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Enrichment for Heterokaryocytes by the Use of Iso-osmotic Density Gradients after Plant Protoplast Fusion

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Summary. Buoyant density differences between different types of protoplasts were used in an iso-osmotic density gradient system to enrich protoplast fusion mixtures for heterokaryocytes. Protoplasts of maize stem and wheat mesophyll, as well as epidermis, stem parenchyma and mesophyll protoplasts of two amphihaploid, light sensitive tobacco mutants were fused with polyethylene glycol using conventional methods and a new rolling tube technique. The protoplast combinations used for fusion involved protoplast types with considerably different buoyant densities. Enriched fractions of maize-wheat heterokaryocytes of intermediate density were recovered which contained up to 31% mostly binucleate heterokaryocytes (a 2-7 fold relative enrichment). Tobacco heterokaryocytes recovered analogously from enriched intermediate fractions readily divided and gave rise to an increased number of light resistant calluses when compared with cultures from non-fractionated fusion mixtures. Maize-wheat fusion products, however, failed to divide.

Key words: Density gradient – *Nicotiana* – Protoplasts – Selection – Somatic hybridization

Introduction

Somatic hybridization via protoplast fusion is considered a powerful tool for both applied and pure genetic studies by providing possible 'alternatives to sex' (Pontecorvo 1975). Its potentialities in plants have been outlined in numerous reviews (Bajaj 1974; Holl 1975; Cocking 1976; Hess 1976; Bhojwani et al. 1977; Melchers 1977). Heterokaryon formation has been observed in a number of interspecific and intergeneric combinations (for a summary see Gamborg 1976). *Intra*specific somatic hybrid plants have been obtained in tobacco (Melchers and Labib 1974; Gleba et al. 1975; Belliard et al. 1977) and in *Datura* (Schieder 1977). *Inter*specific hybridization and regeneration of somatic hybrids have been achieved, involving sexually compatible species, in the genera *Nicotiana* (Carlson et al. 1972; Smith et al. 1976; Melchers 1977; Maliga et al. 1977), *Petunia* (Power et al. 1976, 1977; Cocking et al. 1977) and *Daucus* (Dudits et al. 1977). Recent successes in the culturing of protoplasts from corn (Potrykus et al. 1977) and potato (Shepard and Totten 1977; Butenko et al. 1977; Binding and Nehls 1977) may help extend somatic hybridization to agronomically more important species.

Successful somatic hybridization requires (1) the induction of protoplast fusion, resulting in heterokaryocyte and nuclear hybrid formation, (2) the survival and subsequent division of the fusion products, (3) the selective recovery of the hybrids, and (4) morphogenesis giving rise to hybrid plants. Interest focuses increasingly on the development of selection systems (Power and Cocking 1977). The first example of a somatic higher plant hybrid selected through mutant complementation was recovered by Melchers and Labib (1974) after fusion of protoplasts from two chlorophyll deficient, light sensitive tobacco mutants. In mosses, Schieder (1974) and Grimsley et al. (1977) have demonstrated the effectiveness of complementing auxotrophic mutants in selection systems. The obvious paucity of suitable recessive conditional lethal and dominant resistant mutants in higher plants has led to the proposal of various other procedures, including differential growth in different culture media, drug resistance (Cocking et al. 1974; Power et al. 1976, 1977), and culture of single, isolated fusion products (Kao 1977; Gleba and Hoffmann 1978).

In a previous paper we reported a technique for the fractionation of protoplast populations in an iso-osmotic density gradient system (Harms and Potrykus 1978). The present paper describes the application of this procedure to the enrichment, in protoplast fusion mixtures, for fusion products (heterokaryocytes).

