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Induction of a long-lived fusogenic state in viable plant protoplasts permeabilized by electric fields

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Electropermeabilized tobacco mesophyll protoplasts are shown to fuse by creating cell contact several minutes after electropulsation. Electropermeabilization was analysed by measuring calcein uptake. Experiments were performed at low temperature to avoid resealing of protoplast transient permeation structures. These results confirm that the long-lived permeabilized state induced by the electric field is associated to a fusogenic state, under viability conditions. This is indicative that as for mammalian cells, the electric field-induced membrane modifications, which give the permeable state, are such as to decrease the magnitude of the intercellular repulsive forces between plant protoplasts. Such a fusion method may be useful for somatic hybrids production with protoplasts showing morphological and physiological differences.

Electrical fusion of plant protoplasts firstly demonstrated ten years ago [1,2] is now widely used to produce somatic hybrids among crop species [3,4]. However, little is known about cell and membrane fusion processes induced by the electrical treatment. A more practical use of such a fusion method as well as an improvement of the standard protocols are limited by a lack of understanding of the molecular mechanisms of the fusion process as of many of the molecular events induced by electric fields on biological systems. Usually, the electric field-induced fusion method involves two steps leading to polykaryon formation. The first step is an aggregation process to create cell contact prior to the application of the external electric field to the protoplast suspension. This first step can be performed by three different ways, i.e. dielectrophoresis [5], addition of agglutinating agents like polyethylene glycol (PEG), polyvinylalcohol (PVA) or spermine in the pulsation medium [6], or allowing the protoplasts to settle in the pulsation chamber and to form a monolayer at the bottom of the chamber [6,7]. The second step is a high-field pulse which creates the fusion process. More recently, electrical fusion of mammalian cells was obtained by following the sequence of events in a reverse order. Pulsing was first achieved and close contact of the cells was then induced. Such a method was developped by two different groups using human erythrocyte ghosts [8,9] or CHO cells [10]. In this latter case, cell viability was maintained. These results indicated that a long-lived fusogenic state of the cell membrane was created by the external electric field. This leads to wonder whether the fusion is strictly induced during the pulsation as previously suggested [11,12] and thus strictly dependent on the pulse or whether the field induces a long-lived fusogenic state of the membrane that allows the fusion process to occur after pulsation [8–10]. Another point was to wonder if this fusogenic state was a general property of electropermeabilized membranes and as such would be observed with other systems such as plant protoplasts. In this communication, we indeed report the induction of a fusogenic state of plant protoplasts brought into close contact several minutes after pulsation.

Tobacco mesophyll protoplasts cv *Xanthi* were isolated from plants growing in soil under controlled conditions as previously described [13]. Briefly, after an overnight enzymatic digestion of leaf pieces, protoplasts were washed twice in a saline medium (330 mM KCl, 14 mM $CaCl_2$, 5 mM Mes-KOH (pH 5.8)) and then resuspended in the pulsing medium (PM) similar to that previously developped in our group to fuse plant protoplasts [6] and to obtain viable somatic hybrid plants [14]. In order to demonstrate that the fusogenic state

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induced by the field is long lived, no contact of the protoplasts during electropulsation should be present. This was obtained by ensuring the following conditions (1) use of a PM free from any chemical agent able to induce aggregation of protoplasts before pulsation, like PEG, PVA or spermine, (2) resuspending the protoplasts at a low cell density, (3) pulsing the sample as soon as the protoplasts were brought between the electrodes to avoid settling down of the protoplasts.

The overall protocol is described in Fig. 1. Freshly isolated protoplasts were resuspended in PM modified from Ref. 14: 500 mM mannitol, 50 mM NaCl, 1 mM $MgCl_2$, 5 mM Mes-KOH (pH 5.8) at the concentration of $5 \cdot 10^5$ protoplasts/ml. Two flat stainless steel electrodes (5 mm wide, 20 mm long) dipped in contact with the bottom of a cooled Petri dish were used as the pulsation chamber. A 200-μl aliquot of suspension cooled on crushed ice for ten minutes was pulsed at various pulse intensities, durations and directions using

