High Efficiency Electro-transfection of Tobacco Mesophyll Protoplasts with Tobacco Mosaic Virus RNA

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(Accepted 24 June 1986)

SUMMARY

Tobacco mesophyll protoplasts were transfected with tobacco mosaic virus (TMV) RNA in an electric field using a newly devised chamber constructed with gold-coated glass panel electrodes. Up to 95% of protoplasts subjected to several DC pulses (50 μs, 550 to 800 V/cm) while suspended in 0.5 M mannitol containing 10 μg/ml TMV RNA became infected. The treatment did not affect the viability of the protoplasts or their stability during 40 h of incubation.

The transfection of animal cells with foreign nucleic acids mediated by an electric field was first reported by Neumann et al. (1982). Recently, the method, which we term electro-transfection, has also been used with plant protoplasts. For instance, Ti plasmid DNA (Langridge et al., 1985), the Tn 9 chloramphenicol acetyltransferase gene linked to the nopaline synthase promoter or to the cauliflower mosaic virus 35S promoter (Fromm et al., 1985), and the Tn 5 aminoglycoside phosphotransferase gene linked to the cauliflower mosaic virus 19S promoter (Shillito et al., 1985) have been transfected into carrot or tobacco protoplasts in an electric field. Similar treatment of tobacco protoplasts resulted in 75% transfection with tobacco mosaic virus (TMV) RNA and 46% transfection with cucumber mosaic virus RNA (Nishiguchi et al., 1986). However, the conditions used for these transfections, such as salt concentrations and DC pulse voltages, differ considerably. Electrolyte concentration affects the conductivity, which itself affects the DC pulse voltage required for the transfection. High DC pulse voltages and long pulse durations as used in the published work quoted above are known to cause the disruption of protoplasts (Zimmermann & Scheurich, 1981; Zimmermann et al., 1981; Bates et al., 1983; Zachrisson & Bornman, 1984). Moreover, low concentrations (100 μM) of Ca²⁺ were reported to promote the electro-fusion of protoplasts (Zimmermann, 1982; Watts & King, 1984). On the other hand, for transfection, Mg²⁺ has been used at high concentration (9 to 15 mM) (Potrykus et al., 1985) and reported to be essential (Langridge et al., 1985). We have therefore tried to find suitable conditions for transfection in which almost all protoplasts survive, by a combination of decreasing the pulse voltage and controlling the ionic conditions in the transfection medium.

One proposed mechanism of transfection is a reversible breakdown of the membranes of protoplasts (Neumann et al., 1982), similar to that during fusion as proposed by Zimmermann et al. (1981). Highly efficient (up to nearly 100%) fusion has been achieved with protoplasts of several species of plants using a DC pulse of low enough voltage to cause little damage (Zimmermann & Scheurich, 1981; Zimmermann et al., 1981; Bates et al., 1983; Zachrisson & Bornman, 1984). Moreover, low concentrations (100 μM) of Ca²⁺ were reported to promote the electro-fusion of protoplasts (Zimmermann, 1982; Watts & King, 1984). On the other hand, for transfection, Mg²⁺ has been used at high concentration (9 to 15 mM) (Potrykus et al., 1985) and reported to be essential (Langridge et al., 1985). We have therefore tried to find suitable conditions for transfection in which almost all protoplasts survive, by a combination of decreasing the pulse voltage and controlling the ionic conditions in the transfection medium.

Therefore, we have examined the effects on the efficiency of electro-transfection of different RNA concentrations, protoplasts concentrations, pulse voltages, pulse durations, pulse times and concentrations of Ca²⁺ and Mg²⁺ using tobacco mesophyll protoplasts and TMV RNA. We also describe a newly devised chamber for conducting transfection experiments.
Gold evaporated panel electrode

Fig. 1. Chamber for electro-transfection. Panel electrodes (30 x 20 mm) were made by evaporating gold in vacuo onto one side of the glass plate after pre-evaporation of chromium. The size of the chamber was 10 x 10 mm square on the inside and the distance between the two electrodes was varied from 200 to 1000 μm with polyvinyl chloride spacers.

Fig. 2. Effect of the concentration of TMV RNA on the transfection of tobacco protoplasts (O) and on the pulse voltage in the electric field (▲). Pulse voltage in the electric field was adjusted to 900 V/cm in the absence of TMV RNA. Transfections were carried out in medium without Ca²⁺ or Mg²⁺.

Mesophyll protoplasts were isolated from tobacco leaves (Nicotiana tabacum cv. Xanthi NN) as described by Huber et al. (1977), except that the mannitol concentration was 0.5 M. RNA was prepared from freshly purified TMV (OM strain; Nozu & Okada, 1968) according to the method of Fraenkel-Conrat et al. (1961). Protoplasts were transfected in a newly devised chamber shown diagrammatically in Fig. 1. The chamber consists of two panel electrodes and a spacer which can be readily assembled and disassembled. The distance between the two electrodes and the chamber volume are determined by the thickness of the polyvinyl chloride spacers. Panel electrodes were made by evaporating gold onto glass plates, which results in completely flat surfaces, enables the distance between the two electrodes to be accurately known and causes the intensity of the electric field to be homogeneous.

