Biochimica et Biophysica Acta, 1025 (1990) 87–93
Elsevier

BBAMEM 74884

Facilitated electrofusion of vacuolated × evacuolated oat mesophyll protoplasts in hypo-osmolar media after alignment with an alternating field of modulated strength

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(Received 29 November 1989)

Key words: Electrofusion; Hypo-osmolar medium; Dielectrophoretic alignment; Evacuolated protoplast; (*A. sativa*)

Electrofusion of evacuolated and vacuolated oat leaf protoplasts is difficult because of the different size and density of these cells which results in separation of the two fusion partners during dielectrophoresis. The fusion yield of this cell system was considerably enhanced by electrofusion in hypo-osmolar media containing 0.4 M mannitol, 0.1 mM calcium acetate and 0.1% bovine serum albumin. This increase in yield was only achieved if the dielectrophoretically induced membrane contact between the two fusion partners was enhanced by an initial short 'burst' of higher field strength (500 V/cm, peak to peak, for 5 s followed by a reduction of to 90 V/cm, peak to peak, for 20 s, frequency 1 MHz). Due to the high field strength of the alternating field at the beginning of cell chain formation separation of fusion partners of different size and density was mainly avoided. Simultaneously, the short duration of this high field 'burst' avoided the generation of lethal effects in the cell membranes. The subsequent low field strength of the alternating field was sufficient to keep the aligned cells in position. Optimum fusion was induced by a single square pulse of 750 V/cm and 30 μsec duration. The time required for rounding up of the heterologous fusion products decreased with decreasing osmolarity. Fusion resulted in a 5.7 ± 1.2% yield of heterologous fusion products (compared to 0.7% using the conventional electrofusion protocol) as determined by flow cytometric assay. About 50% of the vacuolated oat protoplasts and 20–50% of the heterologous fusion products regenerated their cell walls within 5 days after hypo-osmolar treatment, but no cell divisions could be observed. Evacuolated oat protoplasts died after 2–3 days in culture without any detectable cell wall regeneration.

Introduction

The fusion of biological membranes is an essential element in somatic hybridisation and regeneration of entire plants [1]. Electrofusion has been extensively utilized in recent years for somatic hybridisation of plant protoplasts [2–9], because of its simplicity, of the possibility of optical and physical control of the fusion process and the high fusion frequency. However, electrofusion and conventional fusion techniques have one problem in common: separation of generated hybrids from unfused parental cells in appropriate selection media often fails because of the lack of protoplasts with suitable genetic markers.

In order to overcome this problem Naton et al. [10] suggested the fusion of an evacuolated protoplast with a vacuole-containing protoplast followed by selection and purification of the interspecific fusion products by iso-osmolar density gradients. Because of large difference in the specific densities of evacuolated and vacuolated protoplasts, the interspecific fusion products have an intermediate density which results in separate bands in a suitable density gradient system. However, application of this technique to electrofused vacuolated and evacuolated tobacco mesophyll protoplasts showed that the yield of heterologous fusion products was very low (about 0.1% to 0.7% ([10], personal communication by Hampp, R.)). This was in contrast to electrofusion of vacuole-containing protoplasts, which resulted fusion frequencies of up to 80% [4]. Analysis of the experimental procedure revealed that the large difference in density and volume of the two types of protoplasts consid-

Abbreviations: ADB, 1,4-diacetoxy-2,3-dicyanobenzene; BSA, bovine serum albumin; DCH, 2,3-dicyanohydrochinon; FITC, fluorescein isothiocyanate.

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erably reduced the interspecific fusion frequency. Due to sedimentation and to the volume-dependence of the dielectrophoretic force, separation of the parental cells occurs, resulting in preferential alignment of protoplasts of the same type. In this communication we have solved this problem in the case of fusion of evacuolated with vacuolated oat protoplasts by modifications of the electrofusion protocol. The first modification is to use a hypo-osmolar fusion medium. As with mammalian cells [11,12] hypo-osmolarity facilitates fusion between differently sized cells. Second, instead of an alternating field of constant strength a modulated alternating field was used for dielectrophoretic alignment of the fusion partners in order to avoid segregation of the differently densed fusion partners. This modified electrofusion protocol resulted in a $5.7 \pm 1.2\%$ yield of evacuolated-vacuolated fusion products as shown by fluorescence labelling and flow cytometry.

