Long-distance movement of cherry leaf roll virus in infected tobacco plants

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The long-distance movement of cherry leaf roll virus (CLRV) in tobacco plants was studied using a tissue printing technique with non-isotopic RNA probes. Time-course analysis revealed that CLRV RNA accumulated in the inoculated leaf at an early stage, such as 20 h post-inoculation. The virus accumulation reached a peak at 8–10 days post-inoculation (d.p.i.) and then progressively decreased. The virus RNA signal was detected before the appearance of symptoms. The virus invaded stem vascular tissues at 3 d.p.i., moving towards the roots before moving to the upper leaves. In systemically infected leaves, the virus appeared first in the basal regions and then moved to the distal parts through the vascular system. The distribution pattern of the virus coat protein in systemically infected leaves was parallel to that observed for the virus RNA, suggesting that CLRV requires the coat protein for long-distance movement. The movement of the virus was influenced by the phyllotactic position of the leaves. The viral symptoms and the virus RNA signal in older systemically infected leaves were asymmetrically distributed, being localized in the side of the lamina closest to the inoculated leaf. Virus distribution in infected plants as well as the susceptibility of the plant to systemic infection were also influenced by the developmental stage of the inoculated leaves. Inoculation of leaves at 95% of their final size resulted in virus replication but no systemic infection. In fully mature leaves the virus did not replicate.

Introduction

To systemically invade a plant, viruses must use two different forms of spread, short- and long-distance movement, in a compatible interaction. In short-distance movement, a virus moves from cell-to-cell through an active process in which the involvement of a specific virus-coded protein (movement protein; MP) is well documented (for reviews see Citovsky & Zambryski, 1991; Maule, 1991; Deom et al., 1992; Mushegian & Koonin, 1993). In long-distance movement, the virus uses the plant vascular system to invade the rest of the plant (Atabekov & Dorokhov, 1984; Hull, 1989; Matthews, 1991; Maule, 1991). In contrast to cell-to-cell movement, many factors are involved in long-distance movement; the requirements are different depending on the virus group. In addition to the MP, a functional coat protein (CP) is required for long-distance movement by some viruses belonging to the tobamo- (Takamatsu et al., 1987), bromo- (Allison et al., 1990; Flasinski et al., 1995), carmo- (Heaton et al., 1991), cucumo- (Suzuki et al., 1991; Talianisky & García-Arenal, 1995) and potex-virus (Chapman et al., 1992) genera. Assembled virions are required for efficient systemic transport of cowpea mosaic virus (CPMV; Wellink & van Kammen, 1989) and tobacco mosaic virus (TMV; Siegel et al., 1962; Dawson et al., 1988; Saito et al., 1990). In the case of tobacco etch potyvirus (TEV), the CP possesses distinct functions required for virion assembly, cell-to-cell movement and long-distance transport (Dolja et al., 1994). The dispensability of the CP for other viruses has been documented for tobra- (Harrison & Robinson, 1986), hordei- (Petty & Jackson, 1990), tombus- (Rochon et al., 1991; Dalmay et al., 1992; Scholthof et al., 1993) and dianthoviruses (depending on the environmental conditions for red clover necrotic mosaic virus; Xiong et al., 1993). In addition, it has been recently shown that long-distance movement can be regulated by sequences within the RNA 2 in the case of brome mosaic virus (BMV; Traynor et al., 1991) or within RNA 1 as described for cucumber mosaic virus (Gal-On et al., 1994), previously thought to be involved only in virus replication.

Less is known about how the plant influences the pattern of accumulation of viruses in their susceptible hosts. Classical experiments of Samuel (1934) showed the rapid long-distance movement of TMV in tomato plants.
Bennett (1940) demonstrated that virus spread is influenced by the flow of metabolites in the plant. Studies with tobacco ringspot virus (TRSV) led Schneider (1965) to suggest that the virus reaches the distant parts of the plant via the phloem transport system. More recently, in cauliflower mosaic virus (CaMV)-infected turnips, Leisner et al. (1992) showed that the pattern of long-distance movement was similar to the translocation of photo-assimilates, indicating that the virus is apparently swept along with the flow of photoassimilates from source leaves to sink leaves. In a later study, the same authors found that developmental changes had a dramatic impact on the long-distance movement of CaMV in Arabidopsis (Leisner et al., 1993).

