Electrotransfection of protoplasts from tomato, wild tomato, barley and chrysanthemum with tobacco mosaic virus RNA

R. Matsunaga,1 K. Sawamura,2 M. De Kok,3 T. Makino,4 K. Miki,2 M. Kojima,3 T. Tsuchizaki1 and T. Hibi5*

1Laboratory of Plant Pathology, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113,
2Fundamental Research Laboratory, Tonen Company, Nishitserugaoka, Ohimachi, Iruma-gun, Saitama-ken 354,
3Laboratory of Plant Pathology, Faculty of Agriculture, Niigata University, Igarashi Nino-cho, Niigata 950-21,
4Plant Pathology Laboratory, Shizuoka Agricultural Experiment Station, Toyoda-cho, Iwata-gun, Shizuoka 438 and
5Laboratory of Applied Microbiology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan

Protoplasts isolated from tomato, wild tomato, barley and chrysanthemum were electrotransfected with tobacco mosaic virus (TMV) RNA under almost the same optimum electric conditions: five square DC pulses of 50 μs duration at 500 to 800 V/cm, with the protoplasts suspended at 2 x 10^5/ml in 0.5 M-mannitol containing 100 μM-MgCl₂ and 10 to 20 μg/ml TMV RNA. ELISAs of these transfected protoplasts showed that the yields and the growth curves of the virus were quite similar, indicating a lack of host specificity in the initially infected cells of these plants.

Introduction

It is known that, in relation to tobacco mosaic virus (TMV) infection, tomato (Lycopersicon esculentum), wild tomato (L. peruvianum), barley (Hordeum vulgare) and chrysanthemum (Chrysanthemum morifolium) are respectively a typical systemic infection host, a conditional resistant host with the Tm-2 gene (Cirilli & Alexander, 1969; Yamakawa et al., 1978), a temperature-dependent subliminal infection host (Hamilton & Dodds, 1970) and an apparent non-host. In the present paper, we show that protoplasts of these four kinds of plant can be electrotransfected with TMV RNA under almost the same optimum conditions as reported previously for tobacco and cowpea mesophyll protoplasts (Hibi et al., 1986, 1988) and that the virus multiplies at the same rate in these transfected protoplasts.

Methods

Preparation of protoplasts. Tomato seedlings (cv. Kurihara) were grown aseptically on TM-1 agar medium (Shahin, 1985), at 28°C during the 14 h light period (approx. 8000 lx) and at 22°C during the 10 h dark period. Fully expanded primary leaves of 22- to 32-day-old plants were cut with a razor, into strips, approximately 2 to 3 mm wide, infiltrated in vacuo with the first enzyme solution containing 0.1% (w/v) Pectolyase Y-23, 2% (w/v) Cellulase Onozuka R-10 (Yakult Honsha) and 0.5 M-mannitol, pH 5.5, and incubated at 30°C with 70 excursions/min. After 1 h the enzyme solution was decanted by filtration through a 150 mesh stainless steel filter and the remaining leaf tissue was incubated for 2 h in the second enzyme solution containing 0.05% (w/v) Pectolyase Y-23, 1% (w/v) Cellulase Onozuka R-10 (Yakult Honsha) and 0.5 M-mannitol, pH 6.0, at 30°C with 60 excursions/min.

Suspension culture cells of wild tomato (L. peruvianum PI 126944) were derived from the first leaf and cultured in Murashige & Skoog (MS) (1962) medium containing 0.2 mg/l 2,4-dichlorophenoxyacetic acid and 0.6 mg/l 6-benzyladenine. The cells were grown with 20 ml of medium in a 100 ml Erlenmeyer flask at 25°C at 80 r.p.m. in the dark, and maintained by regular transfer of 10 ml of 4-day-old cell suspension into 10 ml of fresh medium. Four-day-old cells were collected by centrifugation at 1000 r.p.m. for 5 min, suspended in an enzyme solution containing 0.1% (w/v) Pectolyase Y-23, 2% (w/v) Cellulase Onozuka RS (Yakult Honsha) and 0.5 M-mannitol, pH 5.5, and incubated overnight at 30°C with 60 excursions/min.

Barley seedlings (cv. New Golden) were grown on moistened vermiculite at 20°C, with a 14 h light period (approx. 8000 lx). Mesophyll protoplasts were prepared from 8-day-old primary leaves according to Okuno & Furusawa (1977, 1978) with modifications as follows. The lower epidermis was peeled off with forceps and leaf pieces were infiltrated in vacuo with an enzyme solution containing 0.05% (w/v) Macerozyme R-10 (Yakult Honsha), 1.5% (w/v) Cellulase Onozuka R-10 and 0.5 M-mannitol, pH 5.4, and incubated for 45 min at 30°C with 80 excursions/min.

