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Variation in the inheritance of expression among subclones for unselected (*uidA*) and selected (*bar*) transgenes in maize (*Zea mays* L.)

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Abstract Variation in the inheritance of expression among subclones for an unselected (*uidA*) and a selected (*bar*) transgene was analyzed in two individual transformation events in maize. The unselectable gene (*uidA*) and the selectable gene (*bar*), on two separate plasmids, were transferred to maize (Hi-II derivative) by particle bombardment of embryogenic calli or suspension cells. A total of 188 fertile T1 plants were obtained from one transformant (transformation event BG which integrated *uidA* and *bar*). A total of 98 fertile T1 plants were obtained from a second transformant (transformation event B which integrated *bar*). Through self-pollination and/or cross-pollination in the greenhouse, approximately 10 000 T2 progeny were obtained from event BG, and more than 1000 T2 progeny were obtained from event B. Segregation of transgene expression was analyzed statistically in a total of 2350 T2 progeny from 40 T1 subclones of event BG and in 217 T2 progeny from six T1 subclones from event B. Variation in the inheritance of expression among subclones for the two transgenes (*uidA* and *bar*) was observed in the two transformants. A significant difference was observed between the use of the female or male as the transgenic parent in the inheritance of expression for the two transgenes in event BG. No inheritance through the pollen was observed in two of four T1 subclones analyzed in event B. Co-expression analysis of event BG showed that both transgenes were co-expressed in 67.7% of the T2 plants which expressed at least one of the two transgenes. Of the T2 expressing plants, 30.4% expressed only *bar*, and 1.9% expressed only *uidA*. Inactivation of the unselected (*uidA*) and the selected (*bar*) transgenes was observed in individual T2 plants.

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Introduction

The stability of transgene expression in plants has become a major issue, because the stable expression of transgenes is essential for successful plant molecular breeding by genetic engineering technology. Inactivation of transgene expression has been extensively studied in dicots transformed by *Agrobacterium tumefaciens*, and several factors which may affect the stability of transgene expression have been investigated, including copy number, insertion site, co-suppression, and gene methylation (reviewed by Finnegan and McElory 1994). Inactivation of a transgene in transgenic plants transformed by direct gene transfer was first observed in transgenic tobacco (Potrykus et al. 1985). Recently, inactivation of transgenes has been observed in maize (Register III et al. 1994) and in barley (Wan and Lemaux 1994) transformed by particle bombardment, as well as in rice (Schuh et al. 1993) transformed by electroporation of protoplasts. These results indicate that transgene inactivation may potentially be a problem affecting the application of plant genetic transformation.

Maize is a cross-pollinated crop. However, both self-pollination and cross-pollination are required for the production of inbred lines and hybrids necessary to achieve high productivity. If genetic transformation of maize is to become a successful component of cultivar development, it will be essential to understand the stability of expression of unselected and selected transgenes through self-pollination and cross-pollination. A high level of stability of expression of unselected transgenes is desirable, especially because convenient selection protocols for many agronomically useful genes are not yet available.

Several selectable genes, including the phosphinothricin acetyltransferase (PAT) gene (*bar*), the neomycin phosphotransferase gene (*nptII*), the hygromycin phosphotransferase gene (*hph*), the dihydrofolate reductase (DHFR) gene, and the chlorsulfuron-resistant (ALS) gene have been introduced into maize; and Mendelian segregations of their expression were observed [Fromm et al. 1990 (ALS); Gordon-Kamm et al. 1990 (*bar*); Walter et al. 1992 (*hph*); Spencer et al. 1992 (*bar*); Golovkin et al. 1993 (DHFR); Koziel et al. 1993 (*bar*); Murry et al. 1993 (*npt II*); Register III et al. 1994 (*bar*); Frame et al. 1995 (*bar*); Laursen et al. 1994 (*bar*)]. However, non-Mendelian segregation of transgene expression in progeny was also reported in one of four transformation events for *bar* (Spencer et al. 1992), in two of three events for *hph* (Walters et al. 1992), and in 2 of 45 events for *npt II* (D'Halluin et al. 1992). In a recent report on transgenic maize, segregation of expression of a selected transgene (*bar*) usually followed Mendelian ratios when the gene was inserted at a single locus in the genome of T1 plants which were regenerated from transformed callus (Register III et al. 1994).

Several non-selectable foreign genes, including the β-glucuronidase gene (*uidA*) (Gordon-Kamm et al. 1990; Spencer et al. 1992; Walter et al. 1992; Koziel et al. 1993; Register III et al. 1994), the *Bacillus thuringiensis* (*b.t.*) insecticidal protein gene (Koziel et al. 1993), the firefly luciferase (Luc) gene (Fromm et al. 1990), and the maize dwarf mosaic virus strain B (MDMV-B) coat protein (cp) gene (Murry et al. 1993) have been transferred to maize. In some cases, the unselected transgene (*uidA*) was not expressed (Spencer et al. 1992; Walters et al. 1992). In other cases, unselected transgenes were expressed in T1 plants, but the segregation of their expression in progeny was not shown (Fromm et al. 1990; Gordon-Kamm et al. 1990; Koziel et al. 1993; Murry et al. 1993).

Variation in the inheritance of selected transgene expression among different transformation events was reported in maize (Spencer et al. 1992; Walters et al. 1992; Register III et al. 1994). Understanding the variation in the inheritance of transgene expression among subclones (plants regenerated from a single transgenic event, Potrykus et al. 1985) is valuable for molecular breeding and for studies on the stability of transgene expression. In tobacco transformed by direct gene transfer, this type of data has been extensively analyzed (Potrykus et al. 1985). Variation in the inheritance of transgene exprression among subclones for selected and unselected transgenes through self-pollination and cross-pollination has not been extensively analyzed in transgenic maize.

