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Bialaphos selection of stable transformants from maize cell culture

T.M. Spencer, W.J. Gordon-Kamm, R.J. Daines, W.G. Start and P.G. Lemaux *

Plant Genetics Research, DeKalb-Pfizer Genetics, Groton, CT 06340, USA

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Summary. Stable transformed Black Mexican Sweet (BMS) maize callus was recovered from suspension culture cells bombarded with plasmid DNA that conferred resistance to the herbicide bialaphos. Suspension culture cells were bombarded with a mixture of two plasmids. One plasmid contained a selectable marker gene, *bar*, which encoded phosphinothricin acetyl transferase (PAT), and the other plasmid encoded a screenable marker for β-glucuronidase (GUS). Bombarded cells were selected on medium containing the herbicide bialaphos, which is cleaved in plant cells to yield phosphinothricin (PPT), an inhibitor of glutamine synthetase. The bialaphos-resistant callus contained the *bar* gene and expressed PAT as assayed by PPT inactivation. Transformants that expressed high levels of PAT grew more rapidly on increasing concentrations of bialaphos than transformants expressing low levels of PAT. Fifty percent of the bialaphos-resistant transformants tested (8 of 16) expressed the nonselected gene encoding GUS.

Key words: Microprojectile bombardment – Bialaphos – *bar* gene – Maize – Transformation

Introduction

The inability to genetically transform graminaceous monocots by *Agrobacterium* infection necessitates direct introduction of DNA into cells or protoplasts. Polyethylene-glycol-mediated DNA uptake (Lörz et al. 1985) and electroporation (Fromm et al. 1986) have proven effective techniques for introduction of DNA into graminaceous protoplasts. Both of these approaches require the use of protoplasts as targets for DNA introduction, and plant regeneration from protoplasts of graminaceous species is difficult. Fertile, transformed rice plants were regenerated from electroporated protoplasts (Shimamoto et al. 1989), and two recent reports (Shillito et al. 1989; Prioli et al. 1989) described regeneration of fertile maize plants from protoplasts, but transformed protoplasts were not described. Rhodes et al. (1988) described transformation of maize protoplasts by electroporation, but only infertile maize plants were recovered.

An alternative to the introduction of DNA into protoplasts is its introduction into intact cells. Since the first report of transient expression of an introduced gene in intact onion epidermal cells (Klein et al. 1987), microprojectile bombardment of DNA-coated tungsten particles has been used to deliver DNA into cells of a variety of plant species. Transient expression of an introduced gene was observed in suspension culture cells of rice, wheat, soybean (Wang et al. 1988), maize (Klein et al. 1988a), and barley (Mendel et al. 1989) and in maize embryos (Klein et al. 1988b). Stable transformation of tobacco (Klein et al. 1988c) and maize cells (Klein et al. 1989) was recently reported. A similar technique, involving bombardment of plant tissues with DNA-coated gold particles, was used successfully to obtain stable transformed soybean plants (McCabe et al. 1988).

While microprojectile bombardment provides a mechanism for introduction of DNA into intact cells of monocot species, a reproducible means of identifying viable transformed cells is also needed. Kanamycin selection was used to recover transformed embryogenic (Rhodes et al. 1988) and nonembryogenic (Fromm et al. 1986; Klein et al. 1989) maize cells. Kanamycin, as well as hygromycin and methotrexate, were used to recover stable transformants from several grass species; however,

* To whom correspondence should be addressed

many monocot species show high levels of endogenous resistance to these antibiotics (Hauptmann et al. 1988). In addition, both kanamycin and hygromycin appear to be effective selective agents only if applied soon after protoplast isolation (Hauptmann et al. 1988; Yang et al. 1988). Such a finding suggests that these agents might be less effective in selecting transformed cells within large multicellular clusters, as might occur with microprojectile bombardment of maize suspension cultures.

Introduction of the *bar* gene and selection of bialaphos-resistant cells is an alternative means of identifying stable transformants. Expression of the *bar* gene, isolated from *Streptomyces hygroscopicus* (Thompson et al. 1987), confers resistance to phosphinothricin (PPT), the active molecule in the herbicide bialaphos. PPT-resistant tobacco, tomato, potato (De Block et al. 1987), and *Brassica* (De Block et al. 1989) plants were obtained by *Agrobacterium*-mediated transformation using a chimeric *bar* gene plasmid. The *bar* gene was recently used as a selectable marker for transformation of rice protoplasts (Dekeyser et al. 1989). In this paper, we describe stable transformation of Black Mexican Sweet (BMS) maize suspension culture cells by microprojectile bombardment, and use of the *bar* gene as a selectable marker for the efficient recovery of bialaphos-resistant maize callus. Also described is cotransformation using a second, nonselected gene encoding β-glucuronidase (GUS).

