Infection of Barley Protoplasts with Barley Stripe Mosaic Virus Detected and Assayed by Immunoperoxidase

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SUMMARY

The kinetics of infection of barley protoplasts with barley stripe mosaic virus (BSMV) was followed by enzyme-linked immunosorbent assay (ELISA) using the double-sandwich procedure. Infected protoplasts were identified by an immunoperoxidase method taking precautions to inhibit endogenous peroxidase. About 70% of the protoplasts were infected with BSMV when the inoculum contained 50 μg/ml purified BSMV in 10 mM-potassium citrate buffer pH 4.7 containing 0.65 M-mannitol and 5 μg/ml poly-L-ornithine. It was estimated by ELISA that about 1400 to 1600 virus particles were adsorbed per protoplast. Following an apparent latent period, virus increased linearly between about 14 and 48 h after inoculation. After 48 h the virus concentration reached between 1.62 × 10^6 and 2.0 × 10^6 particles per protoplast.

INTRODUCTION

The barley protoplast system may be useful for studying the infection process of a glycoprotein-containing virus such as barley stripe mosaic virus (BSMV). However, the non-specific fluorescence of healthy barley protoplasts interfered with the use of fluorescent antibody staining of the virus antigen (Takebe, 1977), a phenomenon which is also a problem with protoplasts from other hosts. To overcome this problem, a technique involving unlabelled immunoperoxidase was used to determine the percentage of infection of barley protoplasts by BSMV. Because our BSMV strain has no local lesion host, we used the double-antibody sandwich type of ELISA to measure the amount of virus antigen. This report presents the results of our investigation.

METHODS

Virus. A strain of BSMV isolated from Xinjiang was used (Xie et al., 1980). It was purified from systemically infected leaves of barley as described previously (Chiu et al., 1981 b).

Preparation of protoplasts. Barley (Hordeum vulgare cv. Pidamai) plants were used as the source of protoplasts. The plants were grown in 16 cm diam. pots containing a soil–sand mixture in an insect-proof greenhouse with the temperature averaging 20 °C by day and 15 °C by night. Vigorously growing leaves from plants at the 1 to 2 leaf stage were collected, the lower epidermis was stripped, and the remaining tissues were incubated in 0.5 % cellulase (Dongfeng Biochemical, Shanghai, China) in 0.65 M-mannitol, adjusted to pH 5.4, at 25 °C for 2 to 3 h. After incubation, the medium was filtered through a sheet of gauze to remove the upper epidermis and non-digested tissues. The protoplasts were collected by centrifuging for 3 min at 100 g, washing three times with 0.65 M-mannitol solution, and resuspending in fresh 0.65 M-mannitol solution. A micrograph of isolated protoplasts is shown in Fig. 1 (a).
Inoculation of protoplasts. Purified BSMV was suspended at a final concentration of 100 μg/ml in 20 mM-potassium citrate buffer pH 4.7 containing 10 μg/ml poly-L-ornithine (PLO, mol. wt. 130000; Sigma) in 0.65 M-mannitol. The solution was mixed with an equal volume of protoplast suspension (2 × 10⁶ protoplasts/ml) and incubated for 10 min at 25 °C. Protoplasts were freed from excess inoculum by two washes in 0.65 M-mannitol containing 0.1 mM-CaCl₂. Finally, the protoplasts were resuspended in the incubation medium of Takebe et al. (1968) and the flasks were left in a controlled environment cabinet at 25 °C and illuminated continuously at 3000 lux.

Antiserum. Antiserum against BSMV was obtained by immunizing rabbits with purified virus. The titre was 1:2000 when determined by the tube precipitation test. Antiserum from sheep to rabbit IgG was kindly supplied by the Institute of Biophysics, Academia Sinica. Antiserum from rabbit to horseradish peroxidase (HRP) (Dongfeng Biochemical, Shanghai, China; RZ = 3) was prepared by injecting rabbits with the antigen in Freund's complete adjuvant at multiple sites. The titre was 1:64 determined by dimensional immunodiffusion.