Except where otherwise stated, protoplasts were suspended at approximately 2 x 10⁵ per ml in 0.5 M-mannitol with or without CaCl₂ or MgCl₂, mixed with TMV RNA at 10 μg/ml and transferred into the chamber using a micropipette. The chamber was cooled to 0 °C and several square DC pulses of 50 μs duration at various field voltages were supplied at 1 s intervals by a pulse generator (JASCO PG 001). The protoplasts were taken out of the chamber after 5 min, washed once with 0.5 M-mannitol by centrifuging at 800 r.p.m. for 2 min and incubated in the medium of Aoki & Takebe (1969) at approximately 2 x 10⁵ protoplasts/ml at 28 °C under continuous fluorescent illumination of approximately 3000 lx.
Fig. 3. Effect of protoplast concentrations on the transfection of tobacco protoplasts (○) and on the pulse voltage in the electric field (△). Pulse voltage was adjusted to 900 V/cm in the presence of $4 \times 10^4$ protoplasts/ml. Transfections were carried out in medium without Ca$^{2+}$ or Mg$^{2+}$.

Fig. 4. Effect of the number of pulses given on the transfection (○) and survival (△) rates of tobacco protoplasts. Pulse voltage in the electric field was adjusted to 800 V/cm. Transfections were carried out using $2 \times 10^5$ protoplasts/ml in medium containing 10 μg/ml TMV RNA without Ca$^{2+}$ or Mg$^{2+}$.

After incubation for 40 h, the protoplasts were stained with fluorescein isothiocyanate-conjugated anti-TMV serum as described by Otsuki & Takebe (1969) and examined in a fluorescence microscope. Percentage transfection was the proportion of protoplasts examined that were stained.

Virus yield was determined by local lesion assay as described by Takebe & Otsuki (1969). Protoplasts were fused using the method described by Zimmermann & Scheurich (1981). The fusion chamber of 2.4 μl with parallel, flat-sided platinum electrodes (30 mm length, 400 μm height and 200 μm distance between the two electrodes) was set under an optical microscope. Protoplasts suspended in 0.5 M mannitol with or without CaCl$_2$ at approximately $2 \times 10^5$ per ml were transferred into the chamber, aligned between the two electrodes by supplying AC of 500 kHz (sine wave) at 400 to 500 V/cm (peak to peak) in the electric field by a function generator (Iwatsu Electric Co. Ltd FG 330) and then subjected to one DC pulse of 50 μs duration at various field voltages using the pulse generator used in the transfection experiments. After 5 min, the percentages of protoplasts fused were counted.

In order to find suitable conditions for transfection, the effects of TMV RNA concentration, protoplast concentration and the number of pulses given were examined using 0.5 M mannitol without ions as the transfection medium. The output voltage of the pulse generator was adjusted to 900 V/cm at the lowest concentration of TMV RNA and protoplasts. TMV RNA at 10 μg/ml gave the maximum transfection; 50 μg/ml RNA decreased the pulse voltage and gave a lower transfection rate (Fig. 2). Protoplasts at concentrations from $10^4$ to $2 \times 10^5$ per ml were efficiently transfected, but the rate decreased sharply at concentrations of more than $4 \times 10^5$ per ml, concentrations which also resulted in a drop of the pulse voltage (Fig. 3). More than five pulses (900 V/cm) were needed to attain the maximum transfection. More than 90% of protoplasts exposed to 10 pulses of 50 μs duration survived incubation for 40 h (Fig. 4). Long pulse durations disrupted the protoplasts; whereas a 100 μs pulse caused only 7% of protoplasts to disrupt, a pulse of 250 μs duration disrupted 57% (data not shown).

Both TMV RNA and protoplasts had a negative effect on the pulse voltage in the electric field. Therefore, it seemed useless to increase the TMV RNA concentration above 10 μg/ml and
important to select a protoplast concentration suitable for the capacity of the pulse generator used. From the above results, the concentration of TMV RNA, number of protoplasts and pulse number chosen were 10 μg/ml, 2 × 10^5 per ml and 10 pulses, respectively.

The distance between the electrodes did not affect the transfection rate; supplying 10 DC pulses at 600 V/cm resulted in transfection rates for TMV RNA of 92%, 95% and 94% for separation distances of 200 μm, 500 μm and 1000 μm, respectively.

