J. Phytopathology **153**, 129–136 (2005)

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Characterization of Maize Iranian Mosaic Virus and Comparison with Hawaiian and Other Isolates of Maize Mosaic Virus (Rhabdoviridae)

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Received July 19, 2004; accepted December 17, 2004

Keywords: insect transmission, ultrastructure, virion proteins, enzyme-linked immunosorbent assay, Western blots

Abstract

Maize Iranian mosaic virus (MIMV) was characterized and compared with isolates of Maize mosaic virus (MMV, genus *Nucleorhabdovirus*, family Rhabdoviridae) in insect transmission, cytopathology and ultrastructure of infected maize cells, virion proteins and serologically. MIMV is naturally transmitted by *Ribautodelphax notabilis*, a delphacid planthopper, in Iran. In this study, another planthopper, *Peregrinus maidis*, vector of MMV, transmitted MIMV with an estimated efficiency of 0.4–1.6% following feeding on MIMV-infected maize plants and 64% following injection of MIMV into the hemolymph, suggesting that *P. maidis* gut tissues largely blocked MIMV transmission. MIMV and MMV-HI (Hawaii) induced similar cytopathologies in cells of infected maize leaves, with virions budding through inner nuclear and endoplasmic reticulum membranes. In thin sections, virions of MIMV were significantly shorter than those of MMV-HI. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of virions of MIMV, MMV-HI, MMV-CR (Costa Rica) and MMV-FL (Florida) yielded six proteins of which four were identified as the putative G, N, P and M proteins of plant rhabdoviruses. The N, P and M proteins of MIMV migrated faster in gels than those of the MMV isolates indicating a lower molecular weight, whereas the bands corresponding to the G proteins migrated similarly for both viruses. Polyclonal antibodies to MMV-HI failed to react with virions of MIMV in enzyme-linked immunosorbent assay (ELISA) and with MIMV proteins in Western blots. In contrast, these antibodies reacted strongly with MMV-HI and MMV-FL virions in ELISA and with MMV-HI, MMV-CR and MMV-FL proteins in Western blots. Further, in ELISA, polyclonal antibodies to MMV-MR (Mauritius) reacted weakly with MIMV virions but strongly with MMV-HI and MMV-FL virions. Thus, it is concluded that MIMV is a new virus of the *Nucleorhabdovirus* genus that may be distantly related to MMV.

Introduction

Rhabdoviruses (family Rhabdoviridae) infect humans, other animals and plants. All plant rhabdoviruses replicate in and are transmitted by insects, and therefore have both plant and animal hosts. Plant rhabdovirus vectors are mainly aphids, leafhoppers and planthopper of the order Hemiptera (Jackson et al., 1987). So far, more than 70 plant rhabdoviruses have been described and are divided into two genera *Nucleorhabdovirus* and *Cytorhabdovirus*, depending on whether their forming virions bud from nuclear or only cytoplasmic membranes, respectively, in plant cells. Rhabdoviruses have enveloped bacilliform or bullet-shaped particles that contain a minimum of five proteins (Jackson et al., 1987; Hogenhout et al., 2003).

A rhabdovirus, maize Iranian mosaic virus (MIMV), causing mosaic symptoms on maize (*Zea mays* L.) was reported from Iran (Izadpanah and Parvin, 1979). Symptomatology, host range, and observation of rhabdovirus-like particles in infected maize suggested a relationship between MIMV and maize mosaic virus (MMV, genus *Nucleorhabdovirus*) described from Hawaii (MMV-HI) (Kunkel, 1921; Carter, 1941), Florida (MMV-FL) (Bradfute and Tsai, 1983), Venezuela (Lastra, 1977), Brazil (Kitajima and Costa, 1982), and Mauritius (MMV-MR) (Autrey, 1983). However, MIMV differed from MMV in several characteristics (Izadpanah et al., 1983; Izadpanah, 1989): (i) MIMV infects wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) that are not reported hosts of MMV; (ii) MIMV is transmitted by a delphacid planthopper *Ribautodelphax notabilis*, whereas another delphacid,

Peregrinus maidis, transmits MMV (Herold, 1972; Izadpanah et al., 1983); (iii) serologically, antiserum to MIMV did not react with MMV-HI and an antiserum to a Venezuelan isolate of MMV did not react with MIMV (Milne et al., 1986); (iv) in negatively stained preparations, MIMV particles are shorter (average = 179 nm) compared with those of MMV (204–245 nm) (McDaniel et al., 1985). These differences suggested that MIMV is distinct from MMV.

This publication presents a further characterization of MIMV through comparisons with MMV-HI and other MMV isolates. Brief reports of the present work were previously published (Ammar et al., 1987b; Gomez-Luengo and Gordon, 1987).

Materials and Methods

Virus and vector sources and maintenance

The MIMV isolate was provided by K. Izadpanah (Shiraz University, Shiraz, Iran). The MMV-HI isolate, originally obtained from Hawaii was maintained in our laboratory for several years (Ammar and Nault, 1985; McDaniel et al., 1985). The MMV-CR (from Costa Rica) was supplied by R. Gamez (University of Costa Rica) and L.R. Nault (Department of Entomology, OSU, OARDC, Wooster, OH, USA), and MMV-FL (from Florida) was obtained from J. H. Tsai (University of Florida, Fort Lauderdale). The planthopper vector *P. maidis* and the *Dalbulus* leafhoppers tested were from laboratory cultures of L. R. Nault (OSU, OARDC, Wooster, OH, USA).

