Analysis of the Local and Systemic Movement of Two Isolates of Red Clover Necrotic Mosaic Virus

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L ocal and systemic spread of red clover necrotic mosaic virus (RCNMV)-TpM34 isolate and RCNMV-H isolate were measured quantitatively using Enzyme-Linked Immunosorbent Assay (ELISA) and RNA dot-blot hybridization. RCNMV-TpM34 and RCNMV-H isolates differed in the amount of cell-to-cell movement they are capable of within the inoculated leaves of cowpea plants. The amount of virus coat protein, however, is similar in the inoculated and systemic leaves of *Nicotiana clevelandii*. ELISA results were confirmed by RNA dot blot hybridization.

Key words: ELISA, necrotic mosaic virus, RCNMV, red clover and RNA dot-blot hybridization.

Systemic infection is dependent upon the virus spreading to most, if not all, tissues of the susceptible host. It is generally recognized that plant viruses enter the plant cells through mechanical means, either by damage inflicted by a biological vector (e.g. arthropod) or by abrasion (e.g. breaking of leaf hair). From the initially affected cells the infection proceeds in two ways, namely slow cell-to-cell (short-distance) and rapid migration via vascular tissue (long-distance). However, for the systemic spread of many viruses at least four transport steps must be overcome: initial cell-to-cell transport within the epidermis and parenchyma of the infected leaf (short distance); transfer of the viral genome from the parenchyma to the vascular tissue (transition from short to long distance); transport through the vascular tissue (long distance); and finally the migration from the vascular tissue back to the parenchyma cells with subsequent cell- to- cell spread into the secondarily infected parenchyma tissue (long to short distance) (Atabekov and Taliansky, 1990). For other viruses, spread may be limited to the vascular tissue, usually the phloem, where they are introduced.

Originally, systemic spread of the viral infection was thought to be genetically passive i.e., not virally encoded; accumulated material was believed to migrate passively to the surrounding healthy cells via plasmodesmata. This, however, would theoretically mean that all plant viruses are capable of invading any plant tissue; this of course is not the case, a host-virus interaction had to be occurring.

ELISA was used to measure quantitatively the accumulation of virus coat protein in inoculated and non-inoculated leaves. The presence of viral RNA was shown qualitatively by RNA dot-blot hybridization.

ELISA is a useful method for the detection of plant viruses due to its specificity and speed (Clark and Adams, 1977). This assay is also very sensitive, for instance

CMV can be detected in single viruliferous aphids (Gera *et al.*, 1978). Typically ELISA can be used to detect concentrations in the range of 1-10 ng/ml. It is also convenient for examining large numbers of samples. Moreover, ELISA is a heterogeneous immunoassay, *i.e.* it involves steps during which reacting and non-reacting components can be separated.

The use of ELISA for the detection of plant viruses is well documented (Clark and Adams, 1977). The double antibody sandwich is more commonly used but has the disadvantage that the immunoglobin of each test serum must be purified and coupled to enzyme. Indirect ELISA uses an enzyme-labeled anti-Ig as a second antibody to detect the antigen –antibody complex on the solid phase. This avoids the necessity of making specific enzyme conjugates for each antigen to be tested. Indirect ELISA has been used in the detection of plant viruses (Lommel and Morris, 1982) who found that the indirect ELISA was more sensitive than sandwich ELISA for detecting virus (carnation ring-spot dianthovirus and carnation mottle virus) in crude plant extracts. Therefore the choice of ELISA system used in this work was the indirect assay.

Materials and Methods

1-Propagation and isolation of virus:
a- Propagation of virus:
Two isolates of RCNMV namely, TpM-34 (Musil, 1969) and the English H strain (Hollings and Stone, 1977), were originally obtained from the Crops Research Institute, Littlehampton, U.K. and were purified by three cycles of single lesion isolation on leaves of *Chenopodium quinoa* (Osman *et al.*, 1986). Sap containing the virus was stored at -20°C and was used as inoculum.