Chrysanthemum plants (cv. Shuuhou no Chikara) were grown aseptically on 50% MS agar medium without plant hormones by stem tip culture at 25°C, with a 14 h light period (approx. 4000 lx). Fully expanded leaves of about 1-month-old plants were cut with a razor into strips approximately 4 to 5 mm wide, infiltrated in vacuo with an enzyme solution containing 0.25% (w/v) Macerozyme R10, 1.0% (w/v) Cellulase Onozuka RS and 0.5 M-mannitol, pH 5.6, and incubated for 5 h at 30°C with 70 excursions/min.

After the enzyme digestion the protoplasts were collected by filtration through a 150 mesh filter followed by centrifugation at 500 r.p.m. for 6 to 10 min. The protoplasts were washed twice by
centrifugation at 500 r.p.m. for 4 to 5 min in 0.5 M-mannitol and finally suspended at approx. 2 x 10^7/ml in 0.5 M-mannitol containing 100 μM-MgCl₂.

**Electrotransfection.** The RNA for electrotransfection was prepared from freshly purified TMV (OM strain) according to the method of Fraenkel-Conrat *et al.* (1961). Electrotransfection was performed as described previously (Hibi *et al.*, 1988) with slight modifications as follows. The protoplast suspension was mixed with TMV RNA at 10 to 20 μg/ml, kept at 4 °C for 10 min and exposed at 4 °C to five square DC pulses of 50 μs duration at various voltages (V/cm) in the electric field by a JASCO CET-200 continuous flow electromanipulator. The electrotransfected protoplasts were kept at 4 °C for 10 min, sedimented by centrifugation at 500 r.p.m. for 3 min, suspended in Aoki & Takebe (1969) medium, and incubated for 36 to 48 h at 25 °C under approx. 1000 lx continuous illumination.

**Fluorescent antibody staining and ELISA.** After incubation the transfection percentages were counted by an indirect fluorescent antibody staining method, which was modified from the direct staining method of Hibi *et al.* (1986), using monoclonal anti-TMV OM mouse IgG1 and anti-mouse IgG(H+L) goat IgG F(ab')₂ conjugated with fluorescein isothiocyanate (Tago).

For the ELISA, the transfected protoplasts were sedimented by centrifugation at 500 r.p.m. for 3 min and the supernatant was discarded. The pellet was frozen, thawed and lysed with PBS–Tween (PBST; 8.0 g/l NaCl, 0.2 g/l KCl, 2.9 g/l Na₂HPO₄·12H₂O, 0.2 g/l KH₂PO₄, 0.05% v/v Tween-20), and centrifuged at 8000 r.p.m. for 5 min. The supernatant was assayed according to the ELISA procedures described previously (Hibi & Saito, 1985). The virus concentration of the sample was calculated from the calibration curve plotted with concentrations of purified TMV (ng/ml) against ELISA values (absorbance at 405 nm).

![Fig. 1](image1.png)

**Fig. 1.** Effects of pulse voltage on the TMV RNA transfection (○) and the survival (△) of protoplasts from *L. peruvianum* suspension culture cells (a), tomato mesophyll (b), barley mesophyll (c) and chrysanthemum mesophyll (d). Electrotransfection was performed with 10 μg/ml (for a, b and d) or 20 μg/ml (for c) TMV RNA and five square DC pulses of 50 μs duration at various voltages.

![Fig. 2](image2.png)

**Fig. 2.** Effect of TMV RNA concentration on the transfection of protoplasts from *L. peruvianum* suspension culture cells (a), tomato mesophyll (b), barley mesophyll (c) and chrysanthemum mesophyll (d). Electrotransfection was performed with five square DC pulses of 50 μs duration at 500 V/cm (for a), 800 V/cm (for b and d) or 600 V/cm (for c) with various concentrations of TMV RNA.
Electrotransfection with TMV RNA

Fig. 3. Growth curves of TMV in electrotransfected protoplasts from L. peruvianum suspension culture cells (a), tomato mesophyll (b), barley mesophyll (c) and chrysanthemum mesophyll (d). Electrotransfection was performed under the optimum conditions for each kind of protoplast, i.e. with 10 µg/ml (for a, b and d) or 20 µg/ml (for c) TMV RNA and five square DC pulses of 50 µs duration at 500 V/cm (for a), 800 V/cm (for b and d) or 600 V/cm (for c). Virus particle numbers per transfected protoplast were calculated from the virus concentrations determined by ELISA and percentages of transfected protoplasts determined by the fluorescent antibody staining method. Dashed lines represent the theoretical curves at virus concentrations below the detection limit of ELISA.

