Electrofusion of spermine-treated plant protoplasts

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A new method for the fusion of plant protoplasts is described. It involves the application of short d.c. electric field pulses to protoplasts suspended in an osmotic medium containing defined concentrations of spermine (to induce close contact between protoplasts) and salts. When the physical parameters were set to optimal values, the yield of protoplast fusion was routinely 50%. Moreover, by adjustment of the experimental parameters, pair or multi-fusions can be favoured. The technique can be applied with equal success to the fusion of protoplasts from the same or from two different species and thus represents the first step in obtaining plant somatic hybrids.

Fusion *Electrofusion* *Plant protoplast* *Spermine*

1. INTRODUCTION

Hybrid production obtained by sexual crossing of species has long been used to increase the yield, disease resistance and nutritional qualities of plants. However, in many cases, the production of hybrids from widely divergent species is sexually impossible. Nevertheless, somatic hybridization involving fusion of protoplasts appears to be able to overcome this difficulty. This methodology was previously developed for cultured animal cells [1] and yeast protoplasts [2].

Protoplast fusion is now routinely obtained by treatment with poly(ethylene glycol) (PEG) used at high concentrations [3]. This method, despite spectacular success [4,5], presents many shortcomings: (i) the molecular process remains unknown and thus improvement of the technique is mainly empirical; (ii) multi-protoplast fusions, a phenomenon which remains uncontrolled; (iii) addition of an exogenous chemical to induce the fusion.

This last point is the major drawback in obtaining viable hybrids because it has recently been shown that the fusing agents are in fact the chemical additives present in commercial PEG, that are known to be highly cytotoxic [6].

During the past few years, a new technique, the so-called 'electrofusion method', has been described, in which the fusion is triggered by electrical pulses [7–10]. In this method the cells must be in close contact. This contact occurs spontaneously with some mammalian cells [11] and *Dictyostelium* [7]. In other cases, the presence of PEG [12] or preliminary treatment of the protoplasts or cells in a heterogeneous a.c. field [8,13] in an ion-free medium is necessary to induce aggregation.

This last technique has been described as very efficient for certain plant systems but totally unsuitable in other cases [14]. Taking into account the very successful results obtained in our laboratory in the case of mammalian cell hybridization [11], we have extended this method to plant protoplasts. A d.c. electric field is applied to protoplasts suspended in a medium containing selected ions. The presence of ions appears to be required to avoid any lethal side-effects linked to the electric pulsation and its associated membrane perforation [15].

As discussed above, the spontaneous contact occurring in mammalian cell cultures grown in

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Falcon flasks does not occur in the case of plant protoplasts. Recently, polyamines were described as inducing the aggregation of acidic phospholipid vesicles [16,17] at concentrations below 1 mM. Polyamines are widespread substances among living cells and sometimes appear in plants as biological regulators [18].

Here, we show that spermine (in the 1 mM range) mediates the aggregation of protoplasts of different species. Therefore, the application of a microsecond square-wave electric field in the 1 kV/cm range induces protoplast fusions in the presence of micromolar spermine. Most of the events involve the fusion of two protoplasts and are interspecific or intergeneric.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Cellulase R10 ONOZUKA was obtained from Kinki Yakult (Japan), pectolyase Y23 from Seishin (Japan) and pectinol D from Rohm and Haas (FRG). Pectin acid transeliminase (PATE) was a generous gift from Dr Meyer (University of Perpignan, France).

Mannitol and spermine tetrahydrochloride were purchased from Sigma (USA). Sorbitol and salts were obtained from Merck (FRG).

2.2. *Obtaining of protoplasts*

2.2.1. Plant material

Plants of *Nicotiana tabacum* cv Paraguay 48 were raised in a growth chamber. The photoperiod was 12 h and temperature 25°C during the light period and 19°C in the dark period.

Young but fully expanded leaves were selected from 4–8-week-old plants.

Cell suspension cultures of *Acer pseudoplatanus* and *Catharanthus roseus* were obtained as in [19–21].

2.2.2. Protoplast preparation

Tobacco mesophyll cells were treated following the enzymatic procedure of [22] except that the incubation medium was 5 mM Tris–Mes (pH 5.6) in 0.3 M mannitol and 0.3 M sorbitol.

For *Acer* and *Catharanthus* cell suspension cultures, digestion of the cell walls was carried out for 3 h at 36°C in a solution containing 4% cellulase, 0.1% pectolyase, 25 mM Tris–Mes (pH 5.6) and 0.7 M mannitol.

