Tubule-forming capacity of the movement proteins of alfalfa mosaic virus and brome mosaic virus

D. T. J. Kasteel, N. N. van der Wel, K. A. J. Jansen, R. W. Goldbach and J. W. M. van Lent

Department of Virology, Agricultural University Wageningen, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

The structural phenotype of the movement proteins (MPs) of two representatives of the Bromoviridae, alfalfa mosaic virus (AMV) and brome mosaic virus (BMV), was studied in protoplasts. Immunofluorescence microscopy showed that the MPs of these viruses, for which there has been no evidence of a tubule-guided mechanism, assemble into long tubular structures at the surface of the infected protoplast. Electron microscopy and immunogold analysis confirmed the presence of both MP and virus particles in the tubules induced by AMV and BMV. The significance of the tubule-forming properties of these viral MPs is discussed.

To establish systemic infection of plants, viruses modify the plasmodesmata to allow cell-to-cell movement of the virus particle or the viral genome (Lucas & Gilbertson, 1994). For a growing number of viruses it has been shown that one or more virus-encoded proteins are actively involved in this crucial process, and various biochemical activities have been assigned to these products.

Plant viruses can be categorized by their mechanism of intercellular movement. One category of plant viruses, including tobacco mosaic virus (TMV), does not require coat protein (CP) and moves in a non-virion form through the plasmodesmata. The virus-encoded MP is localized to the plasmodesmata (Tomenius et al., 1987) and causes a significant increase in the size-exclusion limit, thus enabling the passage of viral RNA (Wolf et al., 1989). Another category, including cowpea mosaic virus (CPMV), requires CP for intercellular spread and these viruses move as virions through tubules assembled within the plasmodesmata. In CPMV-infected cells, the viral MP is localized to these tubules (Van Lent et al., 1990). Typically, such movement tubules are formed not only in plant tissue but also in virus-infected protoplasts in the absence of cell walls and plasmodesmata (Van Lent et al., 1991). A third category of plant viruses, including tobacco etch potyvirus (Dolja et al., 1994) and potato virus X (Oparka et al., 1996), also requires the CP for movement but tubule-guided virion transport through plasmodesmata has not been reported.

Recently, evidence was presented that two members of the Bromoviridae, alfalfa mosaic virus (AMV) and brome mosaic virus (BMV), also require CP for successful cell-to-cell movement (Van der Vossen et al., 1994; Rao & Grantham, 1995). Although involvement of the AMV CP in cell-to-cell movement was established using CP mutants, it was not possible to conclude from these studies whether AMV moved as a virion or not. AMV mutant CPN199, with a C-terminal deletion in the CP, could move from cell-to-cell despite the fact that stable virions were not detectable (Van der Vossen et al., 1994).

For BMV, Flasinski et al. (1995) concluded from their analysis of various CP-mutants that CP was not essential for cell-to-cell movement. However, Rao & Grantham (1995) showed that mutants which failed to produce an encapsidation-competent CP also failed to move from cell-to-cell and to induce local lesions, suggesting a requirement for CP for movement.

In view of these observations, we have investigated whether AMV and BMV also move as whole virions through tubule-like structures, by analysing the phenotype of the respective MPs in cowpea protoplasts infected with these viruses.

Cowpea protoplasts (Vigna unguiculata 'California Black-eye') were mock-inoculated with water or inoculated with AMV (strain 425) or BMV (strain M1) at a concentration of 10 µg virus per 10⁶ protoplasts, essentially as described by Eggen et al. (1989). Forty-two hours after inoculation, the protoplasts were analysed by immunofluorescence and negative staining electron microscopy (Van Lent et al., 1991) using antibodies against the respective MPs or CPs.

Antibodies against the CPs of AMV or BMV were generated by injecting purified virus into rabbits. Antibodies against the MP of BMV (3a protein) were obtained by injecting a rabbit with Escherichia coli-expressed protein, and against AMV MP (P3 protein) as described previously (Van Pelt-Heerschap et al., 1987). Controls consisted of samples treated with preimmune sera. Samples were analysed using a...
Leitz Laborlux S fluorescence microscope and a Philips CM12 electron microscope.