RCNMV was propagated in *Phaseolus vulgaris* (cv. Prince). Primary leaves of 10 days old plants were inoculated by first sprinkling on carborundum powder, and then rubbing on infected sap, Excess sap carborundum were rinsed off by spraying with water. The infected plants were incubated at 17°C in a Fitotron 600H growth cabinet, with 18h per day of illumination at intensity of 5 klux (Hollings and Stone, 1977). Leaves were harvested 4-7 days after inoculation, and were picked at the end of a dark period to give lower starch content. The leaves were sometimes stored at -70°C before use.

b- Extraction of virus:
Typically 200g of *P. vulgaris* leaves were processed. The virus was extracted and purified by sucrose density gradient and differential centrifugation (Gould *et al.*, 1981). Leaves (200 g) were homogenized in a Kenwood blender with ml 1x SE and 0.4 ml 2-mercaptoethanol. The homogenate was squeezed through two layers of muslin. To the liquid, 0.5 volume chloroform and 0.5 volume butanol were added and mixed thoroughly. The mixture was separated by centrifugation at 10.000 rpm for 10 min. The aqueous phase was collected and PEG-6000 and NaCl were added to 10% and 15% w/v, respectively. This mixture was then stirred at 4°C for 1.5 h. precipitated virus was collected by centrifugation at 10.000 rpm for 20 min (6x 300 ml rotor) to give two pellets per 200g starting material.

Each pellet was resuspended in 5 ml 1x SE, and after centrifuging for 10 min, the liquid was layered onto a 10-50% w/v sucrose gradient, which was made by freezing 38 ml 25% w/v sucrose in 1x SE in a Beckman SW28 tube at $-70°C$, and thawing at room temperature. The gradient was centrifuged in a Beckman SW28 rotor at 27.000 rpm (82-740g) at $10°C$ for 1.75hr. The gradients were illuminated from above with white light, and the bluish-white opalescent virus band approximately one-third of the way down the tube was collected with a sterile Pasteur pipette. The virus-containing sucrose was diluted 3 to 8-fold with 1x SE, and the virus was pelleted in a Beckman Ti 80 rotor at 50.000 rpm (183 960 g), for 1 hr at $4°C$.

c- Extraction of viral RNA:

RNA was isolated from virus particles by phenol/SDS extraction (Okuno *et al.*, 1983). Virus pellets were resuspended in 500 μl 25 mM Tris-HCl, pH 7.7, 25 mM NaCl, 2.5 mM EDTA, and after the addition of 50μl of 10% w/v SDS, the suspension was incubated at $60°C$ for 10 min. The samples were then extracted three times with phenol and twice with sevag, then 0.1 volume 3M sodium acetate, pH 5.2 and 2.5 volumes of ethanol were added. The RNA was pelleted (Microfuge 10 min), washed twice in 70% ethanol and redissolved in sterile water. The yield was determined spectrophotometrically.

2- Extraction of RNA from plant tissue:
a- Total RNA extraction for dot-blotting:

Two hundred milligrams of leaf tissue were ground to a powder under liquid nitrogen after which 200 μl homogenization buffer (0.2M Tris-HCl pH 8.5, 0.2 M sucrose RNase free, 30 mM magnesium acetate, 60 mM KCl) and SDS to 0.5% were added. The mixture was extracted twice with phenol/sevag and then again sevag until no protein interface remained. The nucleic acids were precipitated using 0.05 vol. 3 M sodium acetate and 2 vol. ethanol. The sample was centrifuged for 15 min and the pellet resuspended in 100ul of 3 M sodium acetate (to remove ethanol and DNA), incubated on ice for 30 min and then centrifuged for 15 min at $4°C$. The RNA pellet was washed with 70% v/v ethanol and resuspended in 100μl RNase-free water then phenol/sevag extracted and ethanol precipitated. The resulting pellet was dried and resuspended in 20μl RNase-free water.

b. Inoculation of plants with viral RNA:

Cowpea and *N. clevelandii* plants were inoculated with 0.5μg per leaf of total virus RNA of RCNMV-TpM34 or RCNMV-H isolates. Inoculated and systemic leaves of plants with TpM34 and H isolates were assayed over several days and the accumulation of virus coat protein was measured. The concentration of virus was estimated from standard curve made from purified TpM-34 and H isolates virus particles at concentration of 2μg/ml serially diluted to 8 ng/ml and the OD_{405} nm.

