**In situ Location of an Alfalfa Mosaic Virus Non-structural Protein in Plant Cell Walls: Correlation with Virus Transport**

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(Accepted 13 February 1987)

**SUMMARY**

The 32000 mol. wt. non-structural protein (P3) of alfalfa mosaic virus (AlMV) has previously been shown to accumulate in the cell wall fraction of tobacco leaves infected with AlMV. We now report the ultrastructural location of this protein. P3 was visualized immunocytochemically in the middle lamella of the walls of those parenchymal or epidermal cells which had just been reached by the infection front and in which viral multiplication had just begun. P3 was not found when AlMV had accumulated to high levels in infected cells. These findings support the concept that P3 is involved in the spread of viral infection from cell to cell, i.e. is the transport factor of AlMV.

Multiplication of a plant virus in its host is not a synchronous process. It starts after virus penetration of a few epidermal cells; thereafter the infection spreads from cell to cell and may eventually invade the entire plant. Spreading (or virus transport) is thus a critical event which largely determines the pathological effects of an infection. There is evidence that virus transport is promoted by a viral protein (Atabekov & Dorokhov, 1984) but tobacco mosaic virus is the only virus for which the transport factor has been identified (as the 30K non-structural protein; Leonard & Zaitlin, 1982; Ohno et al., 1983). For brome mosaic virus and alfalfa mosaic virus (AlMV), the cistron for the equivalent protein has tentatively been assigned to RNA 3 (Kiberstis et al., 1981; Nassuth & Bol, 1983; French et al., 1986). One possible mode of action for the transport factor would be to bind specifically to virions in order to help their transit through plasmodesmata (Langenberg, 1986). Another possibility is that the transport factor modifies the wall or the plasmodesmata between adjacent infected and non-infected cells, making them permeable to the infectious agent. Indeed, we have shown recently that a significant amount of the 32K non-structural protein of AlMV (P3, encoded by RNA 3; Barker et al., 1983; Ravelonandro et al., 1984) is present in a cell wall fraction made from infected tobacco leaves (Godefroy-Colburn et al., 1986). Here we confirm this location by immuneelectron microscopy and we show that, whereas P3 can be visualized in the middle lamella of the walls of newly infected cells that contain only small amounts of viral coat protein, it is never observed in the walls of heavily infected cells.

The leaves of a 4-week-old tobacco plant (*Nicotiana tabacum*, cv. Xanthi nic) were inoculated with the S-strain of AlMV (Pinck & Hirth, 1972) and kept for 80 h at 25 °C with a 16 h day length. Small strips of the inoculated leaves were processed for transmission electron microscopy essentially as described by Garaud et al. (1982). Briefly, they were fixed at 0 °C overnight in 2.5% glutaraldehyde in 100-mM-phosphate pH 7.2, postfixed in 1% osmium tetroxide, dehydrated in acetone and propylene oxide and embedded in Epon. Ultrathin serial sections were mounted on formvar-coated nickel slot grids.
Zone of inoculation: upper epidermis

Lower epidermis

Movement of the infection front

Fig. 1. Drawing illustrating the distribution of coat protein and of P3 in an A1MV-inoculated tobacco leaf, 80 h after inoculation. This diagram summarizes the observations of the AlMV coat protein and of P3 made on successive ultrathin sections after immunolabelling with the anti-A1MV or with the anti-pep3 sera. The intensity of labelling with the anti-A1MV serum is represented by the density of dots along the contours of the cells. Arrows point to areas of the walls in which P3 was observed. Zones 1 and 2 are those from which the micrographs of Fig. 2 were taken.

The antisera were raised in rabbits against a synthetic peptide (pep3), 26 amino acids in length, corresponding to the C terminus of P3, or against AlMV virions. The antisera specifically recognized P3 (Berna et al., 1986) or the coat protein. Sheep IgG against rabbit IgG (Institut Pasteur Production, Paris, France) was labelled with 20 nm gold particles according to Garaud et al. (1982), except that the adsorption of IgG onto colloidal gold was in 5 mM-sodium carbonate pH 9.5, instead of 5 mM-Tris. The sera were diluted in PAT (100 mM-phosphate buffer pH 7.2, 0.05% Tween 20).

Indirect immunolabelling was carried out in triplicate between 15 °C and 20 °C by floating the grids on 20 ~tl drops of the following reagents in succession for the indicated times. (i) Anti-AlMV serum (1:20000) or anti-pep3 serum (1:5000) for 4 h. Control reactions were with a non-immune serum (1:5000) and with the anti-pep3 serum (1:5000) preabsorbed with 5 μg/ml pep3 (Berna et al., 1986). (ii) PAT (4 × 10 min). (iii) Gold conjugate (1:100 in PAT containing 3 mg/ml lysine), for 1 h. (iv) PAT (2 × 10 min), then double-distilled water (2 × 10 min). The sections were counterstained with 2% uranyl acetate in water (10 min), quickly rinsed with double-distilled water and observed with a Hitachi H600 electron microscope.

