A temporal study of the expression of the capsid, cytoplasmic inclusion and nuclear inclusion proteins of tobacco etch potyvirus in infected plants

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Young leaves of tobacco, systemically infected by tobacco etch potyvirus (TEV), were examined for the presence and distribution of four virus encoded proteins [capsid, cytoplasmic inclusion (CI) and two nuclear inclusion (NI) proteins] at various time periods after inoculation of expanded leaves of the plants. The analyses were carried out by ELISA and by immuno-gold electron microscopy of thin sections of the leaves. All four proteins were detected simultaneously in the systemic leaves for the first time on the fifth day after inoculation of the expanded leaves. All four proteins increased in concentration until the seventh day and then showed no further increase with the exception of the capsid protein which continued to accumulate. The CI protein was first detected in association with the plasmalemma/cell wall and was subsequently found mostly in the form of pinwheels in the cytoplasm. The two NI proteins were found at all times after infection within the nucleus, although small concentrations were detected in the cytoplasm. These experiments suggest that both the NIa and NIb proteins are transported into the nucleus immediately after synthesis. At the earliest time periods after infection, high concentrations of these proteins (NIa and NIb) were found in their non-inclusion form in the nucleolus. At 14 days after infection, both proteins were found only as inclusions in the nucleus. The capsid protein was found at all stages of infection only in the cytoplasm.

Introduction

Tobacco etch virus (TEV) is a member of the potyvirus group. The virus particles are flexuous rods 730 nm long and 12 to 13 nm in diameter made up of a single-stranded mRNA of approximately 9500 nucleotides encapsidated by repeating subunits of a capsid protein of Mr 30K (Purcifull & Hiebert, 1982; Allison et al., 1986). Upon infection, the virus RNA is translated into a large polyprotein which is then cleaved into at least eight proteins (Dougherty & Carrington, 1988). Some of these proteins accumulate in the infected cells in the form of inclusion bodies (Edwardson, 1974). These inclusion bodies are: the pinwheel type cytoplasmic inclusion body (CI) made up of a single protein of Mr 70K (Kassanis, 1939; Sheffield, 1941; Hiebert & McDonald, 1973) and a nuclear inclusion body (NI) made up of two proteins of Mr 49K (NIa) and 58K (NIb) respectively (Knuhtsen et al., 1974; Dougherty & Carrington, 1988). At least one other inclusion body, referred to as the amorphous inclusion body, has been found in plant cells infected by some potyviruses such as potato virus Y, watermelon mosaic virus and papaya ringspot virus, but not in TEV-infected cells (DeMejia et al., 1985a, b; Baunoch et al., 1990). Amino acid sequence homology studies have shown that the CI proteins have homology to nucleotide-binding proteins (NTP motif) including helicases (Gorbalenya et al., 1989; Lain et al., 1989a) and the NIa and NIb proteins have homology to viral proteases and viral polymerases respectively (Allison et al., 1986; Domier et al., 1987; Carrington & Dougherty, 1987; Lain et al., 1989b). Electron microscopic studies of thin sections of leaves infected by some potyviruses show a relationship between the CI and the plasmodesmata of plant cells prompting the suggestion that this protein may also be involved in viral transport (Gardner, 1969; Lawson & Hearon, 1971; Andrews & Shalla, 1974; Langenberg, 1986).

In a previous study using older leaves infected by the non-aphid-transmissible (NAT) strain of TEV (14 day infections), we found that the CI protein was found mostly as pinwheels in the cytoplasm and also in association with cell membranes. The two nuclear proteins were found in both inclusion and non-inclusion form in the nucleus (Baunoch et al., 1988). In this more detailed study involving leaf samples infected for many different periods of time, we investigated the following. (i) We determined the relative concentrations of these different virus proteins at various times after infection since no such information is currently available. (ii) We
determined whether any association of the CI protein with the plasmodesmata or with the 58K polymerase protein (NIb) can be found at any stage of the infection in view of the possibility that the CI protein might be involved in viral transport and/or replication (Lawson & Hearon, 1971; Domier et al., 1987; Lain et al., 1989a, b).

(iii) We ascertained whether the almost exclusive presence of the NIa and NIb proteins in the nucleus in older infections also occurs in early infections.

