam*HI as well as *Eco*RI/*Hin*dIII patterns of hybridizations, might have occurred at the time of, and/or soon after, transgene integration. Since no rearrangement was detected in the *Bam*HI site of the transgenes in TR3 and 4P-1, their gene copy numbers were determined from *Bam*HI hybridization patterns. Inconsistency in the copy number of the *bar* gene was observed among plants of all lines, implying the occurrence of gene amplification in R2 plants (Fig. 1a).

Further, homozygous R3 plants of each independent line were selected for an analysis of gene copy number, integration sites and methylation. However, line 2B-2 revealed the very interesting phenomenon of gene deletion. R2 plants of 2B-2 did not show transgene activity but showed hybridization with *bar* and *gus* probes. Both the genes were lost in R3 plants; a total of ten R3 plants of 2B-2 were analysed by Southern hybridization to confirm gene deletion. This observation suggests that transgene integration in line 2B-2 was not stable. It was expressed up to R1, detectable up to R2, and deleted in the R3 plants. This instability may have been a characteristic of the integration site. Unstable transgenic lines have also been reported in maize (Spencer et al. 1992; Register et al. 1994).

Homozygous R3 plants of lines 3P-1, 4P-1, 6P-1 and TR3 were selected for determining the number of gene copies and integration sites (Table 2). For the determination of copy number, the plasmid reconstruction method was employed; therefore, only an approximate copy number is reported. To release the 1.4-kb *bar* fragment, DNA of pAHC25 lines (3P-1 and 6P-1) was digested with *Eco*RI and of pBARGUS lines (TR3 and 4P-1) with *Bam*HI. The sizes of GUS-containing fragments are 6.9 kb and 7.8 kb for pAHC25 and pBARGUS, respectively. Two lines, 3P-1 and 6P-1, were found to have 1–2 copies of the *bar* gene per haploid genome; 4P-1 contained 2–3 copies and TR3 about 5 copies per haploid genome (Fig. 2a). Copy numbers of the *gus* gene in lines 3P-1, 4P-1 and 6P-1 were the same as their respective *bar* copy number, whereas TR3 contained fewer copies of the *gus* gene (2–3) as compared to *bar* (about 5) (Fig. 2b). The range of transgene copy number (1–5) reported here is consistent with the reports on rice

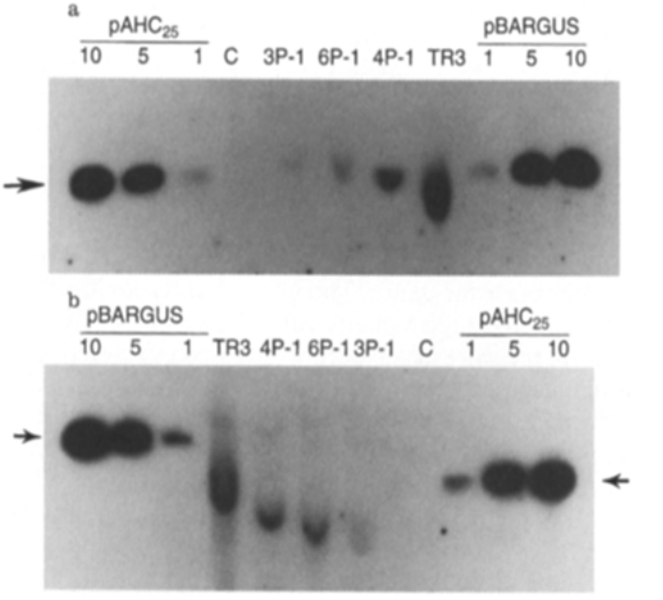


Fig. 2 Determination of copy number of *bar* (**a**) and *gus* (**b**) genes in homozygous plants of transgenic lines. Copy number reconstruction is shown for both the plasmids. To release the *bar* expression cassette, genomic DNA from pAHC25-transformed lines (3P-1, 6P-1) was digested with *Eco*RI and that of pBARGUS-transformed lines (TR3, 4P-1) with *Bam*HI. *Arrow* in **a** shows the 1.4-kb band of pAHC25 and pBARGUS. *Arrows* in **b** show the 7.8-kb *Bam*HI band of pBARGUS and the 6.9-kb *Eco*RI band of pAHC25

