Movement and Intracellular Location of Sonchus Yellow Net Virus within Infected Nicotiana edwardsonii

By IMAD D. ISMAIL, IAN D. HAMILTON, EOIN ROBERTSON and JOEL J. MILNER.

Departments of Botany and Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.

(Accepted 2 June 1987)

SUMMARY

Systemic movement of Sonchus yellow net virus to leaves and roots was first detected by ELISA 24 h after mechanical inoculation. Thereafter, virus levels rose to a maximum 10 days after inoculation; the highest levels were between 4.0 and 7.3 μg/g tissue, in leaves which were not yet fully expanded. Electron microscopy of tissue sections revealed that when the virus content of tissues was greatest, virtually all leaf and root cells were infected. Most of the virions were in the perinuclear space; only a few were scattered in the cytoplasm. Nuclei contained large viroplasms associated with viral nucleocapsids. Between 10 and 20 days after inoculation, levels of virus antigen and viral RNA fell to about 20% of their maximum. By 20 days after inoculation, no more than 10% of cells contained virus particles and almost all the virions were in the cytoplasm. These results suggest that this virus spreads systemically until most or all cells are infected. The plants then undergo a recovery phase during which virus disappears from the nuclei of infected cells and vesiculates into the cytoplasm.

INTRODUCTION

The plant rhabdoviruses are one of the largest groups of plant viruses and include a number of economically important pathogens. In order to understand, and perhaps eventually control, their pathological effects, we need to know the way in which viruses spread systemically through plants and the way virus levels vary within the tissues of infected plants. Spread of many viruses from the initial site of inoculation occurs within as little as 36 h after inoculation and translocation appears to involve the vascular system (for review, see Matthews, 1981). Lettuce necrotic yellows virus, a rhabdovirus, was detected in the xylem sap of infected Nicotiana (Francki & Randles, 1970). Özel (1973) detected by electron microscopy sowthistle yellow vein virus, another rhabdovirus, in the roots of Sonchus oleraceus 48 h after aphid-mediated inoculation of leaves. He suggested that the virus moved initially into the roots through the phloem and subsequently, after multiplication, moved from roots to leaves through the xylem.

Inf ectivity assays have shown that for several rhabdoviruses, virus levels in infected tissues initially increase, reach a maximum, and then decline (Jackson & Christie, 1977; Francki & Randles, 1980). Such assays, however, are only semi-quantitative and are considerably less sensitive than ELISA. We have used a combination of ELISA, dot-blot hybridization and electron microscopy to follow the spread of Sonchus yellow net virus (SYNV) throughout the tissues of Nicotiana edwardsonii following mechanical inoculation.

METHODS

Virus maintenance and purification. SYNV was maintained and propagated in N. edwardsonii as described by Christie et al. (1974). The plants were inoculated when at the eight-leaf stage (about 8 cm across). Approximately 1 g of infected leaf tissues was ground in 10 ml 1% Na2SO4 and rubbed onto three fully expanded leaves from each plant, which had been dusted with carborundum. Plants were kept in a growth chamber at a temperature of 23 °C to 25 °C and illuminated for 14 h per day. Virus was purified as described previously (Jackson & Christie, 1977; Milner & Jackson, 1979) from leaves harvested 10 to 12 days after inoculation. Viral RNA was prepared by lysis of
purified virus with 0.5% SDS followed by sucrose gradient centrifugation (Milner & Jackson, 1979). Virus protein concentration was determined by the Coomassie Brilliant Blue binding assay (Bradford, 1976) using bovine serum albumin as a standard.

For preparation of infected tissue samples, inoculated plants were randomly divided into three batches. At various times after inoculation, samples of (i) inoculated leaves, (ii) unoinoculated leaves which were fully expanded at the time of inoculation (referred to as expanded leaves), (iii) leaves which were less than 1 cm long at the time of inoculation (referred to as unexpanded leaves) and (iv) roots, were taken from one plant out of each batch and assayed for virus protein or examined by electron microscopy.

**Preparation of antisera.** Antiserum was prepared in a New Zealand White rabbit. Purified virus (380 µg) was emulsified in 50% Freund's complete adjuvant and injected subcutaneously at many sites. Six weeks later the animal was boosted with a similar injection of 380 µg virus emulsified in 50% Freund's incomplete adjuvant. Approximately 1 year later the animal was boosted with 250 µg virus in 50% Freund's incomplete adjuvant. After 14 days blood was taken from the animal, allowed to coagulate and antiserum was prepared. Antiserum was pre-absorbed by mixing it with 4 vol. of an extract from healthy leaves. This was prepared as described below for the preparation of sap samples for ELISA. The mixture was allowed to stand 12 h at 4 °C and centrifuged for 10 min at 13000 g in an MSE microcentrifuge.