CLRV belongs to the nepovirus group of plant viruses (Jones, 1985). Its genome is composed of two positive-sense polyadenylated ssRNAs which carry a genome-linked protein (VPg) at their 5’ ends (Hellen & Cooper, 1987). The walnut strain of CLRV (wCLRV) is consistently associated with the causal agent of the lethal disease of graft incompatibility in walnuts known as blackline (Mircetich et al., 1980).

This paper deals with the pattern of accumulation of CLRV in a systemic infection of tobacco plants and the influence of leaf ontogeny and the phyllotactic position of the leaves on susceptibility to virus infection.

Methods

**Virus and plant material.** The walnut isolate of CLRV was purified from frozen leaf tissue as described (Rowhani et al., 1985). Plants of *Nicotiana tabacum* cv. 'Xanthi nc' were grown in a growth chamber with a day length of 16 h, average day and night temperatures of 24 °C and 20 °C, respectively, and relative humidity of 80%. Plants were inoculated with 10 ng/ml of wCLRV by rubbing the leaves with *carborundum* as an abrasive. In time-course experiments and tissue print immunoblotting, two leaves per plant were inoculated, while for the study of the developmental and phyllotactic stage of the leaves on virus distribution, only one leaf per plant was inoculated.

**Tissue print RNA–RNA hybridization and non-radioactive RNA probe.** Tissue prints were performed basically as described by Song et al. (1993) and Más & Pallás (1995). The upper surfaces of the leaves were printed on a membrane using a roller and applying a uniform pressure, beginning from the tip of the leaf. The stem and petiole prints were prepared after sectioning with a razor blade and the surfaces were pressed manually to the membranes for 15-30 s. Nylon membranes (Hybond; Boehringer Mannheim) were used for RNA hybridization. After printing, the membranes were exposed for 3 min to the UV light of a transilluminator. Prehybridization was carried out for 2-3 h at 68 °C in 50% deionized formamide, 5 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate pH 7.0), 2% (w/v) blocking reagent (Boehringer Mannheim), 0.1% (w/v) N-lauroylsarcosine and 0.01% (w/v) SDS. Plasmid clone pClL2.16 was used to synthesize digoxigenin-labelled RNA probe using T7 RNA polymerase as described by Más et al. (1993). Hybridization was performed at 68 °C for 12 h. Membranes were washed after hybridization twice in 2 x SSC and 0.1% (w/v) SDS at room temperature for 5 min and twice in 0.1 x SSC and 0.1% (w/v) SDS at 68 °C for 15 min. The chemiluminescent detection of virus RNA was carried out as described (Más & Pallás, 1995).

**Tissue print immunoblotting.** The second systemically infected leaf and stems of both infected and mock-inoculated tobacco plants were processed at 5 days post-inoculation (d.p.i.; data not shown) and 10 d.p.i. for CP detection. The leaves and stems were printed, as described above, onto nitrocellulose membranes (Schleicher and Schuell). The membranes were washed at room temperature in 3% BSA in TBS (20 mM-Tris–HCl pH 7.5 and 150 mM-NaCl) for 20 min, 3% BSA and 0.1% Nonidet P-40 in TBS for 20 min and 3% BSA in TBS for 20 min. Primary antibody against wCLRV (kindly provided by A. Rowhani, University of California, Davis, Calif., USA) was diluted 1:2000 in 3% BSA in TBS. The primary antibody (2 µl) was pre-incubated with 0.5 µl of a healthy plant protein extract for 30 min on ice. The protein extract was made by homogenizing 1 g of tissue in 1 ml of TBS. After 2 h incubation at room temperature with the primary antibody the membranes were washed and then incubated for 2 h with anti-rabbit IgG alkaline phosphatase (Boehringer Mannheim) diluted 1:1000 in 3% BSA in TBS. Membranes were further washed and colorimetric substrate solution (NBT/BCIP; Boehringer Mannheim) was added. The membranes were incubated in substrate solution until a purple colour appeared (approximately 20-30 min).