transformation by direct gene transfer (Goto et al. 1993; Cooley et al. 1995). In maize, Register et al. (1994) have reported transgenic plants with >10 transgene copies but the majority of their transgenic plants were in the range of 1–10. In *gus* gene hybridization, all of the lines gave a hybridizing band that was lower in molecular size than expected (6.9-kb for pAHC25 and 7.8-kb for pBARGUS), showing that none of the lines have full-length plasmid integration, a common feature of DNA-mediated transformation. It is important to note that all transgenic plants were derived from GUS-positive lines, but most of them lost GUS activity in the R2 plants. In the present study, no significant rearrangement or difference in *gus* copy number was detected. Therefore, the loss of GUS expression cannot be correlated with the molecular data. However, Cooley et al. (1995) have re-

ported an inverse relationship between GUS expression and its gene copy number in rice transgenic lines. In our studies, the loss of GUS expression in R2 plants was seen in the transgenic lines containing 1–5 copies of the *gus* gene. The line expressing GUS up to homozygosity (6P-1) contained only 1–2 *gus* copies.

The number of transgene integration sites was determined by digesting the plant DNA with *Not*I (which does not digest pAHC25 and pBARGUS plasmid DNA) and using the *bar* probe for hybridization. Since *Not*I is a rare cutter, hybridization in high-molecular-size DNA was seen in all the lines. Lines 4P-1 and TR3 in addition showed two distinct bands (Fig. 3). No hybridization was seen in a control DNA sample derived from an untransformed Pavon plant. A faint cross-hybridizing band (about 3 kb) was seen in all lanes (Fig. 3). Line TR3, which showed two bands (about 8-kb and 1.8-kb) and faint hybridization in high-molecular-size genomic DNA, was considered to contain two integration sites. Line 4P-1, which showed two bands (about 4-kb and 1.8-kb) and equally intense hybridization with high-molecular-size DNA, was assumed to contain at least three integration sites (Fig. 3). Because Mendelian segregation of PAT activity was observed in R1 and R2 plants, it was considered that all integration sites of TR3 and 4P-1 were linked and/or only one site was functional. Lines 3P-1 and 6P-1 showed hybridization with high-molecular-size DNA (Fig. 3). Therefore, although the number of integration sites could not be determined by *Not*I digestion, both lines were concluded to have a single integration site because they contained only 1–2 copies of the *bar* gene (described above).

The hybridization patterns of the homozygous R3 plants of 3P-1, 4P-1 and 6P-1 were compared with their R2 parents (Fig. 4). The data showed that the *bar* gene was stably maintained through successive generations in the homozygous plants of the various lines. Copy number reconstruction for only one of the plasmids (pBARGUS) is shown in the figure because both pAHC25 and pBARGUS release the 1.4-kb *bar* expression cassette on digestion with *Eco*RI and *Bam*HI, respectively. The copy number in Fig. 4 was determined for R3 plants only.

One of the attractive parameters to study in gene modification is CpG methylation. Methylation has been implicated in regulating gene expression in eukaryotes (Cedar 1988). By digesting genomic DNA of the transgenic lines with the methylation-sensitive enzyme *Hpa*II, it was observed that wheat genomic DNA is highly methylated in CCGG sequences. Therefore, it was of interest to determine how a transgene is affected by the methylation system of the cell. Plant cells have the capacity to modify a transgene and mark it as foreign. Gene modification is an integral part of homology-based transgene silencing and one of the modification parameters is DNA methylation (for reviews see Finnegan and McElroy 1994; Flavell 1994). The data presented in this paper showed that *bar* sequences are highly

Fig. 3 Hybridization with the *bar* gene to show number of integration sites in four independent transgenic lines of wheat. Genomic DNA from the transgenic lines and a control plant were digested with *Not*I and hybridized with the radiolabelled *bar* probe. Since *Not*I does not cut within pAHC25 or pBARGUS, the number of hybridizing bands indicates the number of integration sites