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Materials and Methods

Protoplast Isolation

Mesophyll protoplasts were isolated from young leaves of 6 week-old greenhouse-grown plants of wheat (*Triticum aestivum* cv. 'Diplomat') according to the methods described previously (Lörz and Potrykus 1976). Protoplasts from maize (*Zea mays* L. cv. 'Inrakorn') stem internode tissue were isolated following the procedures reported by Potrykus et al. (1977). Protoplasts from dihaploid tobacco plants of the light sensitive mutants 'sublethal' (s) and 'virescent' (v) (Melchers and Labib 1974) were isolated as described previously (Harms and Potrykus, 1978). The mutant tobaccos were generously supplied by Prof. Melchers. Epidermis and stem parenchyma protoplasts were recovered from the enzyme solution by floatation on 0,6 M mannitol/0,56 M sucrose (3:1) and then washed in 0,254 M $CaCl_2$, pH 6,0. Mesophyll protoplasts were collected and washed by sedimentation at 50-100 g in 0,6 M mannitol. All procedures were carried out aseptically in an air-flow cabinet using filter-sterilized media. The osmotic pressures of all solutions (except the polyethylene glycol solution and the washing medium used for dilution after PEG treatment) were determined and adjusted to 660 ± 20 mOs/kg H_2O (osmotic pressure of 0,6 M mannitol) using a freezing point depression osmometer (Roebling, Berlin).

Fusion of Protoplasts

Protoplasts were suspended in 0,6 M mannitol + 5 mM $CaCl_2$, pH 6,0 (mesophyll protoplasts) or 0,254 M $CaCl_2$, pH 6,0 (maize stem internode protoplasts; tobacco epidermis and parenchyma protoplasts) and their concentrations were determined in a haemacytometer. The protoplasts were then mixed in equal numbers of each type to give suspensions of approx. 2-5 × 10^5 protoplasts/ml. The fusion combinations were: maize stem internode protoplasts with wheat mesophyll protoplasts; tobacco epidermis and stem parenchyma protoplasts of either mutant with mesophyll protoplasts from the other.

To induce fusion, protoplast mixtures were treated with 40-45% polyethylene glycol (PEG, MW 6000; Merck, Darmstadt) following the protocols of Wallin et al. (1974) and Kao (1975) (slightly modified): protoplasts (200-500 µl) were allowed to settle for 5 min on 18 × 18 mm cover slips placed in 60 mm petri dishes; about one half of the supernatant was pipetted off; 200-400 µl PEG solution were added dropwise; after 15-30 min at room temperature PEG was slowly diluted out by the dropwise addition, at 30 s intervals, of 15 drops of dilution medium (0,4 M mannitol + 10 mM $CaCl_2$, pH 10,5) or culture medium; the supernatant was again pipetted off after 5 min and replaced once by dilution medium (2 ml) and once by culture medium (2 ml). An alternative fusion procedure was devised and also used in these studies: several drops of PEG solution (see above) were placed into a glass centrifuge tube and the tube was rolled to form a thin film of PEG on the inner tube surface. During the rolling, drops of the protoplast mixture were added, thus dispersing the protoplasts in a thin layer over the PEG-coated tube. After 1 min the tubes were incubated stationary for 15-30 min at room temperature before PEG was slowly diluted out. Protoplasts were then washed and incubated in culture medium at 7° C for 1 h.

Density Gradient Fractionation of Protoplast Suspensions After Fusion

Protoplasts were suspended in KMC solution (equal volumes of 0,35 M *K*Cl, 0,245 M *Mg*Cl_2, 0,254 M *Ca*Cl_2, pH 6,0, 660 ± 20 mOs/kg H_2O) and placed on top of iso-osmotic KMC/S-density gradients. Details of the composition and the preparation of these gradients have previously been reported (Harms and Potrykus, 1978). Gradients were run at 20°C for 5 min at 50-100 g. The protoplasts from each interphase were carefully pipetted off using pasteur pipettes and examined under the microscope for heterokaryocytes. Heterokaryocytes were easily identified because of their content of both chloroplasts (from the mesophyll fusion partner) and cytoplasmic markers (from the epidermis and parenchyma type of protoplasts) and their number was determined. In some experiments maize nuclei were stained with acridinorange (0,01% in 0,254 M $CaCl_2$, pH 6,0) prior to fusion. Maize nuclei could thus be recognized in fusion products by their fluorescence. The suspension was adjusted to the population density required for optimal plating efficiency and washed once with culture medium before plating.