**Electron microscopy.** Plant material was fixed in glutaraldehyde, post-fixed in 1% OsO₄, block-stained in uranyl acetate, embedded in Spurr resin, sectioned, stained with lead citrate (Reynolds, 1963) and examined in a Philips EM 301 microscope.

**Preparation of sap samples and ELISA.** One gram of plant material was ground with 10 ml of PBS–TPO (0.136 mM-NaCl, 9.2 mM-Na₂HPO₄, 0.87 mM-KH₂PO₄, 2.68 mM-KCl, 0.05% v/v Tween 20, 2% w/v polyvinylpyrrolidone and 0.2% w/v bovine serum albumin, pH 7.2) (Clark et al., 1986) and clarified at 13000 g for 2 min in a microcentrifuge. All sample preparation steps were performed at between 0 °C and 4 °C and samples were stored at −20 °C. One-hundred µl was taken from each sample, made to 2% in SDS, heated to 65 °C for 15 min and cooled to room temperature. Ten µl of each denatured sample was diluted (1:50 to 1:500) with coating buffer (15 mM-Na₂CO₃, 35 mM-NaHCO₃, pH 9.6) containing 5% normal donkey serum (Scottish Antibody Production Uni0. (0.5 mg/ml) in 25 mta-sodium acetate pH 5.5, 0.03% (v/v) H₂O₂ and the A₄₉₂ was determined.

**Assay for viral RNA.** Sap samples were prepared from tissue as described above. Sap samples, or purified viral RNA as a standard, were applied to nitrocellulose and hybridized as described by Baulcombe et al. (1984). Blots were exposed to preflashed X-ray film at −70 °C for 24 h. 32P-labelled cDNA probes were prepared from recombinant plasmids containing cDNA sequences derived from SYNV mRNAs (P. T. Richardson & J. J. Milner, unpublished). A mixture of three plasmids, pSYN302, pSYN402 and pSYN503, containing non-overlapping inserts of 1.8, 0.8 and 0.5 kbp derived from viral-complementary RNAs 3, 4 and 5 (coding for proteins N, M₁ and M₂) respectively (Rezaian et al., 1983; P. T. Richardson & J. J. Milner, unpublished) were used. The mixture was labelled by nick translation to a sp. act. of approximately 8 × 10⁷ d.p.m./µg (Mackey et al., 1977).

**RESULTS**

*N. edwardsonii* seedlings, mechanically inoculated with SYNV started to develop symptoms 6 days after inoculation. Systemic vein clearing and leaf cupping were observed first on unexpanded leaves. Symptoms spread to inoculated and uninoculated expanded leaves and reached a maximum after 10 days. Unexpanded leaves showed the most severe symptoms. Plants started to recover their normal leaf appearance between 15 and 20 days after inoculation. By 30 to 50 days, plants appeared normal with the exception of those in flower, which showed symptoms on their terminal leaves, underneath the flowers.

Virus antigen levels were measured using ELISA. Addition of SDS to the brei and heating at 65 °C for 10 min increased the amount of virus detected by up to threefold. This procedure also greatly increased reproducibility between replicate samples.

Tissue samples were taken from inoculated, expanded and unexpanded (at the time of inoculation) leaves and roots at 12 h intervals from 0 to 4 days after inoculation, at 24 h intervals from 4 to 6 days after inoculation, at 48 h intervals from 6 to 12 days after inoculation and at 5 day intervals from 15 days and after. Suitable dilutions of each sample were assayed for virus antigen by ELISA. For each time point, samples were taken from three plants and duplicates of
Movement of a plant rhabdovirus

Fig. 1. Concentrations of virus protein in tissues determined by ELISA. (a) Unexpanded leaves, (b) expanded leaves, (c) inoculated leaves, (d) roots. The insets show the same data for the first 6 days on a larger scale. The error bars indicate the standard deviations.

