A New Tobamovirus from *Passiflora edulis* in Peru

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ABSTRACT

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A virus with particles typical of a tobamovirus was isolated from mosaic diseased plants of *Passiflora edulis*. The virus, for which the name maracuja mosaic virus is proposed, produced systemic infections only in *P. edulis* and *Nicotiana benthamiana*. Local lesions were produced in 25 other species from nine different families. The virus had particles 304 nm long that, in infected cells, formed platelike aggregates with a thickness equal to one virion's length. Granular cytoplasmic inclusions were consistently associated with infections. In sodium dodecyl sulfate polyacrylamide gel electrophoresis, purified virus preparations yielded two protein bands with molecular weights of 17.4 and 15.9×10^3. The more rapidly moving protein apparently was not an in situ degradation product. The virus showed only distant serological relationships to other tobamoviruses in the slide-precipitin test, indirect enzyme-linked immunosorbent assay, and immunoelectron microscopy. Serological relationships with other tobamoviruses were readily detectable by electroblot immunoassay.

In 1982, a virus with rigid rod-shaped particles was isolated from *Passiflora edulis* Sims 'Flavicarpa' (Spanish common name: maracuja) plants grown in the coastal area of northern Peru that showed symptoms of leaf mosaic and crinkle. This elongated virus proved to be a new tobamovirus for which we propose the name maracuja mosaic virus (MrMV). Some Passiflora plants with ringspots as well as mosaic symptoms contained tomato ringspot virus in addition to MrMV (13).

MATERIALS AND METHODS

After five successive local lesion passages through *Chenopodium quinoa* Willd., MrMV was maintained in *Nicotiana benthamiana* Domin.

Indicator hosts were grown in pots containing a mixture of sterilized soil and sand and were kept under greenhouse conditions at 22–28 C. Mechanical inoculations were made by rubbing 22 μm (600 mesh) Carborundum-dusted leaves with sap inoculum. Plants were tested for infection by back inoculation to *C. quinoa*. Persistence of virus infectivity in expressed sap of *N. benthamiana* was monitored by inoculation of each treatment to groups of five *C. quinoa* plants.

Purification and serology. MrMV was purified from systemically infected *N. benthamiana* plants by the procedure for potato virus V (7) except that chloroform and butanol in the proportion of 1 ml buffer, 0.2 ml of chloroform and 0.04 ml of *n*-butanol were added in the homogenization step, the first supernatant was not stirred with Triton X-100, and the resuspended high speed pellets were further purified by one or two cycles of differential centrifugation through sucrose cushions prepared by layering 1 ml of resuspended virus on top of 7 ml of 30% sucrose in Type 65 rotor tubes (Beckman Instruments, Palo Alto, CA). Sedimentation coefficient S_{rel} was determined by layering MrMV and marker viruses on top of 5-ml 10–40% linear sucrose density gradients that were centrifuged for 2 hr at 35,000 rpm at 8 C in a Beckman SW 39 rotor. The gradients were analyzed on an ISCO density gradient fractionator and UA-5 ultraviolet absorption monitor. The marker viruses were the top component (53.5S) of turnip yellow mosaic (20), the middle (95S) and bottom (115S) components of cowpea mosaic (25) and tobacco mosaic viruses (194S) (27). Percentage of nucleic acid was calculated as described by Englander and Epstein (5). An antiserum was prepared by injecting a rabbit intramuscularly twice at a weekly interval with 10 mg of virus in 0.5 ml of buffer emulsified with 0.5 ml of Freund's incomplete adjuvant. The slide-precipitin test (2), sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (17), and electroblot immunoassay (4) followed the published protocols. The coat protein for electrophoresis was obtained by heating the purified virus preparations for 2 min at 100 C in 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% 2-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8. Indirect ELISA on nonprecoated plates and plates precoated with $F(ab')_2$ fragments were as described (14) but immune complexes were detected with peroxidase-labelled protein A (1).

