Characterization of a Tombusvirus Isolated from Eggplant

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ABSTRACT

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A virus that produced mottling and malformation of leaves, together with stunting of growth, isolated from eggplant (*Solanum melongena*) growing in the Jiyeh area south of Beirut, Lebanon. Results of morphological, serological, and biophysical analyses, as well as observation of cytopathic effects, enabled identification of the virus as a tombusvirus that is provisionally named eggplant mottled crinkle virus. It is differentiated from other tombusviruses by distinctive serological and electrophoretic properties and host range. The double antibody sandwich form of ELISA, but not the Derrick and decoration immunoelectron microscopy methods, had a rather narrow specificity with tombusviruses.

Eggplant is not a major crop in Lebanon, but it is grown inland and along the coast for local consumption. In a survey on virus diseases carried out earlier (21), two viruses, cucumber mosaic virus and tobacco mosaic virus (TMV), were identified as affecting eggplant.

During the fall of 1978, a new disease was observed on eggplant in the Jiyeh area south of Beirut. Infected plants showed mottling and malformation of leaves, severe stunting, and a poor fruit set. We report here that the causal agent was a tombusvirus provisionally named eggplant mottled crinkle virus (EMCV).

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MATERIALS AND METHODS

Symptomatology and host range. All test plants were mechanically inoculated with a fresh leaf tissue extract from infected *Physalis floridana* Rydb. or eggplant *Solanum melongena* L. 'Black Beauty.' The isolate had been passaged twice through

single lesions on leaves of *Phaseolus vulgaris* L. 'Romano.' Inoculum was prepared by homogenizing infected tissue with 0.01 M phosphate buffer, pH 7.2, containing 2.5% Celite.

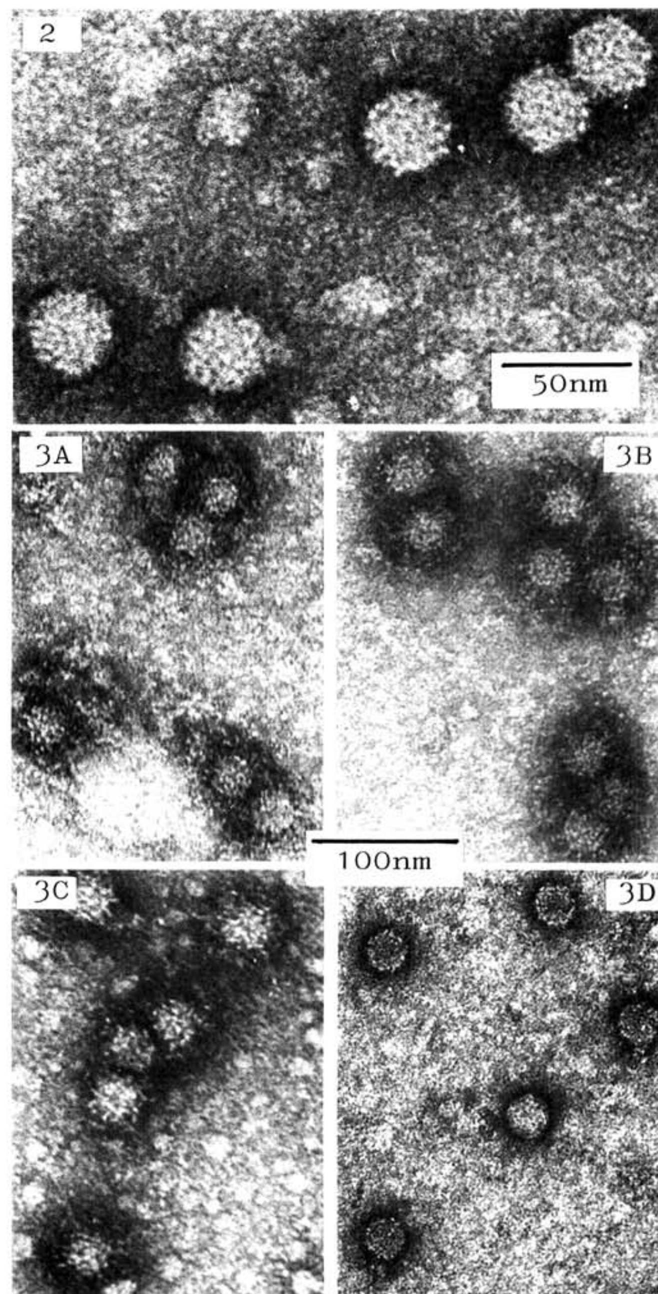
Virus purification. The virus was purified essentially by Steere's method (26) with minor modifications. Infected leaves of *Nicotiana clevelandii* Gray were homogenized in a blender with 0.1 M phosphate buffer, pH 7.2 (4 ml/g), containing 0.05 M sodium sulfite. The homogenate was clarified by adding *n*-butanol-chloroform (1:1) to 25% of the original volume and stirring the mixture for 30 min. The virus was then concentrated by two cycles of differential centrifugation and further purified by sucrose density gradient centrifugation.