For MIMV maintenance, *P. maidis* was injected abdominally with a clarified extract from MIMV-infected maize, maintained for 2 weeks on maize plants and then given several weekly inoculation access periods (IAP) of 7 days each on maize seedlings (inbred Oh28). MMV isolates were maintained in maize inbred Oh28 by weekly transfer of *P. maidis*, previously fed on MMV-infected maize plants for 1–2 weeks, to seedlings exposed to a 7-day IAP. Planthoppers exposed or non-exposed to MMV-infected maize plants were maintained as described (Gingery et al., 1979).

To avoid contamination, plants infected with virus were kept in separate growth chambers (Environmental Growth Chamber, Chagrin Falls, OH, USA) during acquisition access periods and IAPs. After IAPs, the insects were removed from plants within separate transfer hoods, and the plants were sprayed with Resemethrin to kill any remaining insects and then placed in different rooms of an insect-containment greenhouse.

Insect transmission tests

The planthopper *P. maidis* and four species from the cicadellid leafhopper genus, *Dalbulus*, were tested for transmission of MIMV following oral acquisition from MIMV-infected maize plants. Late instar nymphs of *P. maidis*, *D. elimatus* (Ball), *D. longulus* DeLong, *D. maidis* (DeLong & Wolcott) and *D. quevari* DeLong were caged for feeding on MIMV-infected maize plants for 14 days, then transferred to and maintained on healthy maize seedlings for 5 days, and finally tested for inoculativity by feeding on healthy maize seedlings (Aristogold Bantam Evergreen sweet corn) for 7 days (five insects/test plant). Insects were eliminated from test plants by treatment with insecticide. Test plants were maintained in an insect-containment greenhouse for 3–4 weeks and then rated for infection by the presence of disease symptoms.

Further transmission tests were carried out by injecting *P. maidis* and the leafhopper *D. maidis* with clarified extracts from MIMV-infected leaves obtained by the following protocol. Infected maize leaves were ground with a pestle in a mortar containing phosphate-buffered saline–ethylene diamine tetra acetate (EDTA) buffer, pH 7.4 (PBS-EDTA buffer) [0.01 M potassium phosphate, 0.14 M NaCl, 0.01 M EDTA]. The extract was pressed through a double layer of fine-mesh cheesecloth and centrifuged in a Fisher Micro-Centrifuge (Fisher Scientific, Pittsburg, PA, USA) 235.B at $15\,000 \times \boldsymbol{g}$ for 2 min at room temperature. The clarified extract (supernatant) was recovered and injected abdominally under the cuticle of *D. maidis* and *P. maidis* as described (Gingery et al., 1981). The insects were maintained on healthy maize seedlings for 2 weeks and then tested for inoculation effect by feeding on healthy seedlings (Aristogold Bantam Evergreen sweet corn) for 7 days (five insects/two test plants).

The probability of transmission by single insects (P) was calculated as described (Swallow, 1985). Healthy test plants fed upon by non-exposed or non-injected insects of the tested species served as controls.

Electron and light microscopy

The MIMV-infected and healthy maize leaves (Aristogold Bantam Evergreen) were prepared for transmission electron microscopy as described for MMV-HI (McDaniel et al., 1985). Ultrathin sections, stained with uranyl acetate and lead citrate, were examined in a Philips 201 electron microscope (Philips, Eindhoven, The Netherlands) (Figs 1–8). Semithin sections (1–2 μm thick) for light microscopy were stained with toluidine blue and examined by a Zeiss Photomicroscope II or III (Carl Zeiss, Oberkochen, Germany) (Figs 9–11).

To study virus-induced inclusions in whole cells by light microscopy (Figs 12 and 13), epidermal strips from healthy or MIMV- or MMV-HI-infected maize leaves were prepared and stained with orange green (OG) according to Christie and Edwardson (1986).

Virion purification

The procedure for purification of MMV and MIMV from infected maize leaves was developed previously in our laboratory for the purification of MMV-HI (R. G. Gomez-Luengo, L. L. McDaniel and D. T. Gordon, unpublished data), and is described as follows: At 2–3 weeks post-inoculation, infected maize leaves were homogenized in four volumes of 0.1 M sodium citrate

buffer, pH 7.5 (citrate buffer), plus 0.25% thioglycollic acid, pH 7.5. The extract was pressed through a double fine-mesh cheesecloth layer, and 2 g Celite analytical filter aid (Fisher Scientific, Springfield, NJ, USA) was added per 25 g tissue. This slurry was passed through a 3–4 mm thick pad of analytical filter-aid Celite in a Buchner funnel with an aspirator. The filtered extract was then layered onto a linear 100–400 mg/ml sucrose density gradient prepared in Beckman SW28 rotor tubes (Beckman Instruments, Inc., Palo Alto, CA, USA) by layering 5, 7, 7 and 5 ml of 40, 30, 20 and 10% sucrose, respectively, in citrate buffer. The gradients were centrifuged at 82 700 ***g*** for 15 min at 4°C. The two light scattering virus bands located in the middle of the gradient were removed by a hypodermic syringe inserted from the top of the gradient. The recovered 11 ml suspension was then layered onto a linear 250–550 mg/ml sucrose gradient, prepared in SW28 rotor tubes (Beckman Instruments, Inc.) by layering 5, 8, 8 and 5 ml of 25, 35, 45 and 55% sucrose in citrate buffer respectively. Gradients were centrifuged at 82 700 ***g*** at 4°C for 2.5 h. Light scattering virus bands were removed as above, diluted threefold in the citrate buffer, and pelleted in SW28 rotor tubes by centrifugation at 89 500 ***g*** for 30 min at 4°C. The pellets were resuspended in 0.1–0.3 ml of citrate buffer, or 0.05 M Tris–HCl buffer, pH 7.4, per 25 g of tissue.

