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Segregation of transgenes in maize

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Abstract

Progeny recovered from backcrossed transgenic maize tissue culture regenerants (R_0) were analyzed to determine the segregation, expression, and stability of the introduced genes. Transgenic A188 × B73 R_0 plants (regenerated from embryogenic suspension culture cells transformed by microprojectile bombardment; see [9]) were pollinated with nontransformed B73 pollen. Inheritance of a selectable marker gene, *bar*, and a nonselectable marker gene, *uidA*, was analyzed in progeny (R_1) representing four independent transformation events. Activity of the *bar* gene product, phosphinothricin acetyltransferase (PAT), was assessed in plants comprising the four R_1 populations. The number of R_1 plants containing PAT activity per total number of R_1 plants recovered for each population was 2/7, 19/34, 3/14 and 73/73. Molecular analysis confirmed the segregation of *bar* in three R_1 populations and the lack of segregation in one R_1 population. Cosegregation analysis indicated genetic linkage of *bar* and *uidA* in all four R_1 populations. Analysis of numerous R_2 plants derived from crossing transformed R_1 plants with nontransformed inbreds revealed 1:1 segregation of PAT activity in three of four lines, including the line that failed to segregate in the R_1 generation. Integrated copies of *bar* in one line appeared to be unstable or poorly transmitted.

Introduction

Major progress has been made in overcoming the recalcitrance of agronomically important monocots to genetic transformation. Rice yielded to transformation via protoplast-based, direct DNA uptake systems, ultimately giving rise to fertile transgenic japonica [17] and indica [4] rice plants. Recovery of fertile transgenic maize plants [9, 8] was recently accomplished using microprojectile bombardment, a direct DNA delivery system not requiring protoplast isolation [13]. While this progress is encouraging, very little is known about the integration and expression, and ultimately, the inheritance and stability of genes introduced into important monocots.

Fertile transgenic plants have been recovered from numerous dicot species using *Agrobacterium*-mediated transformation. *Agrobacterium* transformation of dicots usually results in a low number (average of 3 copies) of T-DNA insertions into the host plant genome. T-DNA insertions appear to be site-independent and are often unlinked (for review, see [22]). Inheritance

of introduced genes is usually Mendelian [7, 1, 5, 11]. Genes introduced into dicots via *Agrobacterium* appear to be very stable; however, low frequencies of instability have been observed [16, 3].

Inheritance of genes introduced via direct DNA delivery systems, particularly microprojectile bombardment, has not been as well characterized. Genes on gold particles accelerated into soybean meristems resulted in chimeric regenerants containing expressing and nonexpressing sectors; germ-line transformation was observed at low frequencies [15]. Analysis of subsequent generations of two lines of transgenic soybean showed that all copies of foreign genes were inherited in Mendelian fashion as single units [2]. Transgenic tobacco lines recovered from bombarded leaf discs exhibited both Mendelian and non-Mendelian segregation for introduced genes [21]. Here, we describe detailed analysis of inheritance, expression, and stability of genes introduced into cultured embryogenic maize cells by microprojectile bombardment.

Materials and methods

Plant care and backcrossing

Tissue culture regenerants (R_0) were grown to maturity in a greenhouse in 16 liter pots containing modified Promix BX (Premier Brands, Stamford, CT). Plants received a 14 h photoperiod of natural and supplemental light (high-pressure sodium lamps, 250 $\mu E\ m^{-2}\ s^{-1}$). R_0 ears were pollinated with pollen from nontransformed, seed-derived B73 plants. Kernels that developed on R_0 ears were removed 30–40 days after pollination and planted in 0.5 liter pots. R_1 plants were grown to the 1–2 leaf stage in a growth chamber (450 $\mu E\ m^{-2}\ s^{-1}$; 14 h photoperiod) and subsequently transferred to 16 liter pots and grown to maturity in the greenhouse. Progeny (R_1) were crossed, as both the male and female parent, to several nontransformed inbred lines. Kernels recovered as a result of R_1 crosses matured on the plants for 40–45 days, were removed from the ears, and handled as described above.

