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Evaluation of Selectable Markers for Obtaining Stable Transformants in the Gramineae[1]

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ABSTRACT

Cell suspension cultures of *Triticum monococcum*, *Panicum maximum*, *Saccharum officinarum*, *Pennisetum americanum*, and a double cross tri-specific hybrid between *Pennisetum americanum*, *P. purpureum*, and *P. squamulatum* were tested for resistance to kanamycin, hygromycin, and methotrexate for use in transformation studies. All cultures showed high natural levels of resistance to kanamycin, in excess of 800 milligrams per liter, and variable levels of resistance to hygromycin. Methotrexate was a potent growth inhibitor at low concentrations with all species. Kanamycin and hygromycin were growth inhibitory only if added early (within 5 days after protoplast isolation and culture). Protoplasts of *T. monococcum*, *P. maximum*, *S. officinarum*, and the tri-specific hybrid were electroporated with plasmid DNA containing hygromycin (pMON410), kanamycin (pMON273), or methotrexate (pMON806) resistance genes. Resistant colonies were obtained at low frequencies (1×10^{-5} to 2×10^{-6}) when selected under conditions which were growth inhibitory to protoplasts electroporated without DNA. Southern blot hybridization confirmed stable integration of plasmid DNA into *T. monococcum* using hygromycin vectors and *P. maximum* using the methotrexate vector with 1 to 10 copies integrated per haploid genome.

Electroporation has been successfully used for obtaining transient expression in several species of gramineous monocots, including TM[3] (8, 15, 30), *Zea mays* (6), *Pennisetum purpureum*, PM, SC, and a double cross, tri-specific hybrid between *P. purpureum*, PA, and *P. squamulatum* (8), *Oryza sativa* and *Sorghum bicolor* (15). Although transient expression provides a rapid analysis of gene function, the integration frequency of free DNA delivered by chemical (11) or electrical (7) means is low. Even in optimized dicot systems frequencies of no more than 1% can be attained (21). Because of these relatively low frequencies of stable transformation, it is necessary to have good selectable marker genes. These are needed to give high levels of resistance in the transformed cells so that wild type growth is inhibited by addition of the selection agent.

Using kanamycin resistance encoded by the NPTII gene, selection of stable transformants is routine in many dicot systems due to the efficiency of this unambiguous selection procedure. Kanamycin resistance selection recently has been applied to the gramineous species *Z. mays* (7), TM (13), and *O. sativa* (24) with some success. However, *Lolium multiflorum* transformants could be obtained only by using G418 as the selection agent since *L. multiflorum* demonstrated a natural resistance to kanamycin (16).

Other selectable marker genes available include a mutant DHFR gene created by site specific mutagenesis of a wild type mouse DHFR gene (5) which confers resistance to methotrexate and the HPH gene which codes for resistance to the antibiotic hygromycin (12, 19, 29).

We report here the evaluation and use of selectable marker genes coding for resistance to hygromycin, kanamycin, and methotrexate for the isolation of stable transformants in the Gramineae.

MATERIALS AND METHODS

Cell Lines, Protoplast Isolation, and Culture. Cell suspension cultures of the following five established monocot cell lines were used: TM (supplied by Dr. D. Dudits, Szeged, Hungary), PM (14), SC (9, 23), PA (27), and PAPS (4; P Ozias-Akins, W Hanna, IK Vasil, unpublished data). Suspension cultures of PA, PM, and PAPS were grown in a modified MS medium (27) containing 5% coconut milk and 2 mg/L 2,4-D. SC was grown in the same medium but with 2.5 mg/L 2,4-D and an additional 500 mg/L casein hydrolysate. The TM suspension culture was grown in liquid C_8 as previously described (3). All suspension cultures were subcultured once a week with a 2 to 8 ml inoculum in 25 to 35 ml medium except TM and PAPS which were transferred twice weekly.