Analysis of virion proteins

Proteins from purified virions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using 1.5 mm thick resolving gels of 10% or 15% polyacrylamide. Protein bands were stained by incubating gels in 20% isopropanol and 10% trichloroacetic acid for at least 1 h followed by treatment with Coomassie Brillant Blue G-250 (Bio-Rad Laboratories, Richmond, CA, USA), prepared as described by Vesterberg et al. (1977), for 1 h and finally destaining in 7.5% acetic acid overnight. Virion proteins were stained for carbohydrates as described (Falk and Tsai, 1983), and the Schiff's reagent prepared as described (Fairbanks et al., 1971).

Enzyme-linked immunosorbent assay

An indirect $F(ab')_2$-ELISA was performed as described (McDaniel and Gordon, 1989), with the following exceptions: microtitre plate wells were washed with Tris-buffered saline plus Tween 20, pH, 8.0 (TBS-T) [0.25 M TRIS (hydroxymethyl) amino methane, 0.15 M NaCl plus 0.05% Tween 20 (Sigma Chemical Co.)]; unbound sites were blocked with 1% bovine serum albumin (BSA) in TBS-T after coating well surfaces with $F(ab')_2$ fragments; antigen bound antibodies (IgGs) were detected with biotinylated protein A (Amersham Corp., Arlington Heights, IL, USA) diluted 1 : 1500 with TBS-T; bound biotinylated protein A was labelled with a horseradish peroxidase streptavidin conjugate (Amersham Corp.) diluted 1 : 1000 with TBS-T; the colour reaction was developed with the peroxidase substrate [0.1 M citric acid, 1 mM 2,2′-azinobis [3-ethylben-thiazoline sulphonic acid) diammonium salt (ABTS), pH 4.2, plus 0.1% H_20_2]; and reactions were stopped with 5% SDS.

Antiserum to MMV-HI was raised in rabbits. Preimmune serum was recovered before injections with MMV-HI purified as described above. For immunization three intramuscular injections were made at 7-day intervals using 2 ml of a 1 : 1 (v/v) mixture of Freund's complete adjuvant (Gibco Laboratories, Life Technologies, Inc., Chagrin Falls, OH, USA) and 0.8 mg/ml of partially purified MMV-HI for the first injection and 1 ml of a 1 : 1 (v/v) mixture of incomplete Freund's adjuvant (Gibco Laboratories) and 0.5 mg/ml of partially purified virus for the last two injections. Antiserum was recovered from bleedings made at 10–12-day intervals after the last injection. For storage antiserum was diluted 1 : 1 with glycerol and frozen (−20C). Coating $F(ab')_2$ fragments were prepared from MMV-HI and MMV-MR IgGs as described (McDaniel and Gordon, 1989). The polyclonal antiserum to a Mauritian isolate of MMV (MMV-MR) was provided by Jean Claude Autrey (Mauritius) and the antiserum to maize chlorotic mottle virus (MCMV) was produced by us (D. T. Gordon, unpublished data). The concentration of the MMV-HI and MMV-MR $F(ab')_2$ fragments was 1 μg/ml and of the MMV-HI and MMV-MR IgGs, 10 mg/ml.

Extracts for ELISA were prepared by grinding 1 g of MMV-HI-, MMV-FL- or MIMV-infected or healthy maize leaf tissue in 4 ml of TBS-T with a pestle in a mortar. The extract was filtered through a double layer of fine mesh cheesecloth and the filtrate used to prepare twofold dilutions from 1 : 4 to 1 : 4096 with TBS-T as the diluent. Each dilution was tested in two wells of a microtitre plate and dilutions were placed within plates according to a Greco-Latin square design to eliminate location within plate effects on A_{405nm} values. The experiment was repeated three times.

The ELISA results were expressed as an index of relationships that equalled the difference of the means of the $A_{405\,nm}$ readings for the virus-infected and healthy leaf extracts divided by the SD of the mean values of the $A_{405\,nm}$ for healthy extracts. As a control to test for non-specific binding of detecting antibodies to antigen trapped by the $F(ab')_2$ fragments, preimmune serum was substituted for detecting antibodies in the ELISA involving MMV-HI $F(ab')_2$ and antiserum to MCMV for detecting antibodies in the ELISA involving MMV-MR $F(ab')_2$.

