Immunogold Localization of Parsnip Yellow Fleck Virus Particle Antigen in Thin Sections of Plant Cells

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SUMMARY

The distribution of parsnip yellow fleck virus particle antigen in infected cells of Nicotiana clevelandii or Spinacia oleracea was examined by immunogold labelling of ultrathin sections. Best results were obtained by pretreating sections with Decon 75 followed by long incubation times on antiserum (16 h) and gold probe (6 h). The cytoplasmic inclusions induced by infection have three main components: accumulations of 20 to 30 nm diameter tubules, granular bodies and amorphous matrix material. Much gold label was located over the areas of amorphous matrix material whereas the other components of the inclusions were not labelled. Specific but less dense labelling was observed over virus-induced cell wall outgrowths, and over other areas of cell wall and some nuclei in infected cells. Virus-like particles found in 45 nm diameter tubules within the cell wall outgrowths were not labelled, perhaps because they were inaccessible to the antibody. The results indicate that large amounts of virus particle antigen are present in cells. However, the number of recognizable virus particles was considerably less than expected from the amount of virus extracted from leaves.

INTRODUCTION

Parsnip yellow fleck virus (PYFV) (Murant & Goold, 1968; Hemida & Murant, 1989a) has isometric particles approximately 30 nm in diameter and a combination of properties unlike those of any other well characterized virus. On the basis of particle composition [one species of ssRNA, $M_v$ 3-4 × 10$^6$, polyadenylated, probably with a genome-linked protein, and three coat proteins, $M_v$ ($\times 10^{-3}$) approx. 30, 26 and 24] it does not fit into any of the previously recognized taxonomic groups of plant viruses but resembles the picornaviruses of insects and vertebrates (Murant, 1988; Hemida & Murant, 1989a; S. K. Hemida, A. F. Murant, M. A. Mayo & D. J. Robinson, unpublished data). It has therefore been made the type member of a newly created plant virus taxonomic group, called provisionally the parsnip yellow fleck virus group.

PYFV is readily transmitted mechanically but depends on a helper virus, anthriscus yellows (AYV), for transmission in a semi-persistent manner by the aphid Caviariella aegopodii (Murant & Goold, 1968; Elnagar & Murant, 1976). In infected cells of Nicotiana clevelandii, Spinacia oleracea (spinach) and Anthriscus cerefolium (chervil), PYFV induces the formation of large inclusions which comprise mainly small vesicles, 20 to 30 nm diameter tubules and granular bodies (Murant et al., 1975). Despite the fact that the virus is plentiful in leaf tissue (Hemida & Murant, 1989a), virus particles were found by Murant et al. (1975) only in membrane-walled tubules 45 nm in diameter which were usually associated with cell wall outgrowths. This paper describes an investigation of the location and distribution of PYFV particle antigen in leaf cells, using immunogold labelling of thin sections, an approach that has proved successful with several other plant viruses (Tomenius et al., 1983; Lin et al., 1987; Langenberg, 1986; Roenhorst et al., 1988).

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METHODS

Tissue samples. The tissues examined were systemically infected leaves of *S. oleracea* containing PYFV isolates P-121 or A-421 (Murant & Goold, 1968), and of *N. clevelandii* containing isolate P-121.

Fixation and embedding. Healthy and PYFV-infected samples were processed for electron microscopy essentially as described by Murant & Roberts (1979). For conventional electron microscopy the samples were fixed in 5% glutaraldehyde in 0·1 M-pipes buffer at pH 7·4, post-fixed in 0·2% osmium tetroxide in 0·1 M-cacodylate buffer at pH 6·8, and finally fixed and stained in 5% aqueous uranyl acetate at pH 3·5. The pieces of leaf were then washed in distilled water, embedded in 1% agar, cut into cubes and transferred directly into 100% ethanol for 2 × 30 min followed by 100% propylene oxide for a further 30 min. The samples were then infiltrated with Araldite resin for 48 to 72 h and embedded by polymerization for 72 h at 68 °C.

For immunogold labelling the embedding procedure was the same except that the osmium fixation and the uranyl acetate fixation/staining steps were omitted. Ultrathin sections (silver/gold interference colours) were cut on a Reichert Ultratome E ultramicrotome and mounted on pyroxylin-filmed nickel grids (hexagonal, 200-mesh).