Levels of growth inhibition by kanamycin, hygromycin, and methotrexate for the various cell lines were determined by inoculating either 0.3 or 0.5 ml of exponentially growing suspension culture in 2 or 3 ml of suspension culture medium, with and without the selection agent, and culturing for 2 weeks at 28°C in the dark. Alternatively, 0.25 g fresh weight of exponentially growing suspension was inoculated into 50 ml of liquid culture medium with various concentrations of the selection agent and grown for 11 d. Fresh weights were measured after vacuum filtration on miracloth or filter paper and mean values expressed as percent of control growth. Each data point represents at least

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[3] Abbreviations: TM, *Triticum monococcum*; PM, *Panicum maximum*; SC, *Saccharum officinarum*; PA, *Pennisetum americanum*; PAPS, a double cross tri-specific hybrid between *Pennisetum americanum*, *P. purpureum*, and *P. squamulatum*; NPTII, neomycin phosphotransferase; DHFR, dihydrofolate reductase; HPH, hygromycin phosphotransferase; CAT, chloramphenicol acetyl transferase.

three replicates.

Protoplasts were isolated from suspension cultures on the fourth to fifth d following subculture of 5 to 8 ml inoculum in 25 to 35 ml medium. Approximately 4 to 5 ml of settled cells were incubated in 50 ml enzyme mixture. The enzyme mixtures consisted of 1.0% Cellulase RS (Kinki Yakult), and 0.8% pectinase (Sigma) for TM and PM; 2% Cellulase RS and 0.7% pectinase for SC; and 2.5% Cellulase R-10 and 0.75% pectinase for PAPS. All enzymes were dissolved in 3 mM MES, 0.45 M mannitol, 7 mM $CaCl_2 \cdot 2H_2O$, and 0.7 mM NaH_2PO_4 (pH 5.6) as previously described (28).

Plasmids Used and Electroporation. *Preparation and Characteristics of Plasmids Used. Escherichia coli* plasmids containing chimaeric gene constructions expressed by the CaMV 35S promoter used were: (a) pMON145′ containing the CAT gene (8), (b) pMON273 containing the NPTII gene (18), (c) pMON806 containing the DHFR gene (5), and (d) pMON410 containing the HPH gene (19). Plasmid DNA was isolated and quantitated as previously described (8).

Electroporation of Protoplasts. Protoplasts were electroporated at a density of 3×10^6/ml in 1 ml aliquots using a capacitor discharge bank with the electroporation chamber constructed out of cuvettes lined with platinum foil as previously described (8). A 200 V, 1000 μF pulse was applied using 20 or 40 μg of circular plasmid DNA. Transient expression of electroporated DNA was monitored by performing CAT assays to ensure delivery and expression of DNA (7).

The electroporated protoplasts were cultured in the presence or absence of the selection agents (kanamycin, hygromycin, or methotrexate) at various concentrations and at various times of addition. Control protoplasts were electroporated without DNA and cultured similarly. The protoplasts from each treatment were distributed at a density of 3×10^5/ml into two Petri dishes (10 cm), 5 ml per dish, and incubated at 28°C in the dark. The protoplast culture medium was composed of Kao and Michayluk's (10) modified 8p medium (26), containing 0.4 to 0.5 M glucose, 0.5 to 1.0 mg/L 2,4-D, and 0.2 to 0.25 mg/L zeatin. The culture medium for PAPS lacked zeatin. Depending upon growth, the first dilution of the cultures with 2 to 3 ml fresh medium (0.2–0.25 M glucose) was carried out after 6 to 15 d. For TM the cultures were solidified after another week by adding fresh medium with 0.2 M glucose and agarose (SeaPlaque, FMC) to bring the final concentration of agarose to 0.6 to 0.8%. After an additional week, *i.e.* 3 weeks from culture initiation, wedges cut from the cultures were transferred to Petri dishes (10 cm) containing 10 to 20 ml liquid suspension culture medium (20). The liquid medium was replaced every 8 to 10 d. The diluted PM and PAPS cultures were incubated for up to 11 weeks. Any calli which had developed in the liquid culture prior to that time were transferred to agar solidified (0.8%) suspension culture medium containing the growth inhibitor. Other plates with detectable initial growth were diluted with 3 to 5 ml agarose containing medium (0.6% final concentration of agarose) after 3 to 4 weeks. Bead cultures were established from some of these plates. Nearly 6 to 8 weeks after culture initiation the individual resistant colonies were transferred to solid suspension culture medium. Inhibitory levels of the selection agents (kanamycin sulfate or hygromycin for TM and methotrexate for PM) were maintained throughout the duration of the culture.