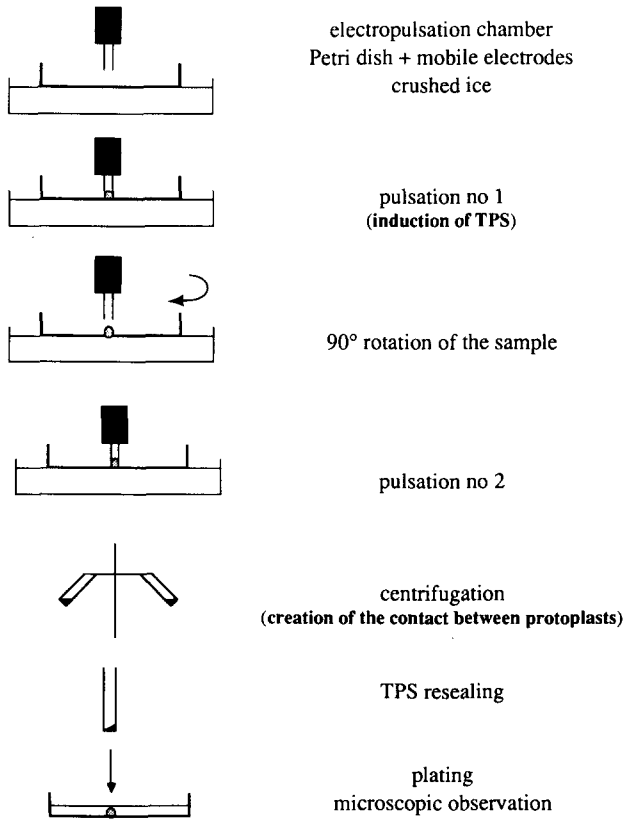

Fig. 1. Experimental procedure used to induce plant protoplast fusion by creating the cell contact after electropulsation. Tobacco mesophyll protoplasts were treated (see text) by a first set of pulses of different intensity and duration of the field. When indicated, a second set of pulses was then given but after a 90° rotation of the sample in order to change the direction of the field. Delay between pulses on one set was 1 s. Delay between two sets of pulses was 20 s. Centrifugation of the protoplasts was then performed in 5 ml plastic tubes previously refrigerated, under rotation speed and time conditions mentioned in the text. The protoplasts were then allowed to reseal for 20 min at room temperature (21° C) before microscopic observation or plating. Fusion yield was then evaluated. One experiment lasted about 30 min.

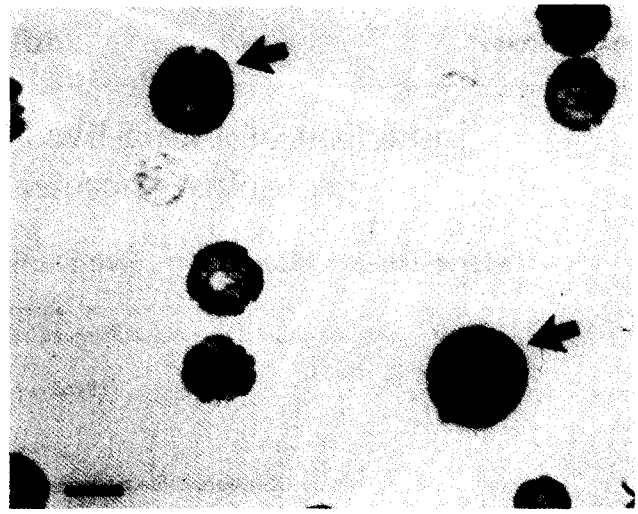

Fig. 2. Micrograph of fused protoplasts obtained through electropulsation followed by centrifugation. Tobacco polykaryons (indicated by the arrows) were obtained after application of two sets of 2×100 μs pulses of 0.8 kV/cm in two perpendicular directions under conditions described in Fig. 1. Cell contact was achieved by centrifuging pulsed protoplasts at $25 \times g$ during 7 min (the bar represented 20 μm).

a square wave CNRS electropulsator (Jouan, France). The shape of the pulse was monitored with an oscilloscope. Immediately after pulsation, close contact of the protoplasts was achieved by centrifugation (Jouan C500, France) of the sample in a 5 ml plastic tube precooled on ice. The protoplasts were then incubated at room temperature (21° C) during 25 min before observation was done using an inverted microscope (Leitz, F.R.G.). Fusion yield was evaluated by the ratio of the number of fused protoplasts (Fig. 2) and the total number of protoplasts as described in Ref. 15. Counting was easily performed by means of a video monitor (JVC, Japan) coupled to a camera (JVC, Japan).