Culture and Selection of Isolated Protoplast Fractions

The protoplasts recovered from each of the gradient interphases were cultured separately in liquid medium. For cereal protoplasts a medium (P) was used which was developed specifically for cereal cultures (Potrykus et al. 1976). Tobacco protoplasts were cultured in NT medium (Nagata and Takebe 1971) containing 1,6 × 10^{-6} M p-chloro-phenoxy acetic acid (pCPA) and 2,5 × 10^{-5} M 6-furfuryl-aminopurine (kinetin) (Harms and Potrykus, 1978) up to the small colony stage when they were transfered, in thin layers to secure optimal oxygen supply, to agar-solidified NT medium with reduced (1/10) organic components and 0,2 M mannitol (Melchers and Labib 1974). After two weeks the tobacco cultures were incubated at 10.000 lX to select for high-light resistant hybrids (Melchers and Labib 1974).

Control Experiments

Several control experiments were run in parallel with the experiments described above:

1) Protoplasts from both parental types were cultured without fusion treatment.
2) Protoplasts from each parental type were cultured after homologous fusion using the fusion procedures described above.
3) Protoplasts from both parental types were fused (heterologous fusion) and cultured without gradient fractionation.
4) Protoplasts recovered from those gradient interphases not containing heterokaryocytes were cultured as viability and sterility controls.

Results

Fusion of Protoplasts

When fusing protoplasts of considerably differing buoyant density they tend to separate, thus escaping the intimate heterologous contact which is essential for fusion. In this respect, neither the fusion protocols of Wallin et al. (1974) nor of Kao (1975) were optimal. The rolling tube technique described was found to be more efficient. Table

Table 1. Effect of fusion procedures on protoplast aggregation and fusion (maize stem internode and wheat mesophyll protoplasts were fused as described in Materials & Methods)

	Procedure I[a]				Procedure II[b]			
	Time after addition of PEG				Time after addition of PEG			
	15 min[c]		85 min[d]		15 min[c]		85 min[d]	
	protoplast number	%	protoplast number	%	protoplast number	%	protoplast number	%
Total	990	100	1312	100	1756	100	1490	100
Single	763	77	946	72	726	41	928	62
Aggregated	227	23	366	28	1030	59	562	38
Fused[e]	21	2	51	4	178	10	122	8

(a) fusion performed in suspension according to Wallin et al. (1974)
(b) fusion performed according to the 'rolling tube technique'
(c) at the end of PEG treatment
(d) 60 min after washing out of PEG solution
(e) binucleated fusion products (homo- and heterokaryons) only

1 summarizes some results obtained with this technique and the one of Wallin et al. (1974). No data obtained with the method of Kao (1975) have been included in this comparison because of the obvious methodological differences which make direct comparison inconclusive. The figures given in Table 1 indicate that both a higher frequency of aggregation between protoplasts and a higher percentage of fusion were obtained with the rolling tube technique. The percentage of aggregates involving just two protoplasts (combinations of type AA, BB, and AB) was also improved from 46% to 61%. As a consequence, the percentage of binucleate fusion products (both homo- and heterokaryons) was 6-9% with the rolling tube technique (Table 1, procedure II) and 1-3% with procedure I (Wallin et al. 1974).

Density Gradient Enrichment for Heterokaryocytes

Fig. 1 demonstrates the density-dependent distribution profiles, in iso-osmotic KMC/S-density gradients, of two protoplast types which were used in fusion experiments described: maize stem internode protoplasts (average buoyant density: 1.033 g/cm^3) banded in interphases 2 and 3 (Fig. 1a); wheat mesophyll protoplasts (average buoyant density: 1.055 g/cm^3) banded in interphases 5, 6 and 7 (Fig. 1b). The average buoyant densities were determined under standardized conditions at 660 ± 20 mOs/kg H_2O and 20°C (Harms and Potrykus, 1978).