Material and Methods

Plants

Seedlings from *Avena sativa* (cv. Flämingsnova, kindly donated from F. von Lochow-Petkus GmbH, Bergen, F.R.G.) were grown for 8 days in potting compost with a light/dark regime of 12/12 h. The temperature was adjusted to 23° C in the light period and 18° C in the dark. Pots were kept well watered, and the relative humidity was kept at 70%. Plants were harvested for protoplast preparation at the beginning of the light phase.

Protoplast isolation

Oat protoplasts were prepared under sterile conditions. Primary leaves were surface sterilized in 10% commercial bleach (Domestos, Lever, F.R.G.) for 2 h and subsequently washed three times with sterile water. Protoplasts were isolated and purified as described elsewhere [13]. Purified protoplasts were suspended in solution I containing 0.5 M mannitol, 1 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA). The pH was adjusted to 6 and the cell density to $5 \cdot 10^6$ per ml. Cells were counted in a Neubauer-type haemocytometer. The protoplasts were stored on ice until required.

Evacuolisation of fluorescence labeled protoplasts

Before evacuolisation protoplasts were labeled with fluorescein isothiocyanate (FITC) according to Ref. 14. Purified protoplasts were sedimented (10 min, $45 \times g$) and resuspended in solution II containing 0.5 M sucrose, 1 mM $CaCl_2$, 1 mM K_2HPO_4 and 0.1% BSA (pH 7, cell density $2 \cdot 10^6$/ml). Then 1 μl of a freshly prepared solution of FITC (5 mg/ml in ethanol) was added per 1 ml of protoplast suspension and the protoplasts were kept at 20° C for 2 h in the dark. After purification by sucrose-flotation [13], the fluorescence-labeled protoplasts were evacuolized according to Griesbach and Sink [15]. 5 ml of this suspension were diluted with Percoll (40 ml, Pharmacia, Lund, Sweden) containing 0.5 M mannitol, 30 mM $CaCl_2$, 5 mM Mes and 0.1% BSA (pH 6.8). Prior to use Percoll had been dialysed overnight against destilled water containing 0.1% activated charcoal and 0.05% Amberlite AG (type MB-1, mesh 20–50; Serva, Heidelberg, F.R.G.). In contrast to the original protocol of Griesbach and Sink [15] the cells were centrifuged at high speed in a vertical rotor (VTI 50, Beckmann, F.R.G.) to obtain short centrifugation times and better separation of evacuolized protoplasts from debris. After 15 min at $95\,000 \times g$ the intact evacuolised protoplasts were collected and diluted with 40 ml of solution I, centrifuged at $75 \times g$ for 10 min and washed twice with solution I. Evacuolized protoplasts were resuspended in solution I at a final density of $7.5 \cdot 10^5$ per ml. Eryrthrosin staining [16] showed that all evacuolized protoplasts were FITC-labeled without significant reduction of viability.

Electrofusion

For electrofusion evacuolated and vacuolated protoplasts were sedimented (5 min, $75 \times g$) and separately resuspended in fusion media containing variable concentrations of mannitol, 0.1 mM calcium acetate and 0.1% BSA (solution III). The osmolarity was determined using a Osmomat 030 (Gonotec, F.R.G.). Cell density of evacuolated and vacuolated protoplasts was adjusted to $1.5 \cdot 10^6$/ml and $1 \cdot 10^6$/ml, respectively. 120 μl of the vacuolated and 240 μl of the evacuolated protoplast suspensions were mixed and pipetted into a helical chamber manufactured in the workshop of the institute. The construction of the helical chamber has been described elsewhere [3,4]. In contrast to previous experiments gold wires (diameter 400 μm) were used which were wound parallel to each other 200 μm apart around the inner cylindrical tubus. The outer jacket of the helical chamber contained a planar window in order to monitor the fusion process under the microscope. The filled chambers were kept in a water bath at 20° C for about 1 min before use. Electrofusion was performed with a Biojet CF (manufactured by Biomed, Theres, F.R.G., and distributed by Braun, Melsungen, F.R.G.). Alignment of cells was achieved with an alternating field of 500 V/cm strength (peak to peak value) at a frequency of 1 MHz for 5 sec followed automatically by an alternating field of 90 V/cm strength (peak to peak value) at a frequency of 1 MHz for 20 s. Finally a square breakdown pulse of 30 μs duration was applied. The field strength of this pulse was varied between 500 V/cm and 1000 V/cm (see below). 60 min after the pulse the helical chamber was opened and the electrodes were rinsed with 200 μl of culture medium. The fused protoplasts were mixed with 1 ml culture medium containing 1.4% molten sea plaque agarose (FMC Marine