The role of centrifugation parameters (rotation speed and duration) was analysed with protoplasts pulsed under pulse duration and intensity previously suggested to be optimal for tobacco protoplasts [6,14]. The results shown in Fig. 3 indicated that fusion was induced when the sample was centrifuged at a low speed during about 5 min. A step of centrifugation of $25 \times g$ for 7 min that gave the highest percentage of fusion was subsequently used in further experiments. Stronger or longer centrifugation treatment led to an increase of altered protoplasts showing lysis without any improvement in protoplast fusion yield. Increase in fusion yield and lysis of the protoplasts are competitive processes along the post-pulse treatment. This is indicative that a fusogenic but fragile state of protoplast membrane was induced by the electric field. These observations were very similar to those reported for the fusion of CHO cells [10].

A crucial electrical parameter is the intensity of the field. We investigated the dependence on field intensity

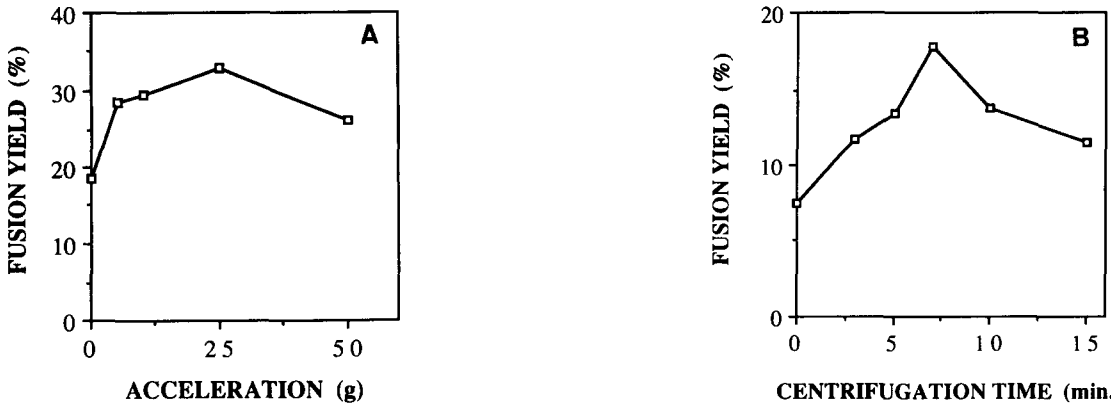

Fig. 3. Effect of centrifugation parameters on protoplast fusion yield. Protoplasts resuspended in PM cooled on ice (see text) were submitted to two pulses of 100 μs at a field intensity of 1 kV/cm as described in Fig. 1. They were then centrifuged under various rotation speed (A) during 5 min or during various times at 25 × *g* (B).

of the fusion yield when pulses of 100 μs were given. As reported in Fig. 4, the fusion percentage increases as a function of field intensity with an optimal value of 0.8 kV/cm. For field intensities stronger than 1 kV/cm, more and more cellular debris appeared in the sample. These observations are indicative that the level of fusogenicity is under the control of the field intensity and that only a narrow range of field strengths is efficient, lysis occurring when strong fields are applied. These results clearly showed that the fusion process is determined by the electric field even when close contact of the plant protoplasts is created after pulsation and did not basically require cell-cell contact during electropulsation to occur.

The main effect of an electric field on a membrane leads to a dramatic increase in its conductance and is associated to the induction of transient permeation structures (TPS [10]). This state allowed exchange of material across the cell membrane. We studied the evolution of the permeation state versus field intensity

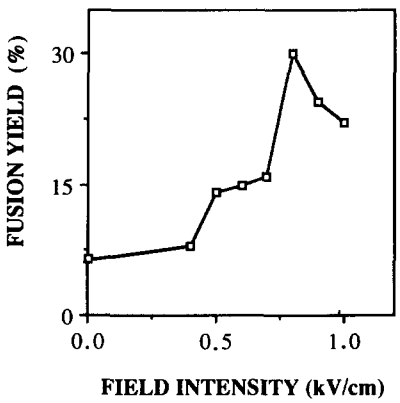

Fig. 4. Role of the field intensity on the fusion yield. Protoplasts resuspended in pulsation medium (see text) were pulsed according to the protocol described in Fig. 1. The intensity of pulses of 100 μs duration was varied. Immediately after pulsation, they were centrifuged at 25 × *g* for 7 min.

