Infection of Turnip Leaf Protoplasts with Turnip Rosette Virus

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SUMMARY

The optimum conditions for infection of turnip protoplasts with turnip rosette virus (TRosV) were 0.2 to 2 × 10^5 protoplasts/ml, 5 to 10 μg virus/ml, 1 μg poly-L-ornithine/ml in 0.6 M-mannitol, buffered with 5 to 10 mM-tris at pH 7.0 to 8.6. Under these conditions, 70% of protoplasts became infected, as indicated by staining with fluorescent antibody. The proportion of protoplasts infected was determined by factors such as pH and buffer ions and by the concentrations of protoplasts, virus and poly-L-ornithine during inoculation. The period of pre-inoculation incubation of virus and poly-L-ornithine was also critical, but mannitol concentration and the inoculation period and temperature had little effect on percentage protoplasts infected. Time course studies showed a single step multiplication of TRosV; the virus was detected on sucrose gradients at 24 h after inoculation and at 60 h reached its maximum concentration, 3 × 10^6 virions per infected protoplast.

INTRODUCTION

The infection of plant protoplasts with plant viruses provides a technique through which synchronous infection can be studied (Takebe & Otsuki, 1969). The technique has been applied to the study of the replication of several plant viruses, with both translated and transcribed products being examined (Sakai & Takebe, 1974; Aoki & Takebe, 1975). In this paper we describe the optimization of conditions for infection of turnip leaf protoplasts with turnip rosette virus (TRosV). This virus belongs to the southern bean mosaic virus group (Hull, 1977a), members of which have small (mol. wt 1.4 × 10^6), apparently single component RNA genomes encapsulated in a protein shell of 180 identical polypeptides (mol. wt 30,000).

METHODS

Isolation of protoplasts. Brassica rapa L. (turnip) cv. Just Right was used as the protoplast source. Plants were grown in a 1:1 mixture of John Innes No. 2 compost and peat in 20 cm pots in a greenhouse at 16 to 25 °C, with GEC Solarcolor sodium lighting (12 to 16 h per day, 2000 to 5000 lux) during the winter months. They were given supplementary nitrogen (Fisons No. 5P) weekly. Further plants were grown in a controlled environment chamber at 20 °C, 14 h illumination (3000 lux) per day and 50 to 80% relative humidity. Plants in the chamber were fed twice weekly with a complete nutrient solution (Hewitt & Smith, 1975).

Plants most suitable for protoplast isolation had large lower leaves (15 to 20 cm lamina length) which felt very thin when rubbed between finger and thumb and were of uniform dark green colour. Fully expanded lower leaves were selected from 30 to 60 day-old plants and protoplasts were isolated using the one-step procedure of Howell & Hull (1978).

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Inoculation of protoplasts. Unless otherwise stated, inoculum TRosV (purified as described by Hull, 1977b) was mixed with poly-L-ornithine (mol. wt. 122000, Sigma Chem. Co.) and incubated in 0.6 M-mannitol for 5 to 10 min at 30 °C in the presence of 10 mM-tris-HCl buffer, pH 8.0. Freshly sedimented protoplasts were resuspended in a minimal vol. of 0.6 M-mannitol and added to the incubated inoculum. The final concentrations in the inoculum mixture were usually: 10 μg/ml TRosV, 1 μg/ml poly-L-ornithine, 1 to 2 x 10⁵/ml protoplasts and 10 mM-tris buffer, pH 8.0. After 5 min at 30 °C the protoplasts were separated from the inoculum by two washes in 0.6 M-mannitol containing 1 mM-CaCl₂. When incorporated in the wash mannitol, and later in the culture medium, CaCl₂ at 1 mM stabilized protoplasts better than that at 0.1 mM; 10 mM-CaCl₂ caused unwanted clumping of protoplasts. Finally, the protoplasts were resuspended in the culture medium of Motoyoshi et al. (1973a) with 0.6 M-mannitol and 1 mM-CaCl₂, containing nystatin (Mycostatin; 6 μg/ml) and carbenicillin (Pyopen; 200 μg/ml). Protoplasts at 1 to 3 x 10⁵/ml in 5 ml portions were transferred to scintillation vials and incubated at 22 or 25 °C under continuous illumination (1000 lux) from fluorescent tubes.

Staining with fluorescent antibody. Antiserum to TRosV (ring precipitin titre 1/8192) was prepared by two subcutaneous injections of a rabbit, a week apart, with virus (4 and 1 mg respectively) emulsified in Freund’s complete adjuvant (Difco Bacto) followed by bleeding 3 to 5 weeks after the second injection.