Initial multiplication of AlMV occurs in a few epidermal cells that have been infected at the time of inoculation, then the progeny invades neighbouring cells. Thus in an infected tissue sample harvested at a given time after inoculation, the degree of infection varies from cell to cell. Fig. 1 is a drawing of a transverse section through such a leaf sample, harvested 80 h after inoculation, which summarizes the observations made after treatment with the anti-AlMV serum and with the anti-pep3 serum. As judged from the density of gold particles after labelling with the anti-AlMV serum, it appears that the cytoplasm of the cells closest to the inoculated surface of the leaf contained the largest amounts of coat protein; this indicates that these cells had reached a late stage of infection; a representative micrograph of this situation (zone 1 of Fig. 1) is shown in Fig. 2(a). In contrast, the cytoplasm of cells located opposite the inoculated leaf surface (i.e. near the infection front) contained on average much less coat protein; therefore
these cells were still at an early stage of infection at the time the sample was taken. Fig. 2(d), showing a cell from zone 2, illustrates this case. The corresponding negative controls (non-immune serum) are shown in Fig. 2(c) and (f). Viral coat protein was never detected in the vascular tissue.

When treated with the anti-pep3 serum, the heavily infected cells of zone 1 did not show significant labelling of the cytoplasm, nor of any other cellular component (Fig. 2(b)). In cells at an early stage of infection, the nucleus, chloroplasts and mitochondria were not significantly labelled but small clusters of gold particles were occasionally observed in the cytoplasm. More conspicuously however, the walls of these cells contained a large number of gold particles, clearly localized in the middle lamella and generally occurring in more or less dense clusters (Fig. 2(e, h)). We ascertained that the immunolabelling was specific for P3 by treating consecutive sections of the same cells with a non-immune serum (Fig. 2(f)) and with the anti-pep3 serum preabsorbed with the peptide (Fig. 2(g)). The sections in Fig. 2(e) and (h) were labelled by the anti-pep3 serum, suggesting strongly that the sections used for the negative controls (Fig. 2(f) and (g)) also contained P3 antigen in the wall.

In view of the possibility that P3 modifies the plasmodesmata, we gave special attention to the areas that contained them. So far we have observed very few plasmodesmata in the newly infected tissue and none of them was labelled by the anti-pep3 serum. This point is still under investigation.

The immunochemical results which have already been reported by Godefroy-Colburn et al. (1986) suggest that P3 is synthesized in the cytoplasm and then moves rapidly to the wall where it accumulates to an apparently constant final level. However, as the immunochemical experiments were performed on whole infected leaves, these results reflect concomitant synthesis and degradation of P3 averaged over different stages of infection. In the present paper the use of immunocytochemistry provides not only a confirmation of the cell location of P3 but also an idea of what happens at the level of a single cell during the multiplication cycle of the virus. The fact that P3 was found only in regions of the tissue which had recently undergone infection and in which the virus content was still low supports the concept that P3 may be involved in virus transport. If this is true several questions remain to be answered. How does P3 perform its function? What is the behaviour of this protein in a local lesion host where the spread of the virus is inhibited by the hypersensitive reaction of the plant? Is the wall localization a general characteristic of all the proteins thought to promote or to control the spreading of plant viruses? Progress in answering one or more of these questions should lead to a better understanding of the intercellular spreading process during plant virus infection and eventually may help to control plant virus disease.

The authors thank Dr L. Stoeckel (L.A. 309 du CNRS, Strasbourg, France) for helpful advice and discussion and for use of the ultramicrotomy facility, Dr J.-P. Briand for synthesizing the peptide, Dr K. Richards for critical reading of the manuscript, Ms M.-J. Gagey for printing the photographs and Mr R. Haas for maintaining the electron microscope. This work was supported in part by grant 84 V 0813 from the Ministère de la Recherche et de la Technologie (Biotechnology program).

REFERENCES


Fig. 2. Gold immunolabelling of ALMV coat protein and of P3 in heavily infected and in newly infected leaf tissue. Ultrathin serial sections of ALMV-infected tobacco leaf tissue were immunolabelled with the anti-ALMV serum (a, d), the anti-pep3 serum (b, e, h), a non-immune serum (c, f) or the anti-pep3 serum preabsorbed with oligopeptide (g). Micrographs (a) to (c) show cells of the heavily infected parenchyma (zone 1 of Fig. 1); (d) to (g) show a newly infected cell of the lower epidermis (zone 2 of Fig. 1). CH, chloroplast; CW, cell wall; CY, cytoplasm; G, Golgi stack; ML, middle lamella; V, vacuole. Bar marker represents 0.5 μm.
Short communication


(Received 24 November 1986)