Enzyme activity determination

Phosphinothricin acetyltransferase (PAT) activity in R_1 plants was assessed by local application of a 2% (v/v) Basta (Hoechst, FRG) solution to leaves as described [9]. The Basta formulation used, Basta TX, contains 200 g/l glufosinate (ammonium salt of phosphinothricin). PAT activity in R_2 plants was assessed by spraying plants at approximately the three-leaf stage with a 1% (v/v) Basta solution (manufacturer's recommended application rate is 0.5–1%) containing 0.1% (v/v) Triton. Herbicide was applied as a fine mist using a Spra-tool aerosol dispenser (Crown Industrial Products Co., Hebron, IL) until all leaf surfaces were wet. Plants were evaluated for resistance to the herbicide one week after spraying.

Southern blot analysis

Genomic DNA isolation from leaf tissue and Southern blot analyses were performed as described [9]. Approximately 5 μg genomic DNA was used per digest. The 0.6 kb *Sma* I fragment from pDPG165 [9] containing *bar* and the 1.8 kb *Bam* HI/*Eco* RI fragment from pCEV5 [18] containing *uidA* were used in random priming reactions ([6], Boehringer Mannheim) to generate probes for detecting PAT- and GUS-coding sequences, respectively.

Results and discussion

In a previous report [9] we described regeneration of 219 plants (R_0) from transformed callus lines recovered from transformation experiments utilizing an embryogenic A188 × B73 maize suspension culture designated SC716. In these experiments, SC716 suspension culture cells were bombarded with tungsten particles coated with two plasmids, one containing *bar* (pDPG165) and the other containing *uidA* (pDPG208). The plasmids pDPG165 (35S-*bar*-Tr7 3′ end) and pDPG208 (35S-*adh1* intron-GUS-*nos* 3′ end)

have been described [9]. Transformed callus lines were selected using the herbicide bialaphos. Transformed SC716 callus lines, previously designated R1-R21 [9], will here be referred to as T1-T21 to avoid confusion with designations for plant generations (e.g. R_0, R_1, and R_2). Plants were regenerated from nine independent SC716 transformants. Two of the ten independent SC716 transformants described by Gordon-Kamm *et al.* [9] were found to be identical upon further Southern analysis (T6, T11; data not shown).

Analysis of R_0 plants

The hybridization patterns of *bar* sequences in SC716 R_0 plants and callus were analyzed to determine if the sequences were stably maintained during plant regeneration and to confirm the clonal origin of the individual callus lines. Southern blot analyses of genomic DNA from each of the nine regenerable callus lines, and two R_0 plants derived from each callus line, are shown in Fig. 1; an additional 18 plants (2–6 plants/callus line) were analyzed for a total of 36 plants (data not shown). Genomic DNA was digested with *Eco* RI and *Hin*d III, which release the 1.9 kb *bar* expression unit (35S promoter-*bar*-Tr7 3′ end) from pDPG165 [9]. Hybridization patterns for all 36 R_0 plants were identical to their corresponding callus lines with the exception of one of the two T5 R_0 plants analyzed (Fig. 1). It is possible that callus line T5 arose from more than one transformed cell and the dissimilar hybridization patterns of the two R_0 plants represent independent transformation events. Or, if callus line T5 originated from a single-cell transformation event, the T5 R_0 plant that exhibited a different hybridization pattern may have resulted from cells that lost some copies of the integrated plasmid DNA.

While only 36 of 219 R_0 plants were analyzed for the presence of *bar*, these 36 plants were randomly selected and all but one exhibited *bar* hybridization profiles identical to that of corresponding callus. These comparisons of hybridization patterns from callus and R_0 plants, as well as similar analyses of four independent transformation events described previously [9], provide strong evidence for lack of genetic chimerism in bialaphos-selected callus lines.