Western blot analysis

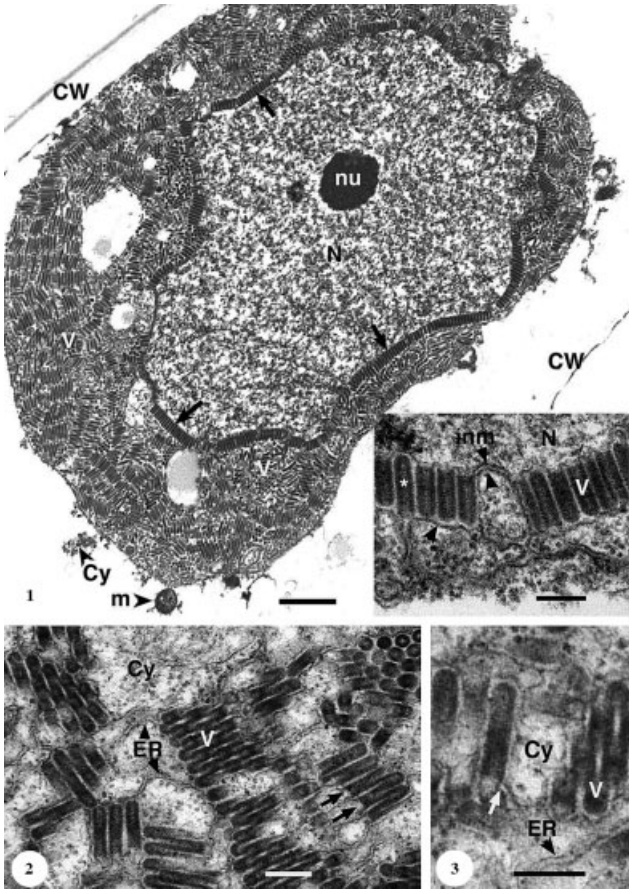
Western blots were performed as described (Towbin et al., 1979), except that 1% BSA + TBS-T [0.25 M TRIS (hydroxymethyl) amino methane, 0.15 M NaCl plus 0.05% Tween 20, pH 7.4] was substituted for the 5% horse serum as a blocking agent. Membranes were incubated in TBS-T for 30 min at 37°C; rinsed with TBS-T and incubated with primary antibody to MMV-

HI, diluted 1 : 1000 with TBS-T. The primary antibody was produced as described above. The conjugate was goat anti-rabbit IgG linked with alkaline phosphatase (Miles-Yeda, Ltd, Rehovot, Israel) diluted 1 : 2500 with TBS-T.

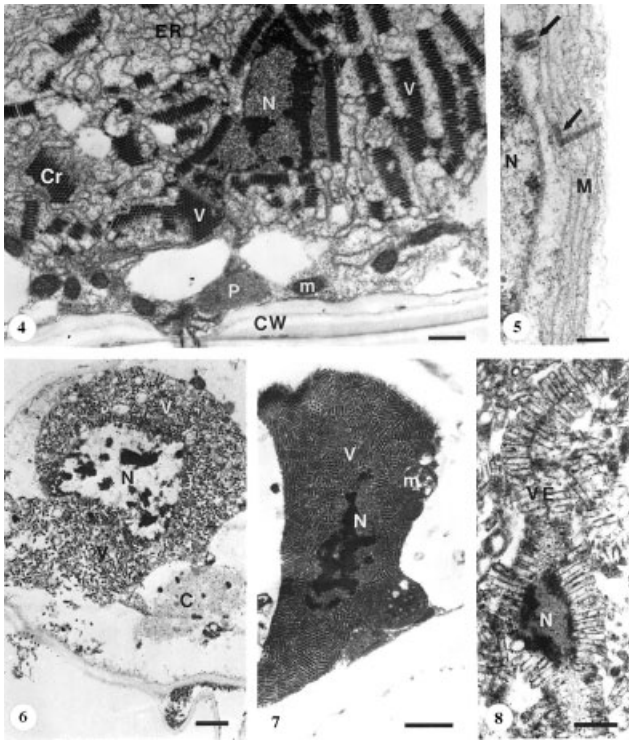
Results

Insect transmission tests

To investigate whether the planthopper *P. maidis* transmits MIMV, late-instar nymphs were either fed on MIMV-infected plants or abdominally injected with clarified extracts from MIMV-infected leaves. *P. maidis* fed for 14 days on MIMV-infected plants transmitted MIMV to 2% and 8% of test plants in two separate tests involving 360 and 500 insects respectively (five insects/plant). For *P. maidis* injected with clarified extract from MIMV-infected maize, 92% of the test plants became infected in a test involving 35 insects (five insects/two plants). For these tests, the probability of MIMV transmission by single *P. maidis* was estimated to be 0.4–1.6% for planthoppers that acquired virus by feeding on diseased plants and 64% for planthoppers that acquired virus from injected clarified extract. Similar high rates of MIMV transmission were observed for *P. maidis* injected with clarified extracts from infected maize in the routine transfer of the virus for maintaining infected plants during this 2-year study.