Materials and methods

Plant material

BMS maize suspension cells (obtained in March of 1979 from V. Tilton, University of Minnesota, St. Paul/MN) were cultured in Murashige and Skoog (1962) medium containing 2 mg/l 2,4-D (MS2). Suspension cultures were maintained in the dark on a rotary shaker (150 rpm) and subcultured into fresh medium every 7 days.

Plant expression vectors

The *bar* gene was cloned from *Streptomyces hygroscopicus* (M. Bibb, John Innes Institute, Norwich, England) and exists as a 559-bp SmaI fragment in plasmid pIJ4104. The sequence of the coding region of this gene is identical to that published (Thompson et al. 1987). To create plasmid pDPG165, the SmaI fragment from pIJ4104 was ligated into a pUC19-based vector containing the Cauliflower Mosaic Virus (CaMV) 35S promoter (derived from pBI221.1, provided by R. Jefferson, Plant Breeding Institute, Cambridge, England), a polylinker, and the transcript 7 (Tr7) terminator from *Agrobacterium tumefaciens* (terminator provided by Calgene, Inc., Davis/CA). The other plasmid used in these studies, pCEV5, contains the maize *Adh1* promoter and first intron, GUS and the *nos* terminator from *Agrobacterium tumefaciens* [construction as described by Klein et al. (1988 b); plasmid provided by Calgene, Inc., Davis/CA].

Microprojectile bombardment

Prior to bombardment, plasmid DNA was precipitated onto tungsten particles (average diameter approximately 1.2 μm, GTE Sylvania) following the protocol described by Klein et al. (1987), using a precipitation mixture that included 1.25 mg tungsten, 25 μg plasmid DNA, 1.1 *M* $CaCl_2$, and 8.7 m*M* spermidine. Log-phase suspension culture cells were pipetted onto 5-cm filters (Whatman No. 4) and vacuum-filtered in a Büchner funnel. Approximately 0.5 ml packed cell volume (PCV) was placed on each filter, and the filters were transferred to petri dishes containing three 7-cm filters (Whatman No. 4) moistened with 3 ml MS2 medium. Cells were bombarded twice with 1 μl aliquots of the DNA-tungsten mixture using a Biolistics bombardment device (DuPont Co., Wilmington/DE). Bombardments were done under partial vacuum (50–100 mm Hg) using Remington A22C1 blank gun powder charges to propel the macroprojectile.

Transformant selection

Following bombardment, the support filter and cells were transferred to MS2 medium solidified with 5.5 g/l Seakem (FMC) agarose. Two protocols were used for selection of bialaphos-resistant cells. In one experiment, cells were grown on filters on solid MS2 medium for 7 days post-bombardment. Proliferating cells were transferred as discrete clumps (~ 5 mm in diameter) to solid MS2 medium containing 1 mg/l bialaphos (Meiji Seika Kaisha, Ltd., Yokohama, Japan). After 10 days, cell colonies were transferred to MS2 containing 0.1 mg/l bialaphos. In a second experiment, bombarded cells were cultured on filters on solid MS2 medium for 14 days after bombardment, gently scraped from the filters, and resuspended in liquid medium. Cells were immediately plated thinly on filters (approx. 0.5-ml packed cells per 7-cm filter) and cultured on solid medium containing 1 or 3 mg/l bialaphos. In both experiments, tissue was subcultured onto fresh selection medium every 10 days until resistant colonies developed, approx. 6–8 weeks after bombardment.

Enzyme assays

PAT activity was analyzed using a procedure modified from De Block et al. (1987). The assay was developed using an *E. coli* strain containing pDPG165 and maize protoplasts electroporated with pDPG165. Protoplasts were isolated from A188 × B73 suspension culture cells according to Heyser (1984), and electroporated in an osmotically balanced, HEPES-buffered saline solution (Fromm et al. 1986) using two, 3-ms square-wave pulses with a 0.5-kV/cm field strength. For PAT activity determinations, the *E. coli* cells and electroporated protoplasts were extracted in 10 m*M* Na_2HPO_4/10 m*M* NaCl, pH 7.0, using a bead beater (Biospec Products). BMS cells (100–500 mg) were extracted using a bead beater in 100–500 μl 50 m*M* TRIS-HCl, pH 7.5, 2 m*M* EDTA, 0.15 mg/l leupeptine, 0.15 mg/l phenylmethylsulfonyl fluoride, 0.3 mg/l bovine serum albumin, and 0.3 mg/l dithiothreitol. To a small amount of plant extract (25 μg total protein in 20 μl buffer), 3 μl ^{14}C-acetyl coenzyme A (48.1 mCi/mmol, NEN) and 2 μl Basta (Hoechst, FRG), diluted 1:10 in extraction buffer, were added. Reactions were incubated at 37 °C for 30 min, spotted onto silica gel thin-layer chromatography plates, and chromatographed in a 3:2 mixture of 1-propanol and NH_4OH. ^{14}C-Acetylated PPT was visualized by autoradiography. GUS activity was assayed histochemically as described (Jefferson 1987). Assays were incubated overnight at 37 °C and scored for blue cells at 18–24 h.