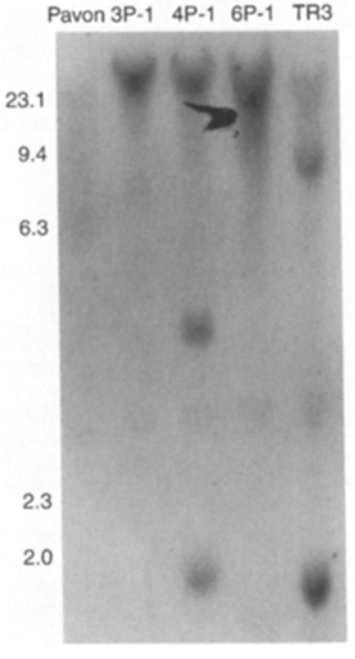
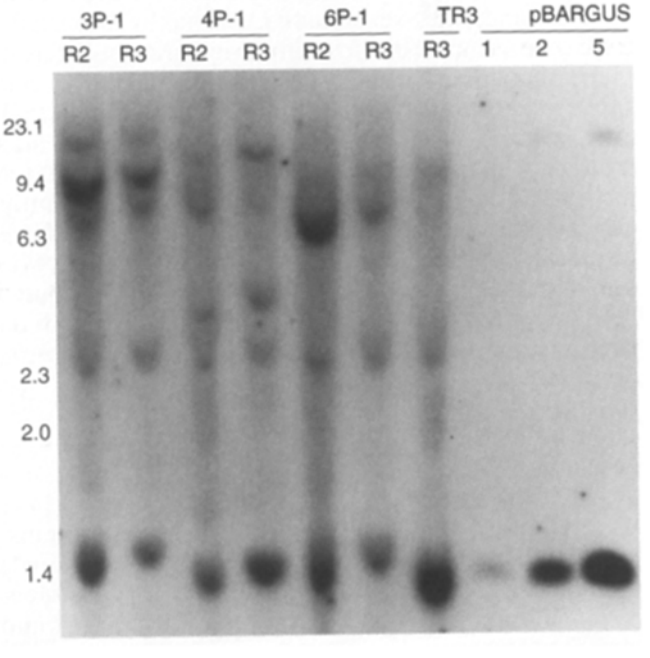


Fig. 4 Comparison of *bar* hybridization patterns of R2 parents with their R3 homozygous progenies in four transgenic lines. Consistency in the patterns was observed between R2 plants and their R3 progenies. Genomic DNA were digested with *Bam*HI (4P-1, TR3) or *Eco*RI (3P-1, 6P-1) and hybridized with a radiolabelled *bar* probe. pBARGUS and pAHC25 release a 1.4-kb band containing the *bar* gene upon digestion with *Bam*HI or *Eco*RI, respectively