Acrylamide gel electrophoresis. Procedures to determine the molecular weight of viral protein by using SDS polyacrylamide gel electrophoresis were followed as described previously (12).

Serology. Procedures for immunizing rabbits, conducting agar gel double diffusion tests, enzyme-linked immunosorbent assay (ELISA), and immunoelectrophoresis were as described before (9,10). Comparative serological tests were done with petunia asteroid mosaic virus (PAMV) (13), pelargonium leaf curl virus (PLCV) (our own isolate), the BS-3 (3) strain of tomato bushy stunt virus (TBSV) and a pepper isolate of TBSV from Morocco (MPV) (5).

Electron microscopy. For negatively stained preparations, a virus suspension was applied to carbon-coated or carbon-formvar-coated copper grids for a few seconds. The grids were then washed with 40 drops of distilled water and stained with a few drops of uranyl acetate. The decoration (20) and the Derrick (4) methods of immunoelectron microscopy (IEM) were done as described (4,19,20) or with a modification using protein A (24). Leaves of *N. clevelandii* infected with EMCV were embedded in Epon, cut, and stained as described recently (25). A Siemens Elmiskop 1A electron microscope operated at 80 kV was used.

Fig. 1. Symptoms produced by the eggplant mottled crinkle virus on leaves of eggplant cultivar Black Beauty showing mottling (upper left) and malformation (upper right) of leaves. Healthy leaf below.



Figs. 2 and 3. Eggplant mottled crinkle virus (EMCV) from crude sap of *Nicotiana clevelandii* negatively stained with uranyl acetate: **2,** particles without antiserum treatment; **3,** immunoelectron microscopial decoration test with particles of EMCV and antisera to: **A,** strain EMCV; **B,** pelargonium leaf curl virus, **C,** tomato bushy stunt virus BS-3, and **D,** with normal serum.

RESULTS

Host range and symptoms. The following test plants produced only local lesions after mechanical inoculation with EMCV: *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Cucumis sativus* L. 'Beit Alpha' (cotyledons), a local variety of *Cucurbita pepo* L. (cotyledons), *Datura stramonium* L., *Gomphrena globosa* L., *Nicotiana glutinosa* L., *N. tabacum* L. 'White Burley,' *Phaseolus vulgaris* 'Bountiful' and 'Romano,' and *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Black Eye No. 5.' Local, as well as systemic, symptoms were produced in *N. clevelandii*, *Physalis floridana*, and *Solanum melongena* 'Black Beauty.' *G. globosa* was a highly sensitive local lesion host, and lesions consistently were produced in 2 days. Systemic symptoms on eggplant were severe mottling and crinkling of leaves and stunting of growth. Leaf blades were markedly reduced in size (Fig. 1).

Particle morphology. Crude sap from EMCV-affected *N. clevelandii* and purified preparations of the virus contained isometric, ~28–30 nm diameter particles, which often had a hexagonal outline (Fig. 2). The particle surface showed indistinct morphological subunits, but the available material allowed no clear observation of details of particle fine structure.