Here we report the analysis of the variation in the inheritance of transgene expression among subclones for an unselectable (*uidA*) and a selectable (*bar*) transgene in two individual transformants of maize. Variations in the segregation of expression for the two transgenes among T1 subclones were investigated in both transformants. The use of female or male as transgenic parent was compared in the inheritance of expression of both transgenes in event BG. Co-expression of the two transgenes was analyzed in T2 progeny of event BG. Inactivation of unselected (*uidA*) and selected (*bar*) transgenes was examined in individual T2 plants. Our result provide additional data that may help researchers to understand the stability of transgene expression in maize.

Materials and methods

Regeneration of transgenic T1 plants with unselectable (*uidA*) and selectable (*bar*) transgenes

Embryogenic callus was initiated from immature embryos of a maize Hi-II derivative (Zhong et al. 1996). The embryogenic calli were bombarded by particle gun bombardment (He/1000, Bio-Rad) with two plasmids in a 1:1 ratio for co-transformation of an unselectable gene (*uidA*) which codes for β-glucuronidase (GUS) (Jefferson et al. 1987) and a selectable gene (*bar*) which codes for phosphinothricin acetyltransferase (PAT) (Thompson et al. 1987). The plasmid p*Act1*-F (McElroy et al. 1990) contained the unselectable gene (*uidA*, 1.87 kb) under the control of the rice actin-1 gene (*act1*) promoter (*act1* 5′, 1.44 kb) and *nos* terminator (*nos* T, 0.26 kb) in pBluescript KS (2.9 kb) (Fig. 1 A). The plasmid pTW-a (Zhong et al. 1996) contained the selectable gene (*bar*, 0.6 kb) under the control of the CaMV 35S promoter (35*s*, 0.8 kb) and *nos* terminator (*nos* T, 0.3 kb), and the potato proteinase inhibitor-II gene (*pin2*) under its own *pin2* promoter (*pin2* 5′) and own terminator (*pin2* T) (Thornburg et al. 1987) with the *act1* intron (*act1* I) of total size 3.0 kb in pUC 19 (Fig. 1 B). From the embryogenic callus, embryogenic cell suspension cultures were established. The suspension cells were bombarded only with the pTW-a construct.

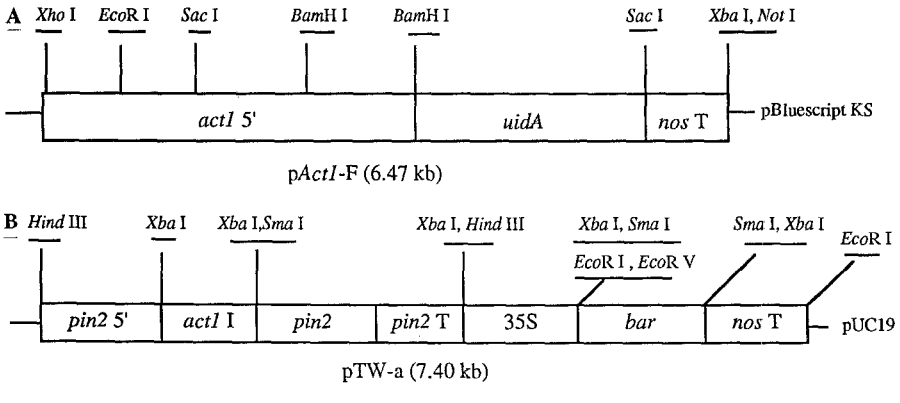

Fig. 1 A,B Schematic diagrams of constructs p*Act1*-F (**A**) and pTW-a (**B**)

Herbicice-resistant colonies were obtained from bombarded embryogenic calli or suspension cells, after selection on 3–5 mg/l of glufosinate ammonium (GA) for approximately 3 months. Resistant callus colonies were transferred to embryogenesis medium (Zhang et al. 1990), and somatic embryos were germinated on MS basal medium (Murashige and Skook 1962) with 2% sucrose. Regenerated plantlets were transferred to MS basal medium supplemented with 5 mg/l of GA in Magenta boxes for 2–4 weeks before transfer to the greenhouse.

Analysis of transgene expression

Expression of the selected transgene (*bar*) was analyzed for PAT activity by in vitro plant selection with 5 mg/l of GA and/or by a foliar spray of 1% (containing 2 g/l GA) of the herbicide Basta AKA (HOE-39866, Hoecht-Roussel Agri-Vet Company, Somerville, N.J.) to greenhouse-grown plants. Histochemical GUS-staining of embryogenic callus, plant roots, leaves, pollen grains, and immature embryos was performed for analysis of the expression of the unselectable gene (*uidA*) (Jefferson 1987).

Genomic DNA analysis of transgenic plants

Leaf genomic DNA samples were purified from greenhouse-grown transgenic plants and untransformed plants. DNA (10 µg per sample) was digested with restriction enzyme(s), separated in a 0.8% agarose gel and transferred (Southern 1975) to a Magna NT nylon transfer membrane (Micron Separations Inc., Westboro, Mass. 01581). To confirm the presence of the transgenes, the blots were hybridized with three ^{32}P-labeled (^{T7} QuickPrime^{TM} kit, Pharmacia Biotech) probes: a *unidA* probe (a 1.87-kb *Bam*HI/*Sac*I fragment containing the *uidA* coding sequence, Fig. 1A), a *bar* probe (a 0.6-kb *Sma*I fragment containing the *bar* coding sequence, Fig. 1B), and a *pin2* probe (a 1.5-kb *Xba*I fragment containing the *pin2* coding sequence and the *pin2* terminator, Fig. 1B).