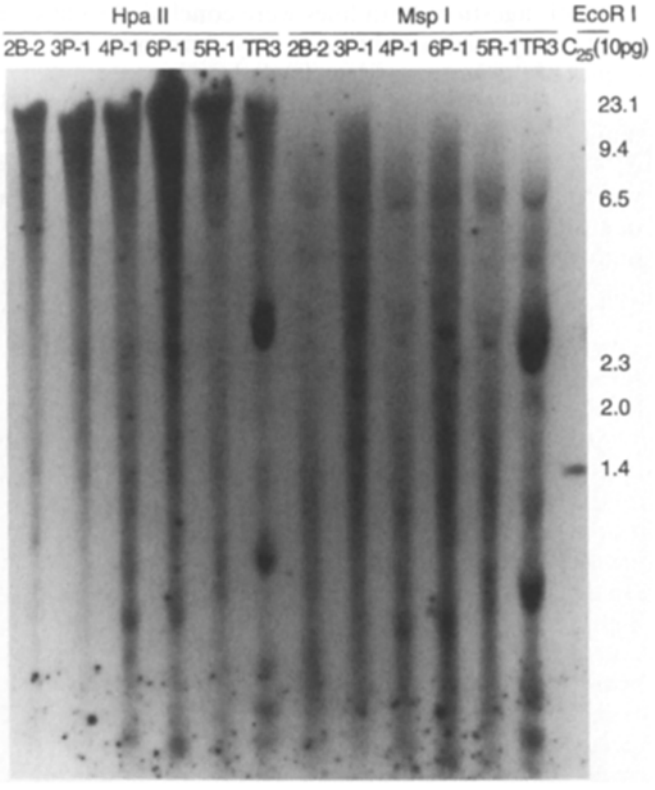


Fig. 5 Methylation of the *bar* gene in six transgenic lines. DNAs from all the lines were digested with *Hpa*II and *Msp*I separately and hybridized with the *bar* probe. *Lane 6P-1* (*Hpa*II) contains double the amount of DNA as compared to rest of the lanes. The figure shows that only one line, TR3, contains some unmethylated copies of the *bar* gene, whereas all the other lines contain methylated copies of the gene

methylated in 3P-1, 4P-1 and 6P-1, whereas some copies in TR3 are unmethylated (Fig. 5). Line TR3, which contained 2–3 integration sites and about 5 copies, was an ideal candidate to examine if DNA methylation in the transgene occurred due to multiple integration. Interestingly, those lines which contained fewer copies and single integration sites were found to be highly methylated, whereas TR3, inspite of containing multiple integration sites, was the only line that contained unmethylated copies of the *bar* gene. More importantly, DNA methylation in the other lines did not shut off *bar* expression. This suggests that transgenic lines with multiple integration sites may not necessarily undergo homology based gene modification and that gene modification may be more characteristic of the site of integration.

Conclusion

We have presented evidence that the selectable transgene *bar* is stably expressed and inherited as a single dominant locus in the wheat genome following Mendelian inheritance. One transgenic line, 2B-2, was found to be unstable and had lost the selectable transgene as well as the reporter gene in R3 plants, suggesting that certain sites in the plant genome are not favorable for inheritable integration of foreign genes. Loss of GUS expression in R2 plants of all lines except one was observed, even though no rearrangement in the *gus* gene was detected. Loss of GUS expression in transgenic lines is a common experience, but its molecular basis is not well understood. Instances of rearrangement of the selectable transgene *bar*, detected by variation in hybridization patterns, did not affect expression. Other modifications, such as amplification and methylation of the transgene, were also detected. Most interestingly, methylation of *bar* sequences did not shut off gene expression. Transgenic lines, having a single-copy gene insertion were identified.

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References

Becker D, Brettschneider R, Lorz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant Jour 5:299–307

Castillo AM, Vasil V, Vasil IK (1994) Rapid production of fertile transgenic plants of rye (*Secalè cereale* L.). Bio/Technol 12: 1366–1371

Cedar H (1988) DNA methylation and gene activity. Cell 53:3–4

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

Cooley J, Ford T, Christou P (1995) Molecular and genetic characterization of elite transgenic rice plants produced by electric discharge particle acceleration. Theor Appl Genet 90:97–104

Finnegan J, McElroy D (1994) Transgene inactivation: plants fight back! Bio/Technol 12:883–888

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific gene duplication. Proc Natl Acad Sci USA 91:3490–3496

Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progenies of transgenic maize plants. Bio/Technol 8: 833–839

Goto F, Toki S, Uchimiya H (1993) Inheritance of a co-transferred foreign gene in the progenies of transgenic rice plants. Transgenic Res 2:300–305

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

Lassner NW, Peterson P, Yoder JI (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol Biol Rep 7:116–128

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 Jour 5: 285–297

Register III JC, Peterson DJ, Bell PJ, Bullock WP, Evans BF, Greenland AJ, Higgs NS, Jepson I, Jiao S, Lewnau CJ, Sillick JM, Wilson HM (1994) Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol Biol 25:951–961

Spencer TM, O'Brien JV, Start WG, Adams TR, Gordan-Kamm WJ, Lemaux PG (1992) Segregation of transgenes in maize. Plant Mol Biol 18:201–210

Vasil IK (1994) Molecular improvement of cereals. Plant Mol Biol 25: 925–937

Vasil V, Castillo AM, Fromm ME, Vasil I K (1992) Herbicide-resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Biol/Technol 10:667–674

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/Technol 11: 1553–1558

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