Transformation efficiency was determined by counting the number of resistant colonies formed after 3 to 4 weeks of culture and expressed as percent of nonelectroporated control grown in absence of selection pressure.

Southern Hybridization Analysis. High mol wt DNA was isolated from callus and cell suspension cultures and purified by CsCl density gradient centrifugation (22). Prior to DNA isolation all callus and cell suspension cultures were examined microscopically and streaked on Luria-Bertani (LB) agar solidified medium to ensure no bacterial contaminants were present. DNA was digested with *Bam*HI, separated on 0.8% agarose gels, and transferred to Gene Screen (NEN/DuPont) using 0.025 M phosphate buffer (pH 6.5) and then the DNA was UV-cross-linked to the membrane (2). Probe DNA was made by nick translation (17) of total plasmid. Hybridization to Gene Screen was performed as previously described (2). *Hin*dIII fragments of λ-DNA were used as size markers.

RESULTS

Resistance of Cell Lines to Antibiotics. Sensitivity studies on the various cell lines showed them to be highly resistant to kanamycin (Fig. 1A). Kanamycin inhibited growth only at high concentrations with 30% of control growth obtained at 800 μg/ml for TM, SC, and PM. Similar natural resistance to kanamycin was seen in PAPS and PA in addition to a cell line of *Zea mays* (data not presented). Methotrexate inhibited growth significantly at low concentrations will all cell lines tested (Fig. 1B). Hygromycin was found to vary in toxicity between the cell lines with SC being the most sensitive, PM and PA were intermediate in response and PAPS and TM were the most resistant (Fig. 1C).

Resistance to growth inhibition by hygromycin and kanamycin was also seen in protoplast derived colonies when the selection pressure was imposed 2 to 3 weeks after protoplast culture when the protocolonies were about the size of the cell aggregates in the suspension cultures.

Selection of TM Transformants. Protoplasts of TM were electroporated using hygromycin (pMON410) or kanamycin (pMON273) resistance vectors as well as the CAT containing plasmid, pMON145′, as a biochemical marker. Positive CAT assays performed 2 d after electroporation confirmed uptake and expression of the introduced DNA. The electroporated protoplasts were cultured under various conditions to maximize selection for stably transformed cultures. Freshly isolated protoplasts were much more sensitive to the antibiotics as compared to addition at later stages in culture (Tables I and II). Addition 3 to 5 d after initiation of culture resulted in the isolation of many more resistant colonies and required higher concentrations for complete inhibition of background cell growth. Addition of the selection agent to control protoplasts (electroporated without DNA) 0 to 3 d after electroporation resulted in no colony formation. Protoplasts electroporated with pMON273 and cultured under identical conditions resulted in the isolation of a total of three colonies out of 6×10^6 plated protoplasts (Table I). Southern analysis of the kanamycin resistant colonies showed hybridization with plasmid DNA 3 months after isolation; however, when one kanamycin resistant line (B3) was retested 12 months later, no hybridization was observed.

When protoplasts electroporated with pMON410 were isolated on hygromycin, eight resistant colonies were obtained out of 21 $\times 10^6$ electroporated protoplasts. No colonies were obtained from a similar number of protoplasts electroporated without DNA. Southern hybridization analysis of DNA from hygromycin resistant colonies showed that several contained sequences which hybridized to pMON410 (Fig. 2).

Selection of PM Transformants. PM was electroporated with pMON806 in two separate experiments. In the first experiment, 0.5 μg/ml methotrexate was added after either 3 or 15 d of culture. Thirty-eight resistant calli were selected from the early addition of methotrexate; however, all of these calli were obtained from one dish of liquid culture and may not represent independent transformation events. Several of these lines showed identical patterns of hybridization with pMON806 plasmid DNA (data not shown). Seventy-three resistant calli were selected from the late addition of methotrexate 10 weeks after culture initiation. These lines were obtained from 'bead' cultures containing