Electron microscopy. Samples prepared from diluted sap of infected *N. benthamiana* or from purified preparations were stained with 2% sodium phosphotungstate, pH 7.0. Particles were measured on enlarged electron micrographs from sap preparations and grouped into classes of 10 nm. The magnification of the electron microscope (JEOL JEM-100S) was checked with a carbon replica of a diffraction grating with 2,160 lines per millimeter.

Immunoelectron microscopy decoration tests (21) used antisera diluted 1:50. Immunosorbent electron microscopy (ISEM) was as described previously (18). Test conditions in ISEM for narrow specificity of reactions comprised antisera dilutions of 1:1,000 and 15-min incubation of antiserum-coated grids with virus samples. For broad specificity reactions, grids were coated with 100 μg/ml of protein A followed by antisera diluted 1:50 and 17-hr incubation. Virus samples for these tests were crude extracts from dried leaves of infected *N. benthamiana*.

Pieces of infected leaves of *N. benthamiana* were fixed in 2.5% glutaraldehyde buffered in 0.1 M phosphate buffer, pH 7 (PB); postfixed in 0.5% OsO_4 in PB; washed with water; stained in 1% aqueous uranyl acetate; dehydrated in a series of 50, 70, and 100% acetone; infiltrated in a rotator at 40 C with a 1:1 acetone-epon mixture (v/v) followed by epon; and polymerized in molds at 60 C for 48 hr. Thin sections cut with a diamond knife were stained with lead citrate or with 1% aqueous uranyl acetate and then lead citrate. Sections were examined under a Zeiss EM 10 C electron microscope.

RESULTS

MrMV induced local infection in 27 species belonging to the Amaranthaceae, Balsaminaceae, Chenopodiaceae, Compositae,

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Cucurbitaceae, Labiatae, Passifloraceae, Scrophulariaceae, and Solanaceae but induced systemic infection in *P. edulis* and *N. benthamiana* (Table 1). The most useful hosts were *C. quinoa, C. amaranticolor* Coste & Reyn., and *Luffa acutangula* Roxb., which developed necrotic local lesions (Fig. 1A). *N. tabacum* L. reacted with necrotic local lesions, incomplete necrotic rings, and lines (Fig. 1C). Leaves of *N. benthamiana* showed systemic necrotic lesions, line patterns, crinkling, and mosaic (Fig. 1B). Plants of *P. edulis* mechanically inoculated under greenhouse conditions showed symptoms similar to those of field-infected plants (Fig. 1D).

The following 22 species in six different families developed no symptoms when inoculated with MrMV, and no virus was detected in them by inoculation to *C. quinoa*: Cucurbitaceae—*Citrullus vulgaris* Schrad., *Cyclanthera pedata* Schrad.; Cruciferae—*Brassica pekinensis* (Lour.) Rupr., *Matthiola incana* (L.) R. Br.; Leguminosae—*Cajanus cajan* (L.) Millsp., *Clitoria ternatea* L., *Dolichos biflorus* L., *D. lablab* L., *Phaseolus aborigineus* Burkart (PI 266-910), *P. acutifolius* Gray (PI 310801), *P. vulgaris* L. 'Monroe' and 'Pinto,' *Vigna unguiculata* (L.) Walp. ssp. *unguiculata* 'Black,' *V. unguiculata* ssp. *cylindrica* (L.) van Eseltine ex Verdc. 'Catjang'; Pedaliaceae—*Sesamum indicum* L.; Portulacaceae—*Montia perfoliata* (Willd.) Howell; Solanaceae—*Capsicum annuum* L., *C. frutescens* L., *C. pendulum* Willd., *C. pubescens* R. & P., *Datura metel* L., *Lycopersicon pimpinellifolium* (Jusl.) Mill., *L. peruvianum* (L.) Mill., *Solanum tuberosum* ssp. *tuberosum* × ssp. *andigena* 'Mariva,' 'Tomasa Condemayta,' and 'Yungay.'

Contact transmission. MrMV was readily transmitted by brushing one systemically infected *N. benthamiana* plant against leaves of three or four healthy plants of the same species; in one experiment, six of 10 plants developed local lesions and systemic symptoms.