Colloids Division, Rockland, NY U.S.A.) at 37 °C and were plated on cell cultures dishes (36 × 10 mm, Nunc, Denmark).

Protoplast culture

The protoplasts were cultured at 20 °C in the dark. Survival of fused oat protoplasts was determined after culture in modified PKM medium [17]. The medium contained a lowered concentration of NH_4NO_3 (2 mM) and additionally 10 mM glutamine and 100 ml/l coconut water (Difco, F.R.G.). The osmotic pressure of the culture medium was adjusted to 530 mosmolar using mannitol. Cell wall regeneration was detected by Calcofluor white ST (0.1%) staining [18] using a Leitz epifluorescence microscope equipped with Leitz filter block A.

Identification of heterologous fusion products

For flow-cytometric identification of heterologous fusion products (evacuolated × vacuolated) a Fluvo II flow cytometer (Heka Elektronik, Lamprecht, F.R.G.) equipped with a hydrodynamically focussing coulter counter and mercury lamp excitation (Osram HBO 100/W2) was used [19]. The orifice was 80 μm × 70 μm and the current 50 μA. The sheath fluid (3–5 ml/min) was 160 mM KCl in 0.2 M mannitol. Usually, fusion yield was determined 2–3 h after fusion by simultaneous measurement of FITC-fluorescence and the relative cell volume [20] (Heka OTFF13 excitation filter: short pass 500 nm, long pass 450 nm, dichroic mirror 510 nm, and Heka OTFF2H emission filter band pass 515–545 nm for FITC measurement and long pass 570 nm for chlorophyll measurement, dichroic mirror 550 nm). Heterologous fusion products exhibited a diameter larger than about 30 μm and showed strong FITC-fluorescence. Chlorophyll-fluorescence was used as internal standard.

The electric cell sizer was calibrated by video-microscopic measurement of the mean diameter of at least 100 each viable vacuolated and evacuolated protoplasts.

Data were stored in list mode on a personal computer. The relative fluorescence intensity per cell was calculated using the Diagnos 1 program system [19].

For microscopic identification of heterologous fusion products in solidified culture medium, cells were stained with 0.1% neutral red. Protoplasts labeled with FITC were visualized with a Leitz Orthoplan fluorescence microscope (E. Leitz, Wetzlar, F.R.G.) equipped with epifluorescence illumination and filter block I2/3. Heterologous fusion products showed FITC fluorescence and red stained vacuoles. Heterologous fusion products could be clearly identified within the first 5 days after fusion.

Cell viability

Viability of cells was determined microscopically after staining with 0.1% erythrosin B (Serva, F.R.G.) [16] or by flow cytometry [19,20]. For flow-cytometric determination, 200 μl of unfused evacuolated, unfused vacuolated or fused mixed-protoplast suspension were mixed with 5 μl 1,4-diacetoxy-2,3-dicyanobenzene (ADB) (1 mg/ml in dimethylformamide). ADB is non-fluorescent and highly membrane permeable. In living cells it is cleaved by esterases to the strongly fluorescent product 2,3-dicyanohydrochinon (DCH), which is membrane impermeable. After 5 min in the dark at 22 °C the DCH-fluorescence in the living cells was measured (Heka OTFFOA exitation filter: band pass 340–380 nm, Leitz A and dichroic mirror 400 nm: Heka OTFF4H emission filter: band pass 415–445 nm, dichroic mirror 470 nm).

Chemicals

Unless otherwise stated, chemicals were from Merck (Darmstadt, F.R.G.) or Sigma (F.R.G.) and of the highest purity available.