Our results showed that all virus proteins studied could be detected in systemically infected leaves simultaneously on the same day after infection and that all of them have a similar pattern of accumulation. The CI protein was found closely associated with the plasmalemma often opposite the plasmodesmata, but not in it. The two NI proteins were found in low concentrations in the cytoplasm, but in higher concentrations first in a non-inclusion form in the nucleolus and nucleus. In older infections, these two NI proteins were found exclusively as inclusions in the nucleus.

Methods

Virus. The virus used in the study was a highly aphid-transmissible strain of TEV (TEV-HAT) obtained from Dr T. P. Pirone of the University of Kentucky, Lexington, U.S.A. The virus was propagated in Nicotiana tabacum L. var. Xanthi n.c.

Time course studies. The expanded leaves of several young tobacco plants (N. tabacum L. var. Xanthi n.c.) about 25 cm tall were inoculated with TEV at a concentration of 10 μg/ml in the presence of Celite at 50 μg/ml. The youngest leaf from the apex of the plant which was not inoculated was tagged at the time of infection in all plants and this leaf was sampled for ELISA and electron microscopy.

Tissue extraction. One gram of the leaf samples was ground in three volumes of sample buffer (Laemmli, 1970) and heated at 100 °C for 5 min. The extracts were then centrifuged at 12000 r.p.m. for 5 min in an RC2B Sorvall centrifuge using the SS34 rotor. Two volumes of cold acetone were added to the supernatant in order to remove SDS and to concentrate and decolourize the proteins. The precipitated proteins were pelleted by centrifugation and repeatedly washed with 70% acetone until all extractable colour had been removed. The proteins were finally resuspended in 3 ml of carbonate buffer pH 9.8 (Winston, 1988) and used for ELISA directly.

Antisera. The antisera were the same as those used earlier (Baunoch et al., 1988). The sera were, however, preabsorbed with leaf proteins from tobacco leaves (Baunoch et al., 1990).

ELISA. One-hundred μl of the protein samples extracted as above was added in duplicate to each well of an ELISA plate and, after incubation, processed for ELISA using antisera to the capsid, CI, NIa or NIb proteins followed after washing by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. Colour was developed using as substrates o-nitrophenyl phosphate in 10% diethanolamine (Engvall, 1980). The ELISA plates were read at 405 nm in a BIOTEK EL340 ELISA reader (Biotek Instruments).

Immunogold labelling. Thin sections for immunogold electron microscopy were cut from Lowicryl K11 (Polysciences)-embedded tissue blocks (Baunoch et al., 1990). Thin sections were cut with a diamond knife, incubated with virus protein-specific rabbit antisera and immunogold-labelled using gold (10 nm)-conjugated goat anti-rabbit IgG (Sigma). Samples were viewed in a Philips EM 201 or 301 electron microscope. Electron micrographs of several cells from infected and uninfected tobacco leaf thin sections exposed to different virus-specific antisera were prepared. These micrographs were used to count the number of gold particles per unit cell area.

Results and Discussion

ELISA studies

The virus capsid protein as well as the three non-structural proteins studied were first detected in the systemic leaves on the fifth day after inoculation. The concentration of all proteins increased until the seventh day and then the concentration of the CI, NIa and NIb proteins dropped slightly between the seventh and fourteenth days whereas that of the capsid protein continued to increase (Fig. 1). However, after the fourteenth day the concentration of the capsid protein stabilized (data not shown). These results are compatible with earlier reports that the yield of inclusion proteins in potyvirus-infected cells is reduced in older infections presumably due to the instability of the inclusions in older infections (Hiebert et al., 1984). The virus capsid protein, in contrast, is apparently stabilized by the formation of virus rods.

The capsid protein

The distribution of the capsid protein at all stages of infection studied was similar to that reported earlier (Baunoch et al., 1988). No capsid protein was found in any of the organelles.

![Graph](image-url)  
Fig. 1. Relative concentration of the TEV capsid (□), CI (○), NIa (△) and NIb (▽) proteins in young uninoculated systemically infected leaves at various times after inoculation of expanded leaves as tested by ELISA.
Expression of non-structural proteins of TEV

The CI protein

Studies on the distribution of the CI protein by immunogold labelling showed that during early stages of infection this protein was found mainly in the form of strands attached to the plasmalemma. These protein strands were often found opposite the plasmodesmata (Fig. 2 a, b and c, Table 1). Subsequently, at later stages in the infection, the CI was found mostly in the cytoplasm as pinwheel type inclusions (Fig. 2d, e). Other investigators in the past have also noted the presence of the CI-type strands in plasmalemma opposite the plasmodesmata (Gardner, 1969; Lawson & Hearon, 1971; Andrews & Shalla, 1974), but this is the first report of localization of the CI protein in free form even before this protein had taken a structural appearance.