Preparation of soluble horseradish peroxidase-anti-horseradish peroxidase complex (PAP). A 5.5 ml amount of saline solution containing 0.5 mg/ml HRP was mixed with 15 ml rabbit antiserum to HRP and allowed to precipitate at room temperature for 1 h. The precipitate was centrifuged at 25000 g for 20 min and washed three times with 10 mM-phosphate-buffered saline (PBS) pH 7.2. It was then resuspended in 6 ml of a solution containing 2 mg/ml HRP and dissolved by adjustment to pH 2.3 with HCl. The solution was immediately adjusted to pH 7.5 with NaOH and 1/10 vol. of 75 mM-sodium acetate and 0.15 M-ammonium acetate were added. An equal volume of saturated ammonium sulphate solution was slowly added to the solution and the resulting precipitate was centrifuged for 20 min at 25000 g, washed once in 50% saturated ammonium sulphate solution, dissolved in 5 ml redistilled water and dialysed against two changes of 2 l each of 75 mM-sodium acetate and 15 mM-ammonium acetate buffer pH 6-7.5. The precipitate which had formed during dialysis was removed by centrifugation at 25000 g for 20 min. The PAP solution was filtered through 0.45 μm Millipore and stored at -10 °C (Sternberger et al., 1970).

Staining protoplasts by PAP. Protoplasts were stained as follows. 1. The protoplasts were spread on a glass slide (previously coated with Mayer's solution) and treated with methanol-containing 0.074% HCl to inhibit the endogenous peroxidase. 2. The preparations were incubated with rabbit IgG to BSMV for 1 h at 37 °C and washed in 10 mM-PBS pH 7.2. 3. The preparations were treated with sheep anti-rabbit IgG for 30 min at 37 °C and washed with 10 mM-PBS. 4. The preparations were reacted with PAP for 30 min at 37 °C followed by a rinse in 10 mM-PBS. 5. The preparations were stained with a freshly prepared solution of 0.05% 3,3′-diamino-benzidine (DAB) and 0.001% hydrogen peroxide for 10 min in the dark followed by a wash in 50 mM-tris-HCl buffer pH 7.6. 6. The slides were examined for brown-stained antigen under a light microscope.

Enzyme-linked immunosorbent assay (ELISA). Anti-BSMV IgG was conjugated to HRP using the method described previously (Mao et al., 1981; Clark & Adams, 1977). ELISA tests were of the double-antibody sandwich type in protein-binding polystyrene plates. The substrate solution was 1.7 ml 1% O-dianisidine in anhydrous methanol, 10 μl 30% H₂O₂ and 100 ml 10 mM-phosphate buffer pH 6; 250 μl was added to each well. Quantitative measurements were made by determining absorbance at 400 nm.

RESULTS

Factors affecting infection

The amount of adsorbed virus particles and percentage of infection of protoplasts inoculated at different pH values of 10 mM-potassium citrate buffer were assayed. The results
Immuno-peroxidase assay of BSMV in protoplasts

Fig. 1. Healthy and BSMV-infected barley protoplasts stained with unlabelled immuno-peroxidase. (a) Barley protoplasts; (b) stained healthy protoplasts; (c) stained infected protoplasts.

Table 1. Effect of pH of the inoculation medium on adsorption of virus and infection percentages of barley protoplasts by BSMV*

<table>
<thead>
<tr>
<th>Inoculation medium pH</th>
<th>Amount of virus adsorbed on protoplasts</th>
<th>Yield of virus after 24 h†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus/10^6 protoplasts (µg)</td>
<td>Virus particles/protoplast (× 10^−3)</td>
</tr>
<tr>
<td>4.7</td>
<td>0.071</td>
<td>1.64</td>
</tr>
<tr>
<td>5.0</td>
<td>0.068</td>
<td>1.58</td>
</tr>
<tr>
<td>5.3</td>
<td>0.060</td>
<td>1.39</td>
</tr>
<tr>
<td>5.6</td>
<td>0.051</td>
<td>1.18</td>
</tr>
<tr>
<td>6.0</td>
<td>0.028</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Inoculation conditions: 10 mM-potassium citrate buffer containing 50 µg/ml BSMV, 5 µg/ml poly-L-ornithine and 0.65 M-mannitol.
† Virus was determined by ELISA immediately and 24 h after inoculation. Average mol. wt. of 26 × 10^8 was used in calculating numbers of particles.
‡ Infection percentages were determined by immuno-peroxidase staining 24 h after inoculation.