3- ELISA for the detection of plant viruses:

An ELISA starter kit (Pierce) was used to examine presence of the tested isolates. The wells were coated with 100μl of antigen solution and the plates were incubated for 1hr at room temperature, then rinsed with 3x 100 μl of wash buffer, tapping the plate empty between each rinse. The wells were than filled with 100 μl of blocking buffer and the plates were incubated at room temperature for 1hr, then

emptied. A 100 µl of primary antibody (antibody to RCNMV-TpM34 coat protein was raised by Osman *et al.*, 1986, and antibody RCNMV-H coat protein was a gift from the Crop Research Institute) was added to each well and incubated at room temperature for 1hr, then washed with 3x 100 µl of wash buffer. A 100 µl of secondary antibody was added to each well. The plates were incubated for 20 hr at room temperature, then rinsed with 3x 100 µl wash buffer. The wells were finally filled with 100 µl of wash buffer and incubated for 5 min, then emptied. A 100 µl of substrate solution was added to each well and incubated at room temperature for 30 min and 50 µl of stop solution (2M NaOH) was added and the absorbance of the reagents measured at 405 nm using an automatic plate reader.

4- Hybridization analysis of nucleic acids:
a- Preparation of random oligodeoxynucleotide primers:
    Random primers were prepared according to Taylor *et al*, (1976). Salmon sperm DNA (Sigma) was dissolved to 5 mg/ml in 10mM Tris-HCL, pH 7.5, 10mM $MgCl_2$, and containing 70 µg/ml DNase-1 (Sigma). After incubation for 2h at 37°C, the DNA was autoclaved for 20 min. The resultant solution was used as a 5mg/ml stock of random primers.

b- cDNA probes:
    In a sterile tube 1-2 µg RNA in 3µl DEPC-treated water was heated to 70°C for 5 min (or 65°C for 15 min) then chilled on ice. To the RNA, the following were added: 3µl inhibitor, 25µl 25mg/ml synthetic hexanucleotides, 5 µl 10x random primer buffer (0.5 M Tris-HCl pH 8.3; 80 mM DTT, 80 mM $MgCl_2$, 0.7M KCl), 2 µl 20 mM dNTPs (bar dCTP), 5 µl (α-^{32}P) dCTP (10 µCi/µl), 10 U AMV-reverse transcriptase and water to 50µl. The reaction mixture was spun down in a microfuge, and then incubated at 42°C for 1hr. The reaction was terminated by adding 1µl 0.5 M EDTA and the RNA was hydrolyzed by the addition of 5µl 1M NaOH and incubation at 68°C for 30 min. The mixture was then neutralized with 5µl KCl and the total volume was brought to 90 µl. To assess incorporation, 1µl of the probe was spotted onto each of two DE-81 discs (Whatman), and one was set aside (to show total radioactive input). The other disc was washed 6x, for 6 min each, in 0.5 M Na_2HPO_4 (20 ml/disc), then briefly in two changes of water, followed by two changes of ethanol. The discs were allowed to dry and radioactivity was monitored by a Geiger counter.

d. RNA dot-blot hybridization:
    RNA was dotted onto Hybond-N paper using a vacuum dot blotter (BioRad) and allowed to dry, then alkali-fixed by soaking the membrane in 0.05 M NaOH for 20 min. The membrane was then rinsed in 2x SSC and then prehybridised in 5x SSC, 5x Denhardt's solution, 0.5% SDS and 1mg/ml denatured salmon sperm DNA for at least 2hr at 65°C. A radiolabelled probe was added and the reaction was allowed to hybridise for 12 hr, after which the filters were washed twice at room temperature in 2xSSC, 0.1%, twice for 20 min at 65°C in 1x SSC, 0.1% SDS and once for 20 min at 65°C in 0.1% SSC, 0.1% SDS. An X-ray film was added for as long as required. 100x Denhardt's solution contains 2% w/v BSA, 2% Ficoll, 2% polyvinylpyrrolidone.

Results

Figures 1 and 3 clear that no virus was found in non-inoculated leaves of cowpea infected with RCNMV-H isolate, while RCNMV-TpM34 isolate was capable of invading cowpea systemically and peaks at day 25 post inoculation. Virus accumulation in inoculated leaves of cowpea peaks at day 7, after which virus concentration slowly decreases. It is clear that the amount of virus found in the inoculated leaves varies according to which virus isolate used. RCNMV-TpM34 was first detected on day 3, but RCNMV-H isolate could only be detected on day 5. It is clear that the two viruses all differ in the amount of cell-to-cell movement they are capable of within the inoculated leaves of cowpea. This could reflect differences in the strength of the movement proteins or differences in the host Hypersensitive Response (HR).