The results obtained do not show that the CI protein is found within the plasmodesmata. However, they do show that the CI protein is found opposite the plasmodesmata in association with the plasmalemma. If indeed these structures found opposite the plasmodesmata are involved in viral transport, then such transport probably occurs in the form of viral RNA since viral capsid protein was found neither in the plasmodesmata nor in the CI protein strands associated with the plasmalemma. These results are in contrast to those of Weintraub et al. (1976) who found intact virions of another potyvirus, namely potato virus Y, in plasmodesmata. Since the CI protein contains NTP-binding and helicase motifs (Domier et al., 1987; Lain et al., 1989a, b) it is reasonable to expect that the CI protein found attached to the plasmalemma at the early stages of infection represents viral RNA replication complexes. If these are replication complexes, one would expect these ribbon-like structures of CI found in the plasmalemma to be associated with the NIa protease-VPg (Murphy et al., 1990) and the NIb putative polymerase (Allison et al., 1986; Domier et al., 1987). Our immunogold labelling experiments did not show any gold labelling of the CI-like structures after exposure to polyclonal NIa or NIb antibodies. These experiments thus suggested that both the NIa and NIb proteins are not associated with the CI protein. However, it is possible that the CI-like structures are associated with only a few molecules of NIa and NIb proteins and our immunogold labelling techniques may not be sensitive enough to detect them. Thus, our results do not rule out the possibility that the CI-like structures attached to the plasmalemma are indeed replication complexes.

Table 1. Distribution of the CI protein in cells*

<table>
<thead>
<tr>
<th>Location of CI protein</th>
<th>Time after infection (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>In cytoplasm</td>
<td>12%</td>
</tr>
<tr>
<td>Attached to cell wall but not opposite plasmodesmata</td>
<td>71%</td>
</tr>
<tr>
<td>Attached to cell wall opposite plasmodesmata</td>
<td>16%</td>
</tr>
<tr>
<td>In plasmodesmata</td>
<td>1%</td>
</tr>
<tr>
<td>In organelles</td>
<td>0%</td>
</tr>
</tbody>
</table>

* At each time period after infection, 100 CI protein bodies from several sections were counted and their distribution in the cell as shown by immunogold labelling with anti-CI antibodies was expressed as a percentage of the total. No counts were made in 14 day infections.
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Fig. 3. Immunogold labelling of NIa protein in cells of young uninoculated systemically infected leaves from plants infected for various lengths of time. (a) NIa protein in the nucleolus of cells of leaves on the fifth day of infection; (b) NIa protein in both nucleoli and rest of the nucleus on the sixth day; (c) immunolabel in association with inclusions and in free form in the nucleus on day 7; (d) immunolabelling of inclusions on day 14; (e) inset showing an enlarged NI body. Bar markers represent 300 nm (a), 275 nm (b), 225 nm (c), 350 nm (d) and 175 nm (e).

Table 2. Distribution of NIa-specific immunolabel based on the number of immunogold particles per micron square*

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>0</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell structure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>1.1</td>
<td>1.1</td>
<td>7.0</td>
<td>14.0</td>
<td>10.4</td>
<td>2.3</td>
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<tr>
<td>Chloroplast</td>
<td>0.36</td>
<td>0.36</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>0.0</td>
<td>0.0</td>
<td>111.3</td>
<td>83.2</td>
<td>54.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.37</td>
<td>0.37</td>
<td>58.4</td>
<td>87.2</td>
<td>39.1</td>
<td>1.65</td>
</tr>
<tr>
<td>Nuclear inclusion</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1800.0</td>
<td>2070.0</td>
</tr>
</tbody>
</table>

* The number of gold particles per micron square of each cell structure above was calculated from counts of several electron micrographs of tissue specimens from 0, 4, 5, 6, 7 and 14 day infections.