Production of T2 and T3 progeny

Transgenic T1 plants were self-pollinated, and/or cross-pollinated, with untransformed seed-derived plants (Hi-II, Hi-II derivative; CML67, an inbred line from CIMMTY) in the greenhouse. Transgenic T2 plants were self-pollinated, or cross-pollinated, with transgenic T2 plants or nontransgenic plant of a Hi-II derivative. Immature seeds and mature seeds were individually collected from T1 and T2 plants and tested for segregation of transgene expression. Genomic DNA from leaf samples of T2 plants was analyzed by Southern-blot hybridization (Southern 1975).

Statistical analysis of segregation ratios of transgene expression in T2 and T3 progeny

A chi-square test (Snedecor and Cochran 1967) with one degree of freedom was used for the statistical analysis of the deviation of observed segregation ratios of transgene expression in T2 progeny from the expected ratios. Chi-squares with less than 0.05 probability were considered significant. Comparison of two samples of unequal sizes (Snedecor and Cochran 1967) was used to analyze the difference in the inheritance of transgene expression between using the male or the female as transgenic parent. The percentage of expressing plants in tested T2 plants obtained from each T1 plant was used in the comparison analysis.

Results

Regeneration of T1 transgenic plants with an unselectable gene (*uidA*) and a selectable gene (*bar*)

The unselectable gene (*uidA*) and the selectable gene (*bar*) were transferred to maize by micropojectile bombardment of embryogenic callus or suspension cells. Herbicide-resistant colonies were obtained from bombarded embryogenic calli and suspension cells after approximately 3 months on selection medium with 3–5 mg/l of glufosinate-ammonium (GA). An intensely GUS-staining colony was isolated after histochemical GUS assay of pieces of herbicide-resistant callus colonies which were obtained from bombarded embryogenic callus. Transgenic maize plants (T1) were regenerated in vitro from this herbicide-resistant and positive GUS-staining colony (event BG). A total of 188 fertile plants were obtained in the greenhouse from event BG. A total of 98 fertile transgenic plants (T1) were regenerated from a second herbicide-resistant colony which was obtained from bombarded suspension cells (event B). Southern-blot hybridization was performed on genomic DNA from leaf samples of the transgenic T1 plants from the two transformation events (Fig. 2 A, B, C). The analysis of event BG (Fig. 2 A) showed that the *uidA* probe hybridized to undigested DNA (lanes 9, 12), to a 1.87-kb *Bam*HI/*Sac*I fragment (the same size as the *uidA* coding sequence) (lanes 11, 14), and to two *Hin*dIII (no site in p*Act1*-F) fragments (lanes 10, 13) of genomic DNA from T1 plants. The *bar* probe hybridized to undigested genomic DNA (Fig. 2B, lanes 7, 10), to a 0.9-kb *Eco*RI fragment (the same size as the *bar* coding sequence plus *nos* terminator) (Fig. 2 B, lanes 9, 12), and to two *Eco*RV (unique site in *pAct1*-F) fragments from genomic DNA samples of transgenic T1 plants (Fig. 2 B, lanes 8, 11). Little hybridization background existed in samples of undigested genomic DNA from untransformed plants in *uidA* (Fig. 2 A, lane 6) and *bar* (Fig. 2 B, lane 4) in Southern blots of event BG. The genomic DNA analysis of event B (Fig. 2 C) revealed that *bar* was integrated into maize genomic DNA (lanes 7, 10), hybridization to 0.9-kb *Eco*RI fragments was observed (lanes 8, 11), and two hybridizing bands were shown after digestion with *Eco*RV (lanes 9, 12) of DNA from transgenic T1 plant samples. Integration of *pin2* was shown in both transgenic events, and *Hin*dIII fragments containing the entire *pin2* gene with an *act1* intron (3.0 kb) were observed in Southern blots of genomic DNA from the T1 plants tested (data not shown). No expression of *pin2* has so far been detected in the two transformants.

Expression of unselected (*uidA*) and selected (*bar*) transgenes in T1 plants

Expression of the unselected transgene (*uidA*) was assayed by histochemical GUS staining of roots from 59 T1 plants of event BG. The roots of 52 T1 plants exhibited a high level of GUS expression in elongation zones. Four T1 plants expressed GUS only in the vasculuar tissues of roots. Three T1 plants expressed GUS only in root tips. A high-level of GUS expression was also observed in younger leaves, leaf bases, and shoot and floral meristems of T1 plants (Zhong et al. 1996).