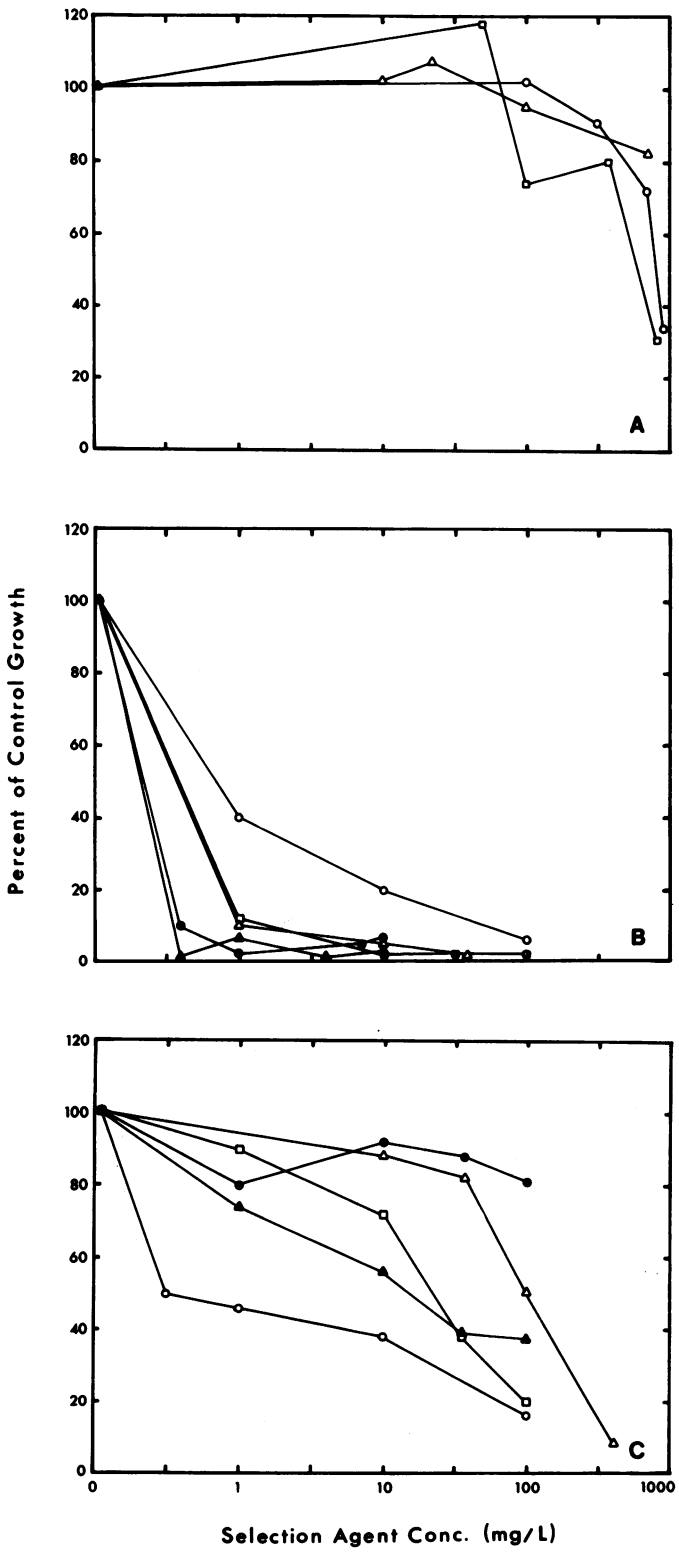

FIG. 1. Degree of growth inhibition by kanamycin (A), methotrexate (B), and hygromycin (C) for monocot cell lines. Growth studies were performed by either inoculating 0.3 or 0.5 ml of exponentially growing suspension cultures in 2 or 3 ml medium, respectively, and culturing for 2 weeks at 28°C in the dark. Fresh weights were measured by vacuum filtration on Miracloth or filter paper and mean values expressed as percent of control growth. Each data point represents at least three replicates. TM (△); SC (○); PM (□); PAPS (●); PA (▲).