**Time course analysis by dot-blot and tissue print RNA–RNA hybridization.** Inoculated and second systemically infected leaves (numbered 0 and 2 in Fig. 4, respectively) and stems and petioles of infected and mock-inoculated tobacco plants were harvested at different times post-inoculation and processed for tissue printing RNA–RNA and dot-blot hybridization. Dot-blot experiments were performed as previously described (Máis & Pallás, 1995). Briefly, total nucleic acids of the leaves, petioles and roots of infected plants were extracted by homogenizing the tissue in a buffer containing 100 mM-Tris–HCl pH 7.5, 100 mM-NaCl, 10 mM-EDTA, 5 mM-2-mercaptoethanol and 0.1% SDS. Nucleic acids were extracted with phenol–chloroform and precipitated from the extracted aqueous phase using ethanol. The samples were denatured with formaldehyde (White & Bancroft, 1982) and hybridized with the non-radioactive RNA probe as described above.

The appearance of typical ringspot symptoms in CLRV-infected tobacco plants was recorded until 15–20 d.p.i. in both inoculated and systemically infected leaves. At the same time, the viral RNA signal was detected by either tissue printing or dot-blot hybridization. The progress of systemic invasion was also recorded in experiments where the inoculated leaf was removed at successive d.p.i.

**Influence of developmental stage and phyllotactic position of leaves on virus distribution.** These studies were carried out in the inoculated and systemically infected leaves, analysed by dot-blot and tissue print RNA–RNA hybridization. The locations of the leaves in the plant are shown in Fig. 4. In the developmental stage experiments, only one leaf was inoculated. The size of the inoculated leaf as a percentage of the final size ranged from 30 % (plants with four fully expanded leaves) to 100% (plants with seven fully expanded leaves). The percentages were calculated by measuring the length of the inoculated leaf at the time of its inoculation with respect to its final size. The inoculated and systemically infected leaves were examined for the appearance of symptoms, recorded at 12–15 d.p.i. The viral RNA signals were determined by dot-blot experiments by extracting total nucleic acids of the apices, systemically infected leaves (other than the first one; leaves numbered 2 and 3 in Fig. 4), first systemically infected leaf (leaf numbered 1 in Fig. 4) and the inoculated leaf (leaf numbered 0) at 12–15 d.p.i. Hybridization and detection were performed as described above.

To study the influence of phyllotactic position of the leaves on virus infection, tobacco plants were inoculated only on a leaf at either 30% or 65% of its final size. The leaves were analysed by the tissue printing RNA–RNA hybridization technique at 10 d.p.i.
Results

Time course analysis of virus accumulation in tobacco leaves as judged by RNA–RNA tissue print hybridization

In inoculated and second systemically infected leaves, stems and petioles of tobacco plants inoculated with CLRV were excised and analysed by tissue printing RNA–RNA hybridization at several times post-inoculation (Fig. 1). In inoculated leaves, the virus RNA signal appeared at an early stage, such as 20 h post-inoculation, giving several infection foci distributed all around the leaf (Fig. 1a, i). By 4 d.p.i. the hybridization concentrated around the main and lateral veins (Fig. 1a, iii). The distribution pattern became more uniform over time, with the hybridization signal preferentially accumulating around the vascular system (Fig. 1a, iv–vii). Virus accumulation reached a peak at 8–10 d.p.i. and then progressively decreased. No signal was obtained when samples from healthy plants were hybridized (Fig. 1a, viii). Fig. 1(b) shows a macroscopic view of the CLRV movement in systemically infected leaves at different stages. The signal clearly first appears at the base of the leaf and then moves towards its tip along the main vein, until it reaches primary veins.