to compare electrical conditions inducing fusion and permeabilization. By using calcein, a fluorescent non permeant dye, as a permeation tracer, electrical conditions promoting fusion were found to induce permeabilization as well (Fig. 5). TPS lifetime of protoplasts which were pulsed under fusion inducing conditions (i.e., low temperature) were longer than 5 min. But the results in Fig. 6 are indicative that in fact two protoplast subpopulations were present, one resealing within 2 min and the other one more slowly. As already observed with other systems, the resealing process was dependent on the incubation temperature. Since the time needed to run the pulsation and centrifugation

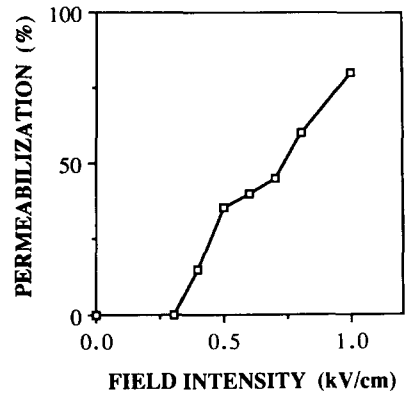

Fig. 5. Role of the field intensity on protoplast electropermeabilization. Freshly isolated protoplasts were resuspended in ice-cold PM containing 5 mM calcein as described in Ref. 16. Constant pulses of increasing intensities were applied to the protoplasts as described in Fig. 1. They were subsequently incubated for 10 min at 4° C (crushed ice) and then for 20 min at 21° C for the resealing process to occur. Four washes with PM were then performed to discard extracellular dye and protoplasts were finally observed under a fluorescence microscope equipped with a H3 filter block (Leitz, F.R.G.). At least 300 protoplasts were counted for each sample. Permeabilization was expressed as the percentage of protoplast exhibiting a yellow-green coloration under UV illumination.

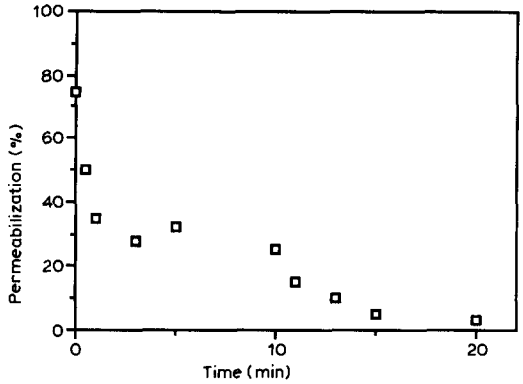

Fig. 6. Lifetime of the electropermeabilized state of protoplasts. Protoplasts were pulsed with 5 pulses (50 μs duration, 0.8 kV/cm) in absence of calcein. Then calcein (15 mM final concentration) was added at the indicated times (the temperature was 4°C during the first 10 min and 21°C thereafter) and protoplasts were incubated for 10 min in presence of the dye. After 20 min at 21°C, they were washed as described in Fig. 4. For the zero-time, calcein was present during electropulsation.

steps was shorter than 4 min, we could conclude that the permeabilized state is also a fusogenic state of the protoplast membrane.

Estimating protoplast viability after the overall treatment is a prerequisite for any application of such a fusion method and for an understanding of the biological processes controlling the induced phenomenon. As a viability measurement performed just after electrical treatment led to overestimated results [17], protoplast survival was monitored during one week on culture under standard conditions as previously described to produce somatic hybrids [14]. In routine experiments, survival was evaluated two days after pulsation and plating efficiency (P.E.) one week later when its maximal value is reached. Viability measurements of protoplasts submitted to the optimal fusion treatment described before are reported in Table I. Centrifugation ($25 \times g$, 7 min) by its own had a negligible lethal effect on protoplasts, whatever the applied field strength. The optimal field strength value that gave the highest viable fusion yield was 0.8 kV/cm. Plating efficiency indicated more accurately than morphological integrity of the protoplasts the electrical conditions which gave the best yield in fusion and viability (0.8 kV/cm). The fusion yield evaluated just after the fusogenic treatment was found to be constant when stronger fields were used, but cell death was larger (1 kV/cm).