Figure 1c demonstrates the KMC/S-density gradient fractionation of a fusion mixture involving maize stem and wheat mesophyll protoplasts prepared with the rolling tube fusion procedure. Parental protoplasts can be seen banding in interphases 2 and 3, and 5 to 7, respectively. In addition, a faint band is present in interphase 4 in an intermediate density position. The protoplasts from each interphase were collected separately for further analysis. When examined microscopically, fusion products could easily be distinguished from parental protoplasts by their content of both chloroplasts (of mesophyll protoplast origin) and cytoplasmic markers (from stem internode

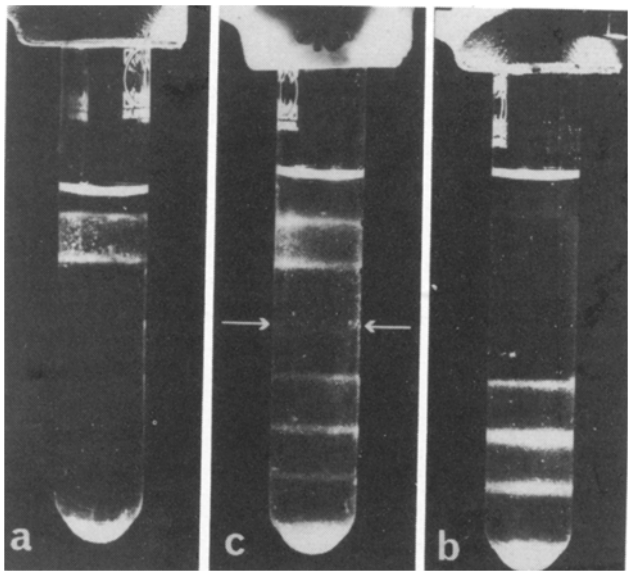

Fig. 1. Density-dependent fractionation profiles in iso-osmotic KMC/S-density gradients of maize stem internode protoplasts (a), wheat mesophyll protoplasts (b), and a mixed population of both types after fusion treatment (c). Note the appearance of a faint additional band in intermediate density position

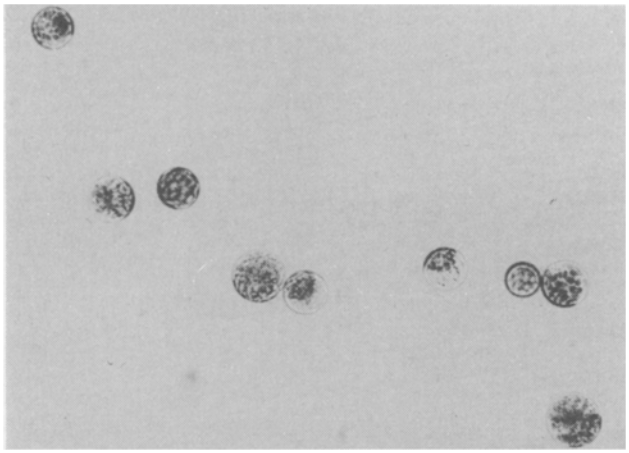

Fig. 2. Heterokaryocytes as recovered from gradient interphase 4 following gradient-mediated enrichment for heterokaryocytes originating from the fusion of epidermis and parenchyma protoplasts of mutant v with mesophyll protoplasts of mutant s

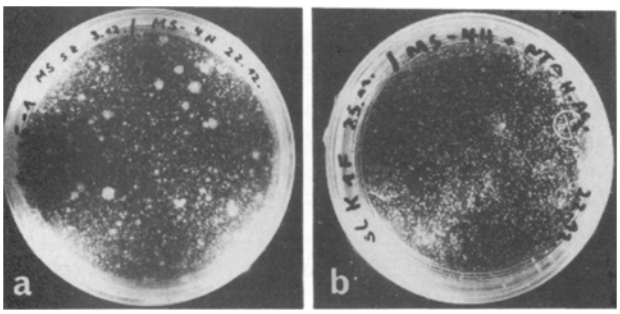

Fig. 3a, b. Appearance of light resistance tobacco colonies (a) under selective high light conditions in cultures derived from interphase 4 after gradient-mediated enrichment for heterokaryocytes originating from the fusion of mesophyll protoplasts of mutant v with epidermis and parenchyma protoplasts of mutant s. No light resistant colonies appeared in infused or homologously fused controls (b)