Figs. 1–3 Electron micrographs of thin sections from maize leaves infected with MIMV. Fig. 1 An epidermal cell packed with virions (V) surrounding the nucleus (N), with fragmented cytoplasm (Cy) and mitochondria (m); arrows indicate budding virions; inset, virions budding through the inner nuclear membrane (inm), unlabelled arrowheads indicate outer nuclear membrane, *bacilliform virion. CW, cell wall; nu, nucleolus. Bars = 1 μm and (inset) 0.2 μm. Figs 2, 3 Masses of virions (V) enclosed in dilated cisternae connected to the endoplasmic reticulum (ER) within the cytoplasm (Cy); black arrows indicate virions apparently budding through the ER; white arrow indicates continuation of the viral envelope with ER membrane. Bars = 0.2 μm

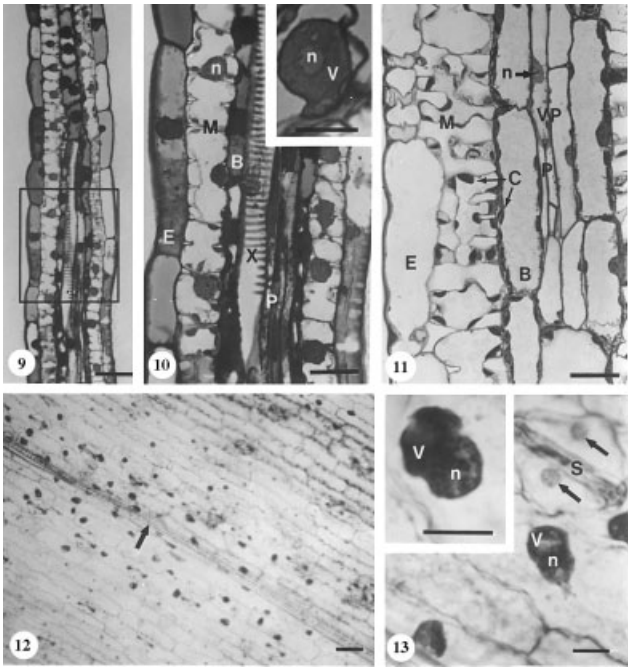


Figs. 4–8 Electron micrographs of thin sections from maize leaves infected with MIMV. Fig. 4 A phloem companion cell with an elaborate network of endoplasmic reticulum (ER) and masses of virions (V), some in crystalline arrays (Cr). Fig. 5 Multi-layered membranous structures (M) near the nucleus (N); upper arrow indicates virions budding through nuclear membranes and lower arrow indicates a partially enclosed virion in the membranous structures
Figs. 6–8 Mesophyll, epidermal, and vascular parenchyma cells, respectively, apparently at various stages of degeneration, with masses of virions (V) or virion envelopes (VE)
C, degenerated chloroplast; CW, cell wall; m, mitochondrion; N, nucleus; P, plastid. Bars = 0.5 μm (Fig. 4), 0.2 μm (Fig. 5, 8), 1 μm (Fig. 6,7)

In tests using the leafhoppers *D. elimatus*, *D. quevari*, *D. longulus* and *D. maidis*, none transmitted MIMV following feeding on MIMV-infected leaves in a single test involving 20–70 insects per *Dalbulus* sp. Similarly, 35 *D. maidis* injected with clarified extracts from virus-infected maize also failed to transmit MIMV.

Ultrastructure and sites of virus assembly and accumulation

To study MIMV particle structure, as well as budding and accumulation sites, thin sections of MIMV-infected maize leaves were studied by transmission electron microscopy. This revealed abundant rhabdovirus particles in almost all cell types, including the epidermis,



Figs. 9–11 Light micrographs of semi-thin sections from maize leaves, either infected with MIMV (Fig. 9,10) or healthy (Fig. 11). Boxed area in Fig. 9 is enlarged in Fig. 10; inset in Fig. 10 shows the nucleus (n) with surrounding virion aggregates (V) comparable to those seen by electron microscopy in Fig. 1. B, bundle sheath cells; C, chloroplast; E, epidermis; M, mesophyll; n, nucleus; P, phloem elements; VP, vascular parenchyma; X, xylem. Bars = 40 μm (Fig. 9), 20 μm (Figs 10, 11), 10 μm (inset in Figs 10)

Figs. 12–13 Light micrographs of orange green-stained epidermal strips from maize leaves infected with MMV-HI. Fig. 12 Enlarged, darkly stained nuclei distributed mainly around a vascular bundle (arrow); Bar = 50 μm. Fig. 13 Higher magnifications showing enlarged nuclei (n), with associated virion aggregates (V); arrows indicate apparently normal nuclei. S, stomata; Bars = 10 μm

mesophyll, bundle sheath cells, phloem elements and vascular parenchyma (Figs 1–8). Most of the virions appeared bullet-shaped but some were bacilliform (Fig. 1, inset). The average length of bullet-shaped and bacilliform virions were 186.5 ± 1.1 nm (standard error) [number of particles (n) = 100], and 212.7 ± 1.5 nm (n = 50) respectively. Particle diameter of either type averaged 60.5 ± 0.2 nm (n = 122).

In infected maize leaf cells, MIMV virions appeared to bud through the inner nuclear membrane (Fig. 1), and on cytoplasmic membranes of the endoplasmic reticulum (Figs 2 and 3). Virions accumulated within the expanded perinuclear space (Fig. 1, inset), and in intracytoplasmic dilated cisternae connected with membranes of the endoplasmic reticulum (Figs 2 and 4). No virions, with or without viral envelopes, were detected inside the nucleus. Extensive proliferation of the endoplasmic reticulum membranes throughout the cytoplasm was frequently observed, particularly in phloem elements and vascular parenchyma of infected cells (Fig. 4). Occasionally, several layers of tubular or vesicular membranes, some of which appeared connected with the endoplasmic reticulum, were seen in the cytoplasm of these cells (Fig. 5).