Considering the vectorial character of the electric field, one should consider that a given cell experienced electric field effects which are position dependent on the cell membrane. The higher the field strength, the larger the permeabilized, i.e., fusogenic surface. But of course the same consequence can be obtained with lower field intensities by changing the direction of the field relative to the cell. Direct evidence of this theoretical prediction was reported with plated mammalian cells electrically fused where the highest fusion yield was reached when cells were pulsed in two perpendicular directions [18]. This result was also obtained with tobacco protoplasts and thus bidirectional pulsing was thus used for subsequent fusion experiments (Fig. 1). In agreement with these results, Sowers et al. [9] demonstrated that the long-lived fusogenic state of erythrocyte ghosts was not laterally mobile. These observations confirmed that the cellular surface really electropermeabilized was indeed determined by the field intensity applied to the cell. In the case of tobacco mesophyll protoplasts treated in our conditions, the minimal threshold field value which created a permeable state was previously found to be about 0.2 kV/cm [16]. The field intensity leading to the highest percentage of viable fusion products corresponded then to the permeabilization of 80% of the protoplast surface area. This was in agreement with the fact that a greater contact probability between permeabilized membranes of cells is achieved.

These results show for the first time in the case of plant protoplasts that electrofusion is not necessarily dependent on the protoplast contact during the pulsation. Similarities in the response of plant protoplast and mammalian cells suggested that the basic process involved in the establishment of the fusogenic state was indeed the same and could be related to a common structural and molecular basis. Nevertheless, it should be noted that recent contradictory results [19] obtained with mouse L cells argued that the fusogenic action of electric field is not mediated by any long-lived fusogenic state, but no control of permeabilization was provided. In comparison, our data showed that plant protoplast fusion was achieved while about 50% of the protoplast population were still able to incorporate ex-

TABLE I

Protoplast viability after optimal fusogenic treatment by electropulsation

Protoplast pulsation was carried out as described in Fig. 3. Two sets of pulses of 100 μs duration were delivered to protoplasts at 0.8 and 1.0 kV/cm field intensities. After centrifugation and resealing for 20 min, culture medium was added under sterile conditions. Plates were incubated for two days at 25°C in the dark. Survival was thus evaluated by counting the percentage of protoplasts that showed no morphologic alteration. Five days later, the percentage of protoplasts in division was counted. At least 300 protoplasts were counted and triplicates were scored for each treatment

E (kV/cm)	Centrifugation ($25g \times 7$min)	Survival yield (%)	Divisions (%)
0	−	85	80
0	+	83	80
0.8	−	78	49
0.8	+	71	47
1	−	16	3
1	+	16	0

ogenous dye, several minutes after pulsation. The break present in permeabilization and fusion profiles versus field intensity (Figs. 4 and 5) led to confirm the presence of two major subpopulations inside the suspension. These observations confirm the association between a long-lived permeabilized state and fusogenicity and explain why the viable fusion percentage was not higher than 30 to 40%. Whatever the quantitative aspects, these results suggested that one first condition for obtaining fusion after pulsation is to control the permeation state maintenance. Since TPS resealing is temperature dependent [16] and moreover cell-type specific, it thus implied that the control of this parameter must be carefully investigated for every cell type allowed to fuse.

Little is known about field primary effects on membrane lipids and proteins in viable cell membrane, particularly on plant protoplasts. However, Hahn-Hägerdal et al. reported that a hydrophobic state of the membrane was created when plant protoplasts were electropulsed under conditions of fusion as well as when a fusogenic PEG treatment was given [20]. More recently, ^{31}P-NMR analysis of permeabilized mammalian cells suggested that the electric field induced a rearrangement of polar heads of membrane phospholipids [21]. The authors suggested that a crucial event induced by electric field was a reorganization of the water hydration layer at the interface membrane/extracellular medium leading to a decrease in the repulsive intercellular hydration forces when cells were in close contact and as such explaining the observed fusogenicity.

From a practical point of view, a long lived fusogenic state of the protoplast membrane induced by electropulsation has a direct consequence, especially when it is needed to fuse protoplasts showing large distinctive differences in their morphology or physiology and thus requiring their own specific field intensity for the induction of the fusogenic state. Protoplasts of different origins, impossible to fuse electrically by using the classical fusion methods should be pulsed separately, then mixed and centrifuged to be brought into contact.

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