Persistence of infectivity in sap. Undiluted sap of *N. benthamiana* stored for 6 mo at 20–24 C induced an average of 150 lesions per half leaf of *C. quinoa*; however, the number of lesions produced increased threefold when sap was diluted 1:100 in distilled water, suggesting the presence of infection inhibitors. Infectivity was lost by heating at 95 C but not 90 C. In several trials, sap was still infectious when diluted 10^{-12} but not 10^{-13}. Because of this abnormally high dilution end point, the following experiment was done: 1-μl drops of sap dilutions 10^{-6} to 10^{-12} were placed in

TABLE 1. Symptoms of maracuja mosaic virus in indicator hosts

Family Species	Symptoms[a]
Amaranthaceae	
Amaranthus edulis L.	CLL
Gomphrena globosa L.	RLL
Balsaminaceae	
Impatiens balsamina L.	CLL
Chenopodiaceae	
Chenopodium amaranticolor Coste & Reyn.	NLL
C. murale L.	NLL
C. quinoa Willd.	NLL
Compositae	
Helianthus annuus L.	SLI
Helichrysum sp.	CLL
Tagetes erecta L.	SLI
Zinnia elegans Jacq.	SLR
Cucurbitaceae	
Cucumis melo L.	NLL
C. sativus L.	NLL
Cucurbita maxima Duch	NLL
C. moschata Duch	NLL
Lagenaria siceraria (Molina) Standl.	NLL
Luffa acutangula Roxb.	NLL
Labiatae	
Salvia splendens Ker-Gawl	SLI
Passifloraceae	
Passiflora edulis Sims	M
Scrophulariaceae	
Anthirrhinum majus L.	SLI
Solanaceae	
Datura stramonium L.	CLL
Lycopersicon chilense Dun.	SLI
L. esculentum Mill.	SLI
Nicandra physaloides Gaertn.	NLL
Nicotiana benthamiana Domin.	NLL, SNL, C, NLP, M
N. glutinosa L.	NLL
N. tabacum L.	NLL, LINR
Physalis floridana Rybd.	SLI

[a]C = Crinkling, CLL = chlorotic local lesions, M = mosaic, NLL = necrotic local lesions, LINR = local incomplete necrotic rings, NLP = necrotic line patterns, RLL = reddish local lesions, SLI = symptomless local infection, SNL = systemic necrotic lesions, SLR = sunken local rings.