Table 3. Distribution of NIb-specific immunolabel in TEV-infected cells based on the number of gold particles per micron square*

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>0</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell structure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cytoplasm</td>
<td>0.73</td>
<td>0.27</td>
<td>8.9</td>
<td>11.1</td>
<td>5.9</td>
<td>0.22</td>
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<tr>
<td>Chloroplast</td>
<td>0.27</td>
<td>0.57</td>
<td>1.9</td>
<td>2.3</td>
<td>1.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>0.0</td>
<td>1.70</td>
<td>86.0</td>
<td>69.6</td>
<td>51.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.24</td>
<td>0.14</td>
<td>49.2</td>
<td>80.1</td>
<td>63.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Nuclear inclusion</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1650.0</td>
<td>1758.0</td>
</tr>
</tbody>
</table>

* The number of gold particles per micron square of each cell structure above was calculated from counts of several electron micrographs of tissue specimens from 0, 4, 5, 6, 7 and 14 day infections.

NIa and NIb proteins

At the earliest time period after infection (fifth day), the heaviest labelling of both NIa and NIb proteins occurred in the nucleolus (Fig. 3a, 4a, and Tables 2 and 3). On the sixth day after infection heavy labelling was found in the nucleolus and the rest of the nucleus (Fig. 3b, 4b, and Tables 2 and 3). On day 7, both virus inclusions as well as free proteins were labelled by both the NIa and NIb antisera (Fig. 3c, 4c, and Tables 2 and 3). By day 14, the labelling of both NIa and NIb was confined to nuclear
Expression of non-structural proteins of TEV

Fig. 4. Immunogold labelling of the NIb protein in cells of uninoculated systemically infected leaves after various lengths of time of infection. (a) NIb protein mostly in nucleoli on the fifth day; (b) NIb protein in nucleoli and rest of nucleus on the sixth day; the arrow shows lack of labelling of chromatin indicating specificity of labelling; (c) NIb protein found mostly in inclusions on day 7; (d) inset showing an enlarged inclusion; (e) NIb protein found in inclusions on day 14; (f) inset showing portion of an enlarged NI labelled with NIb antibodies. Bar markers represent 250 nm (a), 200 nm (b), 350 nm (c), 150 nm (d), 400 nm (e) and 125 nm (f).

inclusions (Fig. 3d and e; 4e and f, and Tables 2 and 3). These inclusions, unlike those of the TEV-NAT strain (Baunoch et al., 1988), were less compact.

Studies on the distribution of immunolabel in the cell showed very little labelling in the cytoplasm at all times after infection. No labelling of any of the organelles other than nuclei was found (Tables 2 and 3). The distribution of immunolabel suggested that both NIa and NIb proteins were transported rapidly after synthesis into the nucleus. The reasons why the potyviral NIa and NIb proteins are transported into the nucleus can only be speculated upon. One possibility is that the accumulation of these proteins in the nucleus is related to their role in viral RNA replication which could be taking place in the nucleus. This would be unusual because all RNA viruses studied seem to replicate outside the nucleus (Rueckert, 1989) and double-stranded TEV RNA has been found in cytoplasmic and not nuclear fractions (Gadh & Hari, 1986). A second speculation is that these proteins are somehow involved in stimulating or modifying host RNA including ribosomal RNA synthesis which takes place in the nucleolus. This would be analogous to the role of the multifunctional large T antigen of simian virus 40 which induces host DNA replication and stimulates rRNA synthesis (Soprano et al., 1983). We have no data at present on whether potyviral infection modifies rRNA synthesis. A third possibility is that the nuclei act as storage regions for the excess polymerase (58K) and protease (49K) which are produced by polyprotein processing. Perhaps the presence of large amounts of these proteins in the cytoplasm could interfere with viral RNA encapsidation and hence these are transported into a different cellular compartment, namely the nucleus. Thus, nuclear transport of the proteins might be a way to assure that viral RNA encapsidation follows replication as otherwise these proteins might be still bind to the viral RNA for initiating new rounds of replication and thus become unavailable for encapsidation into virus particles.

In conclusion, the experiments reported here demonstrated the close association of the CI protein with the plasmalemma, in proximity to the plasmodesmata. Furthermore, the results showed that both the NI proteins are transported rapidly after synthesis into the
nucleolus and nucleus of infected cells before they are polymerized into nuclear inclusions.

References


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