Preparation of IgG. Antiserum to isolates A-421 (titre 1/4096) and P-121 (titre 1/256) were those of Hemida & Murant (1989). In some experiments, to decrease non-specific labelling, antisera were absorbed with protein prepared from healthy *S. oleracea* or *N. clevelandii* leaves by grinding in liquid nitrogen and washing with acetone, hot ethanol and phosphate buffer. The method used was essentially that of Da Rocha et al. (1986) except that low-speed centrifugation was used instead of filtration for the washing steps. The dried powder (5, 10 or 20 mg) was added to 100 µl of a 1/100 dilution of the antiserum, incubated for 1 h at room temperature and then centrifuged for 5 min at approx. 2000 g. The supernatant fluid was used for immunogold labelling. Polyclonal IgG was prepared from crude or absorbed antiserum essentially as described by Van Regenmortel (1982). An equal volume of 4 m-ammonium sulphate was added slowly to the antiserum and the pH adjusted to 7·8. After 1 h at room temperature, the preparation was centrifuged for 5 min at approx. 2000 g and the pellet resuspended in distilled water and dialysed overnight against phosphate buffer pH 7·8. Glycerol was then added to 30% (v/v) and the IgG stored at 4 °C.

Immunogold labelling. All dilutions of antisera and gold probe were made in IGL buffer: 0·07 m-sorensen's phosphate buffer pH 6·5, containing 1% serum albumin, 1% Tween 20 and 0·02% sodium azide. Antisera were used at about 5 to 10 µg/ml and the gold was diluted to give an As20 value of 0·5. Two kinds of gold probe were used: protein A gold (PAG) (10 nm) prepared as described by Van Lent & Verduin (1986) and goat anti-rabbit gold (GARG) (15 nm) obtained from Janssen Pharmaceutica Life Sciences Products or Bio-Cell Research Laboratories.

Attempts to label sections of osmium-fixed tissue were unsuccessful even when sections were pretreated with sodium metaperiodate (Bendayan & Zollinger, 1983) which is reported to restore antigenicity with some samples (Roth, 1986). In preliminary tests, best results were obtained when sections were pretreated with Decon 75 followed by long incubation times with antisera and gold probe. Other pretreatments, which included floating grids on solutions of hydrogen peroxide or sodium ethoxide (sodium hydroxide pellets dissolved in ethanol), were less satisfactory. The following procedure was adopted as standard. All steps were done at 20 °C and all incubations performed in petri dishes coated at the base with silicone rubber and containing moist cotton wool to prevent desiccation. (1) Float grids on 20 µl drops of 2% Decon 75 for 1 h, then wash thoroughly with distilled water and dry. (2) Float grids on 20 µl drops IGL buffer for 1 h. (3) Drain briefly and transfer to 20 µl drops of diluted antiserum. Leave for 16 to 18 h. (4) Wash grids individually in wells of microtitre plates in 450 µl IGL buffer, 2 × 10 min. Agitate occasionally. (5) Drain briefly and transfer to 20 µl drops of gold probe. Leave for 4 to 6 h. (6) Wash grids sequentially (10 min each) in microtitre plate wells containing 450 µl of (i) IGL buffer, (ii) 0·07 m-Sorensen's phosphate buffer pH 6·5 and (iii) distilled water. The grids were drained, dried and post-stained with uranyl acetate and lead citrate and then examined in the electron microscope.

Treatments. The following four test and control treatments were done on sections of infected and healthy *N. clevelandii* and *S. oleracea* leaves to check for the specificity of gold labelling; unabsorbed PYFV IgG homologous to the isolate tested; homologous PYFV IgG absorbed with *N. clevelandii* or *S. oleracea* protein; IgG from normal serum (serum from a non-immunized rabbit), absorbed with *N. clevelandii* or *S. oleracea* protein; omission of the antisera step.

RESULTS

Immunogold labelling technique

Gold labelling was not successful with leaf tissue fixed with osmium tetroxide, despite different pretreatments, but was successful with samples which had been fixed with glutaraldehyde alone. Labelling was improved by pretreating the sections with Decon 75; this gave cleaner sections, a slight increase in label density and a marked decrease in non-specific background labelling. Thorough washing of Decon-treated grids with distilled water and careful background drying was essential to prevent loss of the sections during the labelling procedure.
Fig. 1. Typical inclusion from leaf cell of *S. oleracea* infected with isolate P-121. Fixed with glutaraldehyde, labelled with GARG. The inclusion comprises granular bodies (G), tubules (T) and matrix material (M). The matrix material is heavily labelled and the nucleus (N) lightly labelled. Bar marker represents 2 μm.

Although both the PAG and GARG probes worked well, the latter gave better specificity and label density. Furthermore, the GARG probe, being larger (15 nm), was better for assessing background labelling. Label density was also increased substantially, without any increase in background labelling, by adopting long incubation times (16 to 18 h for the antiserum treatment step and 6 h for the gold labelling step), instead of the 1 to 3 h incubations for each step which were used initially.
The various controls were essential to provide confidence in the specificity of labelling. No labelling was observed when IgG from normal serum was used or when the antiserum step was omitted. Slight labelling of cell walls and some nuclei was observed when sections of healthy *N. clevelandii* or *S. oleracea* were treated with unabsorbed IgG from PYFV antiserum, but this was prevented when the IgG had been cross-absorbed with extracts from healthy plants. The sections remained clean even though the cross-absorbed antiserum was brownish yellow.