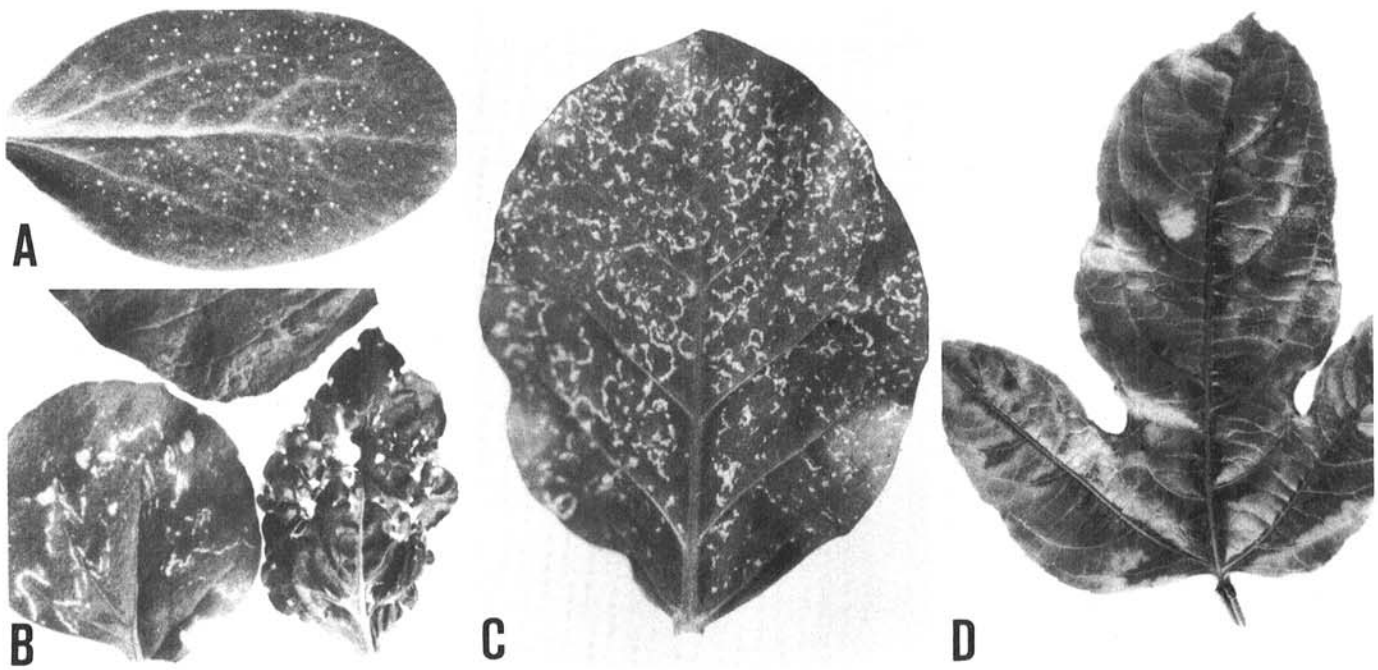

Fig. 1. Symptoms of maracuja mosaic virus. **A,** Necrotic local lesions in *Luffa acutangula*. **B,** Necrotic lesions, line patterns, and leaf rugosity in *Nicotiana benthamiana*. **C,** Incomplete necrotic rings and lines in *N. tabacum*. **D,** Mosaic in *Passiflora edulis*.