Time-course analysis of the virus movement through petioles and stems to invade the distal parts of the plant

In order to determine exactly when the virus is moving out of the inoculated leaf, tissue printing and dot-blot RNA–RNA hybridization of petioles of inoculated leaves and stems at various times post-inoculation (Fig. 2) shows a progressive increase in the virus RNA signal in the petiole and the first systemically infected leaf (Fig. 2a, i–ii). The signal was very weak in healthy plants (Fig. 2a, iii). The virus RNA signal first appears at the petiole base, then moves towards the leaf tip along the petiole main vein, until it reaches the first systemically infected leaf (Fig. 2b, i–iv).
leaves at 2, 3 and 4 d.p.i. were performed (Fig. 2a). A very weak signal was observed at 2 d.p.i. (Fig. 2a, tp 2). This signal was stronger at 3 d.p.i., mainly on the side closest to the leaf (Fig. 2a, tp 3). A more evident signal appeared at 4 d.p.i., being homogeneously distributed along the petiole (Fig. 2a, tp 4). The same results were obtained when dot-blot hybridization, a more sensitive technique, was used (Fig. 2a, column db), confirming that the virus exits the inoculated leaf at around 3 d.p.i. When petioles of systemically infected leaves were analysed at 6 and 8 d.p.i., using either tissue printing or dot-blot hybridization (Fig. 2a), a clear signal was obtained while no hybridization signal was detected at 4 d.p.i.

Longitudinal stem sections, covering approximately 2 cm up and down from the petiole insertion point of inoculated leaves, were analysed by tissue printing RNA–RNA hybridization (at 4–6 d.p.i. this size includes the whole stem). By 4 d.p.i. the hybridization signal appeared initially at the lower part of the section (Fig. 2b, 4). At 6 d.p.i. the virus was present at both the lower and the upper part of the stem section (Fig. 2b, 6) and at 8 d.p.i. the hybridization signal had considerably increased (Fig. 2b, 8). These results indicate that CLRV moved downwards to the root before going up to the apex. When we analysed roots and apices of infected plants at 2, 3 and 4 d.p.i by dot-blot hybridization (Fig. 2c, d), the signal firstly appeared in roots, suggesting that the virus accumulated there before invading the rest of the plant.

A summary of the time-course analysis of the relationship between the symptomatology, systemic infection and virus hybridization signal is shown in Table 1. The appearance of symptoms was delayed in relation to the virus signal. The typical ringspot symptoms were clearly seen at 2 d.p.i. and 6 d.p.i. on inoculated and systemically infected leaves, respectively. However, by tissue printing and dot-blot hybridization, the viral signal can be detected at 20 h p.i. on inoculated leaves and 5 d.p.i. on systemically infected ones.

As stated above, the virus exits the inoculated leaf at 2–3 d.p.i., since viral signal is detected in petioles at 3 d.p.i. (Fig. 2a). This result was confirmed in experiments where the inoculated leaf was detached at 1 day intervals from 1 to 15 days (Table 1). Those plants in which the inoculated leaf was detached at 1, 2 or 3 d.p.i. showed no signs of systemic infection.

Comparison between the spatial distribution of virus CP and virus RNA in infected tobacco plants

In order to study the accumulation pattern of virus CP in infected tissue and its relationship to virus RNA distribution, tissue prints in the second systemically

![Fig. 2. Localization of virus RNAs in petioles, stems, apices and roots. Petioles (a) from inoculated and the second systemically infected tobacco leaves were analysed by tissue-printing (tp) or dot-blot (db) hybridization at 2, 3 or 4 d.p.i. for inoculated leaves and 4, 6 or 8 d.p.i. for systemically infected leaves. Cross- and longitudinal sections of infected tobacco stems (b) were analysed by tissue-printing hybridization at 4, 6 and 8 d.p.i. The arrows on the right indicate the petiole insertion point of the inoculated leaf in the stem. Dot-blot hybridization of apices (c) and roots (d) of infected tobacco plants at 2, 3 and 4 d.p.i. were also performed. Cross- and longitudinal sections of petioles and stems are presented in duplicate. The petiole side closest to the leaf is indicated by the upper arrow in tp 3. The petiole insertion point to the stem is indicated by the lower arrow. Note that signal is more evident at this time in the side closest to the leaf.](image-url)
Table 1. Time-course of symptoms, systemic infection and virus replication signal in infected tobacco leaves