In all cases, after digestion, protoplasts were suspended in a medium consisting of 5 mM Tris–Mes (pH 5.6) and 0.45 M mannitol at 2×10^6 protoplasts $\cdot$ ml^{-1}.

2.3. *Aggregation of protoplasts*

Protoplasts were diluted with the above buffer to a final concentration of $1.25–5 \times 10^5$/ml. Different volumes of spermine were added from a stock solution (4 mM in the same buffer) to give a final concentration ranging from 0 to 2 mM.

Scoring of aggregation was performed under an inverted microscope after at least 10 min incubation at room temperature.

2.4. *Fusion procedure*

One hundred and fifty μl of the spermine-treated protoplast suspension were transferred to the pulsation chamber of an electrofuser (CNRS-ANVAR, project 50717, France) and were submitted to an electronically controlled short-duration homogeneous electric field. The field was generated by a high voltage (up to 1 kV) applied to two flat parallel stainless-steel electrodes at 5 mm distance. The shape and duration of the electric pulses were monitored with an oscilloscope.

At the start of the experiment, the temperature was 21°C and the temperature increase due to Joule heating was computed to be less than 3°C. The interval between repetitive pulses was at least 1 s to avoid heat accumulation.

In these experiments, the pulsation chamber was seated on the stage of an inverted microscope (Leitz, FRG) and the events, occurring in the protoplast suspension, video-monitored (JVC, Japan).

The yield of fusion was estimated from the direct observation of at least 250 treated protoplasts. Using this technique, we were able to discriminate between two and multi-protoplast fusion processes.

The color video-monitoring was useful in interspecific fusions to distinguish homo and hetero events in a mixture of differently colored protoplasts.

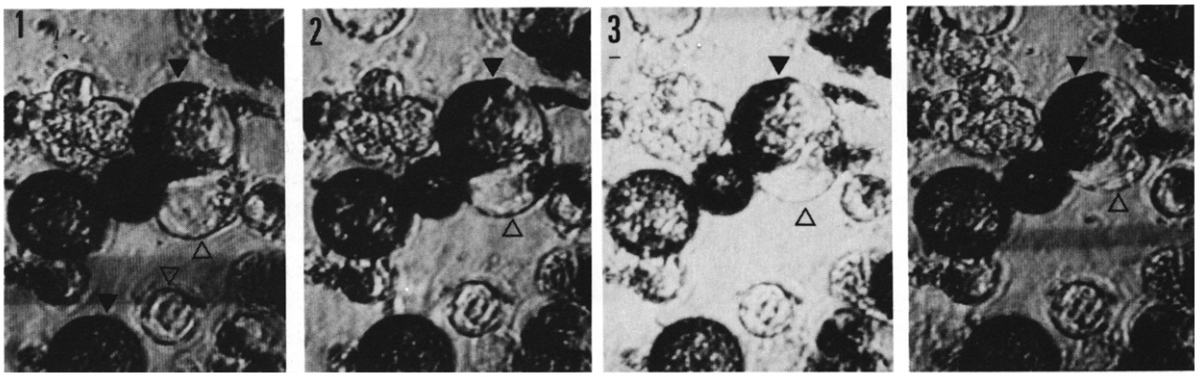

Fig.1. Microphotographs: evolution of the fusion process during the post-pulse incubation of protoplasts of *Nicotiana tabacum* (▲) and *Catharanthus roseus* (△). Pulsing conditions: 3 pulses of 1 kV/cm with a duration of 100 μs; 75000 protoplasts of a 1:1 mixture of *Nicotiana* and *Catharantus* in 150 μl pulsing buffer containing 1 mM $MgCl_2$ and 0.75 mM spermine. 1, 1 min; 2, 8 min; 3, 13 min; 4, 23 min after the pulses.

3. RESULTS

Unless otherwise stated, the optimal conditions leading to protoplast fusion were determined using tobacco protoplasts.

3.1. *Aggregation process*

The presence of spermine induces the formation of small protoplast aggregates. This phenomenon is observed under the microscope 10 min after the addition of 1 mM spermine. No morphological changes of the protoplasts were observed and spermine seemed only to induce close contact between the protoplasts and not to alter their membrane. This is also observed when protoplast suspensions from different species are mixed.

3.2. *Fusion process*

As shown in fig.1, electric pulses induce the fusion of spermine-treated plant protoplasts. The process starts as soon as the field is applied but the structural changes leading to the final spherical shape are slow (about 30 min at room temperature for the complete fusion of two protoplasts).