RCNMV-TPM34/Cowpea/virus coat protein (ng/ml)

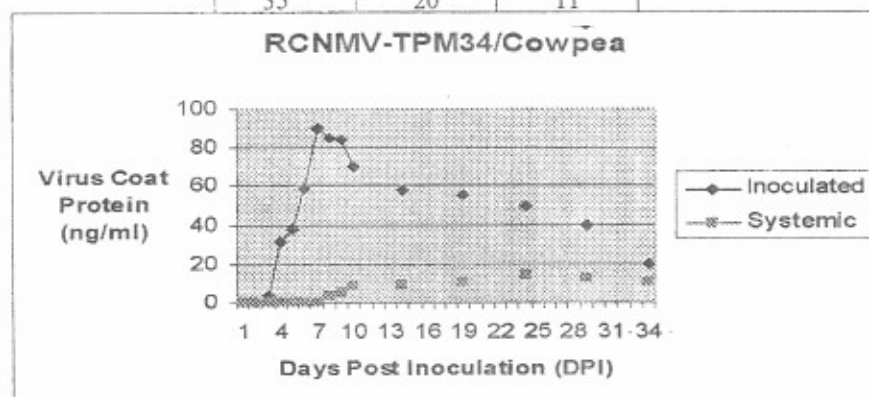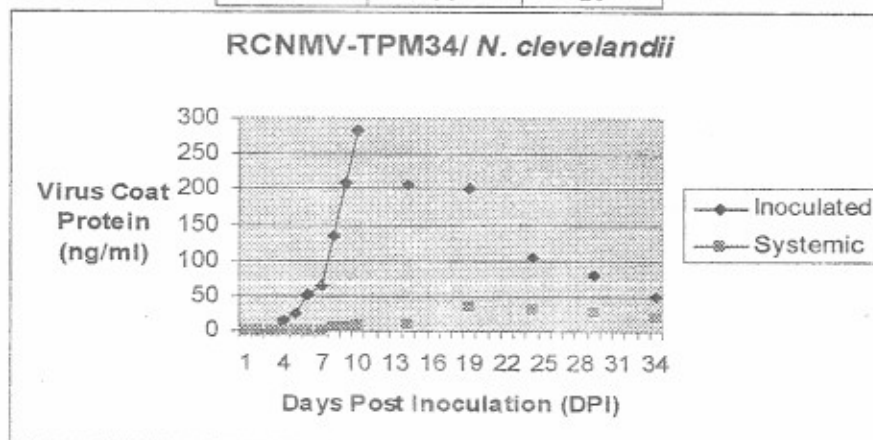| DPI	Inoculated	Systemic
1	0	0
2	0	0
3	3	0
4	31	0
5	38	0
6	59	0
7	90	0
8	85	3
9	84	5
10	70	8
15	58	9
20	55	11
25	50	14
30	40	12
35	20	11

Fig. 1. Virus coat protein accumulation in cowpea.

RCNMV-TPM34/ *N. clevelandii*/ virus coat protein (ng/ml)

DPI	Inoculated	Systemic
1	0	0
2	0	0
3	0	0
4	15	0
5	24	0
6	52	0
7	64	0
8	134	4
9	208	5
10	283	8
15	205	11
20	200	35
25	105	30
30	80	27
35	50	20

Fig. 2. Virus coat protein accumulation in *N. clevelandii.*

It is clear from Figures 1 and 2, that both RCNMV-TpM34 and RCNMV-H isolates capable of invading *N. clevelandii* systemically. Systemic infection was first detected on day 8, and peaks on day 20 in the case of RCNMV-TpM34, while it peaks on day 25 in the case of RCNMV-H and was first detected on day 10. It is also clear that the amount of virus found in the inoculated leaves varies according to virus isolate. RCNMV-TpM34 and RCNMV-H were first detected in inoculated leaves of *N. clevelandii* on day 4, but RCNMV-TpM34 peaks on day 10, while RCNMV-H peaks on day 20 after which, virus concentration slowly decreases.

