Theor Appl Genet (1985) 71:344–350

High frequency callus formation from maize protoplasts

S. R. Ludwig [1], D. A. Somers [2], W. L. Petersen [2], R. F. Pohlman [1], M. A. Zarowitz [2], B. G. Gengenbach [2] and J. Messing [1,*]

[1] Department of Biochemistry, [2] Department of Agronomy and Plant Genetics; University of Minnesota, St. Paul, MN 55108, USA

Received May 7, 1985; Accepted May 16, 1985
Communicated by D. von Wettstein

Summary. A solid feeder layer technique was developed to improve callus formation of Black Mexican Sweet maize (*Zea mays* L.) suspension culture protoplasts. Protoplasts were plated in 0.2 ml liquid media onto a cellulose nitrate filter on top of agarose-solidified media in which Black Mexican Sweet suspension feeder cells were embedded. Callus colony formation frequencies exceeding 10% of the plated protoplasts were obtained for densities of 10^3–10^5 protoplasts/0.2 ml, which was 100- to 1,000-fold higher than colony formation frequencies obtained for conventional protoplast plating methods such as liquid culture or embedding in agarose media. Compared with conventional methods, the feeder layer method gave higher colony formation frequencies for three independently maintained Black Mexican Sweet suspension lines. Differences among the three lines indicated that colony formation frequencies might also be influenced by the suspension culture maintenance regime and length of time on different 2,4-dichlorophenoxyacetic acid concentrations. The callus colony formation frequency reported is an essential prerequesite for recovering rare mutants or genetically transformed maize protoplasts.

Key words: Callus formation frequency – Corn – Feeder layer technique – Protoplasts – *Zea mays* L.

Introduction

Lack of a natural DNA vector system for maize has focused attention on development of other transformation methods. Transformation frequencies obtained by artificial DNA delivery methods, such as polyethylene glycol and liposome-mediated uptake, currently are quite low for protoplasts (Paszkowski et al. 1984; Dellaporta et al. 1981). Therefore, high frequency recovery of callus colonies from protoplasts is required for an artificial gene transfer system. Additionally, if natural systems such as *Agrobacterium tumefaciens* become adapted to maize, optimizing genetic transformation will be facilitated by high frequency recovery of callus colonies from maize protoplasts.

Callus formation from maize stem tissue protoplasts (Potrykus et al. 1977) has not proved repeatable (Harms 1982). Protoplasts isolated from cell suspension cultures originating from maize stem tissue protoplasts divide to form callus (Potrykus et al. 1979). Maize protoplasts isolated from C103 × W155 and Black Mexican Sweet non-morphogenic suspension cultures also divide to form callus at low frequencies (Kuang et al. 1983; Chourey and Zurawski 1981). Approximately 0.1% of C103 × W155 protoplasts gave rise to callus (Kuang et al. 1983). Chourey and Zurawski (1981) reported that nearly half of the Black Mexican Sweet maize protoplasts cultured became eliptical in shape, 30% of the eliptical cells formed a cell plate, and approximately 25% of the eliptical cells with a cell plate formed colonies. We have interpreted their results to depict a callus formation frequency of approximately 4%. However, callus formation frequency was less than 0.01% for Black Mexican Sweet protoplasts when the protoplast culture methods of Chourey and Zurawski (1981) were followed in our laboratory. Assuming a transformation frequency of 10^{-5} events per protoplast (Paszkowski et al. 1984) and maize callus colony recovery frequencies of 10^{-3}–10^{-4}, the recovery of transformed calli would be too low for a reasonable gene transfer experiment. Therefore, other culture methods were evaluated to improve callus formation frequency from maize protoplasts.

Increased callus formation frequency from Black Mexican Sweet maize suspension culture protoplasts was obtained by modifying the feeder layer technique described for culturing Black Mexican Sweet cells at low densities (Smith et al. 1984). Black Mexican Sweet

* To whom offprint requests should be addressed

feeder cells were plated in agarose-solidified medium and overlaid with a cellulose nitrate filter onto which protoplasts were plated. This technique improved callus formation 100- to 1,000-fold when protoplasts were plated at low (5×10^3/ml) and high (5×10^5/ml) densities.