Large accumulations of virions, sometimes in paracrystalline arrays, were observed in MIMV-infected cells (Figs 1–4). Frequently, the cytoplasm of these cells appeared fragmented and rudimentary, and the nucleus had a 'washed out' appearance, indicating a marked decrease in the amount of chromatin (Fig. 1). At a later stage of pathogenesis, nuclei, chloroplasts and mitochondria appeared degenerated (Figs 6–8). In some of these cells, most virions also appeared degenerated and only their envelopes were discernible (Fig. 8).

Virions and associated cytopathological features described above for MIMV-infected maize leaf cells were never observed in cells of healthy maize leaves.

Histopathology at the light microscope level

Cells in semithin sections of MIMV-infected maize leaves, stained with toluidine blue, appeared smaller than those from healthy leaves of comparable age (Figs 9–11). In addition, chloroplasts were generally smaller, but most nuclei appeared larger in MIMV-infected than in healthy leaves. At a magnification of 1000×, the enlarged, semi-opaque nuclei appeared surrounded by more densely stained areas (Fig. 10, inset), comparable with the perinuclear accumulations of the virions seen by electron microscopy (Fig. 1).

In OG-stained epidermal strips from maize leaves infected with either MIMV or MMV-HI, dark green inclusions, presumably composed of accumulated virions, were observed surrounding or bulging to one side of the enlarged nuclei of epidermal cells (Figs 12 and 13). Enlarged nuclei with perinuclear inclusions were easily detected at magnifications as low as 160×, and were much more abundant in cells nearer to vascular bundles (Fig. 12). This suggests that in maize leaves both MIMV and MMV-HI are transported through the vascular bundles from which the virus is spread gradually into the surrounding non-vascular tissues.

Analysis of virion proteins

To compare numbers and molecular weights of proteins of MIMV and MMV isolates, virions were purified and analysed by SDS-PAGE. Purification of MIMV, MMV-HI, MMV-FL and MMV-MR using analytical filter-aid Celite gave reasonably clean and pure virion preparations, whereas with non-acid washed Celite the amount of impurities (green material) was evident and these impurities were difficult to eliminate on sucrose density gradients (data not shown). The typical average yield of partially purified MMV was 0.6–0.8 mg/25 g tissue. Evaluation of the purity of preparations by electron microscopy and electrophoresis on polyacrylamide gels and Western blotting with immunostaining using MMV-HI antiserum or antiserum prepared against healthy tissue revealed no contaminating plant membranes or proteins (data not shown). Thus, the analytical filter-aid celite provided adequate amounts of purified virus suitable for virion protein analysis.

The SDS-PAGE gel showed six bands in virion preparations of MIMV, MMV-HI, MMV-FL and

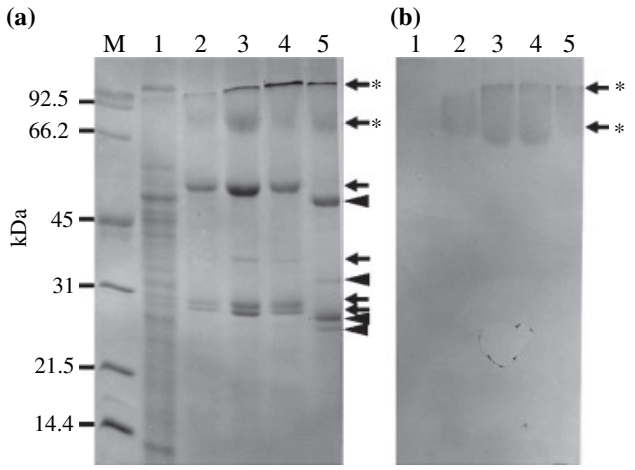


Fig. 14 Relative banding positions of proteins of purified virions of MIMV and MMV isolates in a sodium dodecyl sulphate 15% polyacrylamide gel. (a) Coomassie Brilliant Blue-stained gel showing that MMV and MIMV viruses contain six proteins identified in decreasing molecular weight as tentatively a larger version of the G protein (~100 kDa), the G protein (74 kDa), N protein (56 kDa), P (Ns) protein (39 kDa), and the two lower bands, the M1 (32 kDa) and M2 (31 kDa) proteins (Gomez-Luengo, 1987) (see Discussions for explanation). The four corresponding MIMV proteins that migrate faster than those of MMV are indicated with arrowheads. (b) Gel showing the approximately 100 and 70 kDa proteins positively stained for carbohydrates and probably corresponding to the MMV and MIMV G proteins. Proteins that stain positive for carbohydrates are indicated by an asterisk. Lanes: 1, marker; 2, proteins of uninfected maize leaves; 3, MMV-CR; 4, MMV-FL, 5, MMV-HI; 6, MIMV. The sizes in kDa of marker proteins are indicated at the left of the gels, and the positions of the six proteins are indicated with arrows

MMV-CR (Fig. 14a). The two slowest migrating bands were located at similar positions for all viruses, and stained positive for carbohydrates (Fig. 14b) suggesting that these bands correspond to glycosylated proteins. However, the four remaining bands migrated farther in the lane containing MIMV particles, when compared with the three lanes containing MMV-HI, MMV-FL and MMV-CR proteins (Fig. 14a). Thus, the molecular weights of these four structural proteins of MIMV are smaller than those of MMV. These four bands migrated similarly for the three MMV isolates.