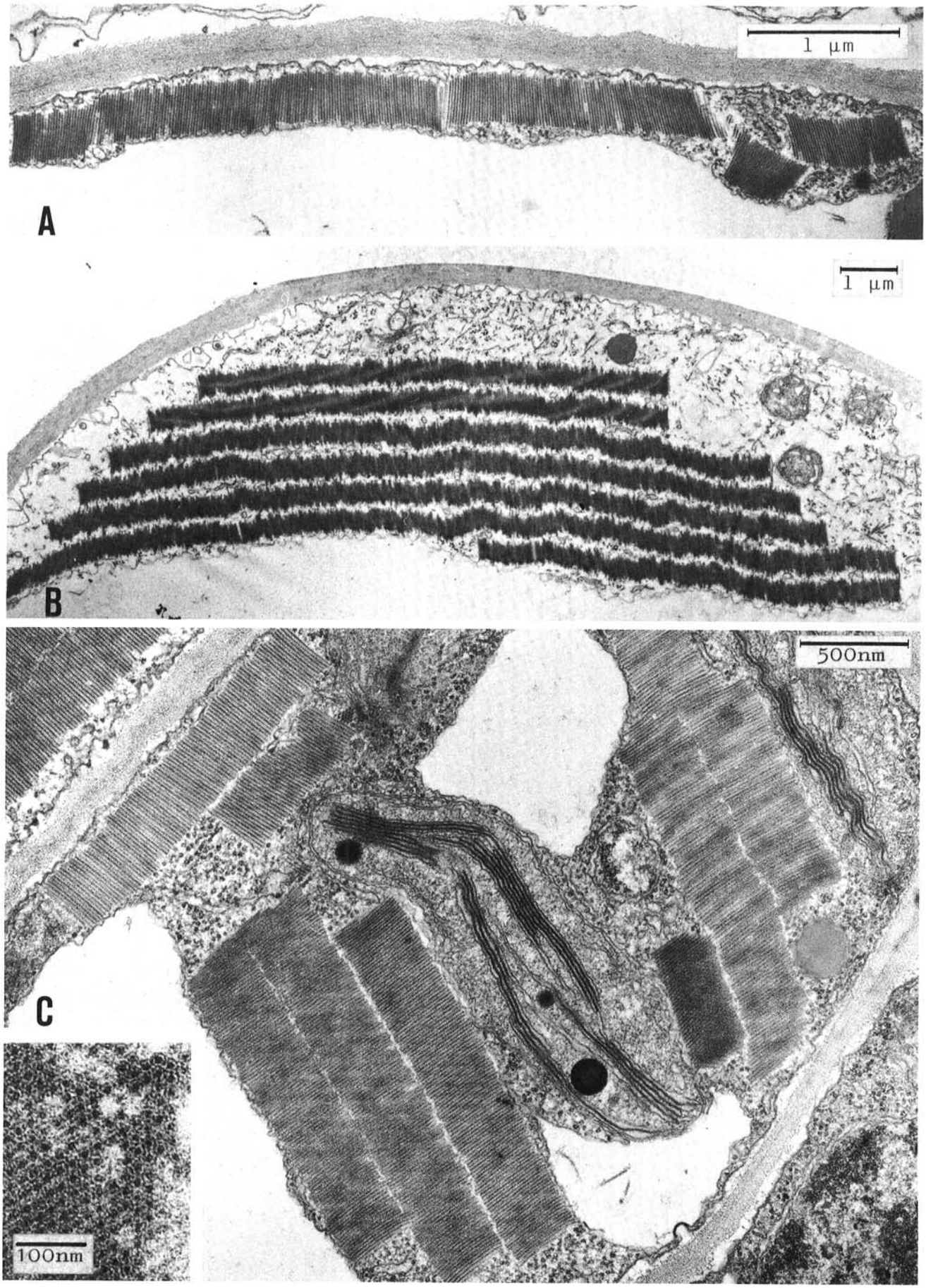

Fig. 2. Virus particle aggregates in leaf parenchyma cells of *Nicotiana benthamiana* infected with maracuja mosaic virus. **A,** Cross section of platelike aggregate. **B,** Seven stacked platelike inclusions in cross section (note scattered virus particles in the cytoplasmic space free of inclusions). **C,** Highly ordered aggregates in vascular parenchyma cells (inset shows crystalline arrangement of particles in the plates in a section perpendicular to the particle axes).

electron microscope grids, allowed to dry, and stained with 2% Na-phosphotungstate, pH 7.0. Virus particles were readily observed in all dilutions up to 10^{-10}, indicating that MrMV is an infectious virus that reaches a high concentration in host tissues.

Particle morphology. Virus particles appeared as rigid rods with an internal canal and a structure typical of tobamoviruses. Their normal length in crude sap preparations was 304 nm, based on 152 particles that occurred in the main peak. Particle width was about 18 nm.

Cytology of infected cells. Systemically infected leaves of *N. benthamiana* contained numerous 300-nm thick, platelike aggregates of virus particles in the epidermis, leaf parenchyma (Fig. 2A and B), and vascular parenchyma (Fig. 2C). Sometimes cross sections of the plates exceeded 12 μm. These virus aggregates were highly ordered and showed a crystal-like pattern when sectioned perpendicular to the long axis of the particles. The ends of the particles were often regularly arranged to form nearly a straight line when sectioned parallel to the long axis of the particles. Stacks of platelike aggregates appeared as banded inclusions in cross sections. Scattered virus particles were seldom seen and were only conspicuous in virus containing xylem vessels (Fig. 3A), where no ordered virus aggregates were found. In addition to virus particle aggregates, cytoplasmic electron dense granular inclusions (Fig. 3B and C), rounded or irregularly shaped, not delimited by membranes, and with diameters of 1–3 μm, were found consistently but sparsely in infected cells. As with other tobamoviruses (6), vesicles budding from the tonoplast into the vacuole were consistently seen. These vesicles were 60–70 nm in diameter and contained fibrillar material that resembled nucleic acid. Similar vesicles have been associated with infections by viruses of other groups, e.g., cucumoviruses and tobacco necrosis virus (9), a potexvirus, and a carlavirus (Lesemann, *unpublished*). No virus-induced alterations were found in nuclei, mitochondria, or microbodies. Lipid globules were seen more often in the cytoplasm than in cells of healthy *N. benthamiana*. Chloroplasts of infected tissues contained abnormally large starch grains.