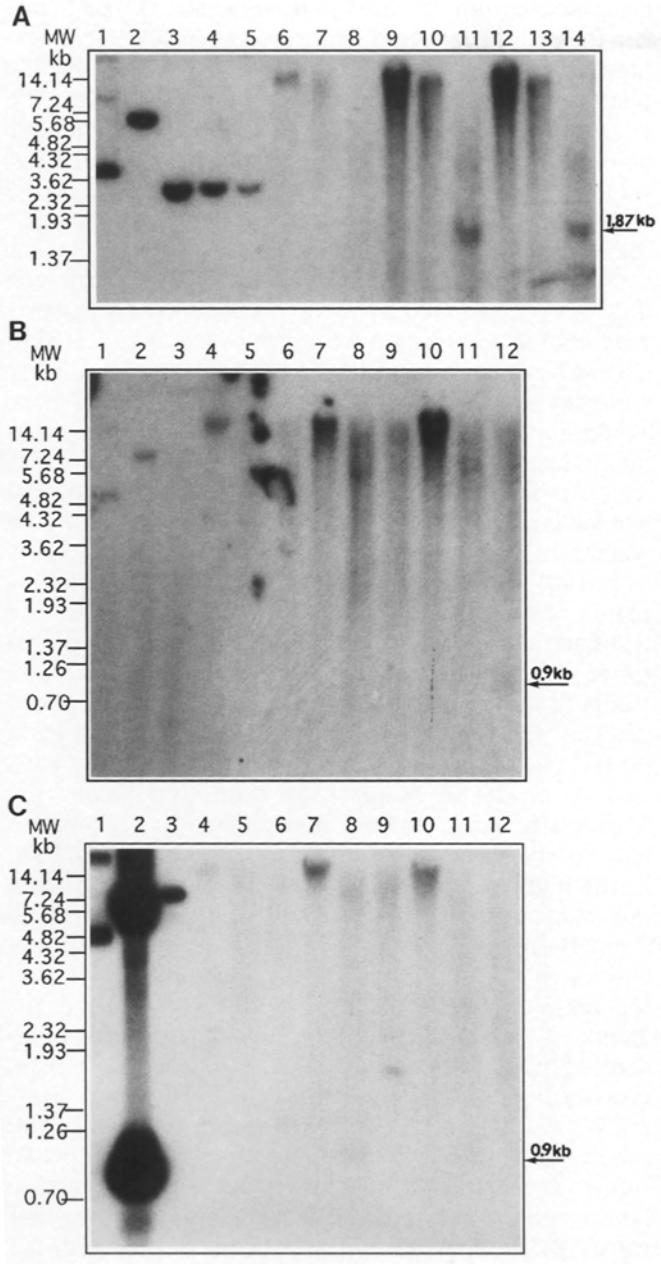

Fig. 2 A–C Genomic DNA analysis of transgenic T1 plants with the two transgenes *uidA* and *bar*. (**A**) Southern blot showing the integration of *uidA* in event BG. *Lanes 1 and 2*, 100 pg of DNA of uncut and *Eco*RI-digested p*Act1*-F; *lanes 3–5*, 100, 50, and 10 pg of *Eco*RI/*Not*I-digested p*Act1*-F; *lanes 6–8*, 10-μg leaf genomic DNA samples of an untransformed plant; *lanes 9–14*, 10-μg leaf genomic DNA samples of two T1 transgenic plants. *Lanes 6, 9, 12*, undigested genomic DNA samples; *lanes 7, 10, 13*, genomic DNA samples digested with *Hin*dIII; *lanes 8, 11, 14*, genomic DNA samples digested with *Bam*HI/*Sac*I. The ^{32}P-labeled *uidA* probe was the 1.87-kb gel-isolated *Bam*HI/*Sac*I fragment of p*Act1*-F containing the *uidA* coding sequence. The *arrow* indicates the 1.87-kb *Bam*HI/*Sac*I fragments of the *uidA* coding region from genomic DNA samples of T1 plants of event BG. (**B**) Southern blot showing the integration of *bar* in event BG. *Lanes 1–3*, 10-pg plasmid DNA samples of pTW-a (undigested, *Eco*RV-digested and *Sma*I-digested); *lanes 4–6*, 10-μg leaf genomic DNA samples of an untransformed plant; *lanes 7–12*, 10-μg leaf genomic DNA samples of two T1 transgenic plants. *Lanes 4, 7, 10*, undigested DNA samples; *lanes 5, 8, 11*, DNA samples digested with *Eco*RV; *lanes 6, 9, 12*, DNA samples digested with *Eco*RI. The ^{32}P-labeled *bar* probe was the 0.6-kb gel-isolated *Sma*I fragment of pTW-a containing the *bar* coding sequence. The *arrow* indicates the 0.9-kb *Eco*RI fragments of the *bar* coding region plus the *nos* terminator from T1 plants of event BG. (**C**) Southern blot showing the integration of *bar* in event B. *Lanes 1–3*, plasmid DNA samples of pTW-a (50-pg undigested, 1-ng *Sma*I-digested, and 50-pg *Eco*RV-digested); *lanes 4–6*, 10-μg leaf genomic DNA samples of an untransformed plant; *lanes 7–12*, 10-μg leaf genomic DNA samples of two T1 transgenic plants. *Lanes 4, 7, 10*, undigested DNA samples; *lanes 5, 8, 11*, DNA samples digested with *Eco*RI; *lanes 6, 9, 12*, DNA samples digested with *Eco*RV. The ^{32}P-labeled *bar* probe was the 0.6-kb gel-isolated *Sma*I fragment of pTW-a containing the *bar* coding sequence. The *arrow* indicates the 0.9-kb *Eco*RI fragments of the *bar* coding region plus the *nos* terminator from T1 plants of event B

Expression of the selected transgene (*bar*) was assayed by in vitro selection of regenerated T1 plants on 5 mg/l of GA and by application of the herbicide Basta AKA (1% foliar spray) to T1 plants in the greenhouse. All the regenerated T1 plants of both events were resistant in vitro to 5 mg/l of GA. Ninety eight greenhouse-grown T1 plants from event BG, 24 greenhouse-grown T1 plants from event B, and 25 untransformed plants were assayed at the 6–8 leaf stage in the greenhouse. All 98 T1 plants from event BG and 24 T1 plants from event B were still alive with no symptoms of herbicide injury 1 week after herbicide application. However, all 25 untransformed plants exhibited foliar necrosis and were completely killed 1 week after herbicide application. These results showed that all T1 plants from both transformation events possessed functional PAT activity.

