Electrically Induced Protoplast Fusion Using Pulse Electric Fields for Dielectrophoresis

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ABSTRACT

The formation of protoplast chains in suspensions of isolated pea (Pisum sativum cv Ran 1) mesophyll protoplasts induced by electric fields was studied. The chain formation induced by a sine-wave field (2 V, peak to peak; 500–0.1 kHz) was compared to that induced by an alternating pulse field (1 V, amplitude; 0.1–0.4 kHz). An increased number of dielectrophoretically paired protoplasts, formation of protoplast chains in the presence of CaCl₂ up to 5 mM, and protoplast fusion in the presence of 3 mM CaCl₂ were found when the pulse field was applied. The present results suggest the possibility of electrically induced protoplast fusion at cation concentrations that prevent fusion when sine-wave fields are applied.

The electrofusion of isolated plant protoplasts has been thoroughly investigated (5, 14, 21–26) and routinely used as an experimental method (1–4, 8–10, 12, 13, 15–20). However, the application of this technique is limited by the composition of the suspension medium. When the medium is relatively conductive, heat development and associated turbulence could break up the protoplast chains. Zimmermann and coworkers (21) avoided side effects by using nonelectrolyte solutions or solutions in which the electrolyte concentration did not exceed 1 mM. Bates et al. (3) showed that dielectrophoresis of oat protoplasts was not prevented by 0.1 mM Ca²⁺, but higher concentrations of salt were inhibitory. Chapel et al. (6) found that Ca²⁺ inhibits electrically mediated protoplast fusion events by 50% at 2 mM and completely at concentrations ≥5 mM. Zimmermann et al. (26, 27) suggested that the difficulties could be overcome technically by applying a pulse field of square pulses or amplitude-modulated sinusoidal fields. Under these conditions, the heat development should be considerably reduced. Experiments have shown that electric field-induced fusion of cells from permanent cell lines by a series of alternating DC² pulses with a frequency of 10 Hz can be carried out in the presence of high concentrations of electrolytes (21).

In the present study, we describe a procedure for improved protoplast fusion in the presence of 3 mM CaCl₂ by application of alternating pulse fields for the dielectrophoretical alignment of isolated pea mesophyll protoplasts.

MATERIALS AND METHODS

Plant Material

The experiments were carried out on mesophyll protoplasts from 10-d-old light-grown pea plants (Pisum sativum cv Ran 1).

Isolation of Protoplasts

The pea mesophyll protoplasts were prepared as reported by Christov and Vaklinova (7). The lower epidermis of the leaves was removed by forceps, and peeled leaves were floated peeled side down on 1% (w/v) Cellulase Onozuka R 10 (Serva), 0.1% (w/v) Macerozyme R 10 (Serva), and 0.4 mM mannitol (Merck). Complete digestion was achieved within 3 h of incubation at 30°C in light. The protoplasts were filtered through a nylon screen 80 μm pore diameter and then centrifuged at 150g for 10 min. The pellet was suspended in 0.5 mM mannitol, layered onto 0.5 mM sucrose (Fluka), and centrifuged once more under the same conditions. The protoplasts were collected from the interface, resuspended in 0.5 mM mannitol, and adjusted to a density of 2 × 10⁵ protoplasts per mL. To the protoplast suspension CaCl₂·7H₂O (Merck) was added at a final concentration of 1, 2, 3, or 5 mM.

Figure 1. Efficiency of sine-wave field-mediated protoplast pair formation at different calcium concentrations. The data are means ± se of six experiments.

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1 This research was supported by grant K-15-1991 from National Foundation "Scientific Research Work."
2 Present address: Michigan State University, Department of Horticulture, East Lansing, MI 48824-1325.
3 Abbreviations: DC, direct current; AC, alternating current.
Electrofusion Technique

The fusion procedure was carried out in a standard open fusion chamber (Zimmermann Cell Fusion System) as described by Zimmermann and Scheurich (23). The distance between the parallel platinum electrodes was 100 μm. The protoplast suspension was injected with a Hamilton syringe into the electrode gap, and the protoplasts were protected by a coverslip during the experiments.

The protoplasts were collected dielectrophoretically using (a) an AC field (sine wave, 2V peak to peak, and frequencies in the range of 500–0.1 kHz) provided by a Tesla oscillator BM 344 (Czechoslovakia) and (b) a field of alternating square pulses (amplitude 1 V and frequencies of 0.4 and 0.1 kHz) provided by a universal electric stimulator, ESU-1 (USSR). A pulse generator (G5 30A; USSR) providing the DC pulses was arranged parallel to the AC field-generating sources and the fusion chamber. Pulse dimensions were monitored with a C8-14 (USSR) memory oscillograph. The fusion process was monitored under the microscope.

RESULTS

In the present experiments, the decrease in the frequency of the sine-wave AC field from 500 to 10 kHz reduced the efficiency of dielectrophoretical alignment of protoplasts suspended in Ca²⁺-free medium (Fig. 1). The additional decrease in frequency caused a slight increase in protoplast pair formation. The addition of 1 mM CaCl₂ to the medium inhibited the process at all frequencies tested. The inhibitory effect was more pronounced when CaCl₂ was added at a final concentration of 2 mM.