Results

Electrofusion in hypo- (or hyper-) osmolar solutions requires adaptation of the field strength of the fusion pulse to the change in cell diameter with variation of the osmolarity of the fusion medium. To this end the average diameter of the evacuolated and vacuolated protoplasts were measured as a function of osmolarity by means of video-microscopy at a temperature of 20 °C to 25 °C. The results are shown in Fig. 1. Each data point represents measurements on at least 100 different cells. It is evident that the diameter of both differently sized fusion partners increased by about 10%

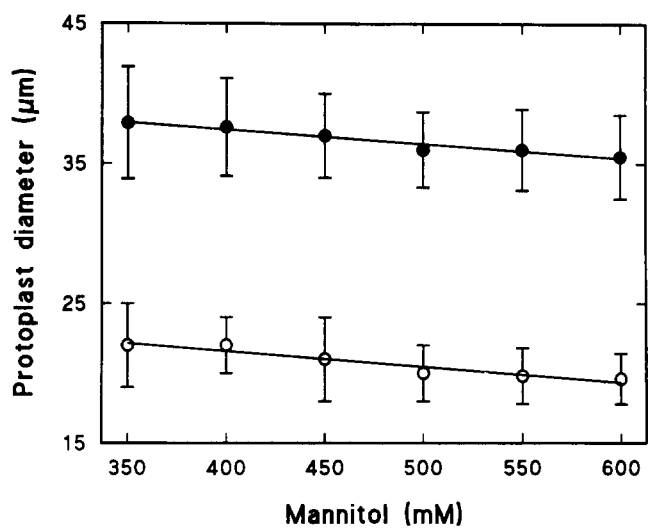

Fig. 1. Diameter of vacuolated (filled circles) and evacuolated (open circles) oat mesophyll protoplasts as a function of the mannitol concentration in the fusion medium (solution III). For each concentration the diameter of at least 100 protoplasts was determined by video-microscopy after 10 min incubation. The data are the mean (± S.D.) of five different protoplast preparations.

when transferred from iso-osmolar solutions (500 mM mannitol) to 350 mM mannitol solutions. Below 350 mM mannitol many of the vacuolated and evacuolated protoplasts burst. Usually, more than 50% of vacuolated oat protoplast subjected to osmotic stress for up to 30 min regenerated their cell walls within 5 days in culture medium provided that the osmolarity was not reduced below 400 mM mannitol. Cell division could not be detected and the cells died after 14 to 20 days. In contrast, evacuolated oat protoplast did not exhibit cell wall regeneration under the same conditions and died after 2–4 days in the culture medium.

Hypo-osmolar stress apparently increased the spontaneous fusogenity of the vacuolated and evacuolated protoplasts. After more than 30 min in media containing 400 mM mannitol, vacuolated protoplasts and/or evacuolated protoplasts tended to fuse to large multicellular aggregates. Homologuous as well as heterologous fusions were observed under this condition. The spontaneous fusion rate was neglible when the cells were incubated for only 10 to 30 min. In order to eliminate spontaneous aggregation and fusion dielectrophoresis was performed within 10 min after transfer of the cells into the hypo- (or hyper-) osmolar fusion medium. The dielectrophoretic force depends on cell volume [22]. Thus, application of an alternating field of constant strength (180 V/cm peak to peak at 1 MHz, see Refs. 10 and 21) resulted within a few seconds in a preferential alignment of large, vacuole-containing cells in chains emanating from the electrodes. The vacuolated protoplasts formed many long chains spanning from one electrode to the other and some of these cells burst (Fig. 2a). The (smaller) evacuolated protoplasts were slower and reached the electrodes only after 10 to 25 s, by which time the formation of the long chains of vacuolated protoplasts was almost complete. They

