The Effect of Charge on Infection of Tobacco Protoplasts by Bromoviruses

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SUMMARY

The susceptibility of freshly isolated tobacco protoplasts to infection by brome mosaic virus (BMV) fell rapidly to a low residual level over a period of 8 h in culture. In contrast, susceptibility to infection by cowpea chlorotic mottle virus (CCMV) fell much more slowly over 15 h in culture to a similar residual level. In the absence of polycations, BMV infected many more protoplasts if CCMV were also present in the inoculum even though CCMV did not infect such doubly inoculated protoplasts. Both effects are thought to be a consequence of the different electrical charges of particles of the viruses and support the view that inoculation of protoplasts depends primarily on physical interactions between virus and protoplast.

INTRODUCTION

The mechanism by which viruses enter and infect plant protoplasts is not well understood; by analogy with the process by which animal cells become infected some have concluded that pinocytosis, or endocytosis, is the principal method of entry (Takebe & Otsuki, 1969; Honda et al., 1974). There is, however, no entirely satisfactory evidence that plant cells show pinocytosis comparable to that observed in animal cells, and Cram (1980) has reasoned that in the walled plant cell pinocytosis is unlikely because of thermodynamic constraints. These restrictions would disappear when the wall is removed to produce a protoplast. There are, however, clear descriptions of coated pits and vesicles both in walled and free protoplasts (Doohan & Palevitz, 1980; van der Valk & Fowke, 1981; Mersey et al., 1982; Traas, 1984) and exocytosis balanced by endocytosis may be normal processes in plant cells. Our own studies have from the beginning suggested that whatever role endocytosis may play in the inoculation of protoplasts with viruses, it is not the limiting process which appears to be insensitive to azide and temperature (Motoyoshi et al., 1974b). The ability of virus and protoplast to interact is governed first by their relative electrical charges and second by the physiological condition of the protoplast (Watts et al., 1981).

During studies of mixed infections of tobacco protoplasts with the bromoviruses, cowpea chlorotic mottle virus (CCMV) and brome mosaic virus (BMV), we have observed differences between the susceptibilities of protoplasts to inoculation with either virus and an interaction between the viruses in mixed inocula. These results throw further light on the mechanism of infection.

METHODS

Protoplasts were isolated from leaves of Nicotiana tabacum (cv. Xanthi), inoculated with virus, and cultured using the methods described previously (Motoyoshi et al., 1974a, b). All inoculations were in 0.7 M-mannitol containing 0.01 M-potassium citrate (pH 5.2). Poly-L-ornithine (PLO; mol. wt. 120000, Sigma) was used at 0.5 to 1 μg/ml. Infection was scored as the proportion of protoplasts surviving culture for 24 h that stained with fluorescent antibody made from rabbit antisera prepared against BMV or CCMV (Motoyoshi et al., 1973).

RESULTS

Inoculation with BMV or CCMV alone

BMV has a net positive charge at pH 5.2 (Bockstahler & Kaesberg, 1962) and infects protoplasts even when polycations are not included in the medium. The percentage infection...
Fig. 1. Changes in susceptibility of protoplasts to infection during culture. Protoplasts were isolated and cultured in incubation medium. At intervals, samples were washed and inoculated with BMV (5 µg/ml) or CCMV (1 µg/ml) in the presence of PLO (1 µg/ml) and cultured once more. Percentage infection was determined by staining with fluorescent antibody 24 h after inoculation. O, BMV; △, CCMV.

Fig. 2. Infection of protoplasts with BMV in the presence of CCMV. Protoplasts were inoculated with BMV (2 µg/ml) in the presence of different concentrations of CCMV but no PLO. Percentage infection was determined by staining with fluorescent antibody after 24 h culture.

increases as virus concentration increases and relatively high concentrations of virus (50 µg/ml) are needed to infect most inoculated protoplasts (Motoyoshi et al., 1974a). The precise relationship between virus concentration and infection depends also on the physiological condition of the protoplasts, the maximum proportion infectible being any value between 0 and 80% (Motoyoshi et al., 1974a). The addition of PLO at about 1 µg/ml to an inoculum of BMV considerably improves the efficiency of infection and reduces the optimal concentration of virus by about an order of magnitude (Motoyoshi et al., 1974a). In contrast to BMV, CCMV has a negative charge at pH 5.2 and must be accompanied by a polycation if it is to infect protoplasts. Moreover, maximum infectivity is attained only after preincubating virus and polycation for 5 to 10 min before inoculation, and there is an optimal ratio of viruses : polycation of about 10:1.

Fig. 1 shows the results of an experiment in which the ability of the two viruses (in inocula containing PLO at 1 µg/ml) to infect protoplasts was studied over a period of hours after isolation of the protoplasts. The two viruses infected virtually the same proportions of freshly isolated protoplasts, but susceptibility to BMV fell very rapidly during the next 8 h. There was, for example, a fall of over 50% in the first 2 h and only about 5% infection was obtained after about 8 h. In contrast, susceptibility to infection by CCMV declined little during the first 2 h and then fell in an approximately linear fashion during the next 12 h so that 15 h after isolation about 10% of protoplasts could still be infected.