Biophysical properties of purified virus preparations. The UV absorption spectrum of the purified virus was typical of tombusviruses with a minimum at 243 nm and a maximum at 260 nm. The A_{260}:A_{280} ratio (average of four separate determinations) was 1.64.

One light-scattering band was observed when virus preparations were centrifuged through a linear-log sucrose density gradient column (2), in a Sorvall AH rotor at 135,200 *g* for 75 min. An estimation of the sedimentation coefficient of EMCV was made by using TMV and bromegrass mosaic virus (BMV) as standards, and it gave an average value of 132 S.

In SDS-polyacrylamide electrophoresis a single protein band was obtained for which a molecular weight of about 41,000 was calculated.

All of the above determinations were in agreement with values reported for other tombusviruses (15).

Serology. The virus cross-reacted with several tombusviruses in agar gel double diffusion tests (Table 1); however, it was not identical to any of them as indicated by spur formation and the serum titer comparisons. In ELISA the cross-reactivity was much lower (Table 2). In the homologous reaction with EMCV, this test was about 5,000 times more sensitive than the agar gel double diffusion test. Heterologous reactions, however, were only barely or not detectable with the EMCV antiserum. The PLCV antiserum gave a definite heterologous reaction only with MPV, but not with EMCV (Table 2) even though its titer with EMCV was only one twofold dilution less than the homologous titer and equal to that with MPV (Table 1).

In the IEM decoration test, EMCV reacted strongly with the homologous antiserum and also with the antisera to PLCV and TBSV BS-3 (Fig. 3A–C). By using the Derrick method as described by Milne and Lesemann (19) with EMCV, we observed a nonspecific trapping of high numbers of particles on normal serum-coated grids. Accordingly, the degree of specific binding by the

TABLE 1. Homologous and heterologous titers[a] of antisera to tombusviruses in the agar gel double diffusion test

	Antigens[b]				
Antiserum to	EMCV	MPV	PLCV	TBSV BS-3	PAMV
EMCV	256	16	16	8	<1
PLCV	128	128	256	32	64
TBSV BS-3	64	128	256	512	128
PAMV	8	32	256	64	512

[a] Reciprocal values of titers.
[b] Crude sap preparations of the eggplant mottled crinkle virus (EMCV), the Moroccan pepper virus (MPV), the pelargonium leaf curl virus (PLCV), the BS-3 strain of tomato bushy stunt virus (TBSV), and the petunia mosaic virus (PAMV) adjusted to a titer of 1:16.

TABLE 2. Absorbencies (A_{405}) in ELISA with tombusviruses[a] in homologous and heterologous reactions

	Antigens[b]					
Antiserum to	EMCV	MPV	PLCV	TBSV BS-3	PAMV	Buffer control
EMCV	>2	0.03	0.06	0.02	0.01	0.01
PLCV	0.02	0.11	0.57	0.04	0.03	0.01

[a] For acronyms see Table 1. Antisera were the same as in Table 1.
[b] Antigens were diluted 100-fold beyond the titers determined in the double diffusion test. Reactions were stopped 1 hr after the addition of the substrate.

TABLE 3. Serological binding of particles of the eggplant mottled crinkle virus (EMCV) to electron microscope grids sequentially coated with protein A and with antisera to several tombusviruses[a]

Antiserum coating[b]	No. of particles per 15 μm^2[c]
EMCV	2,165
PLCV	1,885
TBSV BS-3	1,720
PAMV	303
Normal serum control	2

[a] For acronyms see Table 1. Antisera were the same as in Table 1.
[b] Carbon-formvar-coated grids that had been exposed directly before use to a glow discharge were floated for 5 min on a solution of 0.1 mg/ml protein A in 0.1 M phosphate buffer pH 7.0 (PB), washed with 20 drops of PB, blotted, floated for 5 min on antiserum diluted 1:20 in PB, washed with 20 drops PB, blotted, and floated for 15 min on crude extract of infected *Nicotiana clevelandii* that had been diluted 1:20 with PB. Grids were then washed with 40 drops of distilled water and stained with seven drops of 2% uranyl acetate.
[c] Mean values of counts on 50 random viewing fields on each of two duplicate grids at a magnification of ×40,000.