Materials and methods

Cell lines

Black Mexican Sweet maize suspension cultures were derived from friable callus initiated from germinating seedling stem segments (Green 1977). Two independent subculture routines were followed. A suspension culture designated BM7 was maintained in MS media (Murshige and Skoog 1962) with 0.5 mg/l 2,4-D and 2% sucrose. The BM7 suspension cells were subcultured weekly by transferring 50 ml (ca. 3 g cells) into 200 ml fresh media in a 500 ml Erlenmeyer flask. A second suspension cell culture line, BM8 (obtained from Paul Anderson, Molecular Genetics Inc., Minnetonka, MN), was initiated in January 1982 from the BM7 culture. BM8 was maintained in MS medium with 2 mg/l 2,4-D and 2% sucrose and subcultured weekly by transferring 10 ml (ca. 0.3 g cells) into 100 ml fresh media in a 500 ml Erlenmeyer flask. Similarly, a third suspension cell line was initiated from the BM7 cell line 3 months prior to protoplast isolation. The third cell line was maintained on the same medium and culturing routine as the BM8 line. All cultures were incubated in the dark at 27 °C on a gyratory shaker at 120 rpm.

Protoplast isolation

Protoplasts were isolated 3–4 days following subculture. Approximately 1 g fresh weight of cells was added to 10 ml of filter sterilized enzymatic digestion medium (2% Cellulase, Worthington Diagnostics; 0.25% Pectinase, Worthington Diagnostics; 0.2 M mannitol; 80 mM $CaCl_2$–2 H_2O, pH 6.0) and incubated at 27 °C on a gyratory shaker at 65 rpm for 3 h. Protoplasts were filtered through a sterile 46 um stainless steel mesh filter (Bellco Glass) to remove undigested tissues. Protoplasts were collected by centrifugation (setting No. 1 on an IEC 428 International Clinical centrifuge for 5 min) and washed three times in 20 ml medium (PCM or PRM) (Table 1). All procedures were performed at room temperature.

Cell viability was determined by staining protoplasts with 0.01% fluorescein diacetate (Sigma) dissolved in 50% acetone (Widholm 1972). Cell wall material was detected by staining protoplasts with 0.1% Calcofluor White ST (American Cyanamid Co.) in 0.2 M mannitol (Nagata et al. 1970). Fluorescence indicated the presence of cell wall. Protoplasts were counted using a hemacytometer (American Scientific Products No. B3175).

Protoplast culture

For conventional plating methods protoplasts were cultured at a density of 5×10^5 protoplasts/ml immediately following isolation in: (1) 2 ml of liquid PCM or PRM, (2) 2 ml 0.5% low melting agarose (Bethesda Research Labs) PCM or PRM medium, or (3) 1 ml 0.5% low melting agarose PCM or PRM medium layered on 1 ml of 0.5% low melting agarose PCM or PRM medium (Table 1). All protoplasts plated with conventional plating techniques were cultured in 35 × 10 mm petri plates (Falcon 3001).

Table 1. Culture medium for Black Mexican Sweet maize protoplasts[a]