Enzyme-linked immunosorbent assay

The ELISA tests were conducted to determine whether MIMV and MMV are serologically related. Antibodies to MMV-HI and MMV-MR were tested by $F(ab')_2$-ELISA against series of twofold dilutions of MMV-HI-, MMV-FL- and MIMV-infected and healthy maize leaf extracts respectively. For the MMV-HI antibodies the indexes of relationships for the three experiments were: MMV-HI = 117.4 (SD = 47.7); MMV-FL = 83.1 (SD = 42.0) and MIMV = 4.3 (SD = 7.0). The indexes for the controls involving pre-immune serum in place of MMV-HI IgG were: MMV-HI = 0.0344 (SD = 1.1272); and MMV-FL = 0.2627 (SD = 0.9323) suggesting absence of aspecific binding of rabbit antibodies. Thus, with an index of five considered positive, MMV-HI is serologically related to MMV-FL but not to MIMV.

For the MMV-MR antibodies the indexes were: MMV-HI = 75.2 (SD = 22.2); MMV-FL = 65.8 (SD = 31.1); and MIMV = 14.7 (SD = 4.7). The indexes for the controls involving MCMV antiserum in place of MMV-MR IgG the indexes were: MMV-HI = 0.8095 (SD = 2.6561): MMV-FL = −0.3538 (SD = 2.8132) suggesting absence of non-specific binding of rabbit antibodies. Thus, with an index of five considered positive, MMV-MR was related to MMV-HI and MMV-FL and might be distantly related to MIMV.

In Western blots of SDS-PAGE gels polyclonal antibodies to MMV-HI failed to react with MIMV-virion proteins (data not shown). In contrast, these antibodies reacted strongly with MMV-HI, MMV-CR and MMV-FL virion proteins. Thus, the Western blot analysis provided further evidence that MMV-HI and MMV-FL were not serologically related to MIMV.

Discussion

In this study, the planthopper *P. maidis*, vector of MMV, was able to transmit MIMV by feeding on MIMV-infected maize. Transmission, however, was very inefficient (0.4–1.6%) compared with much higher transmission efficiency (35%) when *P. maidis* was fed on MMV-HI infected plants (Ammar et al., 1987a). A considerably higher transmission rate of MIMV (64%) was obtained when *P. maidis* was injected with extracts from MIMV-infected leaves. This strongly suggests a gut infection or a gut–escape barrier (Ammar, 1994; Hogenhout et al., 2003) for MIMV in *P. maidis*, as injected insects were much more able to transmit MIMV than those that acquired the virus orally.

Ribautodelphax notabilis is a vector of MIMV in Iran and was the only delphacid species collected in maize, sorghum or wheat fields in the Bajgah area where MIMV was common (Izadpanah et al., 1983). It is not surprising that *Dalbulus* species failed to transmit MIMV as rhabdoviruses transmitted by delphacid planthopper vectors usually are not transmitted by cicadellid leafhoppers and *vice versa* (Ammar and Nault, 2002; Redinbaugh et al., 2002).

The purification procedure resulted in relatively large yields of intact purified MIMV and MMV particles without detectable plant protein contaminates. The use of analytical filter-aid Celite in the clarification of infected leaf extracts was critical for eliminating chloroplast material and contaminating membranes, as Celite other than analytical filter-aid failed to eliminate contaminating materials. Celite filtration has also been used successfully as an aid in the purification of MMV-MR (Autrey, 1983), MMV-FL (Falk and Tsai, 1983), MMV-VZ (Lastra, 1977), and sonchus yellow net virus (Jackson et al., 1987). The average thickness of the Celite pads used in the present study was within the range known to give good results. According to Jackson et al. (1987) the thickness of the pad is a critical variable; pads thicker than 7.5 mm seriously

diminish virus yields, whereas pads < 2.5 mm result in serious contamination with chloroplast fragments.

The present work indicates differences between MIMV and MMV-HI in molecular weights of four of the six proteins. The two largest proteins that migrated at the same rate for MIMV and MMV of $\sim$70 kDa and $\sim$100 kDa were glycosylated. However, the larger of the two did not stain with antibodies to MMV-HI, whereas all the remaining proteins did. In addition, the former protein was not seen in electrophoresed gels of extracts from healthy maize leaves. However, this larger glycosylated protein might be a modified form of the G protein as larger G proteins have been reported (Jackson et al., 1987), Thus, the origin of the larger glycosylated protein is uncertain. The smaller glycosylated protein of MMV-HI with an estimated molecular weight of 74 kDa (Gomez-Luengo, 1987) has the predicted size of the rhabdovirus G protein. The estimated sizes of the remaining four proteins of MMV-HI were N (56 000 Da), P (Ns) (39 000 Da), Ml (32 000 Da) and M2 (31 000 Da) (Gomez-Luengo, 1987). A larger virion associated protein was identified in some polyacrylamide gels (not shown in Fig. 15) and had an estimated size of 182 kDa. These findings are consistent with the presence of three major proteins for MMV-FL: G (75 000), N (54 000) and M (30 000) (Falk and Tsai, 1983) or five proteins designated L (150 000), G (75 000), N (56 000), P (Ns) (45 000) and M (33 000) for MMV-VZ (Lastra and Acosta, 1979). All plant rhabdovirus genomes that have been sequenced so far have at least one additional gene positioned between the genes encoding P and M on the viral genome, such as the *sc4* gene of SYNV (Scholthof et al., 1994; Huang et al., 2003; Hogenhout et al., 2003). The protein designated as M2 in the present study may be equivalent to the sc4 protein. An alternative hypothesis is that this protein is another translation product of the M gene, because it has been shown that the M gene may encode additional polypeptides synthesized from downstream methionines in the same open reading frame as the full-length M protein (Jayakar and Whitt, 2002).