Variation in the inheritance of transgene expression among subclones of two transformants

Male and female floral initiation of T1 plants occurred after growth for 3 months in the greenhouse. From event BG, a total of 97 T1 plants were self-pollinated, 16 T1 plants were cross-pollinated with transgenic plants as male, and 43 T1 plants were cross-pollinated with transgenic plants as female. From event B, 14 T1 plants were self-pollinated, and 18 T1 plants were used as males in cross-pollinations.

Segregation of expression of the unselected transgene (*uidA*) in event BG was assayed in pollen grains, in immature embryos, and in T2 plant roots. Pollen grains were collected from four individual T1 plants and stained with X-gluc. The positive:negative GUS-staining ratio of pollen grains was 1:1 for all of four T1 plants tested (Table 1). Therefore, only one copy of *uidA* was expressed, or else all expressed copies were integrated in a single locus in these plants.

A total of 482 immature embryos were isolated from eight T1 plants and stained with X-gluc (Fig. 3 A). A total of 341 in vitro T2 plants were produced from the immature embryos germinated in Magenta boxes from eight T1 plants. The roots of these in vitro T2 plants

Table 1 Segregation of *uidA* expression in pollen grains of T1 plants from event BG

Plant	Total	GUS (+)	GUS (−)	P	$\chi^{2\,a}$
BG87	1079	541	538	> 0.95	0.008*
BG88	821	423	398	0.4	0.76*
BG54	898	450	448	> 0.95	0.004*
BG112	522	251	271	0.4	0.76*
BG146	1022	512	510	> 0.95	0.004*

[a] The chi-square test was used with a 0.05 limit of probablity and one degree of freedom
* Fit the expected ratio (1:1)

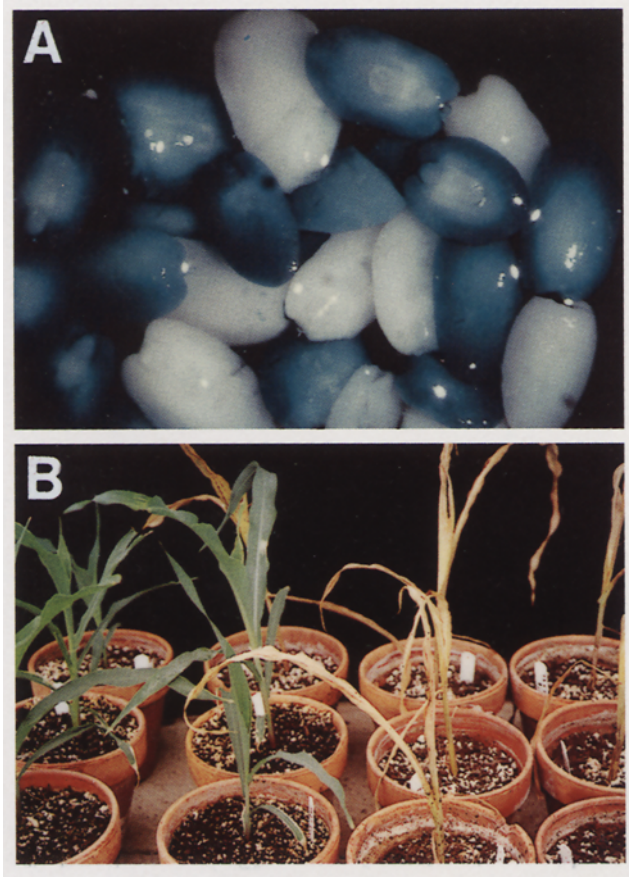

Fig. 3 A,B Segregation of the transgene expression of *uidA* and *bar* in T2 progeny. **A** The immature embryos from a T1 plant after self-pollination were assayed with GUS-staining. **B** The T2 plants obtained from a self-pollinated T1 plant 1 week after spraying with 1% of the herbicide Basta AKA in the greenhouse

were stained with X-gluc. Various segregation ratios of GUS expression were observed from the 16 T1 plants analyzed (Table 2). The expected Mendelian ratios for phenotypic segregation with a single dominant gene at a single locus are 1:1 from cross-pollination and 3:1 from self-pollination. Only 4 of 16 T1 plants followed expected Mendelian ratios. The other 12 T1 plants showed ratios of positive:negative GUS expression that were lower than expected. Our comparison of two types of cross-pollination revealed that transgenic T1 plants as female resulted in significantly greater ($P > 0.90$) inheritance of GUS expression (an average 38.9% of the T2 plants tested) compared to use of the male as transgenic parent (an average 19.5% of the T2 plants tested). Our analysis of self-pollinated progeny revealed that *uidA* was expressed in an average 36.5% of the T2 plants tested, and none of six T1 plants segregated according to the expected Mendelian ratio (3:1).

Segregation of expression of the selected transgene (*bar*) was determined by in vitro selection of T2 plants germinated from immature embryos on 5 mg/l of GA, and/or by herbicide application (1% Basta AKA foliar spray) to greenhouse-grown plants (Fig. 3 B). Transgene expression analysis of T1 plants had shown that either method gave comparable results for *bar*. Seven of sixteen T1 plants from event BG followed the expected Mendelian segregation ratios (Table 3). The female when used as the transgenic parent was significantly ($P > 0.90$) more efficient (an average 47.4% of the T2 plants tested) than when the male was used as the transgenic parent (an average 17.3% of the T2 plants tested) for the inheritance of expression of *bar* (Table 3). Four of seven self-pollinated T1 plants produced progeny that followed the expected segregation ratio (3:1).