each sample were tested. Sap from healthy plants was used as a control. Concentrations of virus protein were determined by including on each plate a standard comprising suitable dilutions of purified virus at a known concentration. The results are shown in Fig. 1. Virus antigen within non-inoculated leaves and roots was detectable at a low but reproducible level of approximately 0.1 μg of virus protein per g of tissue 24 h after inoculation. This quantity of virus gave an absorbance, using buffer as a blank, which was approximately three times that of sap from healthy plants. Virus antigen was detectable in inoculated leaves immediately after inoculation but its concentration declined to near zero within 24 h, perhaps because it was inoculum which had adhered to the leaves and that washed off or degraded within 24 h of inoculation. The concentration of virus in expanded leaves changed little between 24 h and 4 days after inoculation. Virus levels in roots increased slightly, from 0.12 to 0.14 μg/g and levels in unexpanded leaves increased from 0.10 to 0.18 μg/g over the same period.

The amounts of virus antigen detected in all tissues increased from 4 days after inoculation and most dramatically between 6 and 10 days after inoculation. Levels of virus in the same tissues from different plants were similar; standard deviations were usually less than 3% (see below and Fig. 1). Virus antigen reached a maximum in all tissues except expanded leaves after 10 days. In the experiment shown in Fig. 1, maximum levels of virus antigen were: inoculated leaves 2.0 ± 0.07 μg/g, expanded leaves 5.7 ± 0.20 μg/g, unexpanded leaves 7.3 ± 0.02 μg/g and roots 2.8 ± 0.20 μg/g. In several experiments, maximum levels for expanded leaves were in the range 4.0 to 7.3 μg/g; the variation presumably reflects differences in the physiological state of the plants when inoculated. Levels of virus antigen declined rapidly in all tissues (except expanded leaves) between 12 and 20 days after inoculation and declined more slowly thereafter. In expanded leaves virus levels remained constant between 12 and 20 days after inoculation but declined thereafter.

The levels of antigen in leaves closely followed the appearance and disappearance of symptoms (vein clearing and cupping of the leaves). Under our growth conditions, symptoms always appeared first on the unexpanded leaves 6 to 7 days after inoculation; symptoms appeared on the expanded leaves about 2 days later.

Concentrations of viral RNA within unexpanded leaves were estimated by dot-blot hybridization. A series of tenfold dilutions of crude sap was spotted onto nitrocellulose. Sap from uninfected plants was used as a control. Dilutions of purified viral RNA were used as a standard. Viral RNA was detectable in unexpanded leaves 5 days after inoculation and reached a maximum 10 days after inoculation. Levels of hybridizable RNA then fell gradually and were
barely detectable 30 days after inoculation (Fig. 2a). In this experiment the uninfected sap gave a strong positive signal for undiluted, but not diluted samples. Hybridization to samples from uninfected sap was weaker in other experiments (see Fig. 2b). Comparable non-specific binding to undiluted sap samples has been reported by Owens & Diener (1981) using a similar assay. By comparing a series of threefold dilutions of sap from leaves 10 days after infection with dilutions of purified viral RNA (Fig. 2b) we estimated the concentration of hybridizable RNA (genomic plus messenger) to be 40 ng/g tissue.

When ultrathin sections from a variety of tissues from infected plants were examined in an electron microscope, both mature enveloped virions and unenveloped nucleocapsids were observed. The dimensions of the mature virions were 216 ± 3 nm × 74 ± 3 nm and those of the nucleocapsids were 200 ± 3 nm × 51 ± 2 nm (length × diam., average of 70 particles ± standard deviation).

Epidermal and mesophyll cells from unexpanded leaves, 5 days after inoculation contained numerous virus particles. Mature particles were observed in membrane-bound inclusions within the nucleus and within the perinuclear space with a few scattered in the cytoplasm (Fig. 3a). We also observed nucleocapsids associated with densely staining viroplasms within the nuclei (Fig. 3b). Fifteen percent of 180 cells examined in six separate grids contained detectable virus particles. Root cells from the same plants also contained viral inclusions within the nuclei and perinuclear spaces and also a few particles scattered through the cytoplasm (not shown). No virus particles were seen in over 200 cells from expanded leaves examined at this stage of infection.

