Limited Infection of Cereal Leaf Protoplasts by Barley Yellow Dwarf Virus

(Accepted 22 July 1981)

SUMMARY

Oat and barley leaf protoplasts that were readily infected with brome mosaic virus could only be infected with barley yellow dwarf virus (BYDV) at low levels of efficiency. Of the conditions tested for infecting the protoplasts with BYDV, the most reliable combination was 2 μg/ml of virus and 0.2 μg/ml poly-L-ornithine with 10 mM-citrate buffer at pH 5. Tobacco protoplasts could not be infected with BYDV under these conditions.

Barley yellow dwarf virus (BYDV) typifies the luteovirus group (Shepherd et al., 1976) in being phloem-restricted, and aphid-borne but not sap-transmissible. It is also difficult to purify and the yields obtained are low (Rochow & Brakke, 1964; Rochow et al., 1971; Brakke & Rochow, 1974). These factors, and especially the lack of a suitable infectivity assay akin to those applicable with sap-transmissible viruses, have restricted investigation of its multiplication in host tissue. The recent work of Kubo & Takanami (1979) has provided a solution to this difficulty for one luteovirus, tobacco necrotic dwarf virus (TNDV), by demonstrating its efficient infection of isolated tobacco mesophyll protoplasts. Their success suggested investigating whether cereal protoplasts can similarly be infected with BYDV. Reported here are experiments in which this has been achieved, although with only a low level of efficiency.

Primary leaves of 5- to 8-day-old seedlings of BYDV-susceptible oats and barley cultivars (Avena sativa L. cv. Cliftland 64 and Hordeum vulgare cvs. Moore and California Mariout) were used as sources of protoplasts. Seedlings were grown at 10 to 25 per pot of soil either in the greenhouse at about 25 °C, with supplementary fluorescent and tungsten lighting to give a 16 h photoperiod, or in a growth chamber controlled at 24 to 25 °C with fluorescent and tungsten lighting of approx. 3000 lux at a 14 h photoperiod. Plants were watered daily and received fertilizer (10-52-8: N-P-K) to promote rapid growth. Protoplast preparation followed the method of Okuno & Furusawa (1977). Freshly harvested leaf pieces from which the abaxial epidermis had been removed were immersed, at 1 g/10 ml, in enzyme solution containing 1 to 1.5 % (w/v) cellulase ‘Onozuka’ R-10 and 0.05 % macerozyme ‘Onozuka’ R-10 (Yakult Biochemicals, Nishinomiya, Japan) in 0.6 M-mannitol pH 5.6. Protoplast release was efficiently effected by incubation at 23 to 30 °C in a shaking water bath at 60 excursions/min for 2 to 3.5 h. Protoplasts were collected by centrifugation at 100 g for 5 min and then washed twice with 0.6 M-mannitol solution, again collecting by centrifugation.

Prior to inoculation, the washed protoplasts were gently resuspended in 0.7 M-mannitol (Okuno & Furusawa, 1978a) to give 4 × 10^5 protoplasts/ml. For inoculation, 5 ml amounts of the protoplast suspension were mixed with an equal volume of buffered 0.7 M-mannitol containing double the finally required concentrations of virus, poly-L-ornithine (PLO, 100000 to 200000, Sigma) or polyethylene glycol (PEG 6000). Alternatively, protoplasts were washed and pelleted in 0.7 M-mannitol and then resuspended in 10 ml of the inoculation mixture at 2 × 10^5 protoplasts/ml. Pre-inoculation incubation of virus with PLO or PEG ranged from 0 to 20 min, as did the inoculation incubation with protoplasts. Both stages were
done at between 22 and 30 °C in a shaking water bath at 60 excursions/min. For several experiments, protoplasts were purified by floatation on 0-6 M-sucrose prior to inoculation (Hughes et al., 1978a, b) but this made no difference to the results. Protoplasts were collected from the inoculation mixture by low-speed centrifugation, and were then washed twice in 0-7 M-mannitol containing 10 mM-CaCl₂. Finally, sedimented protoplasts were resuspended at 0.5 x 10⁵ to 1.5 x 10⁵ protoplasts/ml in 10 ml of incubation medium containing 0.7 M-mannitol, 0.2 mM-K₂HPO₄, 1 mM-KNO₃, 1 mM-MgSO₄, 10 mM-CaCl₂, 1 μM-KI, 0.01 μM-CuSO₄, together with 500 μg/ml cephaloridine (Sigma), or 25 units/ml nystatin (Sigma) plus 100 μg/ml carbenicillin (Beecham Laboratories, Elmhurst, Ill., U.S.A.), or 10 μg/ml gentamicin sulphate (Sigma), as antibiotics. Samples were then incubated without shaking, at temperatures ranging from 11 to 13 °C or 23 to 30 °C with 800 to 1000 lux of continuous fluorescent lighting for 20 to 90 h. Infection was assessed by direct fluorescence antibody staining (Otsuki & Takebe, 1969) using antisera raised in rabbits as described by Rochow et al. (1971). The titre of the antiserum before labelling with fluorescein isothiocyanate (Clark & Shephard, 1963) was 1/512, and the labelled serum was used diluted to 1/50. The percentage of protoplasts fluorescing was determined from estimates of the number of viable protoplasts obtained, as assessed using the non-permeating pigment, Evan’s blue (Sigma) (Gaff & Okong’O-Ogola, 1971).

