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Infection of tobacco protoplasts with in vitro transcribed tobacco mosaic virus RNA using an improved electroporation method

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We previously established an in vitro transcription system which can produce infectious tobacco mosaic virus (TMV) RNAs from cDNA templates [(1986) Proc. Natl. Acad. Sci. USA 83, 5043–5047]. Here we report a useful system for the infection of tobacco protoplasts with a small amount of infectious RNAs, like in vitro transcripts from cDNA templates, involving an improved electroporation method. This system will facilitate the elucidation of the functions of the genomic RNA and its coding proteins using in vitro manipulated TMV-RNAs.

Electroporation; Transcription; Infectious transcript; Tobacco mosaic virus; (Tobacco protoplast)

1. INTRODUCTION

The success of in vitro transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus (TMV) [1,2] has allowed the synthesis of site-directed mutagenized TMV RNAs by means of manipulation at the level of cDNAs (for example, [3]). To investigate the biological changes of mutagenized viral RNAs, a synchronous virus infection system involving protoplasts would be the most powerful technique. Efficient methods for the viral RNA infection of protoplasts with the aid of liposomes [4,5] have been reported, other than the classical polycation methods [6,7]. However, these procedures require considerable amounts of viral RNAs, about 10 μg at least, and cannot be used for infection with a small amount of RNAs like in vitro transcripts.

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Recently, an electroporation technique was reported as a new and efficient method for the introduction of exogenous substances into eukaryotic cells [8–10]. Some groups have applied this technique to TMV-RNA inoculation to protoplasts [11,12], but the systems still required about 10 μg/ml of TMV-RNA. By refining the electroporation method, we tried to develop an infection system with a small amount of in vitro transcript of TMV RNA of the 0.1 μg order.

2. MATERIALS AND METHODS

Tobacco BY-2 cells were kept as a suspension culture as described [4]. Japanese common strain OM and tomato strain L of TMV were used throughout the experiments. The in vitro transcription reaction from full-length cDNA clones was performed in the same manner as described [1]. After the transcription, the products were treated with DNase I (Worthington, DPRF), followed by phenol-extraction and ethanol precipitation.

Transcripts (or viral RNA) were inoculated to tobacco protoplasts by applying a high-voltage

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pulse to a mixture of protoplasts and RNA as follows. Protoplasts were prepared from suspension cultured tobacco cells by incubation in 1.0% Cellulase Onozuka RS (Yakult Honsha Co.), 1.0% Driselase (Kyowa Hakko Kogyo Co.), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co.) and 0.4 M D-mannitol (pH 5.5) at 28°C for 2 h. The protoplasts were rapidly rinsed with three changes of ice-cold 0.4 M mannitol to remove residual enzyme solution. Then the protoplasts (1×10^6) were suspended in 0.8 ml of ice-cold cell suspension solution (0.3 M mannitol, 5 mM Mes [2-(*N*-morpholino)ethanesulfonic acid] (pH 5.8), 70 mM KCl) [11] and then mixed with the inoculum transcript (of which the infectivity was equivalent to ~0.2 μg viral RNA) or viral RNA (0.05–2 μg) in a flat-sided, open topped plastic disposable cuvette (no.67.742, Sarstedt) cooled on ice. Electroporation was carried out immediately after the two stainless-steel electrodes, which were placed 0.4 cm apart, had been lowered into the cuvette. A toggle switch was used to discharge a bank of a condenser (effective capacitance; 100 μF charged to 300 V with a power supply) through the disposable cuvette. The mixtures of protoplasts and RNA were allowed to stand for 30 min at 0°C after the electric shock. The protoplasts were then warmed at 30°C for 5 min, centrifuged to remove the buffer, suspended in 10 ml of the protoplast culture medium [4] and then cultured at 28°C after being divided into adequate portions.

The infection ratios (the percentage of infected protoplasts) were examined 1 day after virus RNA inoculation by staining protoplasts with fluorescein isothiocyanate (FITC)-labeled TMV antibody [13].

Pulse-labeling experiments with [^{3}H]uridine or [^{35}S]methionine were performed as described [14].

3. RESULTS AND DISCUSSION

The electroporation methods which other groups reported [11,12] all required as much as 10 μg/ml inoculum TMV RNA. However, the in vitro transcription system, from full-length cDNA clones [1], can supply infectious transcripts equivalent to only about 0.2 μg viral RNA on a 150 μl reaction scale.

In model experiments to improve the infection procedures used [11], we used the purified viral RNA as an inoculum at first. Cold treatment for 30 min after an electric shock increased the infection ratio by about 2–3-fold (experiment 1, table 1). To minimize the damage to the inoculum RNA, the enzyme solution had to be carefully and completely washed off from the protoplasts, which were subsequently kept at a low temperature on ice, because the enzyme preparation usually contains a considerable amount of nucleases. When the rinsing of protoplasts was performed only once, the viral infection could not be established with 0.2 μg of the viral RNA (experiment 2). However, repeating the rinsing surprisingly increased the infection ratio and it was a prerequisite for constant infection with 0.2 μg of the viral RNA (experiment 2). In addition, protoplasts and inoculum RNA, once mildly mixed, had to be subjected to the electrical shock as soon as possible.