The globulin fraction of the antiserum was conjugated with fluorescein isothiocyanate (FITC, Koch Light Lab. Ltd.) as described by Otsuki & Takebe (1969) to give a FITC: globulin molar ratio of 1:1.9. The conjugated globulins had a ring precipitin titre of 1/1024 and could be used at a dilution of 1/128 to stain TRosV-infected protoplasts; however, dilutions of 1/8 to 1/32 gave the most easily distinguishable fluorescence. After absorption with acetone-dried turnip leaf powder, no non-specific fluorescence was evident at any dilution. For staining, 15 μl of a protoplast suspension was spread on a glass slide (previously coated with gelatin) and dried with a warm air current. The specimen was fixed, stained, washed and examined as described by Motoyoshi et al. (1973a) using a Vickers fluorescence microscope (M41 Photoplan).

The conjugate globulin was specific for TRosV and did not stain uninoculated protoplasts, or those infected with TRosV but pre-treated with unconjugated anti-TRosV globulin or TRosV antiserum; TRosV-infected protoplasts pre-treated with southern bean mosaic virus antiserum were stained.

Assay of TRosV on sucrose gradients. Protoplast samples were collected by low speed centrifugation, resuspended in 1 to 2 ml of 0.1 M-acetate, pH 5.0 and homogenized in a glass tube with Teflon plunger. The extract was clarified by centrifuging at 3000 rev/min for 10 min and assayed using the sucrose gradient procedure of Motoyoshi et al. (1973b). Samples (0.5 ml) were layered on to 10 to 40 % sucrose gradients in acetate buffer in SW 50.1 tubes and centrifuged at 35000 rev/min for 2 h. The virus yield was quantified by comparison with samples containing known TRosV concentrations, using the 0.25 scale of an ISCO UA-2 density gradient analyser. As little as 0.3 μg of virus was detected by this procedure and at least 80 % recovery was obtained when known amounts of virus were added to uninfected protoplasts.

RESULTS

Factors affecting infection

Inoculation buffer, pH and concentration

The infection of turnip protoplasts with TRosV was greatly influenced by the type, pH and concentration of buffer in the inoculation medium. Tris proved to be the most effective
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Table 1. Effect of different inoculation buffers on infection of turnip protoplasts with TRosV

<table>
<thead>
<tr>
<th>Expt.*</th>
<th>Buffer†</th>
<th>pH</th>
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<th>% infected§</th>
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<td>—</td>
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<td>Potassium phosphate</td>
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<td>8</td>
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<td>2</td>
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<tr>
<td></td>
<td>Tris-HCl</td>
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<td>21</td>
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<tr>
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<td>Unbuffered</td>
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* In Expt. 1, inoculum contained 10 µg/ml virus and 2 µg/ml poly-L-ornithine. Inoculated protoplasts were cultured for 28 h at 25 °C. In Expt. 2, inoculum contained 10 µg/ml virus and 1 µg/ml poly-L-ornithine. Inoculated protoplasts were cultured for 49 h at 22 °C.
† All buffers were at 0.01 M during inoculation.
‡ After culture.
§ Percentage of intact protoplasts infected, as scored by fluorescent antibody staining.

Fig. 1. Effect of (a) pH of inoculation buffer (10 mM) and (b) inoculation buffer concentration (pH 8.0) on TRosV infection. Protoplasts were inoculated with 10 µg/ml TRosV using tris buffer of various pH values and concentrations. Conditions of inoculation were otherwise as described in Methods. Protoplasts were cultured for 32 h (○—○) and 68 h (●—●). Percentage infection determined by fluorescent antibody staining.

buffer (Table 1). Less than 10% infection was obtained using citrate or phosphate buffer, both of which are effective and commonly used in other protoplast inoculation systems (Takebe, 1975). Tris buffer has proved very effective for inoculating tomato protoplasts (Motoyoshi & Oshima, 1976) with tobacco mosaic virus (TMV) and tobacco protoplasts (Mayo, 1978) with tobacco rattle virus (TRV).

The effects of tris buffer pH on the frequency of TRosV infection are illustrated in Fig. 1 (a). Most infection of protoplasts was obtained with inoculum buffered between pH 7.0 and 8.6, which is close to the effective buffering range of tris (pH 7.1 to 8.9). A similar pH range (pH 7.2 to 8.7) of tris buffer is optimal for infection of tomato protoplasts with TMV (Motoyoshi & Oshima, 1976).