Southern blot analysis

Genomic DNA (4 μg per digest) was isolated according to Mettler (1987), digested with a threefold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern 1975) to Nytran (Schleicher and Schuell) filters. Filters were prehybridized at 65 °C in 6× SCP (20× SCP: 2 *M* NaCl, 0.6 *M* disodium phosphate, 0.02 *M* disodium EDTA), 10% dextran sulfate, 2% sarcosine, and 500 μg/ml heparin (Chomet et al. 1987) for 15 min. Filters were hybridized overnight at 65 °C in the same buffer containing 100 μg/ml denatured salmon sperm DNA and ^{32}P-probe labeled using random primed DNA labeling (Feinberg and Vogelstein 1983; Boehringer-Mannheim). The 0.6-kb SmaI fragment from pDPG165 and the 1.8-kb BamHI/EcoRI fragment from pCEV5 were used in the random priming reactions to generate probes for detecting sequences encoding PAT or GUS, respectively. Filters were washed in 2× SCP, 1% SDS at 65 °C for 30 min and visualized by autoradiography using Kodak XAR5 film.

Results

BMS cells were bombarded with tungsten particles coated with equimolar amounts of two plasmids: pDPG165 encoding PAT and pCEV5 encoding GUS (Fig. 1 A and B). A portion of the cells from each bombardment filter was sacrificed for analysis of transient GUS expression 48 h after bombardment. Transient GUS activity was observed as histochemically stained foci containing 1 to about 20 cells. The number of transiently expressing foci was estimated to be 200 per bombarded filter. The cells remaining on the filters were transferred to nonselective medium. In one experiment, tissue was grown in the absence of selection for 7 days, transferred to selection medium containing 1 mg/l bialaphos for 10 days, and subsequently tansferred to medium containing 0.1 mg/l bialaphos. In a second experiment, tissue was grown in the absence of selection for 14 days, resuspended in liquid medium, and plated immediately onto filters and cultured on solid medium containing 1 or 3 mg/l bialaphos. Resistant colonies appeared 6–8 weeks after bombardment at all bialaphos concentrations (Fig. 2). Ten to 20 bialaphos-resistant colonies were recovered from tissue originating from each bombarded filter, with comparable numbers of transformants obtained from each selection scheme. No colonies grew on selection plates containing tissue that was not bombarded. Resistant colonies were removed from the filters and maintained as individual cell lines. The individual cell lines likely arose from independent integration events for two reasons. There was a low density of transformants on the selection filters, and the cells were immobilized during the majority of the selection period, except the first week after bombardment (3–4 generations). This supposition is strengthened by the unique Southern hybridization patterns of all isolates tested (Fig. 5 A). Despite the fact that individual cell lines probably derived from independent integration events, each cell line does not necessarily represent the