Fig. 5 shows the results of experiments on the effects on transfection of different concentrations of Ca^{2+} (a) and Mg^{2+} (b). The output voltage of the pulse generator was adjusted to 1000 V/cm in the electric field in the absence of CaCl_2 and 900 V/cm in the absence of MgCl_2, and the distance between the electrodes was set at 200 μm. The drop in the pulse voltage in the electric field was monitored on an oscilloscope as the concentrations of Ca^{2+} or Mg^{2+} increased. The presence of between 500 μM and 10 mM Ca^{2+} or Mg^{2+} decreased the pulse voltages and transfection rate markedly. However, 10 to 100 μM-Ca^{2+} had no apparent effect on transfection and 10 to 100 μM-Mg^{2+} stimulated transfection. Nishiguchi et al. (1986) found that Ca^{2+} had neither an inhibitory nor a promotive effect on transfection at high voltage (5 to 10 kV/cm). Therefore, the decreased efficiency of transfection with increasing concentrations of Ca^{2+} and Mg^{2+} is probably caused by the reduction of the pulse voltage in the electric field, although the drop of pulse voltage and the decrease in transfection rate were not directly correlated.

It is worth noting that the transfection rate and the effects of these ions depended on the quality of the protoplast preparation used. In one experiment, 95% of protoplasts were transfected with TMV RNA in the presence of 100 μM-Ca^{2+} without Mg^{2+} (Fig. 6) and sometimes over 95% transfection was attained in a medium without ions (data not shown).
However, the addition of low concentrations (10 to 100 μM) of Mg$^{2+}$ to the medium is recommended to achieve reproducibly high rates of transfection, because Mg$^{2+}$ has a promotive and not an inhibitory effect.

Local lesion assays demonstrated that the electro-transfected protoplasts produced approximately $1 \times 10^7$ to $5 \times 10^7$ virus particles/protoplast after 40 to 72 h incubation, a value greater than that reported by Takebe & Otsuki (1969) for similar protoplasts 24 h after inoculation using poly-L-ornithine.

The effects of pulse voltages on transfection and survival and on the fusion of protoplasts were examined by varying the pulse voltages in the electric field in the presence of 100 μM-Ca$^{2+}$, which was shown to have maximum effect on the fusion of protoplasts (Watts & King, 1984). The results (Fig. 6) showed that the rate of transfection of protoplasts began to increase at 400 V/cm and reached a maximum of 95% at 600 V/cm which was maintained up to 800 V/cm, and that the fusion rate of protoplasts began to increase at 400 V/cm, increased rapidly in proportion to the increase in pulse voltage and reached more than 90% at 850 V/cm. There seemed to be a critical voltage, approximately 600 V/cm, for tobacco protoplasts at which transfection and fusion were induced. Although the prior alignment of the protoplasts by AC was necessary for the fusion, the optimal pulse voltage for fusion of protoplasts in the electric field reported by Zimmermann & Scheurich (1981) was confirmed to be almost coincident with that for transfection with TMV RNA, except that the DC pulse voltage that induced the maximum rate of transfection was slightly lower than that which induced maximum fusion. This may be because both processes rely on the breakdown of the cell membrane which is caused by the DC pulse.

At less than 850 V/cm, more than 95% of protoplasts survived the treatment without obvious damage and were stable during incubation for 40 h. At higher voltages some protoplasts were disrupted; a pulse of 1000 V/cm caused about 15% disruption, and more than 50% were disrupted at 1100 V/cm (Fig. 6). The average survival over all the experiments carried out at between 500 and 1000 V/cm pulse voltages was 93 (±3)%.

Fromm et al. (1985), Langridge et al. (1985) and Potrykus et al. (1985) carried out transfection in media containing electrolytes. However, such electrolytes reduce the pulse voltage (Fig. 5) and a decrease in the concentration of electrolytes such as Ca$^{2+}$ and Mg$^{2+}$ might therefore permit the use of a lower voltage from the pulse generator than these authors used.

The optimum conditions in our transfection experiments were different from those of Nishiguchi et al. (1986). They transfected tobacco protoplasts with TMV RNA in the absence of Ca$^{2+}$ and Mg$^{2+}$ at a field strength of 5 to 10 kV/cm, approximately 12-fold higher than ours, using a helical electro-fusion chamber. Perhaps this chamber design necessitated the use of such a high voltage. The homogeneous electric field attainable in our device may well have been an important factor in achieving highly efficient transfection, as it is in the fusion of protoplasts (Zachrisson & Bornman, 1984). Because all the protoplasts were equally exposed to the electric field in our chamber, it was not necessary to change the position of the protoplasts during the electrical manipulation, as was found necessary by Nishiguchi et al. (1986) with their helical chamber. This, as well as the use of lower voltages, probably resulted in less mechanical disruption when using the new chamber.

Electro-transfection may provide a simpler method for the infection of protoplasts than the conventional methods as well as being a potentially useful means for the introduction of cloned recombinant or mutagenized viral nucleic acids as well as other foreign nucleic acids into various plant protoplasts.

The present work was supported in part by a Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries.

REFERENCES


Short communication


(Received 5 February 1986)