Inoculation with mixtures of BMV and CCMV

PLO appears to influence infectivity in several ways: it causes aggregation of negatively charged virus particles so increasing the probability that multicomponent viruses will infect, and at the same time it modifies the charge on virus particles so that they can approach and attach to the protoplast surface (Motoyoshi et al., 1973); there is also a more general phenomenon of activation of the surface of the protoplasts, perhaps by damaging it (Burgess et al., 1973a, b). When BMV and CCMV were mixed at concentrations around 100 µg/ml, visible aggregation and precipitation occurred in a manner analogous to that observed when CCMV, but not BMV, is mixed with PLO (Motoyoshi et al., 1973, 1974a). Experiments were therefore
Injection of protoplasts with bromoviruses

Table 1. Increased infection with BMV in the presence of CCMV*

<table>
<thead>
<tr>
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<th>Infection</th>
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<tbody>
<tr>
<td></td>
<td>BMV</td>
<td>CCMV</td>
</tr>
<tr>
<td>BMV alone</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>CCMV alone</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>BMV + PLO</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>CCMV + PLO</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>CCMV + BMV (no PLO)</td>
<td>57</td>
<td>0</td>
</tr>
</tbody>
</table>

* Tobacco protoplasts (10⁶) in 25 ml citrate-buffered mannitol were inoculated with BMV at 2 μg/ml or CCMV at 4 μg/ml and PLO at 0·5 μg/ml. The percentage infection was scored after 24 h culture using fluorescent antibody staining.

undertaken to see what effect, if any, mixing of the viruses had on their infectivity. Mixed inocula infected many more protoplasts with BMV than did single inocula whereas CCMV, although infective with PLO, did not infect protoplasts alone or when mixed with BMV (Table 1). CCMV clearly potentiated infection with BMV but was not itself rendered infective.

Fig. 2 shows the effect of mixing BMV with different concentrations of CCMV on infection by BMV. The results show that the 6% infection produced by BMV alone was increased most, to 22%, by about 0·5 μg CCMV/ml inoculum and that higher concentrations of CCMV decreased infection by BMV. The optimal ratio (w/w) of BMV:CCMV in this experiment was about 5:1.

The phenomenon of potentiation of BMV infectivity by CCMV was regularly observed. Even at relatively high concentrations of BMV (15 μg/ml) there was a significant increase in infectivity, from 35 to 57% infection in the presence of 1 μg CCMV/ml, although CCMV remained non-infectious. The maximum percentage infection attainable by high concentrations of BMV (50 μg/ml) or at lower concentrations (5 μg/ml) in the presence of PLO (1 μg/ml) was never exceeded but could be attained using intermediate concentrations of virus (e.g. 10 μg BMV and 2 μg CCMV/ml).

Several experiments were also performed using mixtures of BMV inactivated by u.v. irradiation and CCMV, but no infection with CCMV was observed.

**DISCUSSION**

These results fit a model for entry of virus by an initial physical association, controlled by the relative electric charges of virus and protoplast surface at a susceptible region of the plasma membrane (Watts et al., 1981). The number or size of susceptible areas falls with time in culture. The difference in behaviour of CCMV and BMV in the presence of PLO (Fig. 1) is then a consequence of aggregation of CCMV which effectively converts the multicomponent virus to a single particle, thus increasing the probability of infection. This effect becomes increasingly important as the number of sites of entry into the protoplasts falls with time.

The increased infectivity of BMV in the presence of CCMV, but not vice versa (Fig. 2), has a similar origin. Aggregation increases the probability of infection for BMV but not for CCMV because only positively charged aggregates can attach to the protoplast surface. The optimal ratio of BMV:CCMV suggests that small aggregates of about five BMV particles form around a central CCMV particle. A similar aggregate of CCMV particles around BMV particles would be negatively charged and would therefore not be infective.

Exocytosis is a normal process in plant cells, associated for example with transport of wall material (Robinson, 1977). It is balanced by endocytosis (Doohan & Palevitz, 1980; van der Valk & Fowke, 1981; Mersey et al., 1982), so avoiding the thermodynamic constraints discussed by Cram (1980). Endocytosis is thought to be primarily a mechanism for recovery and recycling of membrane, not for uptake of extracellular material (Traas, 1984) but the possibility exists that it may play a role in the uptake of virus. Studies with the electron microscope have been presented as showing (Honda et al., 1974) or not showing (Burgess et al., 1973a, b) evidence of pinocytosis. In no case have coated pits been observed associated with endocytosis of virus particles. The balance of other evidence, including the insensitivity of inoculation to azide and
low temperatures (Motoyoshi et al., 1974b), does not favour a controlled process like
endocytosis. The susceptibility of freshly prepared protoplasts and its rapid decline probably
reflect the trauma of isolation and subsequent recovery (Watts et al., 1981), but the role of
endocytosis via coated pits during this period needs further examination before its part in
inoculation can be established.

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