Relatively high efficiency of chain formation was obtained when a field of alternating square pulses was applied (Fig.
2). No inhibitory effect was found after the addition of 1 mM CaCl₂ to the medium. Moreover, when the field frequency was 0.4 kHz, the number of protoplast pairs formed in the presence of 3 mM CaCl₂ was similar to that obtained with a sine-wave AC field of the optimal frequency of 500 kHz in Ca²⁺-free medium. It is also interesting that protoplast pair formation was observed at a concentration of 5 mM CaCl₂ in the medium.

Most of the protoplast pairs formed with the sine-wave AC field at a frequency of 500 kHz were located close to the electrodes, whereas at the frequency of 0.5 kHz the larger number of the pairs was between the electrodes (Fig. 3). The protoplast pairs obtained by means of the square-pulse field are located preferentially in the middle of the electrode gap.

When protoplast chains were formed, the AC field was switched off, and a DC square pulse was applied to induce membrane breakdown; after 10 s, the AC field was turned on again at half the voltage to maintain the fusion process. No fusion events were detected in the absence of Ca²⁺. At a concentration of 1 mM CaCl₂ in the medium, DC pulses of 20 V and 20 μs duration were efficient, and fusion of two individual protoplasts collected by a sine-wave AC field (2 V, 500 kHz) was completed within 4 min (Fig. 4). Fusion of protoplast pairs formed with a field of alternative square pulses (1 V, 0.4 kHz) provided by DC pulses of 20 V and 20 μs occurred in 1 min (Fig. 5).

The application of the square-pulse AC field (1 V, 0.4 kHz) allowed pea mesophyll protoplasts to fuse in the presence of 3 mM CaCl₂ in the medium (Table I).

**DISCUSSION**

The influence of the AC field frequency on dielectrophoresis has been studied in detail (11, 16). Our data indicate a frequency dependence of protoplast chain formation similar to that established by Pohl and Crane (11). They also support the assumption that the increase in the medium conductivity inhibits the sine-wave AC field-generated dielectrophoresis (11). Using a field of alternating square pulses (1 V, 0.4 kHz) for protoplast alignment, we observed chain formation in the presence of 3 and 5 mM CaCl₂ similar to that obtained with a sine-wave AC field (2 V, 500 kHz) in Ca²⁺-free medium and 1 mM CaCl₂-containing medium, respectively.

The distribution of protoplast pairs in the electrode gap at

*Figure 4. Fusion sequence of a pea mesophyll protoplast pair. Protoplasts were dielectrophoretically collected by sine-wave AC field of 2 V (peak to peak value, 500 kHz) in a 100-μm open fusion chamber (a). Then, a DC pulse of 20 V and 20 μs was applied. Photographs were taken using a Carl Zeiss, Jena camera and microscope (magnification ×240) at the following intervals after the DC pulse: b, 1 min; c, 2 min; d, 3 min.*
frequencies of 500 and 0.5 kHz of the sine-wave AC field is in good agreement with the theory of dielectrophoresis. According to Zimmermann et al. (26), the relative dielectrophoretic constants of cell and medium are frequency dependent, which results in the occurrence of positive or negative dielectrophoresis in different frequency regions (26).

Zimmermann et al. (26) pointed out that it is difficult to carry out electrofusion of protoplasts located in the space between the electrodes because a stable membrane contact could not be achieved there. To fuse protoplasts that are away from an electrode is possible by the method of Watts and King (20). The alternating fields used in their fusion system were too uniform and too weak to cause significant dielectrophoretical movement. In the absence of movement toward the electrodes, the formation of protoplast chains was solely through the mutual attraction of the induced dipoles. We succeeded in inducing electrofusion of protoplasts dielectrophoretically paired in the middle of the electrode gap (Fig. 4).

In summary, we suggest that difficulties inherent in the fusion of protoplasts in the middle of the electrode gap could be overcome with an alternating square-pulse field for the dielectrophoretical arrangement of protoplasts. This manner of protoplast chain formation is also recommended when the Ca\(^{2+}\) concentration in the suspension exceeds 1 mM.

**LITERATURE CITED**


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**Table 1. Yield of Fused Protoplast Pairs, Dielectrophoretically Arranged Using an Alternating Pulse Field (1 V amplitude, 0.4 kHz) after the Application of a Single Breakdown Pulse of 20 V and 20 μs at Different Ca\(^{2+}\) Concentrations**

<table>
<thead>
<tr>
<th>Ca(^{2+}) Concentration (CaCl(_2))</th>
<th>0 mM</th>
<th>1 mM</th>
<th>3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pairs formed</td>
<td>15.8 ± 0.9</td>
<td>16.0 ± 1.3</td>
<td>13.8 ± 0.7</td>
</tr>
<tr>
<td>No. of fused pairs</td>
<td>0.0</td>
<td>1.6 ± 0.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Percentage</td>
<td>0.00</td>
<td>10.00</td>
<td>5.07</td>
</tr>
</tbody>
</table>

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**Figure 5.** Fusion of a pea mesophyll protoplast pair dielectrophoretically formed by an alternating pulse field (1 V amplitude, 0.4 kHz) in a 100-μm open fusion chamber (a) and 1 min after application of a breakdown pulse of 20 V and 20 μs (b).
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