The fusion process depends on the amplitude, duration and number of pulses applied. Fig.2 shows the existence of a threshold of 500 V/cm (for a pulse duration of 100 μs) to induce the fu-

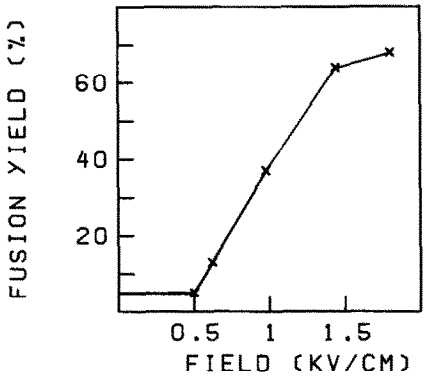

Fig.2. Dependence of the fusion yield on the field strength. Pulsing conditions: 2 pulses with a duration of 100 μs; 75000 tobacco protoplasts suspended in 0.15 ml pulsing buffer complemented with 0.5 mM spermine.

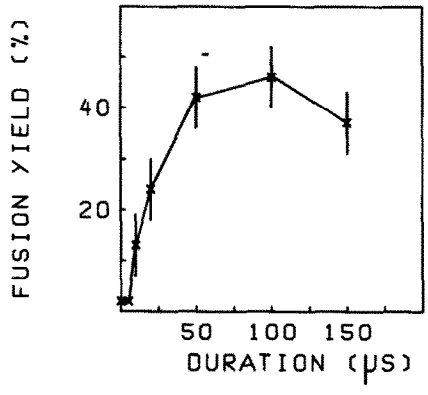

Fig.3. Evolution of the fusion yield with the pulse duration. Conditions: 3 pulses with a 1 kV/cm magnitude; 75000 tobacco protoplasts in 0.15 ml pulsation buffer containing 0.75 mM spermine and 1 mM $MgCl_2$.

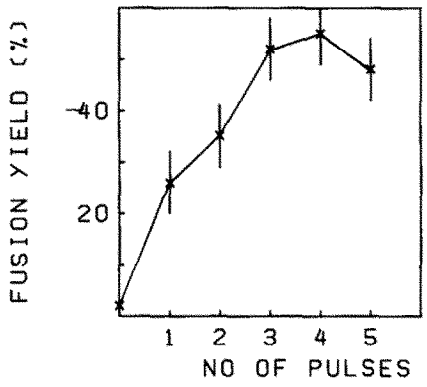

Fig.4. Dependence of the fusion yield on the number of successive pulses. Conditions: 1 kV/cm with a duration of 100 μs; 75000 tobacco protoplasts in 0.15 ml pulsation buffer containing 0.75 mM spermine and 1 mM $MgCl_2$.

sion process. The yield then increases sharply with an increase in the amplitude of the field and levels off for an intensity of 1.8 kV/cm. At amplitudes higher than 2 kV/cm, protoplasts become fragile and burst easily.

In fig.3, the influence of the duration of the pulse on the yield of fusion is displayed. The field (1 kV/cm) must be applied for more than 5–10 μs to induce the fusion process. Above this value, the fusion yield increases up to a maximum observed for durations greater than 50 μs. Beyond 100 μs, 'leaks' of cytoplasmic origin are detected in the medium.

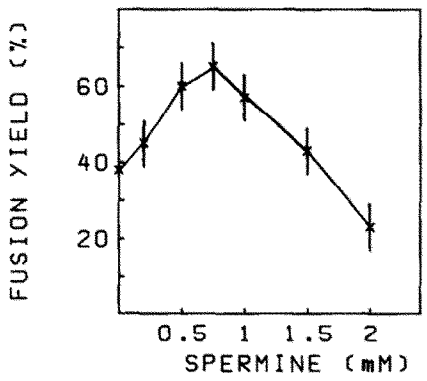

Fig.5. Evolution of the fusion yield with spermine concentration. Conditions: 3 pulses of 1 kV/cm with a duration of 100 μs; 75000 tobacco protoplasts in 0.15 ml pulsation buffer containing the indicated spermine concentration.

The results in fig.4 indicate that the yield of fusion increases almost linearly up to 3 successive pulses, then levels off and decreases when more than 4 pulses are applied. In the latter case, protoplasts burst and debris are produced. It should be noted that the number of events involving more than two protoplasts increases when more than 3 pulses are applied (not shown).