The BYDV used was a 'PAV'-like isolate (Aapola & Rochow, 1971; Rochow et al., 1971) from Indiana, propagated in Clintland 64 oats infected by infestation with viruliferous Rhopalosiphum padi. Purification was essentially by the methods of Rochow & Brakke (1964), using chloroform/amyl alcohol clarification, followed by concentration and, finally, separation on rate-zonal sucrose density gradients. The infectivity of preparations was confirmed by membrane-feeding experiments with non-viruliferous R. padi (Rochow, 1960) using Clintland 64 oats as test plants. The ability of the virus to infect protoplasts seemed unchanged by storage at 4 °C for up to 3 weeks, but preparations frozen at −20 °C failed to infect under the conditions tested. The concentration of BYDV in purified preparations was estimated using the absorbance coefficient A₅₆₀nm of 8.6 calculated for tobacco necrotic dwarf virus (Kubo & Takanami, 1979). Brome mosaic virus (BMV) was raised in Moore barley and purified by the method of Bockstahler & Kaesberg (1962).

As prepared by the methods described, cereal protoplasts from plants raised in either growth chamber or greenhouse appeared intact and in good condition (Fig. 1 a) and behaved similarly in attempts at inoculation. Typically, samples contained 85 to 95% of viable cells. Moreover, when samples of Clintland oat and Moore barley protoplasts were processed by a standard method for infection with brome mosaic virus (Furusawa & Okuno, 1978), between 60 and 70% became infected as judged by staining with specific fluorescent antibody. Thus, a high proportion of the protoplasts were metabolically competent and capable of sustaining and supporting a virus infection.

In the attempts made to infect batches of such protoplasts with BYDV (Table 1), conditions that were varied between experiments included: buffer type, molarity and pH; virus concentration, inoculation and incubation times and temperatures; PLO concentrations. Most experiments were done with oat protoplasts, of which a total of 29 out of 33 batches tested became infected to some degree. Similarly, small proportions of protoplasts from each of the eight batches of barley protoplasts tested (five from cv. Moore and three from cv. California Mariout) became infected. Infections were regularly obtained with oat protoplasts when potassium citrate was used at pH 5. They were also obtained with potassium citrate at pH 4.5 or 6, but not in experiments with potassium phosphate at pH 5.4 (an optimum value with TNDV; Kubo & Takanami, 1979) or with tris-HCl at pH 8.8. By contrast, infection of barley protoplasts was achieved in tests with phosphate buffer at pH 5.4, as well as in several with citrate at pH 5. Preliminary experiments showed that higher buffer molarities than those
Table 1. **Conditions tested for infecting oat and barley protoplasts with BYDV**

<table>
<thead>
<tr>
<th>Protoplast source</th>
<th>Buffer</th>
<th>Concn. (mM)</th>
<th>pH</th>
<th>Virus concn. (µg/ml)</th>
<th>PLO or PEG concn. (µg/ml)</th>
<th>(%) (w/v)</th>
<th>Infections (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oat cv.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clintland 64</td>
<td>Citrate</td>
<td>10</td>
<td>5.0</td>
<td>2</td>
<td>2</td>
<td>0.2-1.0</td>
<td>2</td>
</tr>
<tr>
<td>Clintland 64</td>
<td>Citrate</td>
<td>10</td>
<td>5.0</td>
<td>5-10</td>
<td>0.2-1.0</td>
<td>2</td>
<td>0.1-1.0</td>
</tr>
<tr>
<td>Clintland 64</td>
<td>Citrate</td>
<td>10</td>
<td>4.5 or 6.0</td>
<td>2</td>
<td>0.2</td>
<td>-</td>
<td>0.5-1.2</td>
</tr>
<tr>
<td>Clintland 64</td>
<td>Phosphate</td>
<td>25</td>
<td>5.4</td>
<td>2</td>
<td>0.2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Clintland 64</td>
<td>Tris-HCl</td>
<td>25</td>
<td>8.8</td>
<td>2</td>
<td>0.2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Barley cvs.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moore</td>
<td>Citrate</td>
<td>10</td>
<td>5.0</td>
<td>2</td>
<td>0.2</td>
<td>2</td>
<td>1.0-3.6</td>
</tr>
<tr>
<td>Moore</td>
<td>Phosphate</td>
<td>25</td>
<td>5.4</td>
<td>2</td>
<td>0.2</td>
<td>2</td>
<td>1.0-1.2</td>
</tr>
<tr>
<td>California Mariout</td>
<td>Citrate</td>
<td>10</td>
<td>5.0</td>
<td>2</td>
<td>0.4-0.8</td>
<td>2</td>
<td>0.6-1.0</td>
</tr>
</tbody>
</table>