Results and Discussion

Fig. 1 and 2 show the effects of pulse voltage and of RNA concentration, respectively, on TMV RNA transfection of the four kinds of protoplasts. The growth curves of TMV in the protoplasts electrotransfected using optimum conditions are shown in Fig. 3. The optimum pulse voltages and RNA concentrations for maximum transfection and virus yield are shown in Table 1. These data indicate that the optimum pulse voltages and TMV RNA concentrations are approximately the same for the four kinds of protoplasts, regardless of the plant species, the tissue from which the protoplasts originated and response of whole plants against TMV infections. Previously it was reported that more than 95% of tobacco (Nicotiana tabacum cv. Xanthi NN, a local lesion host) and cowpea (Vigna sesquipedalis cv. Kurodane-Sanjaku, a subliminal infection host) mesophyll protoplasts could be electrotransfected with 10 µg/ml TMV RNA at 850 V/cm and 900 V/cm, respectively, i.e. electrical conditions similar to those in the present study (Hibi et al., 1986, 1988). Therefore the optimum pulse voltage for electrotransfection of various plant protoplasts seems to be in the range of 500 to 900 V/cm, when square DC pulses of 50 µs duration are applied.

Plant protoplasts are fundamentally similar in the structure of the cell membrane. Therefore, if the fluidity of the cell membrane is also similar, the critical breakdown voltage across the cell membrane should be almost the same and the optimum pulse voltage for electrotransfection should in principle be affected only by the size of the protoplast, i.e the voltage should be inversely proportional to the radius (Neumann et al., 1982). Our data indicated a narrow range of pulse voltages with a short pulse duration for the optimum transfection of various kinds of protoplasts (diameter range approx. 20 to 40 µm). However there are several reports stating that considerably higher pulse voltages or longer pulse durations were optimal for protoplast transfection (for review see Hibi, 1989). These discrepancies can be explained by the differences in DC pulse type used (square or exponentially decaying), in the

Table 1. Optimum conditions for maximum electrotransfection with TMV RNA of four kinds of plant protoplasts

<table>
<thead>
<tr>
<th>Protoplasts</th>
<th>Pulse voltage (V/cm)</th>
<th>RNA concentration (µg/ml)</th>
<th>Maximum transfection (%)</th>
<th>TMV yield* (x 10^-5/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. peruvianum</td>
<td>500</td>
<td>10</td>
<td>37</td>
<td>8</td>
</tr>
<tr>
<td>Tomato</td>
<td>800</td>
<td>10</td>
<td>95</td>
<td>6</td>
</tr>
<tr>
<td>Barley</td>
<td>600</td>
<td>20</td>
<td>95</td>
<td>7</td>
</tr>
<tr>
<td>Chrysanthemum</td>
<td>800</td>
<td>10</td>
<td>63</td>
<td>5</td>
</tr>
</tbody>
</table>

* Virus particle numbers per transfected protoplast after 48 h of incubation.
yields comparable to those in tobacco mesophyll protoplasts at almost the same rate, resulting in virus materials, as already discussed (Hibi, 1989).

It seemed that TMV multiplied in these four kinds of protoplasts at almost the same rate, resulting in virus yields comparable to those in tobacco mesophyll protoplasts (approx. \( 10 \times 10^5 \) protoplast: Takebe & Otsuki, 1969; Hibi & Yora, 1972). This happened despite the fact that the plants used in this study show very different responses against TMV infections.

*L. peruvianum* has a TMV resistance gene (*Tm-2*) (Cirilli & Alexander, 1969) and has been reported not to prevent TMV multiplication in mesophyll protoplasts which were infected with virus particles using poly-L-ornithine (Motoyoshi & Oshima, 1977). Tomato and barley are a systemic infection host and a subclinical infection host, respectively, and their mesophyll protoplasts could likewise be infected with TMV using poly-L-ornithine (Motoyoshi & Oshima, 1975; Maekawa et al., 1985). These protoplast infections were confirmed in the present study using electrotransfection with TMV RNA. Chrysanthemum is an apparent non-host of TMV but its mesophyll protoplasts could be infected with TMV RNA, followed by normal virus replication, as shown in this study.

All these results suggest that the host range of TMV is mainly controlled in the whole plant according to whether the virus can translocate into the neighbouring cells from the initially infected cells. However, virus uncoating and the start of replication in the initially infected cells, plant hypersensitive reactions or virus long distance translocation must also be involved in host specificity.

We would like to thank Dr I. Havukkala of the National Institute of Agrobiological Resources for correcting the English.

References


(Received 23 September 1991; Accepted 25 November 1991)