Table 2. Effect of concentration of PLO on adsorption and infection of BSMV in barley protoplasts*

<table>
<thead>
<tr>
<th>PLO concentration (µg/ml)</th>
<th>Amount of virus adsorbed on protoplasts†</th>
<th>Yield of virus after 24 h† (µg/10^6 protoplasts)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus/10^6 protoplasts (µg)</td>
<td>Virus particles/protoplast</td>
</tr>
<tr>
<td>0</td>
<td>0.042</td>
<td>973</td>
</tr>
<tr>
<td>1</td>
<td>0.052</td>
<td>1205</td>
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<tr>
<td>5</td>
<td>0.072</td>
<td>1668</td>
</tr>
<tr>
<td>10</td>
<td>0.096</td>
<td>2224</td>
</tr>
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* Inoculation conditions: 10 mM-potassium citrate buffer pH 4.7 containing 50 µg/ml BSMV and 0.65 M-mannitol.
† Virus was determined by ELISA immediately and 24 h after inoculation.
Fig. 2. Time course of BSMV replication in barley protoplasts, inoculated as described in Methods. The concentration of virus was estimated using ELISA.

Table 3. Effect of concentration of BSMV on adsorption and infection of BSMV in barley protoplasts*

<table>
<thead>
<tr>
<th>BSMV concentration (µg/ml)</th>
<th>Amount of virus adsorbed on protoplasts†</th>
<th>Yield of virus after 24 h†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus/10^6 protoplasts (µg)</td>
<td>Virus particles/protoplast</td>
</tr>
<tr>
<td>1</td>
<td>0.032</td>
<td>741</td>
</tr>
<tr>
<td>5</td>
<td>0.036</td>
<td>834</td>
</tr>
<tr>
<td>10</td>
<td>0.052</td>
<td>1205</td>
</tr>
<tr>
<td>50</td>
<td>0.064</td>
<td>1483</td>
</tr>
<tr>
<td>100</td>
<td>0.066</td>
<td>1529</td>
</tr>
</tbody>
</table>

* Inoculation conditions: 10 mM-potassium citrate buffer pH 4.7 containing 5 µg/ml poly-L-ornithine and 0.65 M-mannitol.
† Virus was determined by ELISA immediately and 24 h after inoculation.

(Table 1) show that the highest numbers of protoplasts were infected at pH 4.7. At this pH, about 70% of the protoplasts were found to be infected 24 h after inoculation (Table 1, Fig. 1b, c). Lower pH values were not tested because the protoplasts were disrupted during incubation. The effect of PLO concentration is shown in Table 2. The amount of virus retained by the protoplasts was proportional to the concentration of PLO. There was aggregation of protoplasts at 10 µg/ml PLO and in all subsequent experiments 5 µg/ml PLO was used. The number of BSMV particles adsorbed by the protoplasts was roughly proportional to the BSMV concentration in inoculation medium (Table 3). Fifty µg/ml BSMV was chosen as the standard concentration for inoculation of barley protoplasts.

**Multiplication of BSMV in protoplasts**

The one-step growth curves of BSMV determined by ELISA (Fig. 2) showed that virus present at zero time represents the inoculum virus adsorbed to protoplasts. The subsequent drop of virus antigen at 6 h after inoculation may be interpreted to reflect virus uncoating. Virus antigen increased rapidly during the period between 12 h and 48 h; thereafter the increase was slow. About 71% of protoplasts were infected 24 h after inoculation. Virus yield after 48 h incubation was 2.07 x 10^6 particles/protoplast.
Immunoperoxidase assay of BSMV in protoplasts

DISCUSSION

The results presented in this paper demonstrate that the unlabelled immunoperoxidase staining technique is a sensitive method for determining the proportion of protoplasts infected by a plant virus, especially when non-specific fluorescence of uninfected protoplasts presents a problem. This method is more sensitive than either the fluorescent antibody or the enzyme-labelled antibody methods (Chiu et al., 1981a). The double-antibody sandwich type of ELISA can be used for estimating the concentration of virus for which no local lesion hosts are available.

The higher concentration of BSMV (50 μg/ml) needed for infection of the barley protoplasts is the same as that used for the infection of barley protoplasts with brome mosaic virus (Okuno et al., 1977). It may be due to the properties of the barley protoplasts or the multipartite genome of these two viruses.

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REFERENCES


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