<table>
<thead>
<tr>
<th>Time (d.p.i.)</th>
<th>Inoculated leaves</th>
<th>Systemic leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viral signal†</td>
<td>Symptoms</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td></td>
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<tr>
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</tr>
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<td>5</td>
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</tr>
<tr>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* Systemic infection visualized at 10–15 d.p.i. after the inoculated leaf was removed.
† Viral signal was recorded by either tissue printing or dot-blot hybridization.

infected leaves and stems were performed. Fig. 3 shows the distribution pattern of virus RNA (b, f) and CP (d, h). The distribution pattern of CP in inoculated leaves was parallel to the signal obtained for the virus RNA. In inoculated leaves, the virus infection foci were uniformly distributed (data not shown). In systemically infected leaves, both the virus RNA (Fig. 3b) and the CP (Fig. 3d) accumulated along the veins and symptom areas. No signal was obtained when mock-inoculated plants were analysed (data not shown).

Longitudinal stem sections of infected and mock-inoculated plants were also hybridized against RNA probe (Fig. 3e, f) or incubated with CLRV antiserum (Fig. 3g, h). A very clear accumulation signal was observed whether RNA or CP was assayed.

* The pattern of virus infection depends on the phyllotactic position of the leaves

The distribution and appearance of symptoms and virus accumulation was studied in relation to the position of the inoculated leaf. Fig. 5 shows the inoculated and the first, second and third systemically infected leaves of a plant inoculated in a leaf at 65% of its final size. The spatial location of these leaves in the infected plant are shown in Fig. 4. The leaves were analysed by the tissue
printing RNA–RNA hybridization technique at 10 d.p.i. The symptoms and the virus RNA hybridization signal on the second and third systemically infected leaves were localized on the half-leaf closest to the inoculated leaf. As can be observed in leaf 2, the foci of hybridization were present in the first and second lateral veins and in a symptom area on the left side of the leaf. In leaf 3 some hybridization foci on the opposite side to the inoculated leaf can be observed, although the foci of hybridization of both secondary veins and symptom area are mainly concentrated on the side closest to the inoculated leaf. The asymmetrical distribution was more evident in older systemic leaves than in the upper and younger leaves (fourth and fifth systemic leaves) where the infection became more uniformly distributed (data not shown). No signal was observed in the first systemic leaf (leaf numbered 1 in Fig. 4) on the opposite side of the plant to the inoculated leaf.

Influence of developmental stage of leaves on the virus infection

In order to study the potential influence of the leaf developmental stage on its susceptibility to the systemic infection, dot-blot analysis of the different parts of the plant, within very narrow limits of plant ontogeny, were carried out.

Fig. 6 and Table 2 show the data obtained from plants
Table 2. Effect of the developmental stage of the inoculated leaf on the symptomatology and virus accumulation in infected tobacco plants

<table>
<thead>
<tr>
<th>Sample</th>
<th>30%</th>
<th>45%</th>
<th>65%</th>
<th>78%</th>
<th>90%</th>
<th>95%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S†</td>
<td>V†</td>
<td>S</td>
<td>V</td>
<td>S</td>
<td>V</td>
<td>S</td>
</tr>
<tr>
<td>Apex</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Systemic leaves§</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>First systemic leaf</td>
<td>+</td>
<td>+</td>
<td>ws</td>
<td>+</td>
<td>ws</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inoculated leaf</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The percentage size was calculated by measuring the length of the inoculated leaf at the time of its inoculation with respect to its final size.
† S, symptoms, recorded at 10–15 d.p.i.
‡ V, viral signal, determined by dot-blot hybridization (see Fig. 6) at 10 d.p.i.
§ Systemic leaves correspond to the second and third systemically infected leaves.
|| ws, Weak signals, as can be observed in Fig. 6 (row 'First systemic leaf').