Two of six T1 plants from event B followed the Mendelian ratios, and no inheritance through pollen was observed in two of four T1 plants which were used as the male parent (Table 4 A). Cross-pollination with the female as transgenic parent was not carried out with event B. The segregation of expression in T3 progeny was tested with six T2 plants of event B. One (B17-1) had the male as transgenic parent, one (B20-4) had the female as transgenic parent, two were from self-pollinated T2 plants (B17-4 and B20-5), and two were from cross-pollination of two transgenic T2 plant (B17-3 × B20-2, B17-6 × B20-7). The results (Table 4 B) showed that all six T2 plants segregated by Mendelian ratios. One (B17-4) of four B17 T2 plants was a homozygote (all resistant in T3 progeny), and the other three (B17-1, 3, 6) were hemizygotes (segregated 1:1 from cross-pollination, and 3:1 from cross-pollination with two transgenic hemizygotes).

Co-expression analysis of unselected (*uidA*) and selected (*bar*) transgenes in T2 progeny of event BG

In order to analyze the co-expression of the two transgenes (*uidA* and *bar*) in T2 progeny, a portion of roots from each in vitro germinated T2 plant was stained with X-gluc for the assay of GUS expression. These plants were then transferred to selection medium containing 5 mg/l of GA to assay for the expression of *bar*. A total of 341 T2 plants from eight T1 plants were assayed for co-expression (Table 5). Three of the eight T1 plants were used as the male in cross-pollination, three were used as the female, and two were self-pollinated. The

Table 2 Segregation of expression of the non-selectable gene *uidA* in T2 progeny of transformation event BG

Cross	Total	GUS (+)	GUS (−)	*P*	$\chi^{2\,a}$
1. Male as transgenic parent (1:1)					
Hi-II × BG2	12	5	7	0.6*	0.33
Hi-II × BG5	100	22	78	0	31.36
Hi-II × BG4	20	5	15	0.025	5
Hi-II × BG7	51	7	44	0	32.96
Hi-II × BG11	27	2	25	0	19.59
Total	210	41	169	0	78.01
2. Female as transgenic parent (1:1)					
BG3 × Hi-II	78	38	40	0.82*	0.05
BG15 × Hi-II	72	26	46	0.015	5.56
BG2 × Hi-II	35	12	23	0.07*	3.46
BG8 × Hi-II	33	11	22	0.068*	3.66
BG7 × Hi-II	11	2	9	0.04	4.45
Total	229	89	140	0	11.36
3. Self-pollination (3:1)					
BG55 × BG55	59	18	41	0	62.29
BG54 × BG54	52	13	39	0	69.33
BG62 × BG62	50	24	26	0	19.44
BG64 × BG64	59	14	45	0	82.72
BG99 × BG99	96	40	56	0	56.89
BG126 × BG126	68	31	37	0	31.37
Total	384	140	244	0	304.23

[a] The chi-square test was used with a 0.05 limit of probability and one degree of freedom
* Fit the expected ratio (1:1 for cross-pollination and 3:1 for self-pollination)

Table 3 Segregation of expression of the selectable gene *bar* in T2 progeny of transformation event BG

Cross	Total	PAT (+)	PAT (−)	*P*	$\chi^{2\,a}$
1. Male as transgenic parent (1:1)					
Hi-II × BG4	20	5	15	0.025	5
Hi-II × BG7	51	7	44	0	26.8
Hi-II × BG11	27	3	24	0	16.32
Hi-II × BG16	151	28	123	0	59.8
Total	249	43	20	0	106.7
2. Female as transgenic parent (1:1)					
BG2 × Hi-II	35	15	20	0.4*	0.71
BG7 × Hi-II	11	4	7	0.35*	0.82
BG8 × Hi-II	33	18	15	0.6*	0.27
BG10 × Hi-II	211	83	128	0	9.6
BG16 × Hi-II	130	79	51	0.015	6
Total	420	199	221	0.3*	1.15
3. Self-pollination (3:1)					
BG51 × BG51	101	51	50	0	32.35
BG56 × BG56	87	63	24	0.6*	0.31
BG58 × BG58	22	16	6	0.8*	0.06
BG66 × BG66	20	9	11	0	9.6
BG69 × BG69	56	38	18	0.2*	1.52
BG94 × BG94	163	102	61	0	13.41
BG126 × BG126	68	43	25	0.05*	4.01
Total	517	322	195	0	44.6

[a] The chi-square test was used with a 0.05 limit of probablity and one degree of freedom
* Fit the expected ratio (1:1 for cross-pollination and 3:1 for self-pollination)

results showed that both transgenes were co-expressed in 107 of 158 (67.7%) T2 plants that expressed at least one of the genes. Of the T2 expressing plants, 48 of 158 (30.4%) expressed only *bar*, and 3 of 158 (1.9%) expressed only *uidA*.

The T2 plants obtained by using the male as transgenic parent had the lowest percent of co-expression. Only 14 of 98 (14.3%) T2 plants inherited co-expression of both genes, one T2 plant inherited only the expression of *bar* and none inherited only *uidA* expression. With the female as transgenic parent, 25 of 79 (31.6%) T2 plants expressed both transgenes, 12 T2 plants expressed only *bar*, and none expressed only *uidA*. From the self-pollinated progeny, 68 of 164 (41.5%) T2 plants expressed both transgenes, 35 T2 plants expressed only *bar*, and three expressed only *uidA*.