MS	mg/l	PRM	mg/l
KNO_3	1,900	PCM+....	
NH_4NO_3	1,650	Casein hydrolysate	200
$CaCl_2$–2 H_2O	440	Fructose	125
$MgSO_4$–7 H_2O	370	Ribose	125
KH_2PO_4	170	Xylose	125
Na_2 EDTA	37.24	Mannose	125
$FeSO_4$–7 H_2O	27.84	Cellobiose	125
$MnSO_4$–4 H_2O	22.30	Myo-inositol	100
$ZnSO_4$–7 H_2O	8.65	Citric acid	10
H_3BO_3	6.20	Malic acid	10
KI	0.83	Fumaric acid	10
Na_2MoO_4–2 H_2O	0.25	Sodium pyruvate	5
$CoCl_2$–6 H_2O	0.025	Nicotinamide	1.0
$CuSO_4$–5 H_2O	0.025	Pyridoxine HCl	1.0
Thiamine	0,5	Ascorbic acid	1,0
PCM		Choline chloride	0.5
MS+....		D-calcium pantothenate	0.5
2,4-D	2.0	Folic acid	0.2
Mannitol	54,700	Riboflavin	0.1
Sucrose	20,000	p-aminobenzoic acid	0.01
Glucose	250	Vitamin B_{12}	0.01
Coconut water (Gibco)	20 ml	Biotin	0.005
pH 5.8			

[a] MS from Murashige and Skoog 1962; PCM from Chourey and Zurawski 1981; PRM adapted from Kao and Michayluk 1975

For the feeder layer method protoplasts were cultured in 0.2 ml of media (PCM or PRM) at various densities on a 47 mm cellulose nitrate filter (0.45 um pore size, Whatman No. 7141 204 WCN) placed on 5 ml of solidified (0.8% low melting agarose) PCM or PRM in 60×15 mm petri plates (Falcon 1007). The solidified feeder layer was prepared with or without BM8 suspension cells at approximate densities of either 4×10^3 cells/ml (low density feeder) or 2×10^4 cells/ml (high density feeder). Suspension cells were allowed to settle, resuspended in PCM or PRM containing 0.8% low melting agarose cooled to 32 °C, poured into petri plates, and gelled at room temperature for 60 min. The high density feeder was the approximate density of a 3–4 day-old suspension containing approximately 500 colonies/ml with the average colony size of 40 cells. Culture dishes were wrapped with Parafilm and incubated at 27 °C in the dark under normal atmospheric conditions.

Callus maintenance

After 10 days, cells cultured on a feeder layer were transferred to fresh MS plates containing 2 mg/l 2,4-D and 2% sucrose. After 4 weeks calli originating from feeder layer cultured protoplasts and conventionally plated protoplasts were transferred to fresh MS plates containing 2 mg/l 2,4-D and 2% sucrose. Five colonies (0.25 g each) were transferred per 100×25 mm plastic petri plate (Lab-Tek No. 4026) containing 50 ml of solid media. Hereafter, callus was transferred monthly to fresh media and maintained on 2 mg/l 2,4-D MS plates.

Results

Characteristics of isolated protoplasts

A dedifferentiated, unorganized, rapidily proliferating suspension of Black Mexican Sweet maize, BM8 ("Materials and methods"), was used for isolating protoplasts (Fig. 1). Approximately 10^7 protoplasts per g fresh weight were isolated from the BM8 suspension cell culture. More than 90% of the cells were converted to protoplasts after a 3 h enzymatic cell wall digestion. Freshly prepared BM8 protoplasts were stained with fluorescein diacetate and at least 95% of the protoplasts were viable. Calcofluor white staining of cell wall material was not detected in association with the protoplast membrane in freshly prepared, filtered protoplasts. After 48 h in liquid protoplast regeneration media (PRM, Table 1), viability decreased to 60% while 85% of all protoplasts appeared to have totally regenerated cell walls. These results indicate that nearly all viable protoplasts formed cell walls, but approximately 30% of the protoplasts subsequently lost viability within the first 48 h.