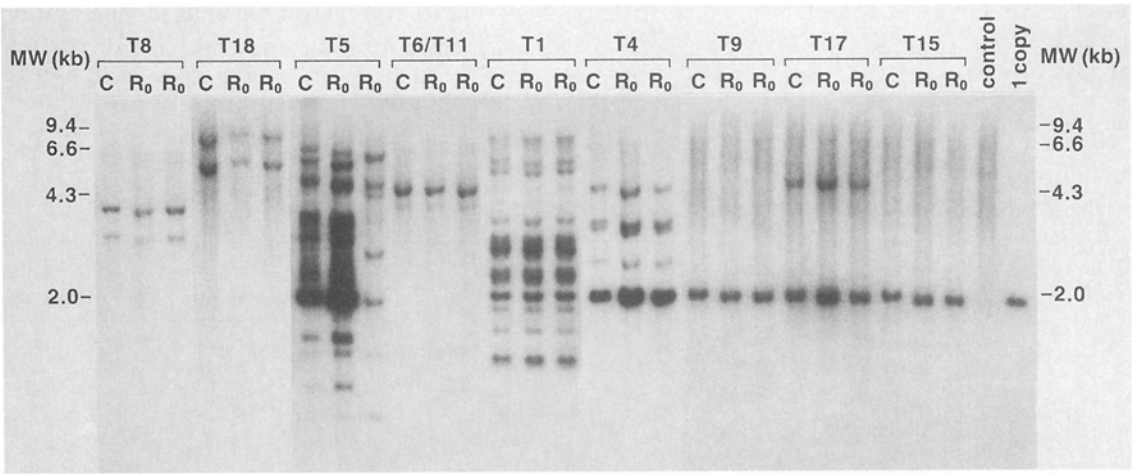

Fig. 1. Southern blot analyses of *bar* in callus (C) and corresponding primary regenerants (R_0). Genomic DNA from the nine regenerable callus lines (T8, T18, T5, T6/T11, T1, T4, T9, T17, and T15) and two plants regenerated from each callus line was digested with *Eco* RI and *Hin*d III and probed with *bar*. Control DNA was from a nontransformed A188 × B73 tissue culture regenerant. Lane designated 1 copy contained 2.3 pg of the 1.9 kb *Eco* RI/*Hin*d III *bar* expression unit from pDPG165, representing one copy per diploid genome. Molecular weights (MW) of standards are shown in kilobases (kb).

Analysis of R_1 plants

As described previously [9], SC716 R_0 plants were backcrossed to nontransformed seed-derived B73 plants. Although pollen capable of germinating *in vitro* was recovered at a low frequency from SC716 R_0 plants [9], no R_1 plants were recovered using this pollen in backcrosses. Ears that developed on SC716 R_0 plants were pollinated with B73 pollen and viable R_1 plants were recovered from R_0 plants representing five independent, transgenic callus lines: T8, T18, T6/T11, T1 and T9 (Table 1). Fertility of R_0 plants was sporadic; progeny were recovered from only 35 of 219 R_0 plants (Table 1). Only a few kernels developed on each fertile R_0 plant; therefore, for analysis, R_1 plants were grouped into populations representing several R_0 plants and the transformed callus lines from which they were derived. R_1 plants were assayed for expression of *bar* by local foliar application of the herbicide Basta. We previously demonstrated that this is a reliable and sensitive method for detection of PAT activity in transgenic maize plants [9]. Segregation of PAT activity was observed in three R_1 populations: T6/T11, T8, and T18 (Table 1). No segregation was observed in the T9 R_1 population; all plants tested were tolerant to Basta application (Table 1). The single T1 R_1 plant was sensitive to Basta application (Table 1).