RCNMV-H/Cowpea/ virus coat protein (ng/ml)

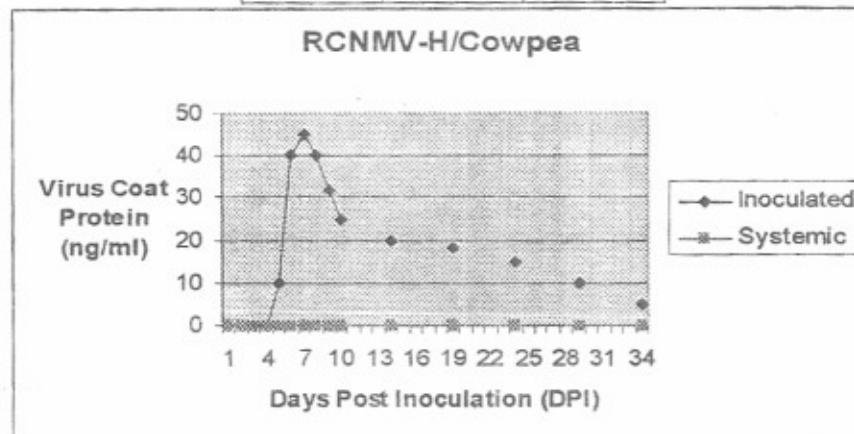| DPI	Inoculated	Systemic
1	0	0
2	0	0
3	0	0
4	0	0
5	10	0
6	40	0
7	45	0
8	40	0
9	32	0
10	25	0
15	20	0
20	18	0
25	15	0
30	10	0
35	5	0

RCNMV-H/Cowpea

Fig. 3. Virus coat protein accumulation in cowpea.

The presence of virus RNA in infected and non-inoculated leaves was examined using dot-blot hybridization. Inoculated leaves were analyzed at 5 days post inoculation and non-inoculated leaves at 20 days post inoculation. RNA was extracted from infected leaves as described before. Spots were applied to a membrane and then hybridized with ^{32}P-labelled first-strand cDNA probe of RNA1 and RNA2 extracted from purified RCNMV-TpM34 virus particles (Fig. 4). The results in Fig. 5 show that virus RNA was present in systemic leaves of cowpea and *N. clevelandii* when RCNMV-TpM34 was used as a source of inoculum but when RCNMV-H isolate was used, virus RNA was present in the systemic leaves of *N. clevelandii* but not in cowpea as seen in ELISA analysis.

RCNMV-H/ *N. clevelandii*/ virus coat protein (ng/ml)

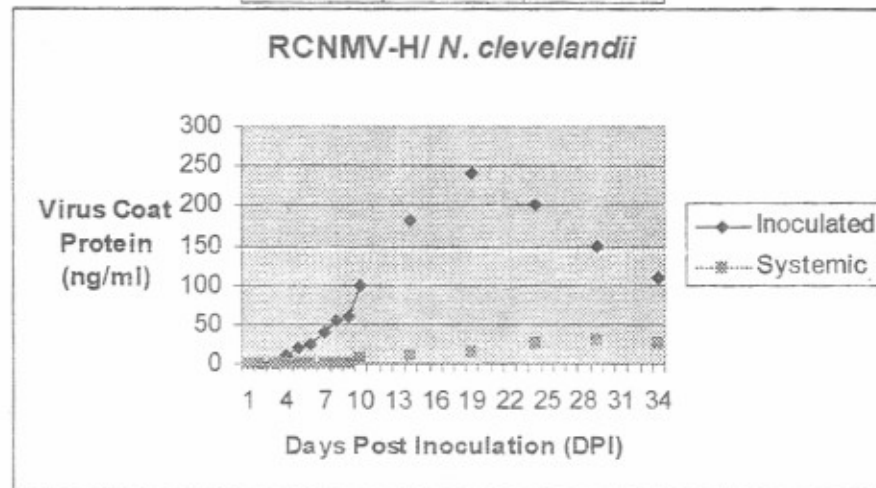| DPI	Inoculated	Systemic
1	0	0
2	0	0
3	0	0
4	9	0
5	20	0
6	25	0
7	40	0
8	55	0
9	60	0
10	100	8
15	180	11
20	240	14
25	200	25
30	150	30
35	110	25