Callus colony formation frequency

Several parameters were tested to determine whether the frequency of colony formation could be increased to a feasible level for gene transfer or mutant selection experiments. Callus colony formation frequency was obtained by counting individual callus colonies growing after 14 days (for feeder layer treatments) or 21 days (for conventional plating treatments) and dividing by the original number of total protoplasts plated. It was necessary to count callus colony formation frequency of feeder layer treatments at 14 days rather than at 21 days due to the rapid proliferation of protoplast-derived callus colonies. After 18 days of culturing, callus colonies plated on a feeder layer were too dense to count whereas conventionally plated callus colonies were not as dense (Fig. 2 a, b). After 4 weeks both treatments yielded callus which proliferated rapidly (Fig. 2 c, d). Each treatment was replicated three times in

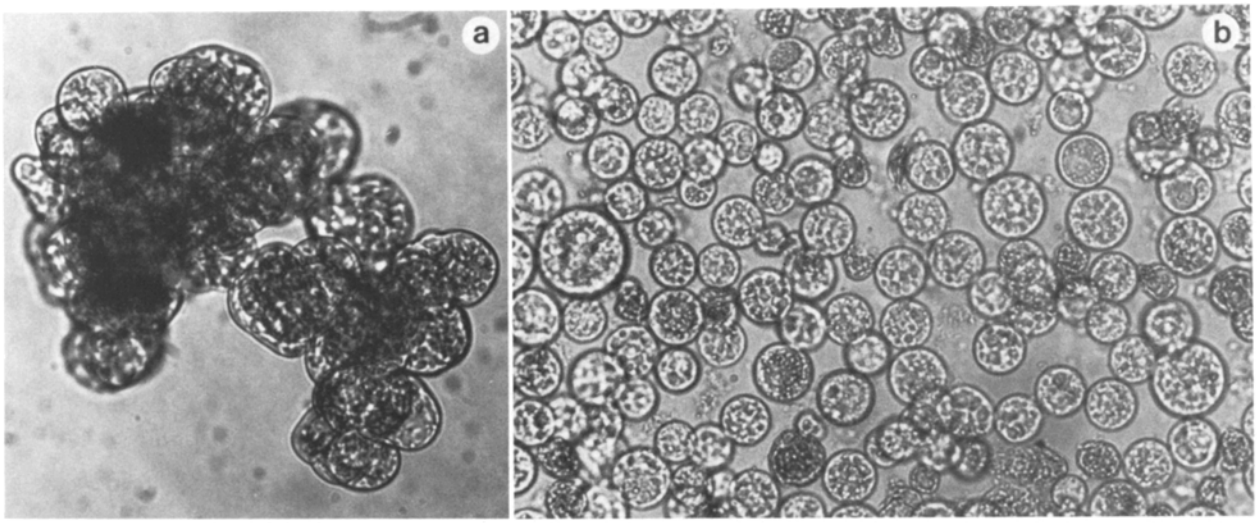

Fig. 1. a) A rapidly proliferating Black Mexican Sweet (BM8) maize suspension colony; **b)** Freshly isolated Black Mexican Sweet protoplasts (BM8)

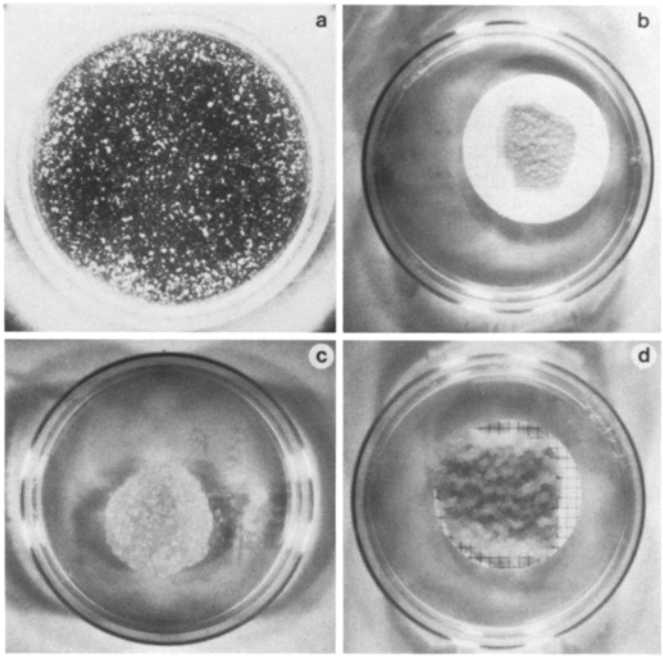

Fig. 2a–d. Callus formation from Black Mexican Sweet maize (BM8) protoplasts comparing conventional vs feeder layer plating techniques. **a** BM8 callus colonies 18 days after plating in low melting agarose with PRM; **b** BM8 callus colonies 18 days after plating on high density feeder layer in PCM; **c** Cell colonies after 4 weeks following plating in agar; **d** and plating on a high density feeder layer