The results of experiment 3 showed that infection was established in 15% of the protoplasts even with a small amount of the viral RNA, i.e. as little as 50 ng, under the standard conditions (see section 2). This indicated that a sufficient infection ratio for the biochemical analysis of the multiplication process would be attained in this system with infectious RNAs of the 0.1 μg order. If a higher infection ratio is desired with small amounts of RNA, less than 1×10^6 protoplasts should be subjected to the electrical shock (experiment 4). The coexistence of 2 μg of *E. coli* rRNA or yeast tRNA with 0.2 μg of viral RNA raised the infection ratios by about 50% (experiment 5). The presence of non-viral RNA would enhance the infection ratios, probably due to lowering of the degradation of the viral RNA.

Before applying the method for infection with an in vitro transcript, we checked the effects of other possible ethanol-precipitable substances derived from the reaction mixture together with the in vitro transcript, that is, premature transcripts, template DNA, ribonucleotide triphosphate and m^{7}GpppG. Template DNA contributed preferably to the infection ratio (experiment 6), but pulse-labeling with [^{3}H]uridine showed that the level of TMV multiplication in infected protoplasts was lowered. The coexistence of rNTPs, m^{7}Gppp and tRNA (the latter two were used in place of m^{7}GpppG and premature transcripts, respectively, in this model experiment) contributed preferably to the multiplication level (not shown) as well as to

Table 1

Model infection experiments

Expt	Inoculum (μg)	Protoplasts	Procedure	Infection ratio (%)
1[a]	2	1×10^6	0 min at 0°C	20
	2	1×10^6	10 min at 0°C	29
	2	1×10^6	30 min at 0°C	55
2[b]	0.2	1×10^6	rinse × 1	<1
	0.2	1×10^6	rinse × 3	33
3[c]	0	1×10^6		0
	0.05	1×10^6		15
	0.1	1×10^6		17
	0.2	1×10^6		28
4[c]	0.2	5×10^5		47
	0.2	2×10^5		65
	0.2	1×10^5		80
5[c]	0.2	1×10^6	no additives	32
	0.2	1×10^6	+ *E. coli* rRNA (2 μg)	50
	0.2	1×10^6	+ yeast tRNA (2 μg)	45
6[c]	0.2	1×10^6	no additives	28
	0.2	1×10^6	+ template DNA (7.5 μg)	40
	0.2	1×10^6	+ template DNA (7.5 μg), rNTPs[d], m^7Gppp[d], tRNA (2 μg)	40
7[c]	W3 transcript[e] (150 μl scale)	1×10^6		30

[a] The protoplasts were rinsed once with 0.4 M mannitol after preparation, mixed with 2 μg of viral RNA, subjected to an electric pulse and then stood at 0°C for the indicated times

[b] The protoplasts were rinsed once or three times with 0.4 M mannitol and then electroporated as described in the text

[c] The experiments were performed as described in the text except that the amounts of protoplasts, RNAs or coexisting substances were varied in the respective combinations

[d] The amounts of rNTPs and m^7Gppp are the residual ones after ethanol-precipitation

[e] The transcripts (150 μl scale reaction) used had an infectivity equivalent to 0.2 μg of TMV-RNA

Protoplasts were mixed with the inoculum (with or without coexisting substances) and then subjected to the electrical shock on a 0.8 ml scale. The protoplasts were stained with a fluorescent antibody 1 day after inoculation and then the infection ratios were determined on fluorescence microscopic observation [13]

the infection ratio. Invasion of these exogenous nucleotide precursors and unrelated RNAs into the protoplasts did not cause any detectable damage to the protoplasts or a decrease in the efficiency of the incorporation of [^{3}H]uridine into the progeny viral RNA. Based on these results, we used the in vitro transcript as an inoculum after digestion of the template DNA with DNase I, phenol extraction and ethanol precipitation. When the W3 transcript [1], which was transcribed from the *Mlu*I-cut pLFW3 DNA template, was used as an inoculum at least 30% of the protoplasts were actually infected (experiment 7). In recent routine work we obtained infection ratios of 40–70% with various

kinds of transcripts. Here, an infection system of protoplasts with in vitro transcripts was established, as described in section 2.