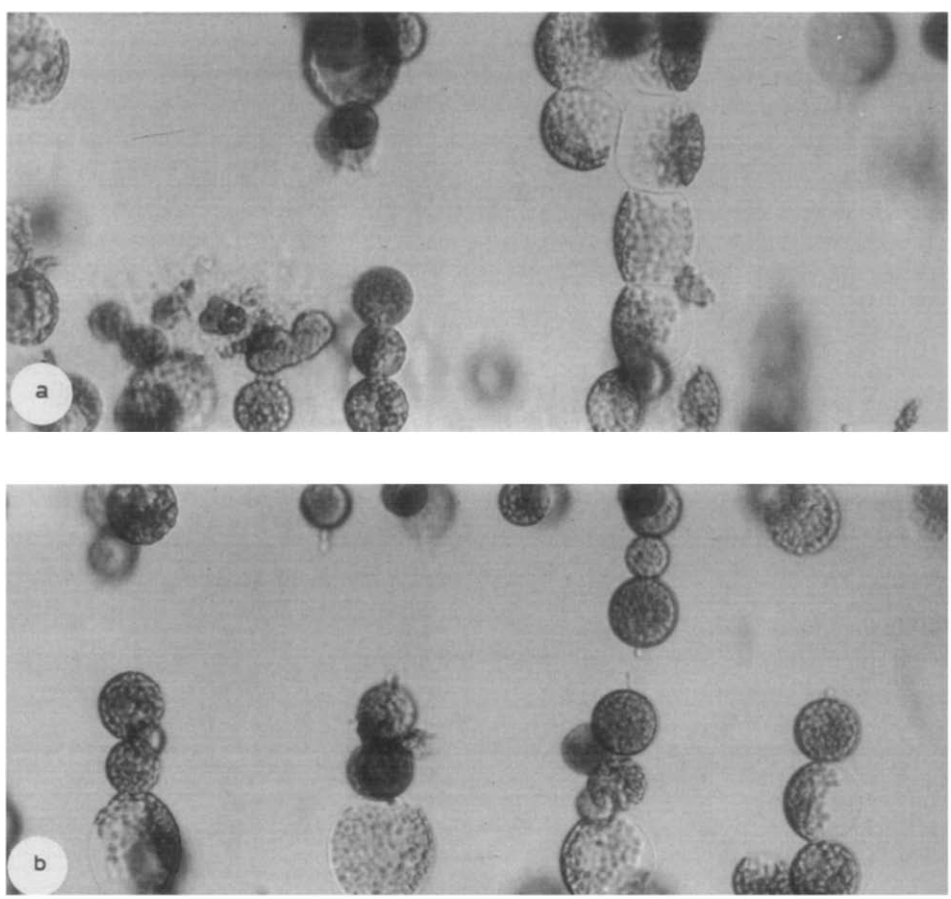

Fig. 2. Dielectrophoresis and cell chain formation in a mixture of vacuolated and evacuolated oat mesophyll protoplasts in an alternating electric field of constant (a) and of modulated (b) field strength. Vacuolised and evacuolised oat protoplasts were suspended in solution III containing 450 mM mannitol (ratio 1 : 3, total suspension density $1.3 \cdot 10^6$ cells/ml). Field conditions: (a) constant field strength of 180 V/cm (peak-to-peak value) and frequency 1 MHz; (b) field strength of 500 V/cm (peak-to-peak value) for 5 s followed by a reduction of the field strength to 90 V/cm (peak-to-peak value) for further 20 s, frequency 1 MHz. Photographs were taken 30 s after application of the electrical field; the magnification was in both cases ×300. Note that in contrast to an alternating field of modulated strength (b) a constant field strength (a) resulted in both long chains of vacuolated protoplasts spanning the electrode gap and in short chains of evacuolated protoplasts. Some of the vacuolated are damaged. Under condition (a) only one chain with heterologous cells was observed, whereas under conditions (b) four chains consisting of evacuolated and vacuolated protoplasts occurred.

