SHORT COMMUNICATION

MOLECULAR AND ULTRASTRUCTURAL PROPERTIES OF MAIZE WHITE LINE MOSAIC VIRUS

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SUMMARY

This paper reports the complete nucleotide sequence of the genome of Maize white line mosaic virus (MWLMV) and describes the ultrastructural features of infected maize cells. The viral genome is an RNA molecule 4293 nt in size with the same structural organization of members of the *Aureusvirus* and *Tombusvirus* genera. It comprises five ORFs flanked by non-coding regions at the 5' (40 nts) and 3' (225 nts) ends which encode, in the 5'-3' direction, proteins with predicted Mr of 30,009 (prereadthrough), 89,140 (readthrough, replication-associated proteins), 35,080 (coat protein), 24,644 (movement protein), and 15,155 (silencing suppressor). Some of ultrastructural features of MWLMV-infected cells, e.g. lobate nuclei, localization of virus particles in tonoplast evaginations bulging into the vacuoles, plentiful occurence of virions and of aggregates of virus coat protein in the cytosol, intramitochondrial patches of electron-dense material, tallied with those reported for a number of members of the family *Tombusviridae.* However, differences were observed with the cytopathology of the aureusvirus Johnsongrass chlorotic streak mosaic virus (JCSMV) which is the viral species more closely related to MWLMV from the molecular point of view. MWLMV was serologically unrelated to JCSMV, *Pothos latent virus* (PoLV), the type species of the *Aureusvirus* genus, and Maize necrotic streak virus (MNeSV), another maize pathogen with similar biological properties, belonging in the family *Tombusviridae*. In a phylogenetic tree constructed with whole genome sequences, MWLMV clustered with all members of the genus *Aureusvirus*. Thus, properties and phylogenetic relationships place MWLMV in the genus *Aureusvirus*. However, molecular differences with all sequenced species of this genus, the lack of serological relationship with PoLV and JCSMV, and the different cytopathology elicited in infected tissues, suggest that MWLMV be regarded as a separate species.

Key words: maize, *Tombusviridae*, *Aureusvirus*, sequencing, cytopathology, serology.

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Maize white line mosaic virus (MWLMV) causes the homonymous disease of maize, which was first reported from New York State (USA) (Boothroyd and Israel, 1980), and extensively investigated biologically and physicochemically in the 1980s (De Zoeten *et al.*, 1980; Louie *et al.*, 1982; Zhang *et al.*, 1991a). Virions are *ca.* 35 nm in diameter, contain a single type of coat protein subunits with Mr of *ca.* 37 kDa and a single-stranded positive sense RNA molecule of *ca.* 4.2 kb in size, accounting for 21% of the particle weight (De Zoeten and Reddick, 1984; De Zoeten, 2004). In addition, MWLMV is one of the four plant viruses supporting the replication of an isometric satellite virus *ca.* 17 nm in diameter (Mayo *et al.*, 2005a), containing single-stranded RNA genome 1.2 kb in size which has been sequenced (Gingery and Louie, 1985; Zhang *et al.*, 1991b).

MWLMV has properties resembling those of members of the family *Tombusviridae* (Lommel *et al.*, 2005) and, like a number of these viruses, is soil-borne but the vector, although suspected to be the plasmodiophorid fungus *Polymyxa graminis* (De Zoeten, 2004), has not been identified. Likewise, the taxonomic allocation of the virus remains undetermined primarily because its genome has not been characterized. Thus, MWLMV is still in the "Unassigned viruses" section of the 8th Report of the International Committee on Taxonomy of Viruses (Mayo *et al.*, 2005b).

We now report the complete sequence and structural organization of MWLMV genome, together with the ultrastructure of infected cells and discuss the taxonomic position of the virus.

MWLMV was propagated in maize (*Zea mays* L.) seedlings cv. Spirit by vascular puncture inoculation of kernels (Louie, 1995) and purified essentially as in Rubino *et al.* (1995). Virus yield was *ca.* 50 µg/g tissue. Virus particles, negatively stained with 2% aqueous uranyl acetate and observed with a Philips Morgagni 282D electron microscope, were isometric *ca.* 35 nm in diameter with a rounded contour and a knobby surface (Fig. 1A).

A polyclonal antiserum against MWLMV was raised in New Zealand white rabbits using standard procedures (Harlow and Lane, 1988) with three intramuscular injections of purified virus preparations mixed with