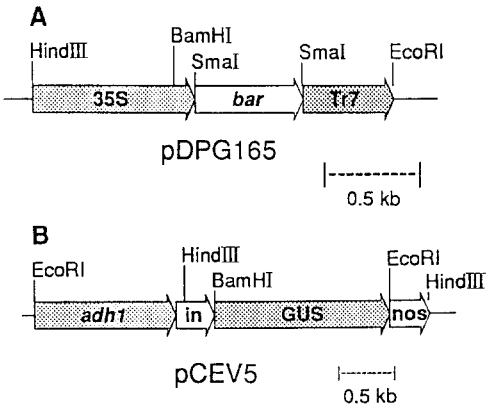

Fig. 1 A and B. Schematic representation of **A** pDPG165 and **B** pCEV5

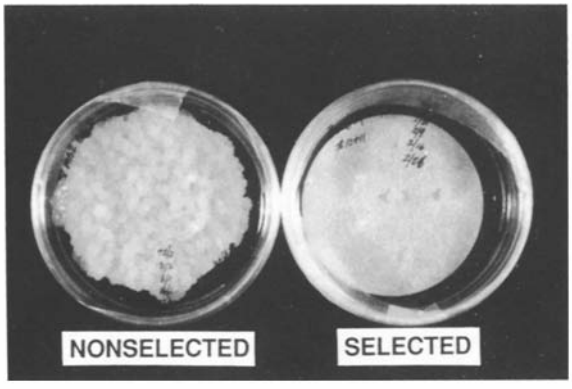

Fig. 2. Selection of bialaphos-resistant BMS cell colonies. Bialaphos-resistant cells recovered on 3 mg/l bialaphos (*right*) and nonselected cells (*left*) 6 weeks after bombardment

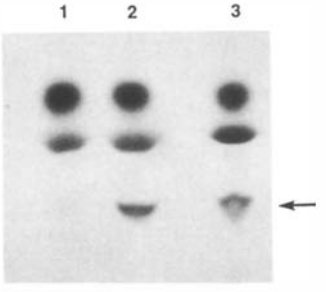

Fig. 3. Analysis of PAT activity in extracts of maize protoplasts 48 h post-electroporation and of *E. coli*. *Lane 1* – A188 × B73 protoplasts electroporated with pCEV5; *lane 2* – A188 × B73 protoplasts electroporated with pDPG165; *lane 3* – *E. coli* strain DH5α containing pDPG165. *Arrow* designates *N*-acetyl-PPT

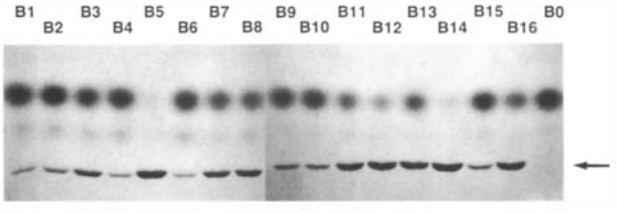

Fig. 4. PAT activity in extracts of BMS transformants (B1–B16) and a nonselected control (B0). Approximately 25 μg of protein per reaction

