Tubular Inclusion Bodies in Plants Infected with Viruses of the NEPO Type

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SUMMARY

Tubular inclusion bodies containing single rows of virus particles were observed by electron microscopy in hosts infected with three NEPO viruses. When Nicotiana rustica L. plants were inoculated with cherry leaf roll virus tubules were found in axillary buds 4 days later and reached a maximum concentration after 2 to 3 weeks. Tubules were also found in root tips, pollen, ovules and mature seeds of these plants, but not in the leaf tissue.

Sections through cherry leaf roll virus infected tissue showed the tubules to be most frequent in the cytoplasm of undifferentiated cells. They occurred singly or in bundles and appeared to pass between cells. The tubule wall usually consisted of a single layer forming a complete tube in cross section.

INTRODUCTION

The presence of tubular inclusion bodies in apical meristematic tissue of Nicotiana rustica L. infected with cherry leaf roll virus was reported earlier (Walkey & Webb, 1968). Tubules were detected in 'squash homogenates' of infected axillary bud-tips examined by electron microscopy. Within each tubule virus particles were arranged in a single row and the tubules appeared similar to those observed in leaf tissues of plants infected with maize rough dwarf virus (Gerola & Bassi, 1966) and turnip yellow mosaic virus (Hitchborn & Hills, 1967, 1968). Recently, Davidson (1969) reported occasional tubules in root-tip cells of Phaseolus vulgaris L. infected with tobacco ringspot virus.

Our early observations showed that the tubules were always present within CLRV infected bud-tips of Nicotiana rustica at definite times after virus inoculation of a healthy plant. Further work was initiated to study the occurrence, development and structure of tubular inclusion bodies induced by cherry leaf roll virus and closely related NEPO (Cadman, 1963) viruses in N. rustica and other hosts. The results of this investigation are reported in this paper and the possible function of the tubules is discussed.

METHODS

Viruses. The cherry leaf roll virus (CLRV) and arabis mosaic virus (AMV) isolates were obtained from rhubarb (Tomlinson & Walkey, 1967) and the strawberry latent ringspot virus (SLRV) from celery (Walkey & Mitchell, 1969). Dr R. W. Fulton, University of Wisconsin, Madison, kindly supplied the tobacco ringspot virus (TRSV), and Dr M. Hollings, Glasshouse Crops Research Institute, Littlehampton, Sussex, the tomato black-ring virus (TBRV). CLRV, TRSV and TBRV were cultured in Nicotiana rustica, SLRV in cucumber and AMV in N. clevelandii Gray.
Culture, inoculation and virus assay of plants. Healthy plants were grown in the glasshouse and subjected to supplementary winter lighting if necessary. When of suitable size they were mechanically inoculated by rubbing the two to three lowest leaves with infected sap, prepared by grinding systemically infected leaves in 0.1 M-potassium phosphate buffer (pH 7.5).

In the sequential studies of tubule development, all *Nicotiana rustica* plants were selected for uniformity and the three oldest leaves inoculated when the plants were 10 to 12 cm. tall. One half of the infected plants were randomly selected for tubule examination. The remainder were assayed for virus by grinding the axillary bud-tips and inoculating the extract obtained to *Chenopodium amaranticolor* Coste & Reyn, as previously described (Walkey, Fitzpatrick and Woolfitt, 1969).

Examination of infected tissues. Tissues from infected plants were dissected under a low-power microscope with scalpels made from pieces of razor blade (Stone, 1963). Axillary bud-tips comprised of the meristematic dome plus one or two pairs of leaf primordia, and root meristems of the last 1 to 2 mm. of the root-tip, including the root cap. Seeds of *Nicotiana rustica* (infected with CLRV) and celery (infected with SLRV) were harvested from plants which had been experimentally inoculated with virus at the seedling stage. All infected tissues were examined with an A.E.I. EM6B electron microscope by the ‘squash-homogenate’ technique (Walkey & Webb, 1968).

Preparation of sections for electron microscopy. Diseased tissues were fixed for 2 hr in 1 % (w/v) glutaraldehyde prepared in 0.1 M-cacodylate buffer (pH 7.2) at 0°C, followed by 2 hr in 2 % (w/v) osmium tetroxide in the same buffer (Sabatini, Bensch & Barnett, 1963). They were then dehydrated through an ethyl alcohol series and embedded in Epikote 812 resin. Sections were cut on an LKB Ultrotome 3 ultramicrotome, using a glass knife, and stained with lead citrate (Reynolds, 1963).