The concentration of tris buffer used during protoplast inoculation affected the proportion of protoplasts that became infected (Fig. 1 b), a narrow concentration range (5 to 10 mM)
Fig. 2. Effect of (a) poly-L-ornithine concentration, (b) TRosV concentration and (c) protoplast concentration on frequency of infection. Protoplasts were inoculated with the appropriate variables, conditions otherwise were as described in Methods. Protoplasts were cultured for 30 h (○—○) and 45 h (●—●). Percentage infection was determined by fluorescent antibody staining.

giving the most infection. For TMV infection of tomato protoplasts, 50 mm-tris buffer was optimal (Motoyoshi & Oshima, 1976). Similar dependence of infection on buffer ion concentrations are observed with phosphate and citrate buffers in other protoplast systems (Kubo et al. 1974).

Poly-L-ornithine concentration

No infection was obtained when protoplasts were inoculated in the absence of poly-L-ornithine. In experiments to investigate the effect of poly-L-ornithine concentration on the percentage infection (Fig. 2a) the optimum was found to be 1 μg/ml; above 2 μg/ml poly-L-ornithine, protoplast survival was adversely affected. Pre-treatment of the protoplasts with poly-L-ornithine 0.1 or 0.5 μg/ml, before inoculation without poly-L-ornithine, produced negligible infection.

Virus concentration

The concentration of TRosV in the inoculum also affected the percentage infection of protoplasts (Fig. 2b); the optimum virus concentration was 5 to 10 μg/ml. At 100 μg virus/ml, 10% infection was obtained, indicating that some other component necessary for infection was limiting, possibly poly-L-ornithine. This was not examined further because protoplast survival, as previously noted, was adversely affected by higher poly-L-ornithine concentrations.

Protoplast concentration

Fig. 2 (c) shows the effect of concentration of protoplasts during inoculation on percentage infection. Maximum infection (80%) occurred with protoplasts at the lowest concentration tested (0.2 × 10⁶/ml). However, because the recovery of protoplasts by sedimentation was less efficient with such a low concentration, a compromise concentration of 1 to 2 × 10⁸/ml protoplasts was used thereafter. Protoplast concentrations in excess of 2 × 10⁹/ml resulted in low percentages of infection.

Mannitol concentration

Infection was not influenced by osmotic shock. Protoplasts, after isolation and washing in 0.6 M-mannitol, were inoculated at several mannitol concentrations between 0.5 and 0.9 M and infection percentages remained around 50%. Some reduction in protoplast survival was occasionally evident with mannitol concentrations above 0.6 M.
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Pre-inoculation incubation of virus and poly-L-ornithine

Pre-inoculation incubation of virus and poly-L-ornithine prior to protoplast inoculation increased the percentage infection (Fig. 3). Few protoplasts were infected without pre-inoculation incubation but the maximum effect was obtained with 5 to 10 min pre-inoculation incubation. This response closely correlated with an increase in turbidity of the virus and poly-L-ornithine solution (Fig. 3).

Inoculation period and temperature

The percentage infection and protoplast survival were unaffected by increasing the inoculation period from 5 to 25 min. No effect on percentage infection was observed with different inoculation temperatures (0, 10, 20 and 30°C); however, during the separation of protoplasts from inoculum, the temperatures almost equilibrated to ambient.

Both the period of inoculation and the temperature can affect infection in other protoplast inoculation systems (Otsuki et al. 1974; Alblas & Bol, 1977).

Method of inoculation

Protoplasts were usually resuspended in a minimal vol. of mannitol (0.5 to 1 ml) and transferred to the inoculation mixture. When this method was compared with both the 'direct' and 'indirect' inoculation methods (Motoyoshi et al. 1974), the percentages of infected protoplasts were 50, 49 and 47 respectively. The 'usual' procedure, involving resuspension in a minimal vol. of mannitol solution was used subsequently for convenience.
TRosV multiplication in protoplasts

The course of TRosV multiplication in turnip protoplasts was followed by assaying the virus at intervals after inoculation. The results, illustrated in Fig. 4, show that TRosV concentration increased exponentially between 24 h and 40 h p.i. and thereafter less rapidly. No virus was detected earlier than 24 h after inoculation using the sucrose density gradient assay, which has limited sensitivity.