three separate experiments. No significant variation was observed among experiments or replicates. However, due to the difficulty of identifying separate colonies in treatments with high protoplast plating densities and high callus formation frequencies, a minimum percentage of callus formation was recorded by counting apparent separate colonies. The actual percentage of callus formation may have been higher than estimated. Protoplast-derived callus colonies appeared similar to the BM8 suspension cultures and completely lacked morphogenetic and plant regeneration capabilities.

Conventional plating methods

Maize protoplasts were plated in liquid medium, in low melting agarose, or by mixing protoplasts with cooled molten agarose and plating over agarose-solidified medium (two agarose layers) according to Chourey and Zurawski 1981. Colony formation frequency due to the three conventional plating methods was less than 1% (Table 2). Protoplasts isolated from BM8 suspension cultures exhibited slightly higher callus formation frequencies than protoplasts from BM7 cultures maintained 3 months on 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in some treatments. Colony formation was not obtained for protoplasts from BM7 cultures maintained continuously on 0.5 mg/l 2,4-D. Conventional plating methods had little effect on colony formation frequency, but some improvement was obtained, especially for BM8 protoplasts, by using an enriched protoplast regeneration medium (PRM, Table 1) (Kao and Michayluk 1981) compared with minimal medium (PCM, Table 1) (Chourey and Zurawski 1981).

Feeder layer plating method

The method of Smith et al. (1984) was adapted for use with maize protoplasts. A sterile cellulose nitrate filter was used to separate the liquid protoplast culture layer from the lower feeder layer in which BM8 cells were embedded in agarose-solidified media. When a 0.2 ml aliquot of medium containing 10^5 BM8 protoplasts was placed on a filter, the liquid either evaporated or was absorbed quickly. When 1 ml of medium containing the same number of protoplasts/ml was placed on the filter, a residual amount of liquid remained throughout the culture period. Callus formation frequency was higher for protoplasts plated in 0.2 ml volumes compared to 1 ml volumes. This difference might have been due to improved oxygen exchange or to higher protoplast density in the smaller volume. Subsequent experiments with the feeder layer plating method were conducted with 0.2 ml sample volumes.

Callus colony formation frequency was at least 3% when 10^5 BM8 protoplasts were plated on filters of feeder plates prepared without feeder cells (Table 3). More importantly, another significant increase in frequency to more than 10% was obtained when BM8 feeder cells were incorporated into the feeder plate agarose (Table 3). No significant difference was observed between a low density feeder layer and a high density feeder layer containing approximately 4×10^3 cells/ml and 2×10^4 cells/ml, respectively. In contrast to the results obtained with conventional plating techniques, the enriched protoplast regeneration medium (PRM, Table 1) appeared to slightly decrease callus formation frequency compared with the minimal medium (PCM, Table 1).

Feeder cells were necessary to obtain callus colonies from protoplasts of BM7 suspension cultures (Table 3). Protoplasts derived from the BM7 line maintained on 0.5 mg/l 2,4-D formed callus when cultured with a feeder layer of BM8 cells, however, the callus formation frequency was 400-fold less than the frequency observed for protoplasts derived from BM8 suspension cells (Table 3). The BM7 and BM8 suspension culture lines have been maintained separately for 33 months on different 2,4-D levels and subculturing routines. Changing the BM7 line to the BM8 maintenance condition for

Table 2. Callus formation frequencies for maize protoplasts cultured with conventional plating techniques[a]