All of the SC716 callus lines that yielded PAT-positive R_1 plants (T8, T18, T6/T11, and T9) were shown previously to have integrated *bar* and *uidA*. These four callus lines all expressed *bar* but lacked GUS activity [9]. Southern blot analyses were performed on R_1 plants derived from each callus line in order to confirm the presence of *bar* and *uidA* and to study segregation patterns and stability following meiosis. Southern blot analyses of *Eco* RI/*Hin*d III-digested genomic DNA from eight T8 R_1 plants showed that the two *bar*-hybridizing fragments detected in T8 R_0 plants segregated together in the R_1 generation (Fig. 2A), indicating that all *bar*-hybridizing sequences were likely integrated on the same chromosome. As expected, PAT activity was detected only in R_1 plants containing *bar*-hybridizing sequence (Fig. 2A). To determine the segregation of *uidA* in T8 R_1 plants, *bar* probe was removed from the filter shown in Fig. 2A, and the filter was reprobed with *uidA*. The restriction enzymes used (*Eco* RI and *Hin*d III) release a 2.1 kb fragment from pDPG208 containing *uidA* and the *nos* 3′ end [9]. GUS-coding sequence cosegregated with *bar* in the T8 R_1 plants (Figs. 2A and B); all T8 R_1 plants that contained *bar*, also contained *uidA*-hybridizing sequence. However, not all *uidA*-hybridizing fragments detected in T8 R_0 plants were present in T8 R_1 plants (Fig. 2B). This may be due to segregation of multiple integrations of *uidA* on more than one chromosome or may be due to the loss of some *uidA* sequence. The lat-

Table 1. Segregation of PAT activity in R_1 populations recovered from backcrossed SC716 R_0 plants.

Calllus line	R_0 plants	R_0 plants yielding viable progeny	Viable progeny recovered	PAT-positive progeny	PAT-negative progeny
T8	24	5	14	3	11
T18	17	6	34*	19	15
T5	3	0	0	–	–
T6/T11	94	4	7	2	5
T1	14	1	1	0	1
T4	2	0	0	–	–
T9	55	19	73	73	0
T17	10	0	0	–	–
T15	10	0	0	–	–

* Does not includes three chlorophyll mutants.

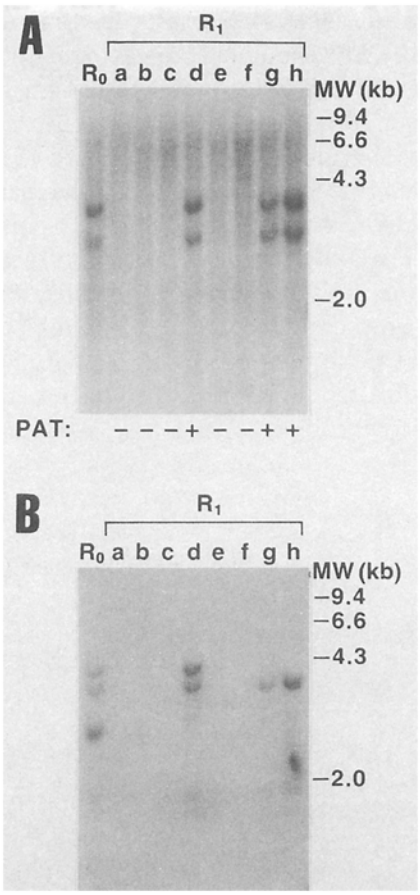

Fig. 2. Southern blot analyses of progeny (R_1) recovered from T8 R_0 plants. A. Genomic DNA from a T8 primary regenerant (R_0) and eight progeny (R_1) was digested with *Eco* RI and *Hin*d III and probed with *bar*. R_1 plants a–d and e–h were recovered from separate R_0 plants. Presence (±) or absence (−) of PAT activity is indicated for each R_1 plant. B. The blot shown in A was stripped and reprobed with *uidA*.