At 42 h post-inoculation, an average infection of 60% (AMV) or 35% (BMV) of inoculated protoplasts was recorded. Approximately 75% of those protoplasts infected with AMV or BMV showed numerous fluorescent tubular structures at the cell surface upon staining with anti-MP sera (Fig. 1 a, b). These tubules were also visible, though to a lesser extent, when anti-CP sera were used, indicating the presence of this protein in these structures (data not shown). Tubules were also observed on AMV-infected protoplasts from *Nicotiana benthamiana* and on BMV-infected protoplasts from *Hordeum vulgare* (not shown).

Electron microscopical examination of AMV- or BMV-infected protoplasts was performed by negative staining with 2% phosphotungstic acid (PTA) at either pH 5.5 or 6.5. These two pH values were chosen as BMV and AMV particles are stable at pH 5.5, but considerably less stable at pH 6.5. At pH 6.5, BMV particles swell and disintegrate (Johnson & Argos, 1985). In preparations stained with PTA at pH 5.5, virus-like particles were observed within the tubules induced by either virus (Fig. 2 b, c, e, f). However, at pH 6.5 no such particles could be seen within the tubules or in the background (Fig. 2 a, d), confirming that the structures observed at pH 5.5 indeed represented virions. The AMV- (Fig. 2 a) and BMV-(Fig. 2 d) infected protoplasts contained several tubules engulfed by plasma membrane. The phenomenon of multiple tubules enclosed by plasma membrane was also reported for CPMV (Van Lent et al., 1991). The average diameter of the tubules was 25 ± 3 nm and 40 ± 4 nm for AMV and BMV, respectively. BMV particles, with an average diameter of 31 nm, were neatly arranged in the wider tubule (Fig. 2 c, f). The particle-like structures observed in the AMV tubules (Fig. 2 b, c) had a diameter of 17 nm and resembled the icosahedral virions of AMV, but were less evident than BMV particles. Occasionally, in the AMV tubules, particles were observed that resembled bacilliform virions in size and shape.

The presence of AMV CP in the tubules was confirmed by immunogold labelling with anti-CP serum. Gold label was found along tubules, in particular at sites where the structure had partly disintegrated (Fig. 3 a). Similarly, BMV tubules could be labelled with the homologous anti-CP serum (not shown). No gold labelling was found on intact BMV tubules, but gold complexes were found at places where virus particles were freely accessible (e.g. at the end of a tubule; results not shown). Apparently, the immunoglobulins cannot reach the CP antigen when tubules are intact, as has been found for CPMV tubules (Van Lent et al., 1991).

The virus-induced AMV and BMV tubules could be labelled to a much better extent using the anti-MP sera (Fig. 3 b, c),...
although in these cases the gold particles were also mainly found at sites where the structure of the tubules had partly disintegrated, thus exposing more antigen.

It is evident that AMV and BMV can induce tubular structures in infected protoplasts and that these tubules contain the MP and occlude virus-like particles. This complements the observations of Van der Vossen et al. (1994) and Rao & Grantham (1995) on the requirement for CP for intercellular movement of AMV and BMV, respectively. Hence, by analogy with CPMV intercellular movement, it is plausible that movement of AMV and BMV particles through tubules assembled in plasmodesmata is a valid mechanism. In this respect, Godefroy-Colburn et al. (1990) noted the transient presence of tubule-like structures, gold labelled with anti-CP, in plasmodesmata of AMV-infected tobacco mesophyll parenchyma cells. Also, for members of two other genera of the family Bromoviridae, tobacco streak ilarvirus (Martelli & Russo, 1985) and tomato aspermy cucumovirus (Francki et al., 1985),

**Fig. 2.** Electron micrographs of tubular structures of AMV (a, b, c) and BMV (d, e, f), negatively stained with PTA at pH 6.5 (a, d) or pH 5.5 (b, c, e, f). Icosahedral and bacilliform (arrows) particle structures are visible within the tubular structure at pH 5.5. Arrowheads indicate the plasma membrane. Bars represent 100 nm.
tubular structures containing virus particles have been observed in plasmodesmata of infected plant cells. However, the presence of virus-containing tubular structures in plasmodesmata of AMV- or BMV-infected plant tissue remains to be established.

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References


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