Treatment	Source of protoplasts		
	BM7 in 0.5 mg/l 2,4-D	BM7 in 2.0 mg/l 2,4-D	BM8 in 2.0 mg/l 2,4-D
PCM in liquid			
Av%	0	< 0.01	< 0.01
Low% to high%	0	0 to 0.01	0 to 0.01
PRM in liquid			
Av%	0	< 0.01	> 0.06
Low% to high%	0	0 to 0.01	> 0.05 to > 0.07
PCM w/ low melting agarose			
Av%	0	< 0.01	< 0.01
Low% to high%	0	0.005 to 0.011	0.003 to 0.012
PRM w/ low melting agarose			
Av%	0	0.016	> 0.18
Low% to high%	0	0.012 to 0.019	> 0.1 to > 0.2
PCM w/ 2 agar layer			
Av%	0	0	< 0.01
Low% to high%	0	0	0 to 0.01
PRM w/ 2 agar layer			
Av%	0	0	< 0.01
Low% to high%	0	0	0 to 0.01

[a] 5×10^5 protoplasts/ml were plated to examine plating techniques and media variations. The experiment was repeated three times with three replicates per treatment

Table 3. Callus formation frequencies for maize protoplasts cultured with a feeder layer technique[a]

Treatment	Source of protoplasts		
	BM7 in 0.5 mg/l 2,4-D	BM7 in 2.0 mg/l 2,4-D	BM8 in 2.0 mg/l 2,4-D
PCM w/ no feeder			
Av%	0	0	> 3.3
Low to high%	0	0	> 3.0 to > 5.0
PRM w/ no feeder			
Av%	0	0	> 3.0
Low to high%	0	0	> 3.0
PCM w/ low density feeder			
Av%	< 0.001	> 0.23	> 10.0
Low to high%	0 to 0.002	> 0.2 to > 0.3	> 10.0
PRM w/ low density feeder			
Av%	0	< 0.001	> 5.0
Low to high%	0	0 to 0.003	> 5.0
PCM w/ high density feeder			
Av%	0.023	> 0.44	> 10.0
Low to high%	0.017 to 0.03	> 0.4 to > 0.7	> 10.0
PRM w/ high density feeder			
Av%	0.017	> 0.42	> 10.0
Low to high%	0 to 0.027	> 0.4 to > 0.5	> 10.0

[a] 10^5 protoplasts were plated in 0.2 ml aliquots. BM8 suspension cells at 4×10^3 and 2×10^4 cells/ml were used for low and high density feeder layers, respectively. The experiment was repeated three times with three replicates per treatment

Table 4. Callus formation frequencies for maize protoplasts cultured at various plating densities[a]

Treatment	No. of protoplasts/0.2 ml				
	10^5	5×10^4	10^4	5×10^3	10^3
PCM + no feeder					
Av%	> 3.3	> 1.0	0.016	0	0
Low to high%	> 3.0 to > 5.0	> 1.0	0 to 0.05	0	0
PRM + no feeder					
Av%	> 3.0	> 1.0	0.022	0	0
Low to high%	> 3.0	> 1.0	0 to 0.05	0	0
PCM + low density feeder					
Av%	> 10.0	ND	> 10.0	14.5	10.5
Low to high%	> 10.0	ND	> 10.0	12.4 to 16.4	8.6 to 13.1
PRM + low density feeder					
Av%	> 5.0	ND	> 5.0	7.3	7.9
Low to high%	> 5.0	ND	> 5.0	5.4 to 8.4	7.1 to 8.9
PRM + high density feeder					
Av%	> 10.0	ND	> 10.0	> 10.0	18.3
Low to high%	> 10.0	ND	> 10.0	> 10.0	16.2 to 22.1

[a] BM8 protoplasts were plated in 0.2 ml aliquots on cellulose nitrate filters placed on an agar layer with and without BM8 feeder cells. The experiment was repeated three times with three replicates per treatment

3 months improved the callus formation frequency 20-fold over the frequency for protoplasts isolated from the original BM7 suspension culture maintained continuously on 0.5 mg/l 2,4-D (Table 3). The callus formation frequency for the BM7 suspension culture maintained on 2.0 mg/l 2,4-D for 3 months, however, was still 20-fold less than that for the BM8 suspension culture.