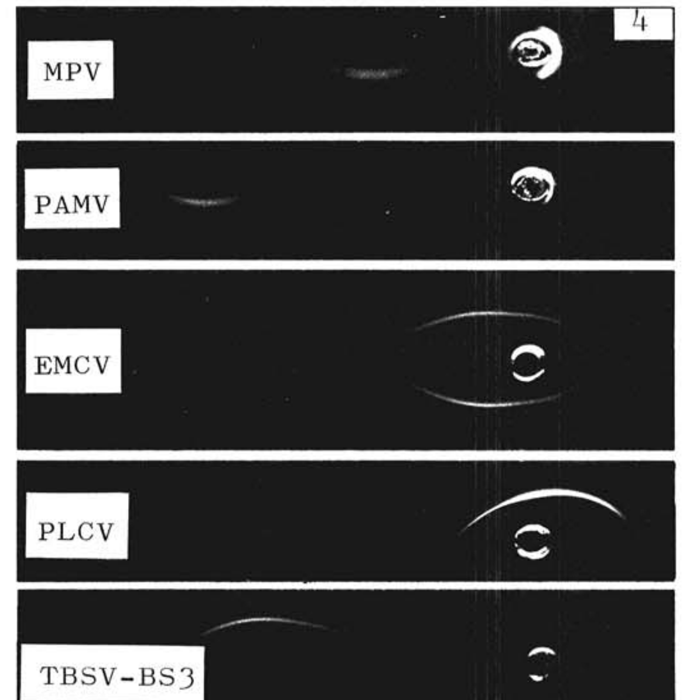


Fig. 4. Immunoelectrophoresis of tombusviruses in 1% agarose containing 0.025 M phosphate buffer, pH 7.0. Antiserum to the pelargonium leaf curl virus (PLCV) was used. The Moroccan pepper isolate (MPV), petunia asteroid mosaic virus (PAMV), the eggplant mottled crinkle virus (EMCV), PLCV and the BS-3 strain of tomato bushy stunt virus (TBSV) were used as antigens. Anode left, cathode right.

antisera could not be determined clearly. This difficulty was circumvented by applying protein A to the grids before coating them with antiserum (24) (Table 3). The highest numbers of particles were obtained with the homologous antiserum. Almost equally high numbers of particles were obtained on grids coated with antisera to TBSV BS-3 and to PLCV. Low particle numbers, which nevertheless were clearly higher than those on normal serum-coated grids, were seen on grids coated with PAMV antiserum.

In agarose-immunoelectrophoresis at pH 7.0, PLCV migrated slowly toward the cathode, whereas EMCV, MPV, TBSV BS-3, and PAMV migrated toward the anode (Fig. 4).

Cytopathology. Ultrathin sections from infected *N. clevelandii* showed numerous cells with pathologic alterations in different grades of severity. Altered cells, in comparison to unaffected cells, showed an increase in volume of the cytoplasm, apparently due mainly to the occurrence of regions containing homogeneous accumulations of small isometric particles that were considered to be virus particles (Fig. 5A). These particle-containing regions appeared distinct from the cytoplasm in electron density and were not delimited from the cytoplasm by a membrane. They contained randomly distributed, more or less densely packed, masses of virus particles with electron-translucent interstices. Often these regions protruded into the vacuole, forming bubblelike virus-filled vesicles that were surrounded by the tonoplast membrane. Apparently these vesicles eventually break, releasing free virus particles into the vacuole.

Virus particles also were found scattered within the cytoplasm and occasionally in the stroma of the chloroplasts (Fig. 5B). It could not be decided whether the particles had indeed been located in intact chloroplasts or whether the outer membranes of these organelles were already broken in the process of chloroplast disintegration, thus allowing the particles to invade the stroma.