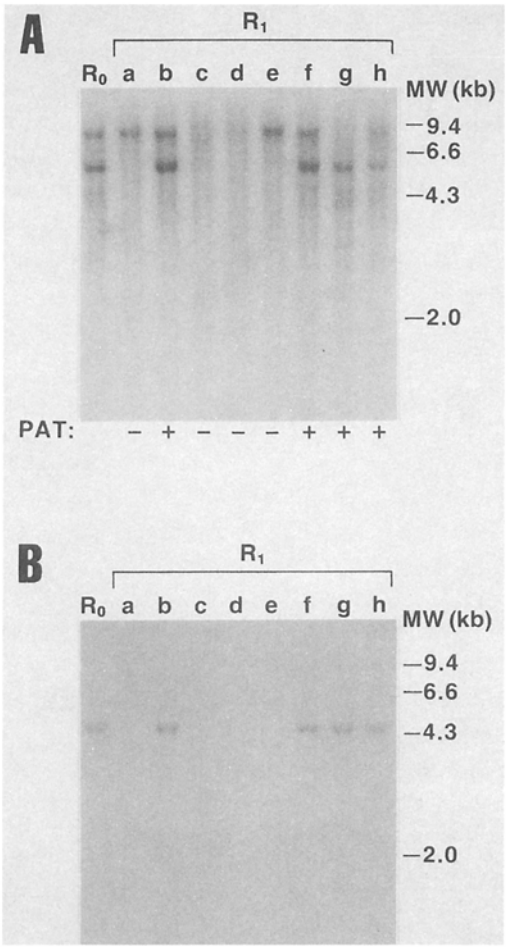

Fig. 3. Southern blot analyses of progeny (R_1) recovered from T18 R_0 plants. A. Genomic DNA from a T18 primary regenerant (R_0) and eight progeny (R_1) was digested with *Eco* RI and *Hin*d III and probed with *bar*. R_1 plants a, b and c–h were recovered from separate R_0 plants. Presence (±) or absence (−) of PAT activity is indicated for each R_1 plant. B. The blot shown in A was stripped and reprobed with *uidA*.

ter seems more likely, given the fact that no hybridization to *uidA* was observed in plants that lacked *bar* (Figs. 2A and B).

Similar analyses of T18 R_1 plants showed that the two *bar*-hybridizing fragments present in *Eco* RI/*Hin*d III-digested R_0 DNA segregated independently in the R_1 generation (Fig. 3A). T18 R_1 plants contained one, both or neither of the *bar*-hybridizing fragments present in T18 R_0 DNA. One of the fragments (ca. 6 kb) cosegregated with PAT activity (Fig. 3A), indicating that this fragment contained a functional copy of *bar* while the larger-molecular-weight fragment (ca. 9 kb) was apparently nonfunctional. Reprobing the filter shown in Fig. 3A with *uidA* showed that the single *uidA*-hybridizing fragment present in T18 R_0 DNA cosegregated with the functional copy of *bar* (Fig. 3A and B).

Genomic DNA from the seven T6/T11 R_1 plants was digested with *Eco* RI and *Hin*d III and analyzed by Southern blot hybridization using *bar* probe. Hybridization to high-molecular-weight (4–5 kb) fragments was observed for two T6/T11

R_1 plants, both of which had PAT activity (Fig. 4A). Segregation of the high-molecular-weight fragments was independent of the segregation of a smaller (ca. 1 kb), apparently non-functional *bar*-hybridizing fragment. Reprobing the filter shown in Fig. 4A with *uidA* probe revealed cosegregation of all *uidA* fragments present in T6/T11 R_0 DNA with the functional copy or copies of *bar* (Fig. 4A and B).