A protoplast gene transfer system with low transformation rates requires high survivability of micro-calli at low callus densities. Therefore, the effect of the feeder layer on callus formation frequency at low protoplast plating densities was examined. Callus formation frequency of BM8 protoplasts plated without feeder cells was reduced as plating density decreased (Table 4). No callus formation was observed when fewer than 5×10^3 protoplasts/0.2 ml were plated. When the feeder layer contained 4×10^3 BM8 suspension culture cells per milliliter of agarose-solidified PCM, an average callus formation frequency of 10.5% was observed with 10^3 protoplasts/0.2 ml. A slight decrease in average callus formation frequency to 7.9% was observed when the feeder layer contained PRM. Increasing feeder layer density to 2×10^4 cells/ml enhanced callus formation frequency 2-fold to 18.3% for 10^3 protoplasts (Table 4).

Discussion

Previous attempts to develop a maize protoplast culturing system have relied on conventional protoplast plating techniques (Chourey and Zurawski 1981), which when tested in our laboratory, resulted in low callus formation frequencies (Table 2). Because maximal cell division of protoplasts from BM8 suspension cultures (Table 4) and other plant species is dependent upon cell density (Shneyour et al. 1984), a feeder-layer technique which consisted of plating protoplasts on cellulose nitrate filters over actively growing suspension cells was developed to increase the effective cell density and protoplast colony formation. This solid feeder layer plating technique increased the Black Mexican Sweet maize callus formation frequency 100- to 1,000-fold over the conventional plating techniques tested. The solid feeder layer technique did not require the special support apparatus described for the liquid feeder layer technique (Smith et al. 1984), thus reducing handling time and the probability of contamination. The cellulose nitrate filter acted as a physical barrier that prevented cross contamination of the cell culture layer from feeder cells and facilitated subculture of protoplast derived callus colonies without disrupting the protoplast callus layer. This will simplify further evaluations of culture media and plating methods for increased callus formation as well as the selection for mutant and transformed protoplast derived callus colonies.

Suspension culture variation

Callus formation frequencies of protoplasts isolated from three independently cultured suspension cell lines of Black Mexican Sweet were compared to examine the possible effects of 2,4-D concentration. The BM7 line cultured continuously in 0.5 mg/l 2,4-D had very low callus formation frequency. After BM7 suspension cells had been cultured on 2 mg/l 2,4-D for

3 months, the callus formation frequency improved slightly. However, the frequencies were not as high as for the BM8 suspension cell line which had been subcultured from the BM7 line and cultured on 2 mg/l 2,4-D for 33 months. A long term effect of 2,4-D or the slighty modified subculturing routine could possibly explain the increased callus formation frequency of BM8. Adaptation of the BM8 suspension cell line to yield high callus formation frequencies might also have been a rare and/or random event and might not be obtained with other Black Mexican Sweet suspension cultures maintained under the same conditions.