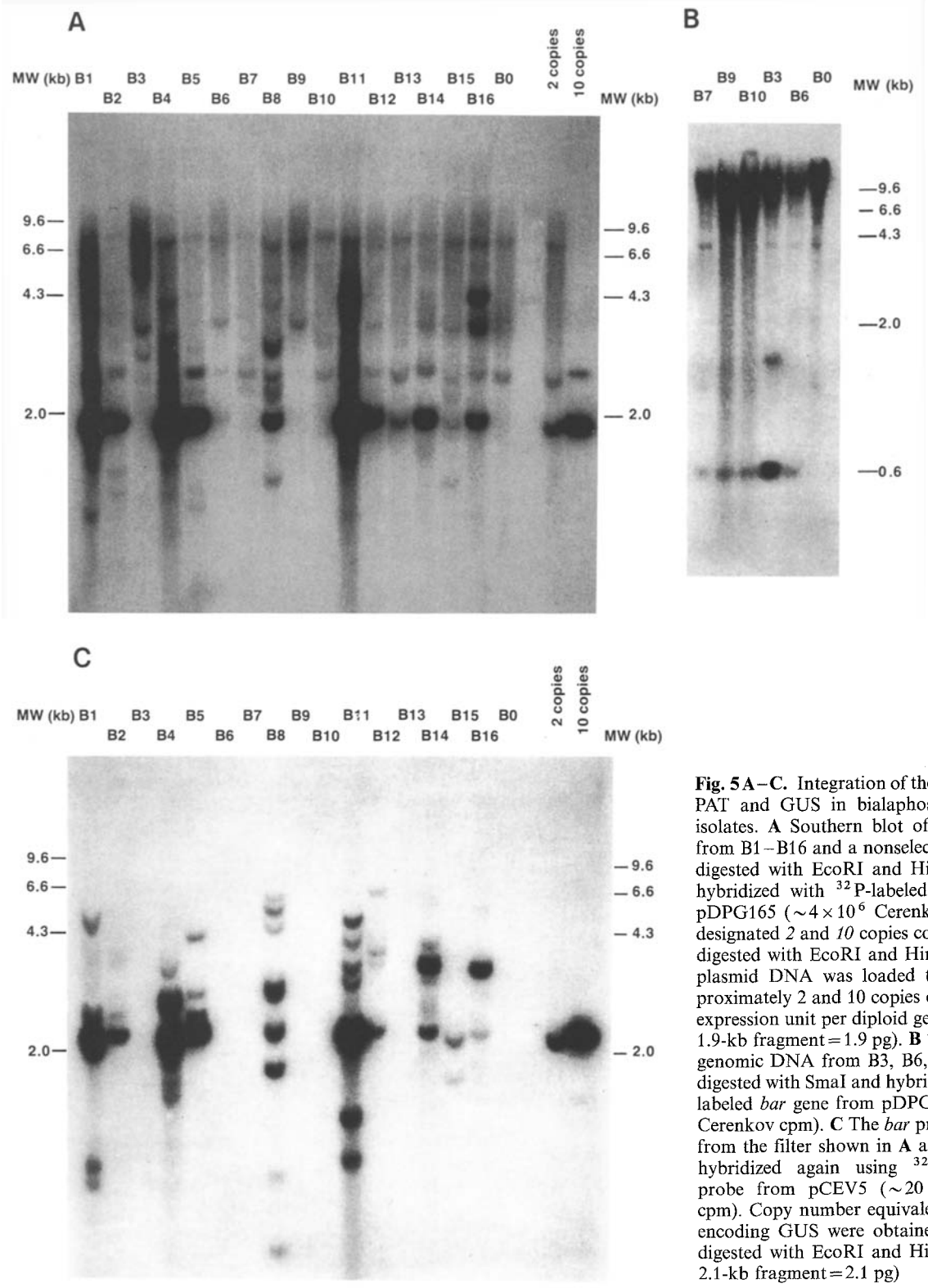

Fig. 5A–C. Integration of the genes encoding PAT and GUS in bialaphos-resistant BMS isolates. **A** Southern blot of genomic DNA from B1–B16 and a nonselected control (B0) digested with EcoRI and HindIII. Blot was hybridized with ^{32}P-labeled *bar* gene from pDPG165 ($\sim 4 \times 10^6$ Cerenkov cpm). *Lanes* designated *2* and *10* copies contain pDPG165 digested with EcoRI and HindIII. Restricted plasmid DNA was loaded to represent approximately 2 and 10 copies of the 1.9-kb *bar* expression unit per diploid genome (1 copy of 1.9-kb fragment = 1.9 pg). **B** Southern blot of genomic DNA from B3, B6, B7, B9, and B0 digested with SmaI and hybridized to the ^{32}P-labeled *bar* gene from pDPG165 ($\sim 33 \times 10^6$ Cerenkov cpm). **C** The *bar* probe was washed from the filter shown in **A** and the filter was hybridized again using ^{32}P-labeled GUS probe from pCEV5 ($\sim 20 \times 10^6$ Cerenkov cpm). Copy number equivalents for the gene encoding GUS were obtained using pCEV5 digested with EcoRI and HindIII (1 copy of 2.1-kb fragment = 2.1 pg)

progeny of a single transformed cell, since there was frequently more than one cell per focus that transiently expressed GUS.

Analysis of the bialaphos-resistant colonies was initially performed by assaying for phosphinothricin acetyltransferase (PAT) activity. The assays were performed using Basta, a commercial herbicide formulation, containing chemically synthesized PPT. PPT, the active moiety in bialaphos, is generated in plant cells through cleavage of bialaphos by intracellular peptidases. PPT, an inhibitor of glutamine synthetase in plants and bacteria (De Block et al. 1987), is acetylated by the *bar* gene product, yielding an inactive product, *N*-acetyl-PPT. The chromatographic assay used to detect PAT activity was developed using extracts from an *E. coli* strain containing pDPG165 and A188 × B73 maize protoplasts electroporated with pDPG165 (Fig. 3). Sixteen randomly chosen bialaphos-resistant BMS isolates (B1–B16) were tested and found to contain PAT activity; the levels varied approximately 20-fold (Fig. 4). In some isolates, there was almost complete conversion of available acetyl-coenzyme A to *N*-acetyl-PPT (B5, B12, and B14).