1.0 μg/ml methotrexate. Resistant calli were selected at a frequency of 1.6×10^{-5} in electroporated-plus-DNA cultures whereas resistant calli from controls (electroporated without DNA) appeared at a frequency of 3×10^{-6}. It was concluded that addition of methotrexate after the first two to three cell divisions but before extensive colony formation had occurred might be optimal for selection of transformants.

On the basis of observations from the first experiment, the selection procedure was modified slightly for the second experiment. Methotrexate (0.5 μg/ml) was added either 3 or 5 d after culture initiation. After 3 weeks cultures were brought to 1.0 μg/ml methotrexate and 0.6% agarose in which they were maintained until resistant calli appeared. After 11 weeks, no resistant calli were recovered from the controls, nor did any survive the 3-d addition of methotrexate. Twenty-five calli were obtained from the 5-d addition of methotrexate for a frequency comparable to that observed in the first experiment.

Southern hybridization analysis of methotrexate resistant PM colonies from the first (PMI) and second (PMII) set of experiments showed homology to nick translated pMON806 plasmid DNA (PMI13, PMI39, PMII37, PMI36, and PMII36) while one colony (PMI28) showed no homology (Fig. 3). Copy number reconstructions using *Bam*HI digested pMON806 plasmid DNA indicated that between 1 and 10 copies of plasmid DNA was present per transformed cell. No hybridization was seen between non-electroporated wild type PM and the nick translated probe (Fig. 3).

Selection for Antibiotic Resistant Colonies in Other Cell Lines. In SC, 2 methotrexate resistant colonies were obtained out of 6×10^6 electroporated protoplasts. Southern hybridization analysis showed no hybridization to nick translated pMON806 probe DNA. This indicates that these cells were not transformed and were selected resistant to methotrexate. Several colonies were obtained from electroporated PAPS selected with kanamycin or methotrexate. Analysis of these colonies by blot hybridization is pending. [Three methotrexate resistant colonies of PAPS electroporated with pMON806 plasmid DNA have been analyzed by blot hybridization and show regions of homology to the plasmid DNA (data not shown).] Attempts have not been made to electroporate and select transformants in PA and PP at this time.

DISCUSSION

Free DNA delivery systems have been shown to be efficient in the delivery of DNA and in the isolation of stable transformants (7, 13, 16, 21). In any transformation system, only a small proportion of plant cells are transformed by the currently available delivery systems and it is crucial to have a selectable marker to recognize the transformed cells and suppress the growth of wild type cells. Using NPTII, stable transformants have been obtained in *Z. mays* (7) and TM (13). The selection for transformants was based on resistance to kanamycin at 80 to 100 mg/L.

All of the gramineous species we have examined showed resistance to kanamycin at least 10 times higher than those previously used to select for transformants. Natural resistance to kanamycin has also been seen in the gramineous monocot *L. multiflorum* (16). In this case, protoplasts transformed using NPTII as the selectable marker could not be separated from wild type colonies using kanamycin resistance selection alone. *L. multiflorum* was less resistant to G418 and this was used as the selection agent.

Some of the reported variation in resistances to kanamycin in members of the Gramineae could possibly be attributed to differences between cell lines. Some differential sensitivity was seen in our studies with hygromycin; however, high levels of kanamycin resistance have been routinely observed.

In the cases of hygromycin and kanamycin, we found that the antibiotics had to be added soon after protoplast isolation to achieve effective inhibition of growth. All cell lines, however, tested sensitive to methotrexate at concentrations approximating

Table I. *Sensitivity and Selection of Kanamycin Sulfate Resistant Callus Lines from TM Protoplasts Electroporated with pMON273 Plasmid DNA*

Kanamycin sulfate (100–400 μg/ml) was added at various times after electroporation (0–5 d). Cell colony formation was estimated at 8 d after culture initiation. At least 3 × 10^6 protoplasts were used for each treatment. At the time of dilution the concentration of kanamycin was brought up to at least 200 μg/ml in all cultures. No resistant colonies developed from the control cultures without electroporated DNA. For large numbers of colonies estimates are given.