The inoculation of the W3 transcript onto tobacco plants resulted in wild-type virus production and indistinguishable pathological symptoms [1]. The previous data did not reveal the phenomena occurring at the early stage of infection, i.e. when TMV infection leads to the production of the 130, 180, 30 kDa and coat proteins as well as the genomic RNA, the 30 kDa protein mRNA and the coat protein mRNA in the replication process [14]. We checked whether or not it was possible to investigate the replication process in the W3 transcript- and the native viral RNA-infected protoplasts, even though the amounts of the inocula were much less than in any previous study. The results of pulse-labeling experiments with [^{3}H]uridine (fig.1) and [^{35}S]methionine (not shown) showed that analysis of the synthesis of the TMV-specific RNAs or viral-coded proteins mentioned above was possible at the early stage of infection as we reported [14]. The results also showed that the infection cycle of the transcript started and proceeded normally, showing almost the same time course as that of the native viral RNA. Also analysis of viral-coded protein synthesis did not reveal any detectable differences in the time courses (not shown). These results showed that the extra nucleotides at the 3′-end of the transcript [1] did not affect the synchronous nature of infection cycle at the early stage of replication. They might be eliminated at the step of the first minus-strand synthesis, the inoculated transcript being used as a template.

This efficiency and feasibility allowed the in vivo analysis of as little as 0.2 μg, or less, of the mutagenized viral transcripts during synchronous multiplication. We can also investigate mutants that are completely devoid of the cell-to-cell movement function and thus will not propagate in plant leaves. Investigations on the functions of RNA structures necessary for replication of the genomic RNA or production of subgenomic RNAs will be possible using this infection system. The techniques refined here will also be applicable to other plant viruses.

Preliminary experiments showed that, when a smaller amount of transcript (e.g., due to a low yield in the transcription reaction) was inoculated into protoplasts, the infection ratio as well as the multiplication level was low as expected from experiment 3 (table 1). For the characterization of a mutant, thus, it would be necessary to investigate replication processes of several independent transcripts at least.

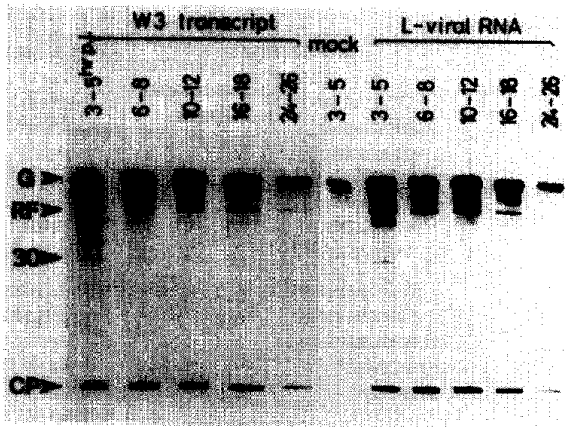

Fig.1. Comparison of the synthesis of TMV-specific RNAs in L viral RNA- and W3 transcript-inoculated protoplasts. Protoplasts (1×10^6) were inoculated with either the W3-transcript (150 μl reaction scale) [1] or L viral RNA (0.2 μg) by the electroporation method, and then pulse-labeled with 20 μCi [^{3}H]uridine/ml for 2 h at the times indicated at the tops of the lanes. RNAs were analyzed on 8 M urea-2.4% polyacrylamide gels as described [14]. G, genomic RNA; RF, replicative form RNA; 30, 30 kDa protein mRNA; CP, the coat protein mRNA [14].

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REFERENCES

[1] Meshi, T., Ishikawa, M., Motoyoshi, F., Semba, K. and Okada, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 5043–5047.
[2] Dawson, W.O., Beck, D.L., Knorr, D.A. and Grantham, G.L. (1986) Proc. Natl. Acad. Sci. USA 83, 1832–1836.
[3] Ishikawa, M., Meshi, T., Motoyoshi, F., Takamatsu, N. and Okada, Y. (1986) Nucleic Acids Res. 14, 8291–8305.
[4] Watanabe, Y., Ohno, T. and Okada, Y. (1982) Virology 120, 478–480.
[5] Nagata, T., Okada, K., Takebe, I. and Matsui, C. (1981) Mol. Gen. Genet. 184, 161–165.
[6] Aoki, S. and Takebe, I. (1969) Virology 39, 439–448.
[7] Motoyoshi, F. and Oshima, N. (1979) J. Gen. Virol. 44, 801–806.
[8] Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) EMBO J. 1, 841–845.
[9] Potter, H., Weir, L. and Leder, P. (1984) Proc. Natl. Acad. Sci. USA 81, 7161–7165.
[10] Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A. and Kucherlapati, R.S. (1985) Nature 317, 230–234.
[11] Okada, K., Nagata, T. and Takebe, I. (1986) Plant Cell Physiol. 27, 619–626.
[12] Hibi, T., Kano, H., Sugiura, M., Kazami, T. and Kimura, S. (1986) J. Gen. Virol. 67, 2037–2042.
[13] Otsuki, Y. and Takebe, I. (1969) Virology 38, 497–499.
[14] Watanabe, Y., Emori, Y., Ooshika, I., Meshi, T., Ohno, T. and Okada, Y. (1984) Virology 133, 18–24.