Southern analyses were performed on the same 16 bialaphos-resistant isolates (B1–B16) analyzed in Fig. 4. Chromosomal DNA was digested with EcoRI and HindIII to release the intact 1.9-kb expression unit (Fig. 1A), electrophoresed in agarose, transferred to nylon membrane, and hybridized to ^{32}P-labeled *bar* gene from pDPG165 (Fig. 5A). In 12 of the 16 samples, a 1.9-kb fragment hybridized to the probe, and the copy number of the intact expression unit (35S-*bar*-Tr7) varied from 1 to approximately 20. Higher molecular weight fragments were also detected in most of the isolates; such fragments may represent rearranged copies of the expression unit or may be the result of partial digestion of chromosomal DNA. Four of the isolates (B3, B7, B9, and B10) lacked an intact expression unit but did contain higher molecular weight fragments that hybridized to the *bar* gene probe. Despite the lack of intact expression units, these isolates did express PAT at levels comparable to transformants containing intact expression units. Therefore, we analyzed the isolates lacking the intact expression unit for an intact copy of the *bar* gene. Chromosomal DNA from these four samples and from transformant B6, which contains an intact expression unit, was digested with SmaI and analyzed by Southern hybridization to the *bar* gene (Fig. 5B). All four isolates lacking the intact expression unit contained intact copies of the *bar* structural gene (0.6-kb, Fig. 1A). Expression of the *bar* gene in these four isolates may not be dependent on the 35S CaMV promoter and/or the Tr7 terminator. As previously report (Nagy et al. 1985), there is no apparent correlation between the copy number of intact or rearranged *bar* genes and the level of expression in transformed callus (Figs. 4 and 5A).

To determine the co-integration frequency of the nonselected marker gene, the Southern blot shown in Fig. 5A was washed and probed again with the gene encoding GUS. The restriction enzymes used to excise the intact 1.9-kb *bar* expression unit also released a 2.1-kb fragment, which did not contain the entire expression unit but did contain the entire structural gene for GUS and approximately 300 bp of the *Adh1* intron. Ten of the 16 *bar* transformants (B1, B2, B4, B5, B8, B11, B12, B14, B15, and B16) contained sequences that hybridized to the gene encoding GUS (Fig. 5C); most contained intact copies as well as higher molecular weight fragments. In these experiments, co-transformation frequency was >50% using an independent plasmid to introduce a second gene for a nonselectable marker. The range in copy number of the 2.1-kb fragment was 1 to approximately 20; however, in this case we cannot make conclusions about the integrity of the expression unit since the digest does not release the intact expression unit. In those isolates containing sequences hybridizing to the GUS probe, the relative copy numbers of these sequences were similar to the copy numbers of the sequences hybridizing to the *bar* probe in that same isolate (Fig. 5A and C). This similarity in integration patterns has been noted previously (Lyznik et al. 1989) and may be related to the competency state of the cell to be transformed. For example, there may be a particular phase of the cell cycle in which DNA is delivered more efficiently to the nucleus (Okada et al. 1986). Alternatively, the similar copy number of the genes encoding PAT and GUS in an individual transformant may be due to the fact that plasmids form concatemers by homologous recombination prior to insertion into the host genome, as has been observed in other systems (Folger et al. 1982).

The frequency of expression of GUS was determined by testing a small amount of tissue from each of the transformants for histochemically detectable GUS activity. Eight of the 16 transformants (B1, B2, B4, B5, B11, B13, B14, and B16) had GUS activity at 11 weeks postbombardment, giving a co-expression frequency of 50%. Two of the transformants expressed GUS uniformly in all tissue tested (B5, B11). Other transformants exhibited GUS expression in approximately half of the cells tested (B1, B14, B16), and two of the transformants had detectable GUS expression in approximately 10% of the cells (B2, B4). With the exception of B13, all transformants that expressed GUS contained sequences that hybridized to the GUS probe. GUS expression in B13 was limited to a few cells in the sample tested (<1%), and may have resulted from contamination of the tissue with tissue from another transformant during culture maintenance, or may be due to the chimeric nature of this isolate. Tissue was tested again 6 months later, and patterns and levels of expression were similar in all isolates except for B13, which contained no cells expressing GUS. Three of