Kanamycin	Application of Kanamycin (time after electroporation)	Surviving Cell Groups after 8 d	Resistant Colonies after 6 Weeks (200 μg/ml)	Resistant Colonies after 8 Weeks (400 μg/ml)
(μg/ml)	d			
100	0	34	1	1
100	3	>1000	>100	None
200	3	>400	>100	2
200	5	>10000	>1000	>100
400	5	>200	>100	>50

Table II. *Sensitivity and Selection of Hygromycin Resistant Callus Lines from TM Protoplasts Electroporated with pMON410 Plasmid DNA*

Hygromycin (100–300 μg/ml) was added at various times after electroporation (0–5 d). Cell colony formation was estimated at 8 d after culture initiation. At least 3 × 10^6 protoplasts were used for each treatment. At the time of dilution the concentration of hygromycin was increased to 200 or 300 μg/ml. No resistant colony developed from the control cultures without electroporated DNA. For large numbers of colonies estimates are given.

Hygromycin	Application of Hygromycin (time after electroporation)	Surviving Cell Groups after 8 d	Resistant Colonies after 6 Weeks	Resistant Colonies after 8 Weeks (300 μg/ml)
(μg/ml)	d			
100	0	100	50	2
100	3	<400	64	3
200	0	None	None	None
200	3	34	8	None
200	5	<200	>100	1
300	3	30	1	1
300	5	>200	49	1

those found to inhibit cell growth in dicots (5). Methotrexate inhibited both at the protoplast and cell level which suggests that this marker may be an efficient selection agent in the Gramineae.

Using culture conditions which were completely growth inhibitory to wild type cells, hygromycin resistant TM and methotrexate resistant PM colonies were isolated following electroporation with plasmid DNAs of pMON410 or pMON806, respectively. Of the resistant cell lines tested by Southern hybridization analysis, the majority showed regions of homology with the electroporated plasmid DNA that varied in copy number from 1 to 10 per cell. Resistant colonies of TM and PM all showed different banding patterns indicating independent sites of insertion of the plasmid DNAs.

Three resistant colonies were isolated when the protoplasts were electroporated with the kanamycin resistant vector pMON273. Early Southern analysis showed regions of homology when probed with plasmid DNA 3 months after isolation; however, 12 months later, one line tested (TMB3) was negative. This could indicate that a mixture of transformed and wild type cells was present in the original isolate and during subsequent subculture the transformant was overgrown by the naturally kanamycin resistant wild type cells. Alternatively, the plasmid DNA was either unstably integrated or lost by chromosomal breakage or rearrangement. Due to the high levels of kanamycin resistance in wild type cell suspensions, the ability to selectively maintain functionally integrated DNA in prolonged cell culture could be a problem.

For highly kanamycin resistant species, such as those of the Gramineae, hygromycin or methotrexate resistance vectors provide alternative selection methods for the isolation of stable transformants.

Direct DNA transfer techniques allow the introduction of DNA into essentially any protoplast culture system (8). Since protoplasts have been isolated and cultured from a variety of plant species where a serious effort has been made (1), this technique has broad applicability for the evaluation of selectable markers *in vitro*.

The regeneration of plants from protoplasts in the Gramineae has been independently reported in a number of laboratories (25) and this, coupled with efficient and unambiguous selectable markers, may make the production of transgenic plants through embryogenic cell and protoplast culture an obtainable goal.

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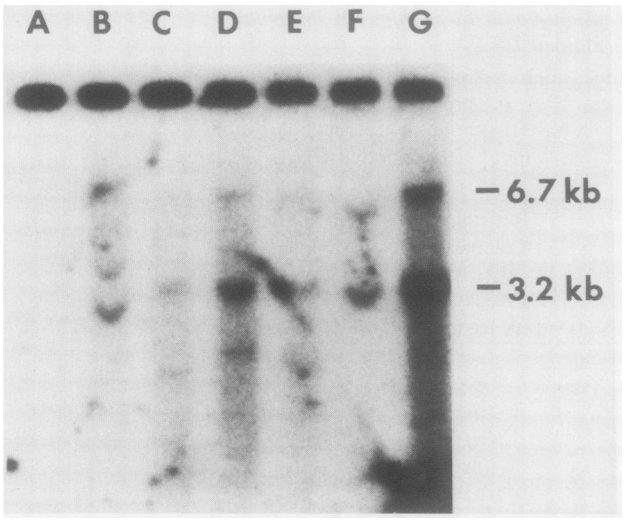