Fusion is also observed in a spermine-free medium if the protoplasts are present at a sufficiently high concentration. However, in this case, they are more fragile and do not withstand the electric field very well. In addition, the yield of fu-

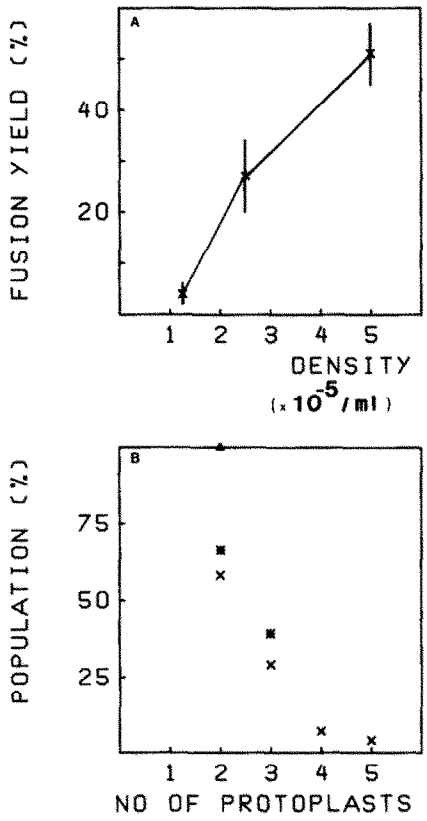

Fig.6. Modulation of fusion by protoplast concentration. Conditions: 3 pulses of 1 kV/cm with a duration of 100 μs; 0.15 ml protoplast suspension in the pulsing buffer containing 0.75 mM spermine and 1 mM $MgCl_2$. A, yield of fusion; B, histogram; fusion events were monitored under the microscope. The total number of fusion events was taken as 100. Each plot represents the percentage of events involving *i* protoplasts as a function of *i*. Protoplast concentration in the medium: (▲) 1.25×10^5, (★) 2.5×10^5, (+) 5×10^5 per ml.

sion remains low. The fusion events are in fact dependent on spermine concentration (fig.5) with an optimal concentration of about 750 μM.

Divalent ions modulate the yield of fusion: optimal fusion is observed in the presence of 1 mM Mg^{2+} ($MgCl_2$); in contrast, Ca^{2+} ($CaCl_2$) inhibits electrically mediated fusion events by 50% at 2 mM and completely at above 5 mM.

The higher the protoplast concentration, the higher the yield of fusion (fig.6A); however, at the same time, an increasing number of multifusion events is observed (fig.6B).

4. DISCUSSION

The application to plant protoplasts of this new electrofusion technique appears to be very successful. Under optimal conditions, the yield of protoplast fusion is routinely 50%. Furthermore by an appropriate choice of the experimental conditions (protoplast concentration, pulsing conditions, presence of $MgCl_2$), our results show that one can favor pair fusions (up to 75% of the events) (fig.6A) which are considered to be more convenient for subsequent obtaining of viable hybrids [3,23]. Moreover, this method can be performed under sterile conditions (under a laminar flow hood).

The spermine used initially as an additive to induce good contact between the protoplasts appears to play a role in the stabilization of the plasmalemma similar to that reported for bacteria [24] and senescent leaf protoplasts [25]. Here, d.c. pulsation of a spermine-free protoplast suspension is observed to cause great damage. The presence of spermine appears to be an advantage for electrofusion. Previous experiments using either dielectrophoresis [8] or spontaneous contact [9,10] to induce cell contact before pulsation suggest that the electric field-mediated perforation of the membrane [15] is a key step in the fusion process. Such pore formation may be very damaging in a membrane which is already rendered fragile by the rupture of the plasmodesmata during plasmolysis and the attack of the outer membrane proteins by fungal enzymes during isolation of the protoplasts from plant cells. Taking into account the striking similarity in the dependence of this electrofusion on the physical parameters of the pulse with our previous results on mammalian cells [9,10], and the fact that such a pore opening has been reported with plant protoplasts [8], we may propose that pore opening is here again the key step in the fusion process. This critical process, which is fully reversible, is thought to be associated with the increase in size of defects in the membrane matrix [26] induced by the membrane potential. Results obtained previously with mammalian cells should contribute to a fuller analysis of the molecular mechanisms involved in the electrofusion currently being studied.

The electrofusion method of spermine-treated plant protoplasts described here is a first step in the preparation of somatic hybrids. The main advantages of this fusion technique, in relation with this problem, are:

(i) The rapidity and ease of the experimental procedure;
(ii) The large number of protoplasts that can be treated under sterile conditions (75000);
(iii) The high percentage of fusion events that permit a large diversity of genetic combination;
(iv) The possibility of inducing mainly dicaryotic rather than polycaryotic units;
(v) The good physiological state of the fused protoplasts, maybe as a consequence of the use of a buffered ion-containing medium;
(vi) The observation that fusion is intra- and interspecific.

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