**Structural observations**

The general ultrastructure of *S. oleracea* and *N. clevelandii* leaves infected with either isolate of PYFV was essentially the same as reported by Murant *et al.* (1975), who used double fixation with glutaraldehyde and osmium. However, in our samples, which had been fixed in glutaraldehyde alone to preserve viral antigenicity for immunogold labelling, membraneous or membrane-bound structures were less well defined. Also the staining and relative contrast of other organelles was less than in osmium-fixed tissues.

Large, clearly defined inclusions were the most obvious ultrastructural abnormality found in infected cells (Fig. 1). They were present in *N. clevelandii* infected with isolate P-121 and in *S. oleracea* infected with either isolate P-121 or isolate A-421, and comprised 20 to 30 nm diameter tubules, granular bodies and areas of matrix material, as well as mitochondria, vesicles and ribosomes.

**Tubules**

The 20 to 30 nm diameter tubules which formed a major component of the inclusions were readily recognizable even in tissues not fixed with osmium and were more numerous and more densely packed within the inclusions in cells infected with isolate A-421 (Fig. 2) than in those infected with isolate P-121 (Fig. 1, 3 and 5). The inclusions always contained these tubules, although the amount varied.

**Granular bodies**

Granular bodies also were found in every inclusion; they were always clearly defined (Fig. 1, 3 and 5) but varied in shape and size and were readily recognizable by their dense electron staining. Sometimes the granular bodies contained other denser material (Fig. 3 and 5), which in
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Fig. 3. Part of an inclusion from a leaf cell of *N. clevelandii* infected with isolate P-121. Fixed with glutaraldehyde, labelled with GARG, antiserum cross-absorbed with 20 mg *N. clevelandii* protein. Note the dense label over the matrix material (M) and the absence of label over the tubules (T) and granular bodies (G), some of which contain dense crystalline material. Bar marker represents 500 nm.

Fig. 4. Different appearances of granular bodies in inclusions from *S. oleracea* cells infected with isolate P-121. (a) Glutaraldehyde and osmium fixation, showing that the bounding membranes of the granular bodies form distinct channels between them. (b) Fixed with glutaraldehyde alone, labelled with PAG, showing the absence of bounding membranes and gold labelling of the channels between the granular bodies (arrows). Bar marker represents 500 nm.

*N. clevelandii* was sometimes crystalline in appearance (Fig. 3). Whether the tissue had been fixed with osmium or not, the granular bodies only occasionally seemed to have bounding membranes. In *S. oleracea* infected with isolate P-121, these membranes often appeared as clearly defined uniform channels 50 nm wide separating adjacent granular bodies (Fig. 4a).

**Matrix material**

The third major component of the inclusions was the matrix material (Fig. 1, 3 and 5), which appeared amorphous in tissues fixed with glutaraldehyde alone, but probably represents the small vesicles previously described in doubly fixed tissue (Murant *et al.*, 1975). This material was not clearly defined or membrane-bounded but occupied the areas of the inclusion between the 20 to 30 nm diameter tubules and the granular bodies.
Cell wall outgrowths

The only other abnormality in PYFV-infected cells was the presence of cell wall outgrowths, as previously described by Murant et al. (1975). They were most commonly found in palisade or mesophyll cells, rarely in vascular tissue, and occurred only in cells that also contained inclusions. They consisted of new cell wall material, often seen to be sheathing single-membraned tubules about 45 nm in diameter containing electron-dense virus-like particles; these tubules seemed to be extensions of enlarged plasmodesmatal tubules. In N. clevelandii the outgrowths were up to 15 μm long and usually contained only one tubule, whereas in spinach they were usually shorter (2 to 3 μm) and broader, irregular in shape and size, and contained many tubules, with no obvious orientation (Fig. 6a). Their nature was determined by serial sectioning and by light microscopy of semi-thin sections (Murant et al., 1975). All virus-like particles observed in these tubules were densely stained and there were no gaps between the particles within a tubule.

Immunogold labelling

The densest labelling was found over the amorphous matrix material in the inclusions (Fig. 1, 3 and 5) and to a lesser extent, in S. oleracea infected with isolate P-121, over the channels separating adjacent granular bodies (Fig. 4b); tubules and granular bodies were not labelled. A uniform but much lighter label was observed over some nuclei in infected cells. Cell wall outgrowths were also lightly labelled (Fig. 6a, b) as were some other parts of cell walls in infected cells, but there was no clear evidence of labelling over the virus-containing tubules or over plasmodesmata. The specificity of gold labelling over nuclei, cell walls and cell wall outgrowths was unaffected by using cross-absorbed antiserum and was not removed by a fourfold increase in the amount of healthy protein used for absorption. No other cell organelles or structures were labelled.