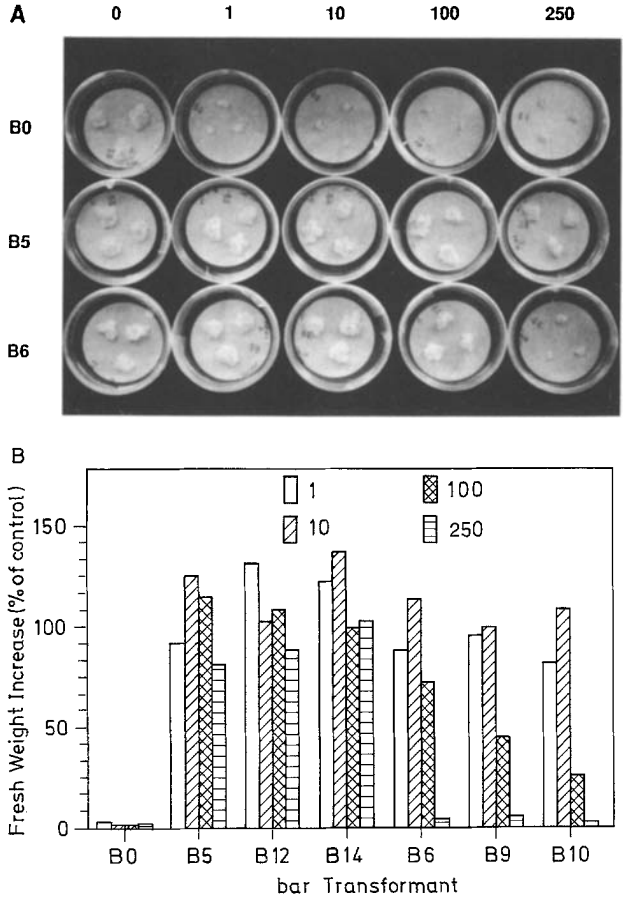

Fig. 6 A and B. Relationship between the level of PAT expression and the fresh weight increase of BMS *bar* transformants grown on a range of bialaphos concentrations. **A** Growth of transformants with relatively high (B5) and low (B6) levels of PAT expression after 4 weeks on a range of bialaphos concentrations. B0 is a nonselected control. **B** Graphic representation of fresh weight increase of transformants B5, B12, B14, B6, B9, and B10 cultured for 5 weeks on 0, 1, 10, 100, and 250 mg/l bialaphos. *Hatched boxes at top* of figure show the patterns used for the various bialaphos concentrations

the transformants (B8, B12, B15) contained intact 2.1-kb fragments but did not express GUS; these isolates may not contain intact expression units.

Transformants were analyzed to determine if higher levels of PAT expression supported faster growth rates on medium containing bialaphos. Six transformants were analyzed: three with high levels of PAT expression (B5, B12, and B14) and three with relatively low expression levels (B6, B9 and B10) (Fig. 6). Approximately 150 mg of each transformant and a nontransformed control was cultured on filters overlaying media containing 0, 1, 10, 100, and 250 mg/l bialaphos. After 5 weeks, the tissue was removed from the filters and weighed. Fresh weight increase of each of the transformants, as a percent of its 0 mg/l bialaphos control, was determined at all bialaphos concentrations (Fig. 6A and B). All six transformants, regardless of PAT expression levels, were not inhibited at 1 and 10 mg/l bialaphos. Growth of low-expressing transformants was inhibited at 100 and 250 mg/l bialaphos, while the growth of the high-expressing transformants was relatively unaffected, The 100- and 250-mg/l levels of bialaphos are much higher than those used for the initial selection of transformants (1–3 mg/l).

Discussion

Our results demonstrate that the *bar* gene is an effective selectable marker for intact BMS maize cells transformed by microprojectile bombardment. Estimating the frequency of stable transformation using this system can be accomplished in at least two ways. It is difficult to estimate the total number of cells on a bombardment filter because maize suspension culture cells often grow in large clusters, containing many cells. Therefore, transformation frequency can be expressed as the number of stable transformants per bombarded filter; this number represents an estimation of the ability to recover bialaphos-resistant transformants from a population of bombarded cells. In the experiments described in this paper, 0.5 ml PCV of cells was plated on each filter for bombardment. From the total number of transformants, we can estimate that approximately 10–20 bialaphos-resistant colonies were recovered per original bombarded filter. This estimation of transformation frequency, however, does not take into account the frequency with which DNA is introduced into cells. In these experiments we can estimate the number of cells (or groups of cells) initially receiving DNA by determining the number of cells transiently expressing GUS. The transformation frequency can then be expressed as the efficiency with which stable transformants arise from a population of cells transiently expressing DNA. The number of cell clusters (foci) expressing GUS at 48 h post-bombardment averaged approximately 200 per bombarded filter (with a range of 1 to 20 expressing cells per focus). From the total number of transformants recovered, we can estimate that about 1 *bar* transformant was recovered for each 10–20 transiently expressing GUS foci observed. Because there were often multiple cells per focus, the transformation frequency on a per cell basis would be lower than on a per focus basis. Estimation of transformation frequency by this method was based on the assumption that all cells that transiently expressed GUS received DNA-coated tungsten particles and remained viable.