The SDS-PAGE results revealed that the four faster migrating proteins of MIMV, presumably equivalent to proteins N, P and M of MMV migrate faster for MIMV than for the MMV isolates. Thus, the N, P and M proteins of MIMV might be smaller than those of MMV, which is consistent with the observation that MIMV particles were shorter and slightly narrower compared with those of MMV-HI in thin sections of maize leaves, prepared by the same procedure used for MMV-HI (McDaniel et al., 1985). Differences in particle sizes of MIMV and MMV were also observed in negatively stained preparations of purified virus (McDaniel et al., 1985; Gomez-Luengo, 1987). Additional studies, such as genome sequencing of MMV and MIMV combined with proteomics, should confirm the identities of these proteins observed on Coomassie-stained SDS-PAGE gels. MIMV and MMV-HI proteins also differed in that those of the former did not react with polyclonal antibodies to the latter when tested by $F(ab')_2$-ELISA or in Western blots in the present study. In addition, in immunosorbent electron microscopy, MIMV-nucleocapsid did not react when tested with antisera to MMV from Venezuela (Milne et al., 1986). MIMV reacted weakly with polyclonal antibodies to MMV-MR in $F(ab')_2$-ELISA, and as expected MMV-HI and MMV-FL reacted strongly to MMV-MR antiserum. MMV-HI has been shown previously to be serologically related to MMV isolates from Venezuela, Florida and Costa Rica (Lastra, 1977; Falk and Tsai, 1983; McDaniel et al., 1985). In the present study; while MIMV failed to react with the polyclonal antiserum to MMV-HI in Western blots and $F(ab')_2$-ELISA, it reacted weakly to a polyclonal antiserum to MMV-MR in $F(ab')_2$-ELISA. By comparison, MMV-HI and MMV-FL reacted strongly to the MMV-MR polyclonal antiserum in this assay. Our serological results suggest that MIMV may be distantly related to MMV-MR but not MMV-HI.

The assembly and accumulation sites of MIMV and MMV appeared to be similar in maize leaf cells, with both viruses budding through the inner nuclear membrane as well as cytoplasmic membranes of the endoplasmic reticulum (McDaniel et al., 1985). This is uncommon among plant rhabdoviruses, most of which reportedly assemble only at either nuclear or cytoplasmic membranes within cells of their host plants (Jackson et al., 1987). Another plant rhabdovirus that apparently possesses similar characteristics is Wheat American striate mosaic virus that assembled at nuclear and cytoplasmic membranes of its host plant (Lee, 1970).

In the planthopper vector, *P. maidis*, budding sites of MMV-HI appears to be tissue dependent, i.e. virions bud through the inner nuclear membrane in most cell types, but mainly through the cytoplasmic plasma membranes in cells of the salivary glands; this is thought to facilitate release of virions with saliva during oral transmission of MMV (Ammar and Nault, 1985). Thus, both MIMV and MMV-HI belong to the genus *Nucleorhabdovirus* based on the observation that both viruses bud from inner nuclear membranes, a phenomenon not observed for members of the genus *Cytorhabdovirus*. Nevertheless, as sites of assembly may differ between plant and insect hosts as well as between different vector tissues, as indicated for MMV-HI (Ammar and Nault, 1985), it seems that classification of plant rhabdoviruses into genera based on nuclear or cytoplasmic sites of assembly is problematic and another basis for classification may need to be established.

The cytopathologies induced by MIMV and MMV-HI in maize leaf cells were generally similar when examined either by light or electron microscopy. The multi-layered membranous structures associated with the endoplasmic reticulum in MIMV-infected cells are similar to those reported for the Brazilian isolate of MMV (Kitajima and Costa, 1982), and are probably similar (but differently oriented) compared with the

membranous vesicles found in MMV-HI infected maize cells (McDaniel et al., 1985).

In conclusion, the present work reveals differences and similarities between MIMV and MMV. Our results show that MIMV and MMV are distinct viruses based on differences between them in molecular weights of four of six proteins, particle lengths, and in serology. Similar characteristics of MIMV and MMV-HI are budding and accumulation sites in maize cells, cytopathology in maize leaves and transmission, though at different efficiencies, by *P. maidis*.

Acknowledgements

We thank L.R. Nault for providing cultures of leafhoppers and planthoppers, K. Izadpanah for providing the MIMV isolate, R. Gamez for the MMV-CR isolate, J. H. Tsai for the MMV-FL isolate, and J. C. Autrey for the polyclonal antiserum to MMV-MR. We also thank W. E. Styer for transmission of MIMV by needle-injected vectors, D. Fulton for technical assistance, and C. Rivera for preparing the epidermal strips shown in Figs 12 and 13. Salaries and research support were provided by State and Federal funds appropriated to the OARDC/OSU and USDA NRI grant 2002–35302–12653.

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