The same pattern of immunogold labelling was observed in all three types of tissue examined.
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Fig. 6. (a) Cross-section of a large cell wall outgrowth (CO) adjacent to matrix material (M) in the cytoplasm of a *S. oleracea* leaf cell infected with isolate P-121. Fixed with glutaraldehyde, labelled with GARG, antiseraum cross-absorbed with 5 mg *S. oleracea* protein. (b) Higher magnification of part of the outgrowth showing labelling of the cell wall material. Bar markers represent (a) 1 μm and (b) 200 nm.

Fig. 7. Part of a large cell wall outgrowth in *S. oleracea* with numerous virus-containing tubules. Fixed with glutaraldehyde, unlabelled. Bar marker represents 100 nm.

DISCUSSION

The ultrastructural changes found in infected leaf cells were similar in two host plants and with both isolates of PYFV used, and were essentially the same as described by Murant *et al.* (1975). However, tissues which had been fixed with glutaraldehyde alone for immunogold labelling lacked structural definition and visible membranes, in contrast to tissues fixed with both glutaraldehyde and osmium (Murant *et al.*, 1975). Immunogold labelling provided valuable information on the distribution of PYFV antigen in infected plant cells but absorbing the antisera with protein from healthy plants was essential to eliminate non-specific labelling. A similar problem was encountered by Brakke *et al.* (1987) in studies of wheat leaf tissue infected with wheat streak mosaic virus. Tomenius *et al.* (1983) reported gold labelling of the walls of xylem vessels in pea leaf tissue infected with red clover mottle virus, although it is not clear whether this labelling was regarded as virus-specific.
In our experiments, although absorbing the antiserum with protein from healthy plants prevented immunogold labelling of cell wall material of healthy plants, it did not prevent labelling of cell wall outgrowths and light labelling of other parts of cell walls, and of nuclei, in virus-infected plants. Moreover, such labelling, and indeed the outgrowths themselves, were found only in those cells of infected plants which contained virus-induced inclusions. These observations suggest that the labelling is virus-related. Immunogold labelling of cell walls has been reported with antibody to the 30K transport proteins of tobacco mosaic (TMV) and alfalfa mosaic (AMV) viruses (Tumenius et al., 1987; Moser et al., 1988; Stussi-Garaud et al., 1987) and with antibody to the P1 protein of cauliflower mosaic virus (CaMV) (Linstead et al., 1988) which is thought to have a transport function. With CaMV and TMV, the labelling was over the plasmodesmata, whereas with AMV it was confined to the middle lamella of newly infected cells. As far as we know, specific labelling of the walls of virus-infected cells has not previously been reported with antibody to virus particles. However, PYFV is unusual among small isometric plant viruses in having three particle proteins, of $M_r (\times 10^{-3})$ 30, 26 and 24 (Hemida & Murant, 1989a), and a possible explanation of our results is that one of these proteins might also function as a transport protein. In our studies the immunogold labelling over the cell wall outgrowths seemed to occur only over the cell wall material itself and not over the particles within the tubules. Possibly the virus particles in the tubules are unavailable for labelling because either they are shielded by the tubule membrane or they are not at the surface of the section and so cannot be reached by the labelling antibodies. However, the relatively small number of virus particles found in the tubules seem unlikely to account for the amount of virus shown by Hemida & Murant (1989a) to be extractable from leaves and it is difficult to explain why intact virus particles are not visible elsewhere in the cell.

In contrast, labelling did occur over other structures in the cells, especially over the matrix material found in the inclusions. Immunoblotting experiments (S. K. Hemida, A. F. Murant & M. A. Mayo, unpublished data) have shown that the PYFV antiserum used in the present study can detect all three particle proteins of homologous isolates. Thus the labelled areas contain at least one of these proteins, possibly all three. The labelling of matrix material is similar to that found in protoplasts infected with cowpea mosaic virus (CPMV) by Van Lent (1988), who used immunogold labelling with antibodies both to structural and to non-structural proteins and found both antigens localized in the same place. The fact that in CPMV (Goldbach & Van Kammen, 1985), and perhaps also in PYFV (S. K. Hemida, A. F. Murant, M. A. Mayo & D. J. Robinson, unpublished data), both kinds of protein are produced by post-translational cleavage of a large polyprotein, suggests that the matrix material may represent the site of protein synthesis in the cell, as well as probably being the site of virus particle assembly.

REFERENCES


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