FIG. 2. Southern hybridization analysis of hygromycin resistant TM. High mol wt DNA was isolated from hygromycin resistant and control colonies of TM. DNA (15 μg) was digested with *Bam*HI, separated on agarose gels, transferred to Gene Screen, and probed with nick translated pMON410 plasmid DNA as described in "Materials and Methods." Lane A, wild type TM; lanes B to G, hygromycin resistant TM; B, TMA; C, TMA2; D, TMA3; E, TMA4; F, TMA5; G, TMA6.

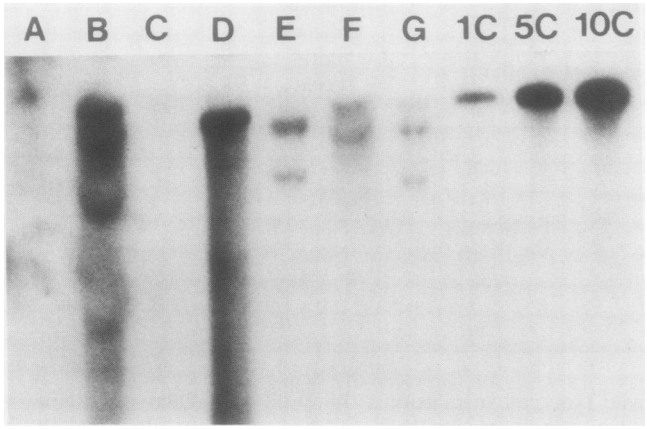

FIG. 3. Southern hybridization analysis of methotrexate resistant PM. High mol wt DNA was isolated from methotrexate resistant and control colonies of PM. DNA (15 μg) was digested with *Bam*HI, separated on agarose gels, transferred to Gene Screen and probed with nick translated pMON806 plasmid DNA as described in "Materials and Methods." A, Wild-type PM; lanes B to G methotrexate resistant PM; B, PMI13; C, PMI28; D, PMI39; E, PMII37; F, PMI36; G, PMII36. Copy number reconstructions using *Bam*HI digested pMON806 plasmid DNA corresponding to numbers of haploid genomes: 1C, 5C, 10C.