Virus recovered on sucrose gradients from TRosV inoculated protoplasts was found to be infectious when rubbed on to leaves of turnip cv. Snowball.

The production of virus antigen was first evident by fluorescent antibody staining at 18 h p.i. (Fig. 4). The intensity of fluorescence and the proportion of protoplasts stained increased rapidly until 40 h p.i. TRosV-infected protoplasts, stained with fluorescent antibody, showed a general distribution of antigen throughout the cytoplasm with none of the organelles being specifically stained (Fig. 5).
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Fig. 5. Fluorescence micrograph of a turnip protoplast, sampled at 32 h after inoculation with TRosV, and stained with fluorescent antibody to virus.

Effect of antibiotics

The antibiotics nystatin (Mycostatin) and carbenicillin (Pyopen), 6 and 200 μg/ml respectively, were usually included with incubated protoplasts to reduce growth of microbial contaminants. The amount of TRosV detected 45 h post inoculation on sucrose gradients was slightly reduced by the inclusion of these antibiotics and also by inclusion of actinomycin D at 10 μg/ml. Nystatin and carbenicillin reduced virus yield by about 10% in protoplasts sampled at 45 h p.i. and actinomycin D reduced the yield by about 25 to 30%. Neither actinomycin D (1 to 30 μg/ml) nor chloramphenicol (100 to 200 μg/ml) affected the proportion of protoplasts that stained with fluorescent antibody at 45 h p.i. Reduced virus multiplication in protoplasts in the presence of actinomycin D has been observed with alfalfa mosaic virus, pea enation mosaic virus and potato virus X (Motoyoshi & Hull, 1974; Otsuki et al. 1974; Alblas & Bol, 1977).

DISCUSSION

In this investigation up to 70% of protoplasts isolated from turnip leaves, by a one-step enzyme procedure, were infected with TRosV under appropriate inoculation conditions. The efficiency of infection was affected by several components of the inoculum mixture, including the type of buffer, its pH and concentration, and the concentrations of poly-L-ornithine and virus. The infection of turnip protoplasts with TRosV was dependent on the presence of poly-L-ornithine, presumably because the virus and protoplasts had the same type of charge (Takebe, 1975); TRosV had a negative charge at pH 7.0 when electrophoresed on polyacrylamide gels (unpublished observation). Furthermore, pre-inoculation
incubation of virus and poly-L-ornithine was essential to produce the most infection. An increase in the turbidity of the inoculum was noted during pre-inoculation incubation which was similar to that observed with TRV (Kubo et al. 1976), and was presumably caused by the formation of aggregates or complexes between poly-L-ornithine, buffer anions and virus (Motoyoshi et al. 1974; Otsuki et al. 1974; Kubo et al. 1976). Complexes of virus and poly-L-ornithine may therefore be important for the infection of protoplasts with TRosV. The infection of protoplasts with TRosV also appeared to depend on the tris buffer concentration and excess buffer ions possibly interfered with the formation of TRosV aggregates capable of infecting protoplasts. Thus buffer ions and TRosV may compete for poly-L-ornithine, and indeed a reaction between poly-L-ornithine and buffer ions in the absence of virus was observed by Kubo et al. (1976). A reduced proportion of protoplasts became infected with TRosV with increasing inoculum concentrations of protoplasts; a similar effect has been reported by Mayo (1978).

Subjecting turnip protoplasts to 'osmotic shock' by increasing the mannitol concentration during inoculation did not increase infection with TRosV, in contrast to what was observed with barley protoplasts and brome mosaic virus (Okuno & Furusawa, 1978) and cowpea protoplasts and alfalfa mosaic virus (Alblas & Bol, 1978). There appears to be no correlation between osmotic shock effects and the structure or net surface charge of the viruses.

Exponential accumulation of TRosV in turnip protoplasts is completed by 40 h p.i. which is later than reported for many other viruses in protoplasts (e.g. Takebe & Otsuki, 1969; Motoyoshi et al. 1973a; Otsuki et al. 1974; Hibi et al. 1975) including turnip yellow mosaic virus in Brassica protoplasts (Renaudin et al. 1975). Whether this is a reflection of a longer lag period before synthesis of TRosV begins has yet to be determined. No virus was detected earlier than 24 h p.i. by the sucrose gradient assay procedure used in the present study though staining of protoplasts with fluorescent antibody was obtainable 18 h p.i. Further work is under way to examine virus synthesis prior to 24 h p.i. using other assay procedures.

REFERENCES


Turnip rosette virus in protoplasts


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