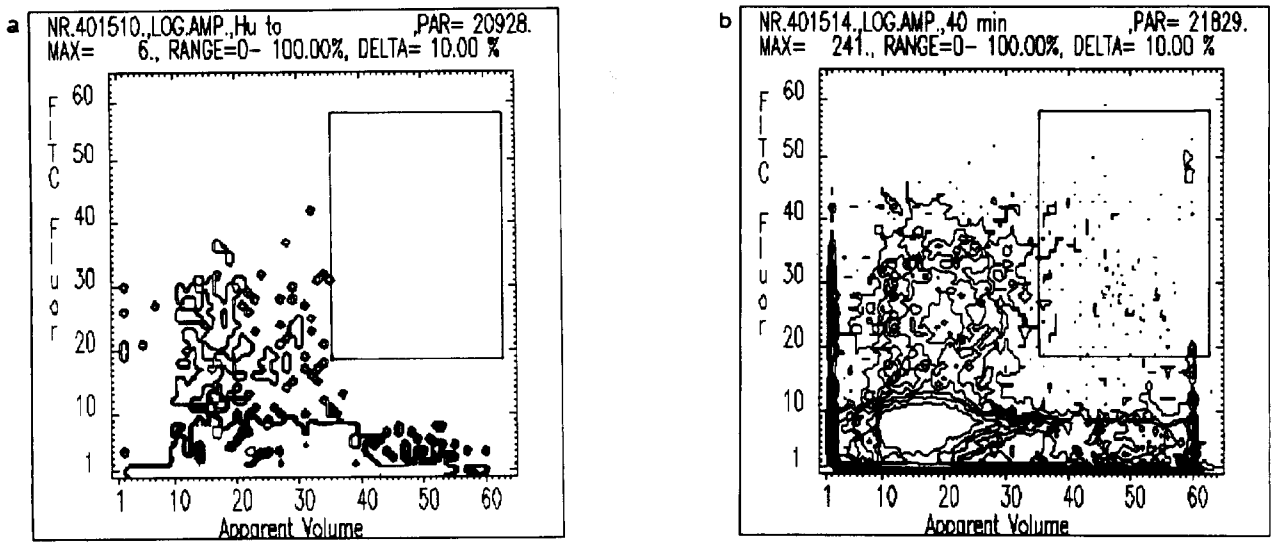

Fig. 3. Flow cytometry diagrams of the FITC-fluorescence intensity as a function of the relative cell volume (indicated as channel number) of a mixture of vacuolated and evacuolated (FITC-labeled) oat protoplasts before (a) and after (b) electrofusion. Vacuolated and evacuolated oat protoplasts (ratio 1:3, total suspension density $1.3 \cdot 10^6$ cells/ml) were suspended in solution III containing 400 mM mannitol and aligned by the modulated alternating field (see Fig. 2). Fusion was induced by the application of a single rectangular field pulse of 30 μs duration. The field strength of the fusion pulse was 750 V/cm in this particular experiment. Heterologous fusion products could be identified by their large size (channel number ≥ 35) and their FITC-fluorescence (section in Fig. 3b). In this particular experiment 1203 of the total 21829 protoplasts were identified as fusion products from vacuolated × evacuolated protoplasts. For further explanations, see text.

aligned mainly with each other, because there were only a few free vacuolated protoplasts left. In contrast, in the presence of an alternating field of an initial strength of 500 V/cm (peak-to-peak value) both vacuolated and evacuolated protoplasts moved towards the electrode at comparable high speeds. If the suspension density of the evacuolated protoplasts was made a factor of three higher than that of the vacuole-containing protoplasts, then chains consisting of almost equal proportions of the fusion partners formed within 5 s. Only very few cells burst during this short period of field application. In addition, subsequent cell viability tests showed that this high field strength did not result in lethal cellular or membrane effects provided that the duration was limited to 5 s. The length of the cell chains formed at this high field strength depended on the total suspension density. Screening experiments showed that optimum fusion yields were obtained when the suspension density of the evacuolated and the vacuolated protoplasts were adjusted to $1 \cdot 10^6$ and $3 \cdot 10^5$ cells/ml, respectively.

Subsequent reduction of the field strength of the alternating field to 90 V/cm (peak-to-peak value) was enough to maintain these chains and to keep the aligned cells in the correct position (Fig. 2b). Almost no formation of long chains spanning the electrodes was observed and only very few vacuolated protoplasts burst at this low field strength. The formation of short cell chains consisting of heterologous fusion partners in response to the modulated alternating field was observed over the whole osmolarity range between 400 and 700 mM mannitol. Fusion was initiated after 25 s by injection of a single field pulse of 30 μs duration. The radius-dependence of the critical field strength required for breakdown of the cell membranes (see footnote *) makes it difficult to predict the optimum field strength for fusion of two differently sized cells. On the one hand, the field strength must be high enough to permeabilise the membrane of the smaller fusion partner in the contact zone. On the other hand, it has to be considered that the larger cell may be irreversibly destroyed by the applied field strength. Due to the angular dependence of the generated voltage (see Laplace equation in the footnote) the membrane of the large cell will be permeabilised over a large area. If the ratio of the permeabilised area to the intact membrane surface is unfavourable the reversible membrane breakdown will become irreversible [2–4].