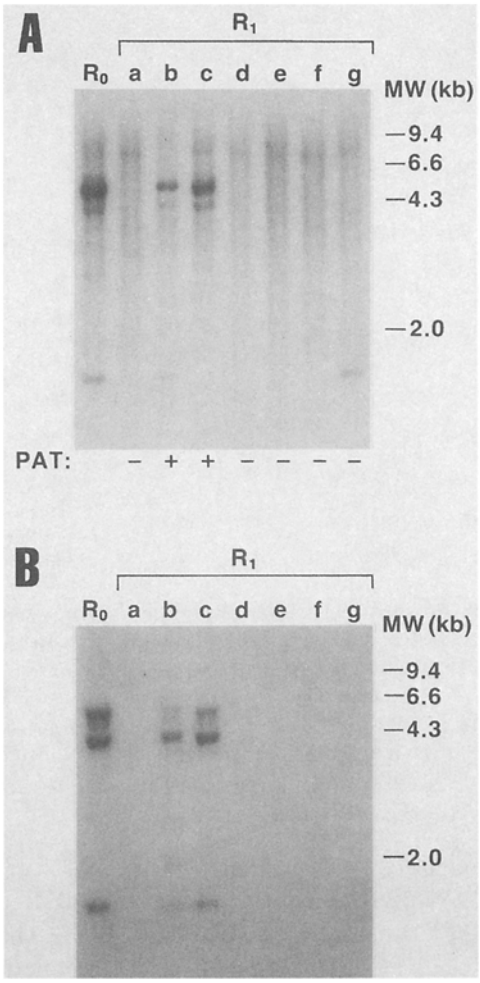

Fig. 4. Southern blot analyses of progeny (R_1) recovered from T6/T11 R_0 plants. A. Genomic DNA from a T6/T11 primary regenerant (R_0) and seven progeny (R_1) was digested with *Eco* RI and *Hin*d III and probed with *bar*. R_1 plants a–c, d, e, f and g were recovered from four separate R_0 plants. Presence (±) or absence (−) of PAT activity is indicated for each R_1 plant. B. The blot shown in A was stripped and reprobed with *uidA*.

Southern blot analyses of *Eco* RI/*Hin*d III-digested T9 R_1 genomic DNA demonstrated that the intact 1.9 kb *bar* expression unit fragment(s) present in T9 R_0 plants did not segregate and was inherited by all T9 R_1 plants tested (Fig. 5A). This is consistent with the lack of segregation for PAT activity observed in the T9 R_1 population (Table 1). To determine if the lack of *bar* segregation was a result of two or more integrations on homologous chromosomes, Southern blot analyses were performed on T9 R_0 and R_1 genomic DNA digested with either *Eco* RI or *Hin*d III;

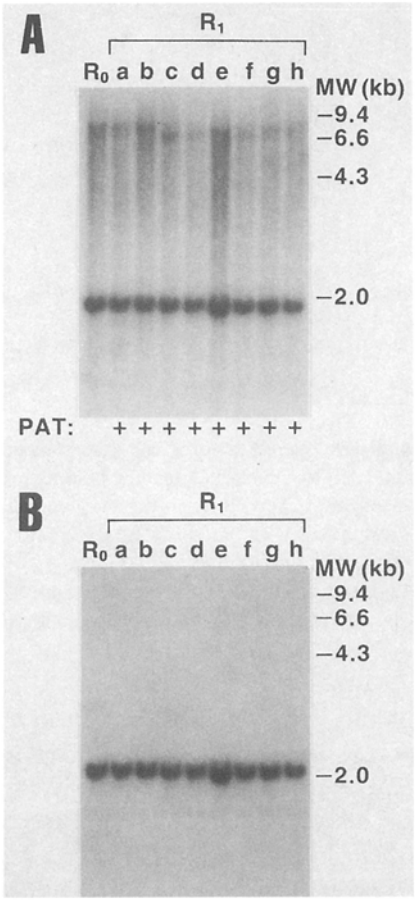

Fig. 5. Southern blot analyses of progeny (R_1) recovered from T9 R_0 plants. A. Genomic DNA from a T9 primary regenerant (R_0) and eight progeny (R_1) was digested with *Eco* RI and *Hin*d III and probed with *bar*. R_1 plants a–d and e–h were recovered from separate R_0 plants. Presence (±) or absence (−) of PAT activity is indicated for each R_1 plant. B. The blot shown in A was stripped and reprobed with *uidA*.

both of these enzymes have unique sites on pDPG165 [9]. If multiple integrations had occurred, two or more fragments of varying size would be expected in T9 R_0 DNA, and these fragments would be expected to segregate in T9 R_1 plants. Only one fragment was observed for R_0 and R_1 plants using either *Eco* RI or *Hin*d III (data not shown). Lack of segregation was also observed for *uidA* in T9 R_1 plants; reprobing the filter shown in Fig. 5A with *uidA* probe demonstrated the inheritance of a 2.1 kb *uidA*-hybridizing fragment by all T9 R_1 plants (Fig. 5B).