protoplast origin) (Fig. 2). Heterokaryocytes were found predominantly in interphase 4, indicative of their intermediate buoyant density. The content of heterokaryocytes in this particular fraction was up to 31%, depending upon the experimental conditions. When compared with the fusion mixtures prior to gradient-mediated fractionation and enrichment, the content of heterokaryocytes in the enriched fraction was increased by a factor of 2-7 (relative enrichment). Between 70 and 85% of the heterokaryocytes recovered from the intermediate interphase 4 were found to be binucleate rather than multinucleate (Fig. 2). About 70% of all heterokaryocytes recovered from the gradients were re-isolated from the intermediate interphase 4. Some were found in the adjacent fractions 3 and 4, and only very few were recovered from the other gradient fractions. The microscopical examination of both stained and unstained fusion products recovered from these fractions revealed that they were mostly multinucleated having irregular numbers of nuclei.

Similar results were obtained using tobacco protoplasts from epidermis and stem parenchyma, and mesophyll. These protoplast types were chosen because of their essentially differing average buoyant densities (mesophyll: 1.052 g/cm^3; epidermis and stem parenchyma: 1.030 g/cm^3) and their density-dependent fractionation profiles in iso-osmotic KMC/S-density gradients which are similar to those obtained with wheat mesophyll and maize stem protoplasts, respectively (Harms and Potrykus, 1978). The culture conditions for optimal plating efficiency had been worked out previously (Harms et al. in prep.).

Culture and Selection of Isolated Protoplast Fractions Enriched for Heterokaryocytes

The parental cereal protoplasts from both maize stem and wheat mesophyll regenerated a rigid cell wall within two days of culture in P2 medium. Under these conditions cell divisions have only been observed in a few cases when culturing parental protoplasts either separately (control 1) or after fusion (controls 2 and 3, and experiments involving gradient-mediated enrichment). When cultured in a modified P2 medium, however, maize stem internode protoplasts have been induced to undergo sustained divisions (Potrykus et al. 1977). Maize-wheat heterokaryocytes from both fractionated and control fusion experiments (control 3) also formed new cell walls but failed to divide regularly.

Tobacco fusion mixtures, involving epidermis and mesophyll protoplasts from either mutant, were also enriched for heterokaryocytes in iso-osmotic KMC/S-density gradients as was described above for maize-wheat heterokaryocytes. In cultures derived from intermediate density fractions (interphases 4, enriched for heterokaryocytes) and from control fusion experiments (control 3), mesophyll protoplasts and s + v heterokaryocytes readily regenerated into cell clusters, whereas the plating efficiency of the parental epidermis and parenchyma protoplasts was comparatively low (ca. 10%). When the protoplast-derived colonies are exposed to selective conditions at 10.000 lx (Melchers and Labib 1974) only those survive which have complemented to give the wild-type genotype exhibiting light resistance. Under these conditions, in cultures derived from gradient-enriched fractions (interphases 4) and from heterologous fusion controls (control 3), numerous colonies became light to dark green and continued growing (Fig. 3a). However, most of the colonies, originating from unfused parental protoplasts or homologous fusion

events, remained whitish and stopped growing. Light resistant colonies were found exclusively in cultures derived from gradient-enriched fractions and from heterologous fusion experiments (control 3). In a series of 5 experiments no such light resistant colonies could be detected in any of the controls (controls 1 and 2; Fig. 3b) run in parallel, indicating both the high specificity and effectiveness of the selection procedure and the non-occurence, in these experiments, of revertants. The number of light resistant colonies per petri dish (containing approx. 10-15.000 colonies) was 0-3 in heterologous fusion experiments without further enrichment for heterokaryocytes (control 3), but 20-35 per petri dish when the gradient-mediated enrichment procedure was employed. This was true for cultures derived from the intermediate interphases 4. However, the number of light resistant colonies was considerably lower in cultures originating from fractions 3 and 5 of the KMC/S-density gradients.