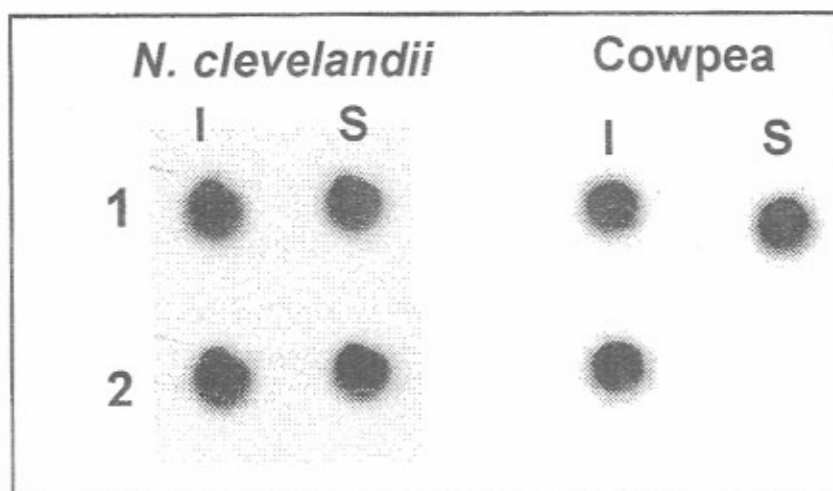Fig. 4. Virus coat protein accumulation in *N. clevelandii*.

Fig. 5. Detection of RCNMV RNA in local and systemic leaves of plants. Whereas: I= inoculated; S= systemic; 1= RCNMV-TpM34 and 2= RCNMVH.

Discussion

The initial viral infection of plants is limited to a small number of infected cells; to systemically invade a plant, where the virus requires a means of moving between cells. The plant cell wall prevents virus entry by fusion with membranes or by endocytosis, leaving the intercellular connections, the plasmodesmata, as the only feasible route. However, the size-exclusion limit (SEL) of plasmodesmal channels is far below that of virus particles or even folded viral nucleic acid (Wolf et al., 1989). Transport through the palsmodesmata is therefore likely to be an active process, mediated by specific movement proteins. Indeed, it has been shown that movement proteins are capable of increasing SEL (Ding et al., 1992). The movement of the ribonucleoprotein complex through the palsmodesmata may represent the major cell-to-cell transport mechanism of plant viruses (Citovsky et al., 1990 and 1992 and Osman et al., 1992). The formation of necrotic lesions following infection with some plant viruses is part of the Hypersensitive Response (HR). It has been shown that lesion morphology on cowpea is dependent on RNA2 (Osman et al., 1986). The fact that RCNMV-H isolate produces different lesion type compared to RCNMV-TpM34 and does not support a systemic infection in cowpea but does in N. clevelandii may be due RCNMV-H movement protein (P2) induces a stronger host response, which could limit the spread of virus (Ponz and Bruening, 1986) or may be P2 is a weaker movement protein than that of RCNMV-TpM34.

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تحليل للإصابة المحلية والجهازية في نوعين من
النباتات وذلك بعد الإصابة بعزلتين مختلفتين
لفيروس تبرقش البرسيم الأحمر
توبة أبو السعود محمد عثمان
قسم النبات الزراعي كلية الزراعة بالفيوم – جامعة القاهرة.

تم استخدام تقنية الـ ELISA كمقياس كمي للإصابات المحلية والجهازية
في نباتي اللوبيا والدخان N. clevelandii   وذلك بعد الإصابة بعزلتين
مختلفتين لفيروس تبرقش البرسيم الأحمر.

اختلفت حركة فيرس البرسيم الأحمر من خلية إلى أخرى وذلك تبعاً
لعزلة الفيروس المستخدمة في العدوي وذلك في حالة الإصابة المحلية لأوراق
اللوبيا ولكن كان هناك تشابه في كمية الغطاء البروتيني للفيروس في حالة
الإصابة المحلية والجهازية في أوراق الدخان.

أكـدت النتائج التــي تــم الحصـول عليهـا باستخـدام تقتيـة
Dot blot hybridization النتائج السابقة.