Although the incidence of fertility of backcrossed R_0 plants was low (Table 1), generalizations about the inheritance of the introduced genes can be made from the analysis of R_1 plants. Three fertile lines, T8, T18, and T6/T11, segregated for PAT activity and integrated plasmid DNA in the R_1 generation. However, the small number of T8, T18, and T6/T11 R_1 plants recovered precludes definitive conclusions about the modes of inheritance. Given the evidence that the functional copy or copies of *bar* segregate as a single unit in each of these lines, a 1:1 segregation for PAT activity would be expected in crosses with nontransformed plants, if sufficient numbers of progeny were recovered. In the T9 R_1 population, lack of segregation for PAT activity, *bar*, and *uidA*, as well as the evidence for a single *bar* integration event, suggest that either the transformation was nonnuclear (maternally inherited) or a single nuclear integration event was replicated in T9 callus yielding T9 R_0 plants that were homozygous for the introduced DNA.

Molecular analysis of the inheritance of foreign DNA in these four transgenic lines (T8, T18, T6/T11 and T9) was somewhat simplified by the fact that all four lines were the result of relatively low-copy-number transformations. Progeny expressing introduced plasmid DNA were not recovered from transgenic lines containing more than 5–6 copies of integrated plasmid DNA.

Analysis of R_2 plants

To further elucidate the modes of inheritance of introduced DNA in the transgenic lines, PAT-positive T8, T9, T6/T11, and T18 R_1 plants were crossed to nontransformed inbred plants. The fertility of R_1 plants was substantially improved over that of R_0 plants; viable R_2 plants were recovered from all four lines using PAT-positive R_1 plants as either the female or male parent. Seedset as the result of R_1 crosses, regardless of the direction of the cross, was usually normal. In a previous report [10], we described dramatic improvements in the fertility of transgenic maize lines after one or two backcrosses. We do not, as yet, have any evidence to suggest that integration or expression of foreign genes affects fertility or plant vigor.

Although callus lines T8, T9, T6/T11, and T18 all lacked GUS activity [9], molecular analyses of R_1 plants demonstrated that *uidA* was present and apparently linked to functional *bar* in all four lines (Figs. 2, 3, 4, 5). R_2 plants were assayed fluorometrically [12] for GUS activity to determine if expression was differentially regulated in callus and plants. Several R_2 plants from each line were tested; all were negative (data not shown).

PAT activity in R_2 plants was determined by spraying seedlings at the 2–3 leaf stage with a 1% (v/v) Basta solution. For each line, numerous R_2 progeny of single PAT-positive R_1 plants were

Fig. 6. Herbicide application to T18 R_2 plants segregating for *bar*. The plant on the left was sprayed with water and four plants on the right were sprayed with a 1% (v/v) Basta solution. The photograph was taken seven days after herbicide application.

Table 2. Analysis of PAT activity segregation in R_2 plants derived from crosses of transgenic R_1 plants with nontransformed inbred plants.