* Determined by direct fluorescent antibody staining of duplicate samples of approx. 2 × 10⁴ protoplasts each after post-inoculation incubation for periods of 20 to 90 h.

recorded in Table 1 damaged protoplasts, particularly of oat. Virus was routinely used at 2 µg/ml, as increasing the concentration used to 5 or 10 µg/ml actually reduced infection efficiency. The proportion of infections obtained was not clearly influenced by variations in any of the pre-inoculation, inoculation or post-inoculation treatments used as described above, including varying the PLO concentration between 0·2 and 1 µg/ml (increments of 0·2 µg/ml). PEG at 2% (w/v) was used (Cassells et al., 1978) instead of PLO in some experiments and gave a similarly low proportion of infections. Omission of both additives also
gave low infection. Of the conditions tried, the combination most efficiently infecting oat protoplasts was: 2 µg/ml virus, 0.2 µg/ml PLO and 10 mM-citrate buffer at pH 5. Under these conditions, the estimated percentage of infections obtained ranged from about 0.3 to 5.0, but was typically 1% or less. Under similar conditions, protoplasts of Moore and California Mariout barley also became infected, although again to low levels. Fig. 1 (b to e) illustrates fluorescent protoplasts typical of those recorded as infections. Especially with barley, occasional faint yellowish autofluorescent specks, quite distinct from the bright green specific fluorescence, were observed in both virus-inoculated and control protoplasts (compare Okuno & Furusawa, 1978 b) and in protoplasts treated with unlabelled antiserum. That shown in Fig. 1 (d) was selected for photography because it was unusually intense. No fluorescence due to the presence of virus antigen was observed in protoplasts stained and examined after inoculation, but before incubation.

Although these results conclusively establish for the first time that cereal leaf protoplasts can be infected with BYDV, they indicate that the proportion of infections obtained by the methods described is too low and variable to be useful as an infectivity assay or for examination of virus synthesis. This contrasts with the results of Kubo & Takanami (1979), who were able to efficiently infect tobacco leaf protoplasts with TNDV by essentially the same methods. In case such material might be more widely susceptible to luteoviruses, protoplasts were prepared by the method of Loebenstein et al. (1980) from Nicotiana tabacum L. cvs. Xanthi and White Burley, and from Nicotiana × edwardsonii (Christie & Hall, 1979), and attempts made to infect them with BYDV by the procedure found to be most efficient with oat protoplasts (see above) and by that of Kubo & Takanami (1979) also, but without success. Possibly conditions other than those tested are required for efficient infection of cereal protoplasts with BYDV, but at present, the technical difficulties of virus purification and infectivity assay preclude more exhaustive examination. Alternatively, it may be that only relatively few of the protoplasts in cereal leaf preparations are competent to sustain infection with BYDV. If so, these could conceivably be the protoplasts from cells associated with the phloem, for example companion cells, which have been shown by electron microscopy of infected cereal tissue sections to contain virus particles (Gill & Chong, 1975, 1976). In relation to this, however, in several tests when protoplasts were prepared from pre-infected leaf tissue of both Clintland 64 oat and Moore barley and processed directly with fluorescent antibody, no infections were detected.

We wish to thank Dr J. E. Foster for supplying infected plants, Dr R. L. Nicholson for help with photography, Dr C. Schaller for seed of California Mariout barley, and Drs A. O. Jackson and J. F. Tuite for helpful discussions. The work was funded by Special Research Grant No. 901-15-42 of the USDA/SEA. Journal paper no. 8380 Purdue Agricultural Experiment Station.

Department of Botany and Plant Pathology

Purdue University

West Lafayette, Indiana 47907, U.S.A.

A. Barnett†

J. Hammond

R. M. Lister*

† Present address: K.A.R.I., P.O. Box 30148, Nairobi, Kenya.

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(Received 13 May 1981)