assayed at different inoculated leaf stages. Dot-blot hybridization (Fig. 6) was performed in apices, systemically infected leaves (other than the first one), first systemic leaf and inoculated leaves, at different developmental stages. When very young leaves were inoculated at 30% of their mature length (plants having four fully expanded leaves) a general infection occurred in the whole plant (all the dots with a strong signal in Fig. 6, column 30%). This invasion decreased as the leaf became more mature. From 45–78% of the final length, a very weak signal was observed in the first systemic leaf (Fig. 6). The limit of systemic infection seems to be a stage where the inoculated leaf had a size of 90% of its final length. At 95% of the final size there was a clear hybridization signal in the inoculated leaf, while in the apex and systemically infected leaves no signal was observed, indicating that at this leaf developmental stage the virus loses its ability for systemic spread (Fig. 6). However, the virus was still able to replicate in the inoculated leaf (Fig. 6). When fully mature leaves were used, no signal was observed even in the inoculated leaf, indicating that the virus was unable to replicate (Fig. 6). These differences between the results at 95% and 100% of the final length of the inoculated leaves are based on replicated data, either in the number of experiments or in the number of plants analysed per experiment. With regard to the first systemic leaf, in all cases (except at 30% of the length of the mature inoculated leaf) no apparent symptoms were observed and a very weak signal was obtained when a dot-blot hybridization was performed (Fig. 6). This is probably due to its developmental stage at the time the virus reaches it, which results in faulty import of the virus into this leaf.

The relationship between the symptomatology and the viral signal can be observed in Table 2. When plants with leaves at 30% of their final length were inoculated, the symptoms closely corresponded with the positive viral signal (except in the apices, which did not show symptoms although the viral signal was positive). In the case of the plants inoculated in leaves at 45–100% of their final size, the relationship between the appearance of symptoms and the positive viral signal is maintained except in apices (where no symptoms were observed) and in the first systemic leaves (where a weak signal was obtained by dot-blot hybridization and no symptoms were observed).

Discussion

A macroscopic view of virus accumulation and pattern of translocation in whole plants was achieved by the application of the tissue printing technique. In conjunction with dot-blot hybridization and biological assays, this technique was used to follow the routes that a plant RNA virus, CLRV, used to move in a systemic infection, and to study the influence of the phyllotactic position of the leaves and their developmental stage on susceptibility to infection.

Time-course analysis revealed that the virus RNA signals were observed in the inoculated leaf as early as 20 h p.i. indicating rapid virus replication and cell-to-cell movement. Both the number and the diameter of the virus foci increased over time until 8 d.p.i. and then progressively decreased. From the inoculated leaves the virus moved out through the vascular system at 3 d.p.i. These conclusions are drawn not only from experiments where the inoculated leaf is detached at 1 day intervals but also by tissue printing and dot-blot analysis. Once CLRV invades stem vascular tissues, it moves first to the roots, accumulating there (Fig. 2), before invading the rest of the plant. This invasion route seems to be different to the one observed for viroids. Palukaitis (1987) has shown that potato spindle tuber viroid was always detectable first in the shoot tip and in leaves that were...
very close to the tip in inoculated tomato plants. No previous replication stage in the inoculated leaf seems to be necessary in this case for a systemic infection to occur, indicating that cell-to-cell and long-distance movement are very much easier events for viroids to achieve than for viruses. Thus, in the case of viruses, a minimum input of virus RNA would be necessary to afford an active process such as cell-to-cell movement and a critical level in the phloem may be necessary in establishing a systemic infection. The high virus concentration within roots suggests that virus multiplication occurs in this organ. Atchison & Francki (1972) have shown that virus replication takes place in the root tip cells of bean plants for only about 1 day following invasion of TRSV. Using the tissue printing technique in cross sections of stem and petiole, CLRV RNA has previously been detected in parenchymal and outer phloem tissues (Más & Pallás, 1995). This suggests that from the roots the virus moves, probably via phloem, to the systemic leaves. CLRV enters the non-inoculated tobacco leaves at 4–6 d.p.i. The pattern of virus distribution in systemically infected leaves is quite different to that in the inoculated ones. Very clear hybridization signals were observed within the major vein of the leaves, indicating that the virus probably replicates in vascular tissue. This is contrary to observations by Leisner et al. (1992) in turnip plants infected with CaMV and by Dolja et al. (1992) in tobacco plants infected with TEV where virus accumulation was significantly less in major than in minor veins and mesophyll cells. The foci of hybridization resulting from a non-vascular symptomatic area were only present when the lateral vein situated immediately downward had a viral signal, indicating that minor veins are the most frequent pathways for invasion of parenchyma (note that in the right part of leaf 3 in Fig. 5, where no symptom foci are present, there is no hybridization signal in the minor vein located downwards). This would be in agreement with the idea that the viral route of infection is similar to the photoassimilate flow since phloem unloading of imported photoassimilates occurs primarily from large minor veins in sink leaves (Ding et al., 1988).