R_1 parent	Cross	R_2 plants sprayed with Basta		Expected ratio	χ^{2a}	P^b
		Resistant	Sensitive			
T8	male	0	36	1:1	34.02	<0.001
T8	female	2	34	1:1	26.69	<0.001
T9	male	15	15	1:1	0.03	0.70
T9	female	13	17	1:1	0.30	0.50
T6/T11	male	24	30	1:1	0.46	0.50
T6/T11	female	31	23	1:1	0.91	0.30
T18	male	31	29	1:1	0.02	0.90
T18	female	33	27	1:1	0.42	0.50

[a] χ^2 values calculated using the Yates correction term as described by Strickberger [20].
[b] $P = \chi^2$ probability with 1 d.f.

analyzed, half derived from a backcross using the R_1 as the female, and half using the R_1 as the male. PAT activity was easily assessed; sensitive plants began to appear necrotic two to three days after herbicide application and were completely necrotic after one week, while most resistant plants were indistinguishable from water-sprayed controls (Fig. 6). Occasionally, small lesions appeared on leaf tips or at the base of the whorl of resistant plants, however plant vigor was unaffected. Applications of up to 21% (v/v) Basta were tested on T18 R_2 plants. Some small lesions and leaf cupping were occasionally observed 1–2 weeks after application on resistant plants, but subsequent growth and fertility were unaffected (data not shown).

As assayed by Basta application, PAT activity segregated 1:1 in the R_2 generations derived from individual transgenic T9, T6/T11, and T18 R_1 plants (Table 2). Recovery of Basta-resistant T8 R_2 plants was sporadic (Table 2). Results from additional Basta applications to R_2 plants recovered from several transgenic R_1 plants from each line were consistent with data presented in Table 2 (data not shown). One-to-one segregation of functional *bar* in T18 and T6/T11 R_2 plants is consistent with molecular and enzyme activity analyses of R_1 populations (Figs. 3 and 4). The reason for the low frequency of PAT-positive T8 R_2 plants was investigated by performing Southern analyses on twelve T8 R_2 plants. The twelve R_2 plants, including one PAT-positive plant, were all derived from a single PAT-positive T8 R_1 plant. If gene inactivation were responsible for the low frequency of PAT-positive T8 R_2 plants, some of the PAT-negative T8 R_2 plants would be expected to contain *bar*. Southern analyses revealed the presence of *bar*-hybridizing fragments in only the single PAT-positive R_2 plant (data not shown). These data indicated that loss of the gene, or poor transmission of the gene was responsible for the aberrant segregation ratio of T8 R_2 plants (Table 2). Given the molecular evidence for the loss of some *uidA* sequence in T8 R_1 plants (Fig. 2B), instability of the T8 integration event seems likely. It is also possible that the T8 integration event was linked to a deleterious chromosome abnormality such as a small duplication or deletion and therefore transmitted at a low frequency. Segregation of PAT activity in T9 R_2 plants (Table 2), coupled with the molecular evidence for a single integration event, supports the hypothesis that a single integration event was replicated in T9 callus, yielding T9 R_0 plants that were homozygous for *bar* and *uidA*. This finding also rules out the possibility of a nonnuclear transformation event. Possible mechanisms responsible for this homozygosity include mitotic recombination [19] and gene conversion [14]. Gene conversion is a particularly attractive ex-

planation, given the possibility that integration of plasmid DNA and the replication event may have been concomitant, both the result of the same nick in chromosomal DNA.

Conclusions

Data in this report demonstrate Mendelian inheritance of a functional gene introduced into maize by microprojectile bombardment. Functional *bar* sequence(s) segregated as single units in all four transgenic lines analyzed; however, integrated copies of *bar* appeared to be unstable or poorly transmitted in one line. Independent segregation of apparently nonfunctional copies of *bar* was also observed. GUS-coding sequence, introduced simultaneously on a separate plasmid, was inherited by all four lines and appeared to be linked to functional *bar* in all cases. However, expression of *uidA* was not detected in any of the lines described here. These lines were recovered from experiments using separate plasmids encoding the selectable and nonselectable markers. Coexpression frequencies in these experiments, analyzed at the callus level, were approximately 20% [9]. Currently, we are improving coexpression frequency by bombarding with single plasmids encoding both a selectable and a nonselectable marker.

Using microprojectile bombardment, we can now study the processes involved in heterologous gene function in maize. An increased understanding of the mechanisms of gene integration, as well as the effects of gene position and copy number on expression, should facilitate significant genetic manipulation of maize in the future.

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