Table 1. Incidence of tubule formation with different NEPO viruses

<table>
<thead>
<tr>
<th>Host</th>
<th>CLRV</th>
<th>TRSV</th>
<th>SLRV</th>
<th>AMV</th>
<th>TBRV</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiana rustica</em> L.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>N. tabacum</em> L. ‘White Burley’</td>
<td>+ *</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>N. clevelandii</em> Gray</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Chenopodium quinoa</em> Wild.</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. amaranticolor</em> Coste &amp; Reyn</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Spinacia oleracea</em> L. (Spinach)</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Cucumis sativus</em> L. (Cucumber)</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Apium graveolens</em> L. (Celery)</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>

* Not tested.

RESULTS

Occurrence of tubules in hosts infected with viruses of the NEPO group

Tubular inclusion bodies were present in ‘squash-homogenates’ of axillary bud-tips taken from various host plants infected with the NEPO viruses CLRV, TRSV and SLRV (Table 1). Tubules have not yet been seen in plants infected with AMV and TBRV.

The average width and structure of the tubules induced by CLRV, TRSV and SLRV (Fig. 1a, b, c) were similar in all plant species used. They measured from 44 to 50 nm. in width and their walls were 5 to 6 nm. in thickness. Tubules were unbranched and contained...
a single row of virus particles. Accurate measurement of the tubule length was not possible as the tubule usually fractured transversely during homogenization. Some of the longer fractions were 3 to 5 μm in length and contained 100 to 150 virus particles.

**Sequence of tubule development in Nicotiana rustica infected with CLRV**

In this experiment (Table 2) and in two others daily sampling of axillary bud-tips of CLRV infected *Nicotiana rustica* showed that no tubules could be found during the first 72 hr following virus inoculation. After 4 days a few tubules were detected in the axillary buds of the second and third inoculated leaves, and at 5 days in the axillary buds of the first uninoculated leaf and in the terminal bud. At 8 days they were present in the axillary buds of all inoculated leaves and in those of most of the uninoculated leaves that had developed.

**Table 2. Sequential development of tubules in axillary-buds at different leaf positions**

<table>
<thead>
<tr>
<th>Bud position</th>
<th>Number of days after inoculation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Apex</td>
<td></td>
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<tr>
<td>Uninoculated leaves</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Inoculated leaves</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
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<td>4</td>
<td></td>
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<td>5</td>
<td></td>
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<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
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</tbody>
</table>

* Oldest leaf.

Free virus particles, not enclosed in tubules, were detected in some buds 24 hr before the appearance of tubules in buds taken from corresponding leaf positions. In other cases free virus and tubules were observed simultaneously but the period between observing unenclosed particles and tubule occurrence was never more than 24 hr.

The results of these experiments show that if infected plants are grown for periods of up to 8 weeks the greatest concentration of CLRV tubules in *Nicotiana rustica* bud-tips occurs 2 to 3 weeks after virus inoculation. After this period they were found far less frequently.

The detection of virus in similar bud-tips from the remaining plants showed good correlation with the observation of tubules; virus was detected only at the stages when tubules could be seen in the tips.
Occurrence of CLRV and SLRV tubules in various plant parts

Tubules were observed in the following meristematic and embryonic tissues of *Nicotiana rustica* infected with CLRV; apical meristems of axillary and terminal buds, root tips, undehisced and dehisced pollen, undeveloped and mature ovules and mature seed (Fig. 1d). The

Fig. 1. (a) Single CLRV tubule from *Nicotiana rustica*; (b) bundle of TRSV tubules from *N. clevelandii*; (c) single SLRV tubule from *N. rustica*; (d) bundle of CLRV tubules from mature *N. rustica* seed.
Fig. 2. Section through undifferentiated meristematic cells of *Nicotiana rustica* showing fractions of numerous CLRV tubules.
tubules were also seen in mature celery seed infected with SLRV. The concentration of tubules was greater in mature seed than in most other tissues with both CLRV and SLRV. No tubules were observed in leaf or stem tissues.