BMS maize suspension cultures have been used as a model system for studying maize transformation. Stable kanamycin-resistant transformants have been recovered from BMS suspension culture cells bombarded with the *neo* gene (Klein et al. (1989); present authors' unpublished results). Our results demonstrate that bialaphos can be used to select transformed BMS cells from a pop-

ulation of nontransformed cells. Additionally, we have demonstrated that co-transformation and co-expression frequency was 50% or greater in BMS using independent plasmids to introduce a nonselectable marker. The utility of any selection scheme for introducing traits into corn, however, will depend upon its effectiveness as a selective agent in regenerable maize cell cultures. Nonembryogenic BMS cells differ from many embryogenic maize cell cultures in their sensitivity to kanamycin. Hauptmann et al. (1988) described insensitivity to kanamycin of cell cultures from numerous monocot species. We have been unsuccessful in our laboratory in reproducibly selecting transformed kanamycin- or G418-resistant cells from embryogenic maize cell cultures bombarded with the *neo* gene. We recently repeated bialaphos selection of *bar* transformants using regenerable embryogenic maize cell cultures. These data will be presented in a subsequent paper.

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References

Chomet PS, Wessler S, Dellaporta SL (1987) Inactivation of the maize transposable element *Activator* (*Ac*) is associated with its DNA modification. EMBO J 6:295–302

De Block M, Botterman J, Vandiwiele M, Dockx J, Thoen C, Gossele V, Rao Movva N, Thompson C, Van Montagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513–2518

De Block M, De Brouwer D, Tenning P (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol 91:694–701

Dekeyser R, Claes B, Marichal M, Van Montagu M, Caplan A (1989) Evaluation of selectable markers for rice transformation. Plant Physiol 90:217–223

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

Folger KR, Wong EA, Wahl G, Capecchi MR (1982) Patterns of integration of DNA microinjected into cultured mammalian cells: Evidence of homologous recombination between injected plasmid DNA molecules. Mol Cell Biol 2:1372–1387

Fromm ME, Taylor LP, Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. Nature 319:791–793

Hauptmann RM, Vasil V, Ozias-Aikins P, Tabaeizadeh Z, Rogers SG, Fraley RT, Horsch RB, Vasil IK (1988) Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiol 86:602–606

Heyser JW (1984) Callus and shoot regeneration from protoplasts of Proso millet (*Panicum miliaceum* L.). Z Pflanzenphysiol 113:233–239

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

Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73

Klein TM, Fromm M, Weissinger A, Tomes D, Schaaf S, Sletten M, Sanford JC (1988a) Transfer of foreign genes into maize cells with high-velocity microprojectiles. Proc Natl Acad Sci USA 85:4305–4309

Klein TM, Gradziel T, Fromm ME, Sanford JC (1988b) Factors influencing gene deliver into *Zea mays* cells by high-velocity microprojectiles. Bio/Technol 6:559–563

Klein TM, Harper EC, Svab Z, Sanford JC, Fromm ME, Maliga P (1988c) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. Proc Natl Acad Sci USA 85:8502–8505

Klein TM, Kornstein L, Sanford JC, Fromm ME (1989) Genetic transformation of maize cells by particle bombardment. Plant Physiol 91:440–444

Lörz H, Baker B, Schell J (1985) Gene transfer to cereal cells mediated by protoplast transformation. Mol Gen Genet 199:178–182

Lyznik LA, Ryan RD, Ritchie SW, Hodges TK (1989) Stable co-transformation of maize protoplasts with *gusA* and *neo* genes. Plant Mol Biol 13:151–161

McCabe DE, Swain WF, Martinell BJ, Cristou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technol 6:923–926

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

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Rep 5:346–349

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

Nagy F, Morelli G, Fraley RT, Rogers SG, Chua N-H (1985) Photoregulated expression of a pea *rbcS* gene in leaves of transgenic plants. EMBO J 2:3063–3068

Okada K, Takeve I, Nagata T (1986) Expression and integration of genes introduced into highly synchronized plant protoplasts. Mol Gen Genet 205:398–403

Prioli LM, Sondahl MR (1989) Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L.). Bio/Technol 7:589–594

Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer JJ (1988) Genetically transformed maize plants from protoplasts. Science 240:204–207

Shillito RD, Carswell GK, Johnson CM, DiMaio JJ, Harms CT (1989) Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technol 7:581–587

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274–276

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Thompson CK, Rao Movva N, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J 6:2519–2623

Wang Y-C, Klein TM, Fromm M, Cao J, Sanford JC, Wu R (1988) Transient expression of foreign genes in rice, wheat, and soybean cells following particle bombardment. Plant Mol Biol 11:433–439

Yang H, Zhang MH, Davey MR, Mulligan BJ, Cocking EC (1988) Production of kanamycin-resistant rice tissues following DNA uptake into protoplasts. Plant Cell Rep 7:421–425