Inactivation of unselected and selected transgenes in individual T2 plants

The inactivation of transgenes was tested by Southern hybridization to non-expressing T2 plants. Sixteen greenhouse-grown T2 plants obtained from a self-pol-

Table 4 Segregation of expression of the selectable gene *bar* in T2 and T3 progeny of transformation event B

Cross	Total	PAT (+)	PAT (−)	*P*	χ^{2} [a]
A. T2 progeny					
1. Male as transgenic parent (1:1)					
CML67 × B3	41	0	41	0	41
CML67 × B11	12	4	8	0.25	1.33*
CML67 × B20	59	8	51	0	31.34
Hi-II × B7	36	0	36	0	36
Total	148	12	136	0	103.9
2. Female as transgenic parent (1:1) – no data					
3. Self-pollination (3:1)					
B7 × (B1 + B55)	54	27	27	0	18
B17 × B17	15	9	6	0.2	1.8*
Total	69	36	33	0	19.17
B. T3 progeny					
1. Male as transgenic parent					
Hi-II × B17-1	119	51	68	0.15	2.4*
2. Female as transgenic parent					
B20-4 × Hi-II	176	82	94	0.36	0.82*
3. Self-pollination					
B17-4 × B17-4	4	4	0	1	0*
B20-5 × B20-5	15	11	4	0.9	0.02*
B17-3 × B20-2	15	11	4	0.9	0.02*
B17-6 × B20-7	10	7	3	0.7	0.13*

[a] The chi-square test was used with a 0.05 limit of probablity and one degree of freedom
* Fit the expected ratio (1:1 for cross-pollination and 3:1 for self-pollination in T2 progeny; 1:1 for hemizygote in cross-pollination, 3:1 for hemizygote in self-pollination, and all:0 for homozygote in T3 progeny)

Table 5 Co-expression of *bar* and *uidA* in T2 progeny of event BG

Cross	Total	PAT (+)/GUS(+)	PAT (+)/GUS(−)	PAT(−)/GUS(+)
1. Male as transgenic parent				
Hi-II × BG4	20	5	0	0
Hi-II × BG7	51	7	0	0
Hi-II × BG11	27	2	1	0
Subtotal	98	14	1	0
2. Female as transgenic parent				
BG2 × Hi-II	35	12	3	0
BG7 × Hi-II	11	2	2	0
BG8 × Hi-II	33	11	7	0
Subtotal	79	25	12	0
3. Self-pollination				
BG126 × BG126	68	29	14	2
BG99 × BG99	96	39	21	1
Subtotal	164	68	35	3
Total	341	107	48	3

linated T1 plant were analyzed by Southern-blot hybridization of undigested genomic DNA with *bar* as the probe. Genomic DNA samples from 10 of 16 T2 plants hybridized with the *bar* probe (Fig. 4 A, lanes 5, 9, 11, 12, 13, 14, 15, 16, 17, 18). Among these ten plants, six expressed (Fig. 4 A, lanes 5, 9, 13, 14, 17, 18), and four (lanes 11, 12, 15, 16) did not express, PAT as determined by a herbicide (1% Basta AKA) spray to the 16 T2 plants in the greenhouse. Therefore, the selected transgene (*bar*) was inactivated in the four of the ten transgenic T2 plants. The transgene (*bar*) was actually transmitted by a Mendelian ratio (3:1), and non-Mendelian segregation of the expression of *bar* in T2 progeny resulted from inactivation of *bar* in individual transgenic T2 plants.

Genomic DNA samples from 16 *bar*-expressing T2 plants, which were obtained from a self-pollinated T1 plant, were analyzed for the presence of the unselected transgene (*uidA*). In the 16 *bar*-expressing T2 plants, only four of them (Fig. 4 B, lanes 17, 18, 19, 20) expressed the unselected transgene (*uidA*) as determined by GUS-

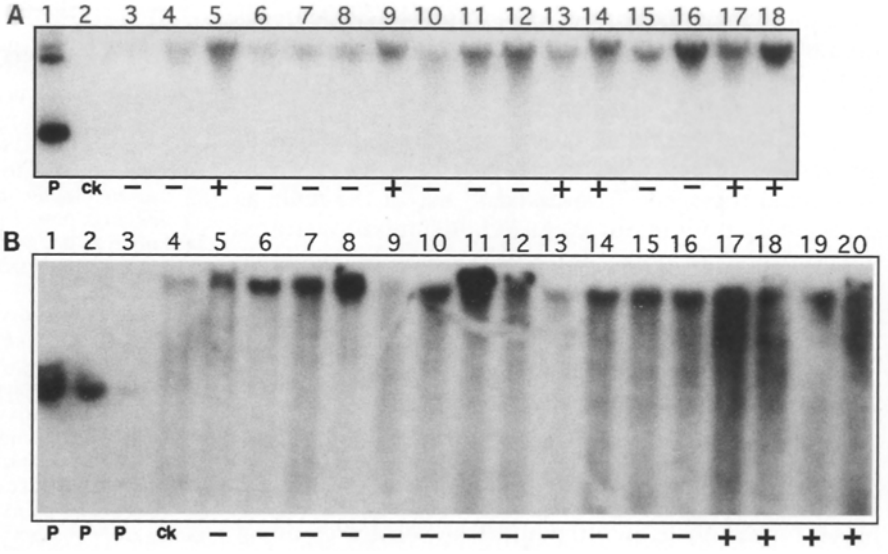

Fig. 4 A,B Genomic DNA analysis of T2 plants with *bar* and *uidA* probes. **A** Southern-blot analysis of undigested genomic DNA samples of 16 T2 plants obtained from a self-pollinated T1 plant with a *bar* probe. **B** Southern-blot analysis of undigested genomic DNA samples of 16 *bar*-expressing T2 plants in which 4 of 16 expressed *uidA* and 12 of 16 did not express *uidA* with a *uidA* probe. Abbreviations: *p* plasmid, *ck* untransformed plant, (+) positive expression, (−) negative expression

staining of the roots of the 16 T2 plants. However, genomic DNA analysis showed that 14 of them hybridized with the *uidA* probe (Fig. 4 B, lanes 5, 6, 7, 8, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20). All 16 plants were tested later for GUS expression in leaves and in pollen grains. The expression or lack of expression of *uidA* was consistent through all stages of each T2 plants.