![Image](image-url)

**Fig. 3.** (a) Section through meristem of *Nicotiana rustica* showing portions of what appears to be the same bundle of CLRV tubules within adjacent cells. (b) Transverse section through bundles of CLRV tubules showing single wall and ribosome-free area surrounding the tubules.

**Structure and location of tubules within cells**

Ultrathin sections of CLRV-infected axillary bud-tips and young ovule tissues were used to study the structure and location of tubules within the cell. The highest concentration of tubules was found in relatively undifferentiated cells (Fig. 2) and the lowest in older cells that had developed recognizable organelles.
Tubules associated with three NEPO viruses

Tubules occurred singly or in bundles of as many as 15 (Fig. 3a, b). In older cells single tubules were more frequent than bundles. Whether single or in bundles, the tubules were sinuate and only a portion of the structure could be seen in longitudinal section (Fig. 3a). Because of this it was difficult to follow the passage of the tubules through adjacent cells, but some sections (Fig. 3a) showed what appeared to be portions of the same bundle on either side of the wall separating adjacent cells.

Individual tubules and bundles were seen only in the cytoplasm and were surrounded by a zone free of ribosomes (Fig. 3b).

Longitudinal and transverse sections (Fig. 3a, b) showed the tubule wall to consist of a single, dark staining layer that formed a complete tube. Although the upper and lower surfaces of the tube were difficult to see in negative stain, their presence was readily detected by optical diffraction. Tubules with a double wall were occasionally seen.

No differences were detected in the structure or location of tubules occurring in axillary bud-tips or ovule tissue.

**DISCUSSION**

The widespread occurrence of tubules within the meristematic tissues of plants infected with at least three NEPO viruses suggests that tubules may play a part, at present unknown, in the processes of infection by these viruses. It is not known whether this type of inclusion body is induced by other viruses of this group. The possibility that the NEPO viruses could be sub-divided on the basis of tubule occurrence is being investigated.

Superficially, NEPO-type tubules resemble those of maize rough dwarf virus (MRDV) (Gerola & Bassi, 1966) and TYMV (Hitchborn & Hills, 1968) in that they consist of a tube containing a single row of virus particles. The MRDV and NEPO tubules have diameters only slightly larger than those of the virus particles within, whereas the TYMV tubules vary in size with diameters considerably greater than those of the enclosed particles. The length of MRDV tubules is not known but those of CLRV and TYMV are similar and up to several microns in length. Unlike MRDV and TYMV tubules, which are common in leaf tissues, the CLRV tubules were only found in undifferentiated meristematic tissue, pollen and seed.

Hitchborn & Hills showed that the tubules of TYMV were virus-associated structures and not artifacts. They were also able to show by optical diffraction studies that the wall of the TYMV tubule had an ordered array of sub-units consisting mainly of protein which was related serologically to the virus protein. Similar optical diffraction experiments with CLRV tubules showed them to have a definite structure differing from that of TYMV tubules and from any virus structure previously studied. This suggests that the tubular inclusion body induced by a virus has a specific structure and is characteristic for the virus or virus-type concerned.

Numerous attempts have been made to detect a 'pre-tubule' stage which might associate the inclusion body with virus assembly but, although opaque tubular structures were seen at a time when tubule development would be expected, they have not yet been shown to be related to mature tubules.

The fate of tubules and of the virus particles they contain is not known. Our evidence from sections shows that the tubules become less frequent as the cells become older and that CLRV tubules probably pass through cell walls into adjacent cells. The passage of tubules through cell walls was confirmed by Davidson (1969), who found that TRSV tubules passed through the plasmodesmata of infected *Phaseolus vulgaris* cells. Based on this and our own evidence it is reasonable to speculate that if the virus is synthesized in meristematic cells then the tubules may be involved in virus dissemination to older, adjacent cells. The tubules could
transport the virus between cells, probably via the plasmodesmata, and eventually liberate the particles by breakdown of the tubule wall.

Finally, it is of interest that very high concentrations of tubules were found in seeds of *Nicotiana rustica* plants infected with CLRV and from celery plants infected with SLRV. In both cases young seedlings grown from the seeds were always infected. The high incidence of tubules in these seeds may indicate their involvement in the seed transmission of these viruses.

We are grateful to Mr G. J. Hills, John Innes Institute, for the optical diffraction studies

REFERENCES