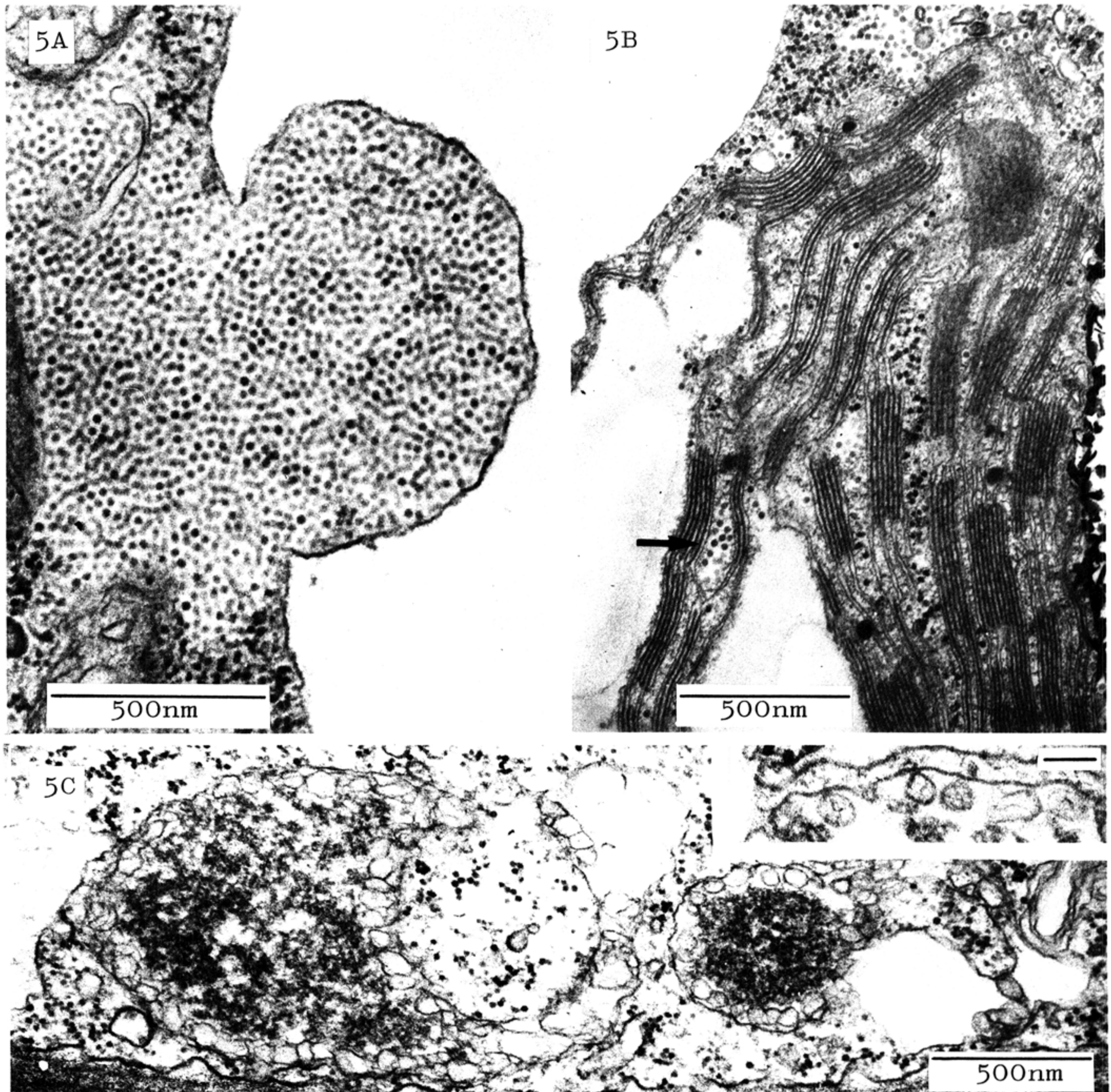


Fig. 5. Cytological alterations observed in *Nicotiana clevelandii* infected with the eggplant mottled crinkle virus. **A,** Portion of cytoplasm in a mesophyll cell with a virus-containing region that protrudes into the vacuole. **B,** Part of a chloroplast that contains virus particles in the stroma between the grana (arrow). **C,** Multivesicular cytoplasmic inclusions showing numerous small vesicles at the periphery. Inset shows detail of arrangement of small vesicles at the membrane surrounding the inclusion. Bar equals 100 nm.