The optimum field strength was found empirically as followes. The critical field strength required for breakdown of membrane sites in field direction ($\cos \alpha = 0$) was calculated for the evacuolated and vacuolated protoplasts, respectively using the appropriate average values for the radii of these cells in the 400 mM mannitol fusion medium. These calculations yielded field strengths of 600 V/cm for the evacuolated and 350

* The generated membrane potential is given by the integrated Laplace equation: $V_g = 1.5 \cdot a \cdot E \cdot \cos \alpha$ [2], where a is the radius of a spherical cell, E the applied external electrical field and α the angle between the normal vector of a given membrane site and the direction of the external field vector.

V/cm for the vacuolated protoplasts. These values were multiplied with a factor of 1.5 because it is known that initiation of fusion requires 50% higher field strengths, due to the angular dependence of the breakdown voltage [2–4,17]. Taking these calculations into account the field strength was varied between 500 V/cm and 1000 V/cm and the fusion yield determined as a function of the osmolarity of the fusion medium. This was done by flow cytometry (Fig. 3 shows a typical flow cytometric recording). Evaluation of the flow cytometric recordings indicated that the yield of heterologous fusion products was very low (and variable) if the field strength of the fusion pulse was adjusted to 500 V/cm (Fig. 4). This result is expected if the average critical field strength for the evacuolated protoplasts is considered. It is obvious that with this field strength the breakdown voltage of only the very large protoplasts was exceeded. Under this condition maximum yield was obtained in fusion media containing 400 mM mannitol (Fig. 4). A dramatic increase in yield was observed when the fusion pulse was increased to 750 V/cm (Fig. 4). The rapid increase in yield of heterologous fusion products to a value of $5.7 \pm 1.2\%$ in 400 mM mannitol indicates that at this osmolarity optimum field conditions had been selected. Towards lower and higher osmolarities (up to 650 mosmolar) the yield decreased significantly. An average value of 5.7% for the yield of heterologous fusion products corresponds to an enhancement by a factor of about eight if we compare this result with that obtained using the protocol of Naton et al. [10]. For this model system conventional electrofusion only yielded 0.7% fusion products on average. Increase of the field strength to 1000 V/cm again resulted in a significant decrease of yield over the whole osmolarity range (Fig. 4), as predicted from the angular dependence of the generated membrane voltage. Under this field condition maximum fusion yield was obtained in media containing 500 to 550 mM mannitol. This is expected because of the decrease in cell radius in these solutions (see footnote p. 91). Therefore the field strength of 1000 V/cm is not as lethal as for cells incubated in solutions of 450 mM mannitol or less. It is also interesting to note that the time required for rounding up of fusion products decreased with increasing osmotic pressure of the fusion medium: in 400 mM mannitol fused protoplasts rounded up in less than 5 s, whereas under iso-osmolar conditions 10 to 20 s were required. In strongly hyper-osmolar solutions (700 mM mannitol) rounding required even longer (150 to 180 s). The viability and the capability for cell wall regeneration were investigated for a large number of fusion products obtained by application of a breakdown pulse of 700 V/cm to the fusion partners incubated in 400 mM or 500 mM mannitol solutions. The results showed that 20–50% of the heterologous fusion products were viable and regenerated their cell walls within 5 days.