LITERATURE CITED

1. BINDING H 1986 Regeneration from protoplasts. *In* IK Vasil, ed, Cell Culture and Somatic Cell Genetics of Plants, Vol 3. Academic Press, Orlando, pp 259–274
2. CHURCH GM, W GILBERT 1984 Genomic sequencing. Proc Natl Acad Sci USA 81: 1991–1995
3. DUDITS D, G HADLACZKY, E LEVI, O FEJER, Z HAYDU, G LAZAR 1977 Somatic hybridization of *Daucus carota* and *D. capillifolius* by protoplast fusion. Theor Appl Genet 51: 127–132
4. DUJARDIN M, W HANNA 1984 Cytogenetics of double cross hybrids between *Pennisetum americanum-P. purpureum* amphiploids and *P. americanum* × *Pennisetum squamulatum* inter-specific hybrids. Theor Appl Genet 69: 97–100
5. EICHHOLTZ D A, S G ROGERS, RB HORSCH, H J KLEE, M HAYFORD, N L HOFFMANN, S B BRAFORD, C FINK, J FLICK, K M O'CONNELL, R T FRALEY 1987 Expression of a mouse dihydrofolate reductase gene confers methotrexate resistance in transgenic petunia plants. Somatic Cell Mol Genet 13: 67–76
6. FROMM M, L P TAYLOR, V WALBOT 1985 Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82: 5824–5828
7. FROMM M E, L P TAYLOR, V WALBOT 1986 Stable tranformation of maize after gene transfer by electroporation. Nature 319: 791–793
8. HAUPTMANN R M, P OZIAS-AKINS, V VASIL, Z TABAEIZADEH, S G ROGERS, R B HORSCH, I K VASIL, R T FRALEY 1987 Transient expression of electroporated DNA in monocotyledonous and dicotyledonous species. Plant Cell Rep 6: 265–270
9. HO W J, I K VASIL 1983 Somatic embryogenesis in sugarcane (*Saccharum officinarum*) L.: growth and plant regeneration from embryogenic cell suspension cultures. Ann Bot 51: 719–726
10. KAO, K N, M R MICHAYLUK 1975 Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta 126: 105–110.
11. KRENS F A , R A SCHILPEROORT 1984 Ti-plasmid DNA uptake and expression by protoplasts of *Nicotiana tabacum*. *In* IK Vasil, ed, Cell Culture and Somatic Cell Genetics of Plants. Vol 1. Academic Press, Orlando, pp 522–534
12. LLOYD A M, A R BARNASON, S G ROGERS, M C BYRNE, R T FRALEY, R B HORSCH 1986 Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. Science 234: 464–466
13. LORZ H, B BAKER, J SCHELL 1985 Gene transfer to cereal cells mediated by protoplast transformation. Mol Gen Genet 199:178–182
14. LU C Y, I K VASIL 1981 Somatic embryogenesis and plant regeneration from freely-suspended cells and cell groups of *Panicum maximum* Jacq. Ann Bot 48: 543–548
15. OU-LEE T-M, R TURGEON, R WU 1986 Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplasts of rice, wheat, and sorghum. Proc Natl Acad Sci USA 83: 6815–6819
16. POTRYKUS I, M W SAUL, J PETRUSKA, J PASZKOWSKI, R D SHILLITO 1985 Direct gene transfer to cells of a graminaceous monocot. Mol Gen Genet 199: 183–188
17. RIGBY P W J, M DIECKMANN, C RHODES, P BERG 1977 Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Microbiol 113: 237–251
18. ROGERS S G, K O'CONNELL, R B HORSCH, R T FRALEY 1985 Investigation of factors involved in foreign protein expression in transformed plants. *In* M Zaitlin, P Day, A Hollaender, eds, Biotechnology in Plant Science. Academic Press, Orlando, pp 219–226
19. ROGERS S G, H J KLEE, M BYRNE, R B HORSCH, R T FRALEY 1987 Improved vectors for plant transformation: expression cassette vectors and new selectable markers. *In* R Wu, L Grossman, eds, Methods in Enzymology, Recombinant DNA, Parts D and E. Academic Press, New York. In press
20. SHILLITO R D, J PASZKOWSKI, I POTRYKUS 1983 Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. Plant Cell Rep 2: 244–247
21. SHILLITO R D, M W SAUL, J PASZKOWSKI, M MULLER, I POTRYKUS 1985 High efficiency direct gene transfer to plants. Bio/Technol 3: 1099–1103
22. SHURE M, S WESSLER, N FEDOROFF 1983 Molecular identification and isolation of the waxy locus in maize. Cell 35: 225–233
23. SRINIVASAN C, I K VASIL 1986 Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L.). J Plant Physiol 126: 41–48
24. UCHIMIYA H, T FUSHIMI, H HASHIMOTO, H HARADA, K SYONO, Y SUGAWARA 1986 Expression of a foreign gene in callus derived from DNA-treated protoplasts of rice *Oryza sativa* L. Mol Gen Genet 204: 204–207
25. VASIL I K 1987 Developing cell and tissue culture systems for the improvement of cereal and grass crops. J Plant Physiol 128: 193–218
26. VASIL V, I K VASIL 1980 Isolation and culture of cereal protoplasts. Part 2: embryogenesis and plantlet formation from protoplasts of *Pennisetum americanum*. Theor Appl Genet 56: 97–99
27. VASIL V, I K VASIL 1981 Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). Ann Bot 47: 669–678
28. VASIL V, D Y WANG, I K VASIL 1983 Plant regeneration from protoplasts of Napier grass (*Pennisetum purpureum* Schum.). Z Pflanzenphysiol 111: 233–239
29. WALDRON C, E B MURPHY, J L ROBERTS, G D GUSTAFSON, S L ARMOUR, S K MALCOLM 1985 Resistance to hygromycin B. Plant Mol Biol 5: 103–108
30. WERR W, H LORZ 1986 Transient gene expression in a Gramineae cell line: a rapid procedure for studying plant promoters. Mol Gen Genet 202: 471–475