Virus particles were not found in mitochondria or nuclei, nor did these organelles appear specifically altered by the virus infection apart from degenerative alterations in necrotizing cells.

The cytoplasm of infected cells contained many organellelike multivesicular inclusions in sizes between those of mitochondria and chloroplasts. They were seemingly bounded by a unit membrane and contained along their peripheral membranes many small vesicles, ~50–100 nm in diameter; the vesicles contained fibrillar structures that resembled nucleic acidlike material (Fig. 5C). In some places the small vesicles resembled invaginations of the membrane surrounding the multivesicular inclusions (Fig. 5C, inset). The central parts of the inclusions contained granular-amorphous material of varying electron density.

In most infected cells chloroplasts were rather weakly affected, showing mainly a rounded shape with swollen stroma regions and poorly developed thylakoid systems. In cells near the necrotic part of tissues many chloroplasts seemed to be completely distintegrated in the cytoplasm after losing the outer membrane envelope.

DISCUSSION

The morphological, serological, and biophysical properties of EMCV and its cytopathic effects are those typical of tombusviruses. Host range, electrophoretic, and serological properties differentiate EMCV from known tombusviruses.

Unlike the type strain of TBSV (16), EMCV did not produce lesions on inoculated leaves of tomato, nor did it invade this host systemically. Pelargonium leaf curl virus produces lesions on *P. vulgaris* and *D. stramonium* followed by systemic spread (6), whereas EMCV also produced lesions on the inoculated leaves of these two hosts but without systemic invasion. Petunia asteroid mosaic virus infects *Petunia* systemically (16), but EMCV did not. The carnation strain of TBSV produces local and systemic infections in *C. amaranticolor* and *C. quinoa* (7), whereas EMCV produced lesions on these hosts without systemic spread. Many of the host reactions produced by EMCV resembled those produced by artichoke mottled crinkle virus (AMCV) (14) except that the latter, contrary to EMCV, does not produce lesions on inoculated leaves of *P. vulgaris* cultivar Bountiful.

The most detailed study of serological interrelationships among tombusviruses has been made by Hollings and Stone (8). EMCV is apparently only rather distantly related to PAMV (Table 1), which together with AMCV as well as cherry and grapevine isolates (1) of TBSV form a group of closely related viruses (8). EMCV seems to be more closely related to MPV and PLCV although it is clearly distinguishable from those viruses.

Different suggestions have been made concerning the taxonomic status of serologically related viruses, such as PAMV, PLCV, and TBSV, within the tombusvirus group. Earlier they were regarded as strains of TBSV (16), but in more recent reports (8,15) including that of the International Committee on Taxonomy of Viruses (18) they are listed as separate viruses. Since EMCV is more distantly related to TBSV than PAMV and PLCV (Table 1), it should also be regarded as a separate virus. A future reevaluation of the virus species concept (R. I. Hamilton, J. R. Edwardson, R. I. B. Francki, H. T. Hsu, R. Hull, R. Koenig, and R. G. Milne, *unpublished*) may possibly necessitate a reconsideration of EMCV, PAMV, PLCV and TBSV as strains of a single tombusvirus.

Antisera to tombusviruses, like those to tymoviruses (10,11), strains of barley yellow dwarf virus (22) and certain short elongated viruses (10), but unlike those to certain potyviruses (27,28), tend to have a rather narrow specificity in ELISA. In the IEM decoration (19,20) and Derrick (4) tests, on the other hand, a broad specificity with tombusviruses was obtained, which was comparable to that of the agar gel double diffusion test.

The cytopathic changes induced by EMCV are in all important details typical for alterations induced by tombusviruses (17) although they seem to be less severe. An association of the multivesicular inclusions to chloroplasts or mitochondria was not found in EMC-affected cells. Budding of the chloroplasts was not observed. The internal material of the inclusions resembled that of microbodies, although this organelle was not observed in infected tissues in contrast to its presence in healthy *N. clevelandii*. It seems possible, therefore, that the multivesicular inclusions represent altered microbodies. A similar consideration already has been discussed by Russo and Martelli (23).

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