Progress towards gene transfer in maize

Various techniques exist for the genetic transformation of dicotyledonous plants. Paszkowski et al. (1984) reported the transformation of *Nicotiana tabacum* protoplasts with a hybrid gene in the presence of polyethylene glycol at a frequency of 10^{-5}. Another study has shown that the Ti-plasmid can be encapsulated in liposomes to transform protoplasts at a frequency of less than 10^{-6} (Dellaporta et al. 1981). The most common method of plant transformation employs *Agrobacterium tumefaciens* to transfer and stably integrate T-DNA into the plant genome (Zambryski et al. 1984). Although, *A. tumefaciens* may be useful in the transformation of some monocotyledonous plants (Hooykaas-Van Slogteren et al. 1984), no natural host-vector system is presently available for the transformation of maize. Therefore, the best current prospects for developing a gene transfer system for maize rely on uptake and stable maintenance of exogenous DNA by protoplasts that can be cultured to callus. Our previous attempts to transform Black Mexican Sweet protoplasts were limited by low callus formation frequencies of 0.1% (unpublished results). When the callus formation frequency of 0.1% is coupled with the expected low transformation rate of 10^{-5} for the polyethylene glycol DNA uptake method (Paszkowski et al. 1984), the possibility of detecting a transformed cell is extremely unfavorable, 10^{-8}. However, with the development of an efficient system giving protoplast to callus formation frequencies of at least 10%, the possibility of finding a transformant becomes more reasonable, greater than 10^{-6}.

We have presented here the first step in the development of tissue culture techniques for a maize gene transfer system. The next step will be regeneration of plants from protoplast-derived callus. Although the probability of regenerating Black Mexican Sweet callus is questionable, the above techniques should facilitate the development of an embryogenic maize suspension culture protoplast system which could be used to regenerate a plant. Additionally, a Black Mexican Sweet protoplast transformation system might indicate which vector constructions would be most efficient for transformation of maize.

Acknowledgements. We thank Kris Kohn and Maureen Lowe for their help in preparing the manuscript. We are grateful to Paul Anderson (Molecular Genetics Inc., Minnetonka, MN) for the gift of BM8 suspension cells. This work was supported by NIH grant No. GM31499 (JM and BGG) and Minnesota Agricultural Experiment Station project No. 0302-4813-56 (DAS). RFP is a recipient of a NSF post-doctoral fellowship in plant molecular biology (PCM-8312545). MAZ is a recipient of a DeKalb-Pfizer Plant Genetics post-doctoral fellowship.

References

Chourey PS, Zurawski DB (1981) Callus formation from protoplasts of a maize cell culture. Theor Appl Genet 59: 341–344

Dellaporta SL, Fraley RT (1981) Delivery of liposome-encapsulated nucleic acids into plant protoplasts. PMB Newslett 2:59–66

Green CE (1977) Prospects for crop improvement in the field of cell culture. HortSci 12:131–134

Harms CT (1982) Maize and cereal protoplasts – facts and perspectives. In: Sheridan WF (ed) Maize for biological research. University of North Dakota Press, Grand Forks, pp 373–384

Hooykaas-Van Slogteren GMS, Hooykaas PJJ, Schilperoort RA (1984) Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature 311:763–764

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta 126: 105–110

Kuang VD, Shamina ZB, Butenko RG (1983) Use of nurse tissue culture to obtain clones from cultured cells and protoplasts of corn. Fiziol Rast 30:803–812

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

Nagata T, Takebe I (1970) Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92:301–308

Paszkowski J, Shillito RD, Saul S, Mandak V, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722

Potrykus I, Harms CT, Lorz H, Thomas E (1977) Callus formation from stem protoplasts of corn (*Zea mays* L.). Mol Gen Genet 156:347–350

Potrykus I, Harms CT, Lorz H (1979) Callus formation from cell culture protoplasts of corn (*Zea mays* L.). Theor Appl Genet 54:209–214

Shneyour Y, Zelcer A, Izhar S, Beckman JS (1984) A simple feeder-layer technique for the plating of plant cells and protoplasts at low density. Plant Sci Lett 33:293–302

Smith JA, Green CE, Gengenbach BG (1984) Feeder layer support of low density populations of *Zea mays* L. suspension cells. Plant Sci Lett 36:67–72

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol 47:189

Zambryski P, Herrera-Estrella L, De Block M, Van Montagu M, Schell J (1984) The use of the Ti plasmid of *Agrobacterium* to study the transfer and expression of foreign DNA in plant cells: new vectors and methods. In: Setlow JK, Hollaender A (eds) Genetic engineering, vol VI. Plenum, New York, pp 253–278