Eight days after inoculation, about 65% of over 200 cells from unexpanded and expanded leaves contained virus particles. Large clusters, containing mature virions, were observed in the
Fig. 3. Electron micrographs of the distribution of viral particles in unexpanded leaves 5 days post-inoculation. (a) Mature particles (indicated by V₁, V₂ and V₃, respectively) within the nucleus (N), perinuclear space and cytoplasm (CY). (b) Nucleocapsids (VN) associated with viroplasm (VP) within a nucleus. The inner and outer nuclear envelopes are indicated by IE and OE, respectively and cell wall by CW. Bar markers represent 1 μm.
perinuclear spaces. Virions could clearly be seen budding from the nuclei through the inner nuclear envelope into the perinuclear spaces. Nucleocapsids were present, scattered within some nuclei as well as being associated with densely staining viroplasms. A few virions were scattered within the cytoplasm, often associated with rough endoplasmic reticulum (Fig. 4).

Ten days after inoculation, sections from all tissues showed the highest proportions of cells containing virus particles. The location and numbers of particles were similar to those in sections from plants 5 and 8 days after inoculation. In unexpanded leaves and expanded leaves, virus particles were seen in 90% of over 1000 mesophyll palisade and epidermal cells examined.
in a large number of independent sections. We also observed virions within guard cells. Cells of both primary and secondary roots contained mature virions in the cytoplasm, as well as large clusters within the perinuclear space. Some of the virus particles within the cytoplasm of both root and leaf cells were in small groups bounded by a membrane. The time at which most cells contained virus thus coincided with the time at which the concentration of virus antigen, as determined by ELISA, and viral RNA as determined by dot-blot hybridization were greatest.

Phloem companion and phloem parenchyma cells from plants 8 to 19 days after inoculation consistently contained large numbers of virus particles (Fig. 5) and scattered virus particles were present in the phloem sieve elements. On rare occasions we observed virus within xylem parenchyma cells.

The numbers of virus particles observed within cells and the proportion of cells examined that contained virus fell dramatically between 10 and 20 days after inoculation; after 20 days, virus particles could be observed in less than 10% of cells from either unexpanded leaves or roots. When leaf and root material from plants 15 and 20 days after inoculation was examined, most of the virus had disappeared from the nucleus and perinuclear space. Scattered particles were observed in the cytoplasm, sometimes in small membrane-bound inclusions (Fig. 6). Cells from plants infected for longer than 20 days only rarely contained virus, nearly always within the cytoplasm.

Terminal (unexpanded) leaves of flowering plants were examined 30 days after inoculation. These leaves showed symptoms of mottling and vein clearing. Sections showed up to 90% of cells containing virions, with large numbers of viral inclusions within the perinuclear space and
Fig. 6. Virus particles within cells from unexpanded leaves from plants 20 days post-inoculation. Virus particles within the cytoplasm are indicated by V. A cytoplasmic viral inclusion surrounded by a membrane is arrowed. M, mitochondrion; other abbreviations are as in the legend to Fig. 3. Bar marker represents 1 μm.

scattered through the cytoplasm. Various parts of the flowers (petals, stamens, anthers and filaments) also contained virus particles within the nuclei and cytoplasm. The numbers of virions observed decreased when corresponding sections from plants 50 days after inoculation were examined; the great majority of virions in these sections were observed in the cytoplasm. No virus was observed in pistils (stigma, ovary and style) either 30 or 50 days after inoculation.

DISCUSSION

Our ELISA assays indicate that movement of virus away from the initial site of inoculation is detectable after as little as 24 h. Although the levels of antigen detectable within roots and uninoculated leaves were low, they were consistently threefold above the background absorbances obtained with healthy sap. Synthesis of viral proteins can be detected between 9 and 13 h after inoculation of cowpea protoplasts (Van Beek et al., 1986); mature virus is assembled between about 12 and 56 h after inoculation (Van Beek et al., 1985a, b). If multiplication occurs at a similar rate in cells of N. edwardsonii, virus can start to spread systemically, presumably via the vascular system, after no more than one cycle of replication. Indeed it is possible that virus from the inoculum is transported directly through the vascular system. Özel (1973) has reported spread of sowthistle yellow vein virus to roots 2 days after inoculation by the aphid vectors. However he detected virus by electron microscopy and would not have seen low levels of virus occurring sooner after inoculation.

Rising levels of virus antigen within roots and unexpanded leaves between 1 and 4 days after inoculation suggest that virus multiplication occurs within these tissues. Within expanded
leaves, the lack of increase in levels over the same period may indicate that antigen detected after 24 h consists of virus circulating within the vascular system and that this virus multiplies less efficiently within expanded leaves. Using electron microscopy, however, we have not been able to see virus within the vascular tissues at this stage of infection. We are puzzled by our inability to detect virus in inoculated leaves for 4 days after inoculation since virus must a priori be present from the time of inoculation. It is possible that virus is restricted to a few initially infected cells and that our tissue samples did not contain these particular cells.