The observation that the CP distribution pattern parallels the one observed for virus RNA in stem and leaf tissue suggests, although it does not demonstrate conclusively, that this virus needs CP for long-distance movement. Wellink & van Kammen (1989) have shown that virions are required for the efficient systemic transport of CPMV, the type member of a virus genus closely related to the genus Nepovirus, which forms part of the family Comoviridae. In addition, CLRV has been observed to form tubular structures in the cell walls of the differentiated leaf cells of Nicotiana clevelandii Gray (Jones et al., 1973) which would be in agreement with the idea that CLRV moves as virions to distant parts of the plant.

The virus infection is influenced by leaf developmental stage, in accordance with the sink to source leaf transition. If CLRV moves via phloem with photoassimilates, sink leaves (that import photoassimilates) will be easily infected, while source leaves (exporting photoassimilates) will not become infected. Using a different system, Leisner et al. (1992) showed that only those parts of the plant into which photoassimilates flow are accessible to CaMV during systemic infection. In that sense, the analysis of the developmental stage of infected plants confirms this hypothesis (Fig. 6). At 4–6 d.p.i., the virus probably reaches the first systemic leaf when the net photosynthetic flow is directed out of this leaf, so the virus is unable to enter and replicate (Fig. 6). Only when the virus reaches the first systemic leaf at a very young stage (i.e. when the inoculated leaf is only 30% of its final size) will this leaf become infected (Fig. 6). This could be the reason why in Nicotiana benthamiana plants infected with BMV, the one or two lowest uninoculated leaves consistently escaped virus infection (Mise et al., 1993). When leaves were inoculated at 30–90% of their final size, the other systemic leaves became infected, although the plants became less susceptible to infection (Fig. 6). However, with inoculated leaves at 95% of final size, the virus is unable to spread systemically. In mature leaves (100% of final size), CLRV is apparently unable to replicate. However, the possibility that the virus causes a subliminal infection in the fully mature leaves cannot be excluded even though tissue print analysis of these leaves showed no signal at all (data not shown). Our results are in agreement with previous observations that showed that as soybean plants age, the chance of their becoming systemically infected with tobacco necrosis virus decreases greatly (reviewed by Schneider, 1965). The observed pattern of movement through the different plant developmental stages is typical of the movement of metabolites in the phloem. The residual virus signal in the first systemic leaf (Fig. 6) may be due to the cell-to-cell movement through non-phloem cells associated with the vasculature. In fact, the virus accumulated not only in phloem tissue in stem cross-sections but also in the parenchymatous tissue (Más & Pallás, 1995). This relatively abundant accumulation in non-phloem cells of the stem tissue was also observed by Wisniewski et al. (1990) in a stem cross-section of tobacco plants infected with TMV. Moreover, the final distribution of the virus in the infected plants is determined by the phyllotactic position of the leaves; foci were mainly concentrated at the side closest to the inoculated leaf. This phyllotactic influence is preferentially observed in the leaves situated immediately upward of the inoculated leaf but only when the virus reaches those leaves in a sink stage. This viral
distribution pattern may be explained if the virus is transported with the net photosynthetic flow, directed from the source to the closest side of the sink leaves. The upper systemically infected leaves are not influenced by their phyllotactic position. The various observations given above indicate that the accumulation and translocation of a RNA virus are influenced by the phyllotactic position of the leaves and plant developmental stage.

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