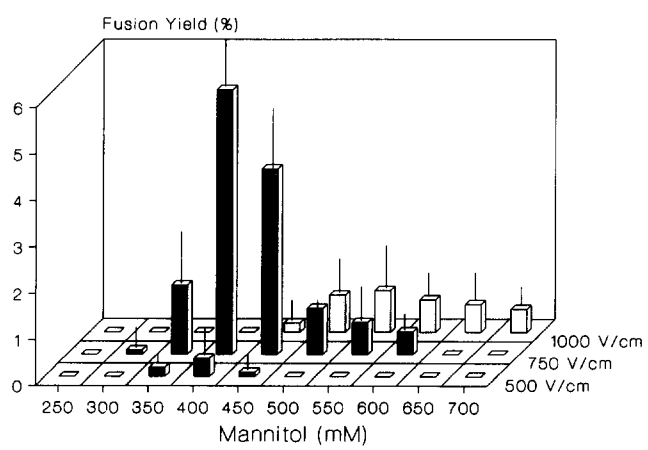

Fig. 4. Yield of heterologous fusion products after electrofusion as a function of the mannitol concentration in the fusion medium. A single fusion pulse of 500 V/cm (black columns), 750 V/cm (hatched columns) or 1000 V/cm (white columns) was applied. Otherwise the field conditions were the same as in Fig. 3. The yield of heterologous fusion products at a given osmolarity and field strength of the fusion pulse was determined after 2–3 h by flow cytometry. The data are the mean (± S.D.) of four experiments.

Discussion

The yield of heterologous fusion products could be considerably increased by combination of preferential alignment of vacuolated with evacuolated protoplasts and by fusion in hypo-osmolar media. Flow cytometric analysis of the cell suspension after electrofusion suggested an increase in yield by a factor of about eight compared to the value of 0.7% when using the experimental conditions reported by Naton et al. [10]. In conventional electrofusion membrane contact between freely suspended cells is achieved by an alternating field of moderate, but constant amplitude [2–5]. The field strength usually used for alignment of plant protoplasts was about 180 V/cm (peak-to-peak value [10,21] and the alignment times were in the range of 15 to 30 s. Field strengths higher than 250 V/cm resulted in irreversible changes in the membrane structure when applied for this period of time [2–4]. As shown by means of measurements of cell viability and cell wall regeneration, application of high field strengths for only a few seconds did not lead to detrimental side effects. However it is important to note that application of the modulated alignment field only increased the fusion yield by a factor of 2–3 when fusion was performed in iso-osmolar solution (Fig. 4). This indicates that additionally to the establishment of a membrane contact between cells of the two fusion partners changes in the membrane structure are required to facilitate the subsequent electric field-mediated fusion process. As with electrofusion (and electrotransfection) of mammalian cells [11,12] and vacuolated plant protoplasts [23] hypo-osmolarity of the medium apparently also in-

creased the fusogenity of plant protoplast membranes. This is evident both from the increase of the spontaneous fusion rate at high protoplast density and from the accelerated formation of a spherical fusion product once the breakdown pulse had been applied.

The significant increase in fusion products of evacuolated and vacuolated plant protoplasts is also interesting from another point of view. Recently, Mehrle et al. [24] reported a comparable increase in fusion yield form evacuolated and vacuolated protoplasts of *Nicotiana tabacum* electrofused in iso-osmolar solutions under short-term (5 min) microgravity conditions. In weightlessness sedimentation and convection effects are minimised. Therefore, when electrofusion is performed under microgravity, the establishment of membrane contact between cells of different size and density is enhanced. The elimination of gravitational effects is thus expected to enhance fusion yields to a similar extent (i.e., by a factor of 2–3) as has been observed after alignment with the modulated alternating field in iso-osmolar solutions (see also Refs. 25 and 26). The finding that the use of microgravity resulted in much higher yields of fusion products (even when performed in iso-osmolar solutions) suggests that microgravity resulted in changes of cellular and membrane properties which are comparable to those induced by hypo-osmolarity under 1 g conditions. This interesting conclusion has to be proved in future microgravity experiments. The yield reported here for the evacuolated/vacuolated protoplast system is large enough to apply this modified electrofusion technique to the fusion of other cells with considerable differences in specific density and volume, e.g., for fusion of protoplasts from embryogenic tissues with protoplasts from other tissues.

Acknowledgements

The authors are very grateful to Ms. J. Erb for her skilful technical assistance. This work was supported by grants of the Federal Ministery for Technology and Research (grant No. DFVLR 01QV354), the Deutsche Forschungsgemeinschaft (SFB 176) and of the Fond of the Chemical Industry to U.Z.

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