Properties of purified preparations. Purified virus preparations had an A_{260}/A_{280} ratio of 1.1 and an extinction coefficient of 2.4 $(mg/ml)^{-1}\ cm^{-1}$ at 260 nm, both corrected for light scattering. The percentage of nucleic acid, assumed to be RNA, was calculated at about 7%. S_{rel} obtained by centrifugation with marker viruses in sucrose gradients was 192. SDS polyacrylamide gel electrophoresis showed two closely spaced protein bands of 17.4 and 15.9 kD (average of seven determinations). Prolonged contact of the virus with plant sap during purification, e.g., by overnight incubation of the sap, did not increase the more rapidly moving band at the expense of the more slowly moving one. Because our isolate was highly infectious and had been obtained after only five single lesion passages on *C. quinoa*, there was a possibility that the isolate contained a mixture of strains with different protein molecular weights. A 10^{-7} dilution of sap of infected *N. benthamiana* was therefore inoculated onto *C. quinoa*, and two new local lesion isolates were obtained after two successive single lesion passages. These isolates were propagated and after purification, each again yielded the 17.4 and 15.9 kD proteins. In electroblot immunoassay (Table 2), both proteins reacted with homologous and heterologous antisera.

Serology. Antisera to MrMV from different bleedings had homologous titers up to 1:1,024 in slide precipitin tests. They did not react with other tobamoviruses, i.e., cucumber green mottle mosaic (CGMMV), odontoglossum ringspot (ORV), tobacco mosaic (TMV), and the tobamovirus from *Ullucus* described by Brunt et al (3). Antisera to other tobamoviruses reacted in slide precipitin tests only at low dilutions or not at all with MrMV. In indirect ELISA on nonprecoated plates to which the virus was adsorbed directly, we observed some reactivity of MrMV with antisera to CGMMV, Frangipani mosaic virus (FMV), and TMV but not with those to a number of other tobamoviruses. Indirect