During the period between 5 and 10 days after inoculation, the virus appears to multiply and spread rapidly, as indicated by levels of virus antigen, viral RNA and the proportion of infected cells observed by electron microscopy. Neither ELISA nor the dot-blot assay indicate how much of the protein or RNA is encapsidated. The probes used to detect RNA would be expected to detect both genomic (minus sense) RNA and mRNA. It is nevertheless of interest to note that our estimates suggest that 10 days after inoculation, unexpanded leaves contain 200 times more viral protein than viral RNA by mass. The relative proportions found in the mature virion are approximately 140:1 (Jackson & Christie, 1977).

During the phase of rapid virus synthesis, virions and unenveloped nucleocapsids are present in large numbers. The slight differences in their sizes from those reported by Jackson & Christie (1977) presumably reflect differences in preparation techniques between tissue sections and negatively stained purified virus. We consistently observed nucleocapsids within viroplasms inside nuclei. Van Beck et al. (1985b) report the presence of a densely staining granular matrix within the nuclei of cowpea protoplasts infected with SYNV. This structure is presumably similar to the viroplasms which we observed in infected Nicotiana and both are presumably the sites of assembly of nucleocapsids.

After 10 days, nearly every cell examined contained virus. SYNV thus resembles the majority of other members of the rhabdovirus group in being able to infect a wide variety of cell types (Peters, 1981). The maximum levels of virus (approximately 2.0 to 7.3 μg/g depending on tissue) can be compared to yields of some of the positive-stranded viruses, e.g. 2 mg/g for tobacco mosaic virus and 0.7 mg/g for potato virus X (Bawden, 1964). The low yields of SYNV and presumably some other rhabdoviruses must therefore reflect the numbers of virus particles per cell rather than a failure of the virus to spread to the majority of cells.

Following the peak of virus concentration 10 days after inoculation, the levels of virus antigen and viral RNA fall dramatically in most parts of the plant. This fall is accompanied by changes in the location of most virus particles, from nucleus to cytoplasm, and a marked reduction in numbers of particles per cell. The considerable decrease in the amount of virus in nuclear or perinuclear inclusions suggests an active mechanism whereby virus is either degraded or released/transported into the cytoplasm.

Assembly of the type II rhabdoviruses is believed to occur in the nucleus (Francki & Randles, 1980). Although our observations, as well as previous reports (Jackson & Christie 1977; Van Beek et al., 1985b), demonstrate that this is the case for SYNV, we consistently observed virions scattered throughout the cytoplasm sometimes within small membrane-bound inclusions (Fig. 6). Similar observations have been made for other type II plant rhabdoviruses including wheat striate mosaic virus (Sinha, 1971) and clover enation mosaic virus (Vela & Rubio-Huertos, 1974). Van Beek et al. (1985b) detected nucleocapsids within the cytoplasm of SYNV-infected cowpea protoplasts; we also observed such species. The numbers of both enveloped and unenveloped particles within the cytoplasm increased between 10 and 20 days after inoculation presumably as a result of rupture or vesiculation of the outer nuclear envelope surrounding perinuclear inclusions of virus.

On the basis of these observations we can propose a model for the spread of SYNV through mechanically inoculated tobacco. Virus deposited at the infectible site(s) undergoes one or more cycles of replication. Progeny from these initially infected cells are transported, presumably via the vascular system, throughout the plant. Virus multiplies in all or nearly all tissues until all susceptible cells have been infected. This acute phase of infection takes 10 days. The plants then undergo a recovery phase which involves loss of virus from the perinuclear space of infected cells. The extent of this loss is such that we believe that it must involve active degradation of
virus and/or vesiculation or release of virus into the cytoplasm. Ultimately, the plants enter a phase of chronic infection in which the number of virus particles within most infected cells has decreased to a level at which virions are no longer detectable by electron microscopy. However, virus antigen remains present at low levels and extracts of such tissue remain infectious, indicating that at least some virus is present.

We should like to thank Ms R.-A. Millar for technical assistance and Mr N. Tait for help with the photography. We are indebted to Mr I. M. Roberts for his help in the interpretation of the electron micrographs. Part of the work was supported by S.E.R.C. grant GR/B/81090. Imad D. Ismail was supported by a scholarship from Tishreen University, Lattakia, Syria.

REFERENCES


(Received 26 March 1987)