Discussion

In the absence of sufficient physiological and biochemical selection systems of more general applicability which remove undesired parental protoplasts and favour the selective development of heterologous fusion products, all methods leading to an absolute or relative enrichment for fusion products must be exploitated. The possibilities include more efficient fusion technologies as well as physical methods such as density gradient fractionation, amongst others. We focused our studies onto the improvement of fusion techniques and iso-osmotic density gradients as a means to enrich for heterokaryocytes.

A rolling tube technique for the fusion of protoplasts was developed which resulted in higher fusion frequency, and, more important, higher frequency of binucleate heterologous fusion products with high viability. Bilateral aggregation involving only two protoplasts was increased from 46% to 61%. Binucleate homo- and heterologous fusion products must be exploited. The possibilities in-procedure of Wallin et al. (1974) to 6-9% with the rolling tube technique. Presumably the restricted mobility of the protoplasts in the thin film in which they are moved over the PEG-coated tube surface makes contact and aggregation with other protoplasts more likely. This quasi-stationary state resembles the situation in the fusion method of Kao (1975), where protoplasts settle onto a glass surface prior to fusion, and contrasts with the freely suspended mixture characteristic of the method of Wallin et al. (1974).

The fractionation results described support the view that physical characters of protoplasts, such as their buoyant densities, can be used to select for fusion products. When maize and wheat protoplasts with considerably differing buoyant densities were fused and then fractionated in iso-osmotic KMC/S-density gradients, a separation of the mixed population was achieved which resulted in highly purified fractions of parental protoplasts and a fraction rich in heterokaryocytes. Most of the heterokaryocytes were recovered from a fraction of intermediate specific density. Compared to the non-fractionated fusion mixture prior to the gradient-mediated fractionation, there was not only a 2-7 fold relative enrichment for heterokaryocytes but also a preferential increase in the number of binucleate (AB type) fusion products. Possibly this type of fusion product may have some advantage in further development due perhaps to its more balanced gene dosage. The low frequency occurence of light resistant colonies in cultures derived from gradient fractions containing mostly multinucleated fusion products gives some preliminary indication to this view.

If protoplast types differ in their average buoyant densities by about 20 mg/cm^3 or more, they may be separated in iso-osmotic KMC/S-density gradients to give highly purified fractions (Harms and Potrykus, 1978). Overlapping of the fractions and contaminations by the respective other protoplast type are reduced when using protoplasts with sufficiently differing buoyant densities. Recent preliminary experiments indicate that the heterokaryocyte content of the intermediate density fraction may be increased by the use of previously purified parental protoplast fractions with more distinct buoyant density rather than using whole protoplast populations which exhibit a more diffuse buoyant density distribution. In those cases where protoplast types of sufficiently differing buoyant densities are not readily available, the buoyant density of one type may be altered experimentally (Harms and Potrykus, in preparation).

Besides the microscopical examination of the fractions obtained by gradient-mediated enrichment for heterokaryocytes, a second approach was used to identify the somatic hybrids. To study whether or not the enrichment for heterokaryocytes by means of iso-osmotic density gradient fractionation actually increased the recovery of developing somatic hybrids, protoplasts from two complementing chlorophyll-deficient, light sensitive amphihaploid tobacco mutants were fused and fractionated. Upon sexual or somatic hybridization, these mutants complement to give green and light resistant hybrids of wild-type genotype. Somatic hybrids can thus be identified and recovered at an early stage when developing calluses are exposed to high light intensities. This selection system allowed a rapid estimation of the number of fusion products originating from the enriched gradient fractions which developed into light resistant colonies of presumed somatic hybrid origin. The results obtained indicate that the enrichment for heterokaryocytes by means of iso-osmotic density gradient fractionation was correlated with

an significant increase in the number of selected light resistant colonies.

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