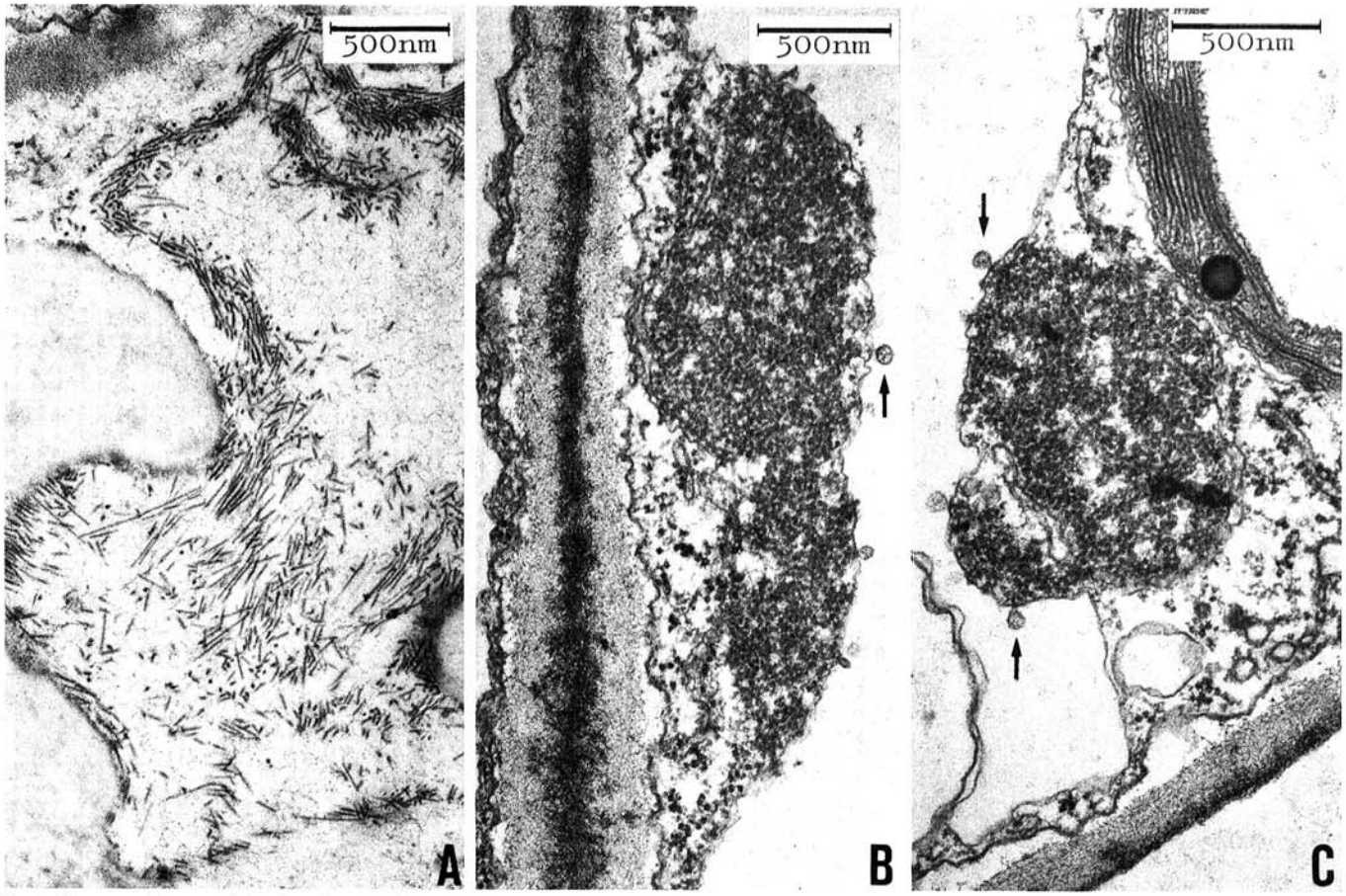

Fig. 3. Virus particles and inclusions in cells infected with maracuja mosaic virus. **A,** Scattered particles in xylem vessel. **B and C,** Granular inclusions and virus-induced vesicles associated with the tonoplast (arrows).

TABLE 2. Detectability of serological relationships of maracuja mosaic virus[a] in serological tests

Serologic test[b]	Measurement	Antiserum to virus[c] MrM	CGMM	FM	OR	RM	SO	TM	U	Normal serum
Slide precipitin	Serum titer (reciprocal)	1,024	2 (512)[d]	4 (256)	2 (256)	0 (256)	0 (256)	4 (512)	2 (1,024)	0
Indirect ELISA on nonprecoated plates	E_{490}	0.82×5^{e}	0.12	0.05	0.02	0.01	0.00	0.30	NT[f]	0.00
Indirect ELISA on plates precoated with 3 μg/ml $F(ab')_2$ fragments of MrMV antibodies	E_{490}	0.90×5^{e}	0.02	0.00	0.00	0.00	0.00	0.00	NT	0.00
Immunoelectron microscopic decoration	Visibile binding of antibodies	strong	none	none	none	NT	NT	none	NT	none
ISEM, antiserum 1:1,000 15-min incubation	Virus particles (no./1,000 μm^2)	>20,000	460	140	80	NT	NT	96	NT	102
ISEM, protein A, antiserum 1:50, 17-hr incubation	Virus particles (no./1,000 μm^2)	>20,000	1,710	380	84	NT	NT	450	NT	128
Electroblot immunoassay	Color intensity of protein bands at serum dilution of									
	1:500	strong	strong	NT	strong	NT	NT	strong	NT	none
	1:5,000	strong	medium	NT	medium	NT	NT	medium	NT	none