Discussion

Variation in the inheritance of expression among subclones for an unselectable gene (*uidA*) and a selectable gene (*bar*) was observed in our two maize transformation events. This variation among subclones can not be explained by any developmental dependency, because silencing or expression of the unselected transgene (*uidA*) and the selected transgene (*bar*) in individual T2 plants was consistent throughout the entire growth of plants from in vitro germination to mature flowering in the greenhouse. Variation among subclones in the inheritance of a selectable gene (*bar*) was reported in one transgenic event in which one of four maize T1 plants showed a segregation ratio different from the other three (Wan et al. 1995). In transgenic tobacco transformed by direct gene transfer, 3 of 20 subclones in one event and 2 of 21 subclones in a second event deviated from the other subclones in their segregation after self-pollination (Potrykus et al. 1985).

Both the unselected transgene (*uidA*) and the selected transgene (*bar*) were expressed at a high level in all T1 plants regenerated from two transformations event in our experiment. Transgenic T1 plants which did not express the selected transgene (*bar*) were eliminated by in vitro selection on 5 mg/l of GA. In a previous report (Register III et al. 1994), it was shown that T1 plants regenerated from a single transformation event were not all identical in transgene expression. In that study, 8 of 97 transformation events resulted in T1 plants expressing the selected transgene (*bar*) in less than 25% of the plants. Sixteen of forty one transformation events resulted in T1 plants that expressed the unselected transgene (*uidA*) in less than 25% of the T1 plants.

Various segregation ratios for the expression of the two transgenes (*uidA* and *bar*) were observed among subclones in our two transformants. In event BG, 4 of 16 T1 subclones segregated by Mendelian ratios for the unselected transgene (*uidA*), and 7 of 16 T1 subclones segregated by Mendelian ratios for the selected transgene (*bar*). The observed segregation ratios were lower than expected for the rest of the T1 subclones analyzed. In event B, 2 of 6 T1 subclones followed Mendelian segregation ratios for the expression of *bar*, and the other four segregated in ratios lower than the expected. In previous work, various segregation ratios were observed from different maize transformation events (Spencer et al. 1992; Walters et al. 1992; Register III et al. 1994). Lower than expected Mendelian segregation ratios for selected transgenes (*bar*, *nptII*, and *hph*) were observed in some transformation events (Spencer et al. 1992; Walters et al. 1992; D'Halluin et al. 1993), although no explanation has been provided for these results. One possibility is that the transgenes may have been inactivated or silenced in some transformation events (Register III et al. 1994). The presence of multiple copies (Linn et al. 1990) and/or multiple insertion sites (Hobbs et al. 1990) was shown to influence the degree of gene inactivation in plants transformed by *Agrobacterium tumefaciens*. In transgenic maize, it was reported that two-thirds of the transformants which were assayed followed expected Mendelian inheritance ratios when the transgene was inserted at a single locus (Register III et al. 1994). In our experiment, Southern-blot data indicated that more than one copy of *bar* was integrated.

Also, more than one copy of *uidA* was inserted. Segregation analysis indicated that each of the two transgenes had only one copy expressed, or all expressed copies were inserted at a single locus.

Use of the female as transgenic parent resulted in greater inheritance of expression for both selected and unselected transgenes compared to use of the male as transgenic parent in our event BG. A similar low transmission through pollen for a selected transgene (*hph*) was observed in maize (Walters et al. 1992). A low level of transmission through pollen when two or more bands were present in Southern hybridization was reported in petunia transformed by *Agrobacterium tumefaciens* (Ulian et al. 1994). No transgene transmission through the pollen was observed in one line of soybean (Christou et al. 1989) and in six lines of oat (Somers et al. 1994). Therefore, low pollen transmission or maternal bias is not unique either to plants transformed by direct gene transfer or to cross-pollinated crops. It may be that transgenic pollen generally has poorer germination, elongation, or fusion competence compared to non-transgenic pollen, or transgenes may be inserted within a gene that affects the competence of the pollen grains.

Our co-expression analysis of T2 plants from event BG showed that expression of the two unlinked transgenes (*uidA* and *bar*) before transformation was co-inherited in 67.7% of the T2 plants that expressed at least one gene. Co-inheritance of unlinked transgenes was observed in rice (Goto et al. 1993; Peng et al. 1995) and tobacco (Saul and Potrykus 1990).

More T2 plants expressed the selected transgene (*bar*) than the unselected transgene (*uidA*) in our experiment. This difference was greater in T2 plants obtained from self-pollination than from cross-pollination, although the reasons are unclear. In a transgenic rice report, more progeny expressed *bar* than *uidA* even though both genes were unselected, possibly because of gene- and promoter-specific expression. Alternatively, *uidA* expression may be more susceptible to truncation, position effects, and/or co-suppression than is *bar* expression (Cooley et al. 1995).

Inactivation or silencing of selected (*bar*) and unselected (*uidA*) transgenes was observed in individual T2 plants in our experiment. In an earlier study (Register III et al. 1994), inactivation of a selected transgene (*bar*) was observed in many, or all, maize T2 plants in 5 of 14 transformation events. Several possible mechanisms or factors affecting transgene inactivation have been proposed, including site of insertion, copy number, rearrangement, amplification, methylation, and co-suppression (reviewed by Finnegan and McElory 1994). However, much remains to be learned about the factors and mechanisms influencing the silencing of transgene expression.

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