[a] Antigens for the immunoelectron microscopic tests were infectious crude extracts from dried leaves of *Nicotiana benthamiana*; for other tests, purified preparations at a dilution optimal for each test were used.
[b] ELISA = enzyme-linked immunosorbent assay, ISEM = immunosorbent electron microscopy.
[c] MrM = Maracuja mosaic, CGMM = cucumber green mottle mosaic, FM = Frangipani mosaic, OR = odontoglossum ringspot, RM = ribgrass mosaic, SO = Sammon's opuntia, TM = tobacco mosaic, U = *Ullucus* virus.
[d] Homologous titers in parentheses.
[e] In indirect ELISA procedures, the MrMV antiserum had to be at a 1:5,000 dilution to give measurable E_{490} values; other antisera were at a 1:1,000 dilution. If the MrMV antiserum had also been used at a dilution of 1:1,000, E_{490} values would presumably have been five times as high as those actually measured.
[f] NT = Not tested.

ELISA in which the virus was trapped on plates precoated with $F(ab')_2$ fragments of MrMV antibodies was more specific. In this test MrMV reacted only with its homologous antiserum. In immunoelectron microscopic decoration tests, MrMV also reacted only with its homologous antiserum. In ISEM under narrow specificity conditions, a weak reaction with antisera to CGMMV and FMV was detectable. Under broad specificity conditions, the heterologous reactivity of MrMV was strongest with antiserum to CGMMV but was also clearly detectable with antisera to FMV and TMV. No reactions were seen in either ISEM test with antiserum to ORV. In electroblot immunoassay, heterologous reactivities of the two coat proteins of MrMV were readily detectable with antisera to all other tobamoviruses, i.e., those to CGMMV, ORV, and TMV, but no reactions were seen with antisera to beet necrotic yellow vein virus, a possible tobamovirus, and with antisera to viruses in other groups, i.e., tymoviruses, tombusviruses, comoviruses, nepoviruses, potexviruses, carlaviruses, and potyviruses (4).

DISCUSSION

The virus isolated from *P. edulis* has properties typical of a tobamovirus (8): straight rod particles 304 nm long, transmissibility by contact, thermal inactivation point around 90 C, sedimentation coefficient of 192, and formation of plate-shaped inclusions in the cytoplasm of infected cells. In SDS gel electrophoresis, it yielded one protein band corresponding to a coat protein molecular weight of 17.4 kD, which is similar to that of other members of this group. Unlike other tobamoviruses, however, it showed an additional more rapidly moving band that was apparently not produced by degradation of the virus in crude plant sap as is the case with other viruses (10,16) and was not the product of contamination with a different strain.

Although MrMV had a wide host range, infecting species in nine families, it induced predominantly local infection. In this respect, it does not resemble TMV or ribgrass mosaic virus, both of which induce systemic symptoms in many hosts (22,27). Similarly, unlike CGMMV or sunn-hemp mosaic virus, it does not systemically infect cucurbits or legumes (11,12). The only definitive tobamovirus (8) that resembles MrMV is ORV (23), which infects several hosts locally; however, they differ in symptomatology and serology. MrMV also differs from a tobamovirus found in *Passiflora caerulea* in India (19), which systemically infects several hosts in the Chenopodiaceae, Leguminosae, and Solanaceae.

In most serological tests, even in indirect ELISA on plates not precoated with antibodies and in ISEM using protein A and long incubation periods, only distant serological relationships of MrMV with other tobamoviruses were detected (Table 2). In previous experiments with other viruses, the latter two tests were especially suitable for sensitive detection of distant serological relationships (14,15,26). As with other viruses, the ability of indirect ELISA to detect distant serological relationships was diminished when the plates were precoated with $F(ab')_2$ fragments (14,15). The results (Table 2) again demonstrate that electroblot immunoassay is especially sensitive in detecting distant serological relationships (4,24).

MrMV is thus a new tobamovirus that is only distantly related to other members of this group.

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