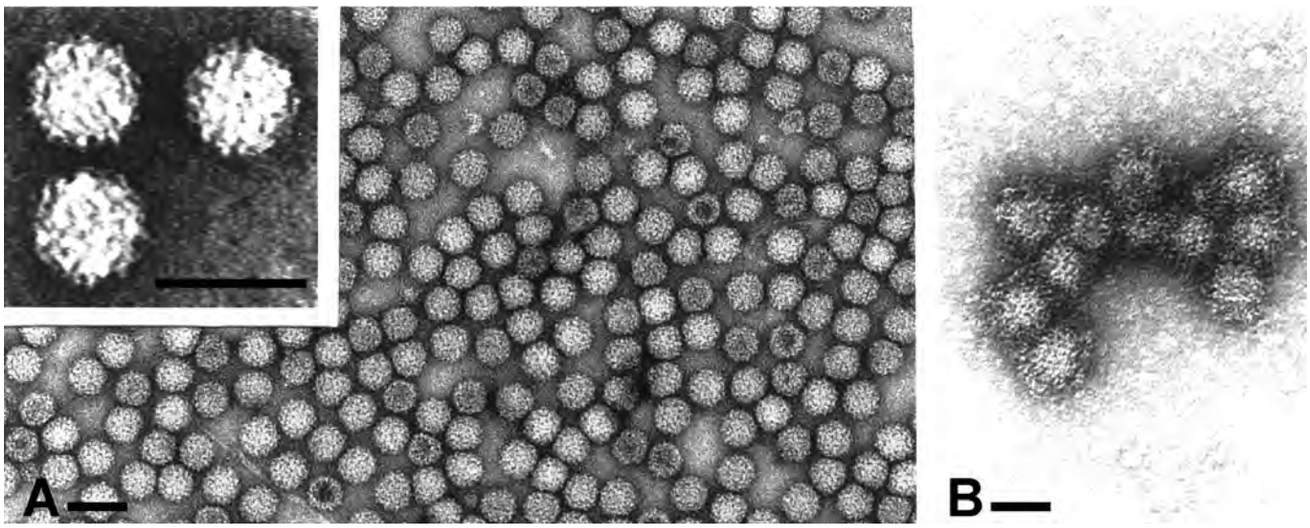

Fig. 1. A. Purified preparation of MWLMV particles. B. A clump of MWLMV particles decorated by the homologous antiserum. Bars = 50 nm.

an equal volume of Freund's incomplete adjuvant (*ca*. 0.1 mg/ml nucleoprotein) followed, after about a month, by two additional intramuscular injections at weekly intervals. In gel double diffusion test, the antiserum reacted with the homologous antigen to a dilution of 1:128, but not with Maize necrotic streak virus (MNeSV), proposed as unassigned species in the family *Tombusviridae* (S.A. Lommel, personal communication) and *Pothos latent virus* (PoLV), the type species of the genus *Aureusvirus* (not shown). Furthermore, MWLMV particles were decorated by the homologous antiserum (Fig. 1B) but not by a polyclonal antiserum to Johnsongrass chlorotic stripe mosaic virus (JCSMV), proposed as a definitive species in the genus *Aureusvirus* (Winter *et al.*, 2002).

For thin sectioning, tissue pieces were excised from symptomatic leaves 5, 12, 27, and 50 days after germination of infected seeds and processed according to standard procedures at 4°C throughout (Martelli and Russo, 1984), i.e. fixation in 4% glutaraldehyde in 0.05 M potassium phosphate buffer for 2 h, post-fixation in 1% osmium tetroxide for 2 h, staining overnight in 2% aqueous uranyl acetate, dehydration in ethanol, and embedding in TAAB low viscosity resin (Agar Scientific, UK)**.** Thin sections were stained with lead citrate prior to viewing. Leaf tissue fragments from healthy maize processed as above served as controls.

The architectural organization of mesophyll cells at 7 days post infection (d.p.i) was rather well preserved, but single organelles were variously altered in infected plants. For instance, nuclei were deeply lobed and mitochondria suffered partial or total loss of the internal structure and contained clumps of amorphous or finely granular electron-dense material within dilated cristae (Fig. 2B). Clumps of similar material were plentiful also in the cytoplasm of some infected cells of the same (Fig. 2A) or older age. Chloroplasts were apparently undamaged at 7 d.p.i., but by 12 and 27 d.p.i. could be heavily modifed or completely disrupted. Vesicular structures within dilations of the endoplasmic reticulum were seen in some cells (not shown). Virus particles were plentiful in all samples, regardless of the age of the infection. Particles were present in the cytoplasm of all leaf cell type, including the epidermis, mesophyll and vascular bundles. They formed disorderly aggregates within the cytoplasm and were rarely in crystalline arrays. In the vacuoles, particles were often within bleb-like evaginations of the tonoplast (Fig. 2C).

For immunogold labeling of infected cells, tissue fragments were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, dehydrated in graded ethanol dilutions, and embedded in London Resin White. MWLMV antiserum was pre-absorbed with 2 vol of healthy maize sap, incubated overnight at 4°C and the supernatant collected after centrifugation at 20,000 *g* for 10 min. Thin sections from healthy and infected tissues, collected on grids, were floated for 30 min at room temperature on a drop of 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 2% bovine serum albumin (BSA) and 0.05% Tween-20 (PBST-BSA). Grids were then incubated for 60 min at room temperature on 50 µl drops of a 1:50 dilution of the MWLMV antiserum in the same buffer. After rinsing three times by floating on drops of PBST-BSA for 5 min each, the grids were incubated with a 1:20 dilution of 15 nm colloidal gold particles conjugated with goat antirabbit antibodies (Amersham Biosciences, UK) for 60 min at room temperature in PBST-BSA. Prior to electron microscope observation, sections were extensively rinsed with PBS and distilled water and stained with 2% aque-

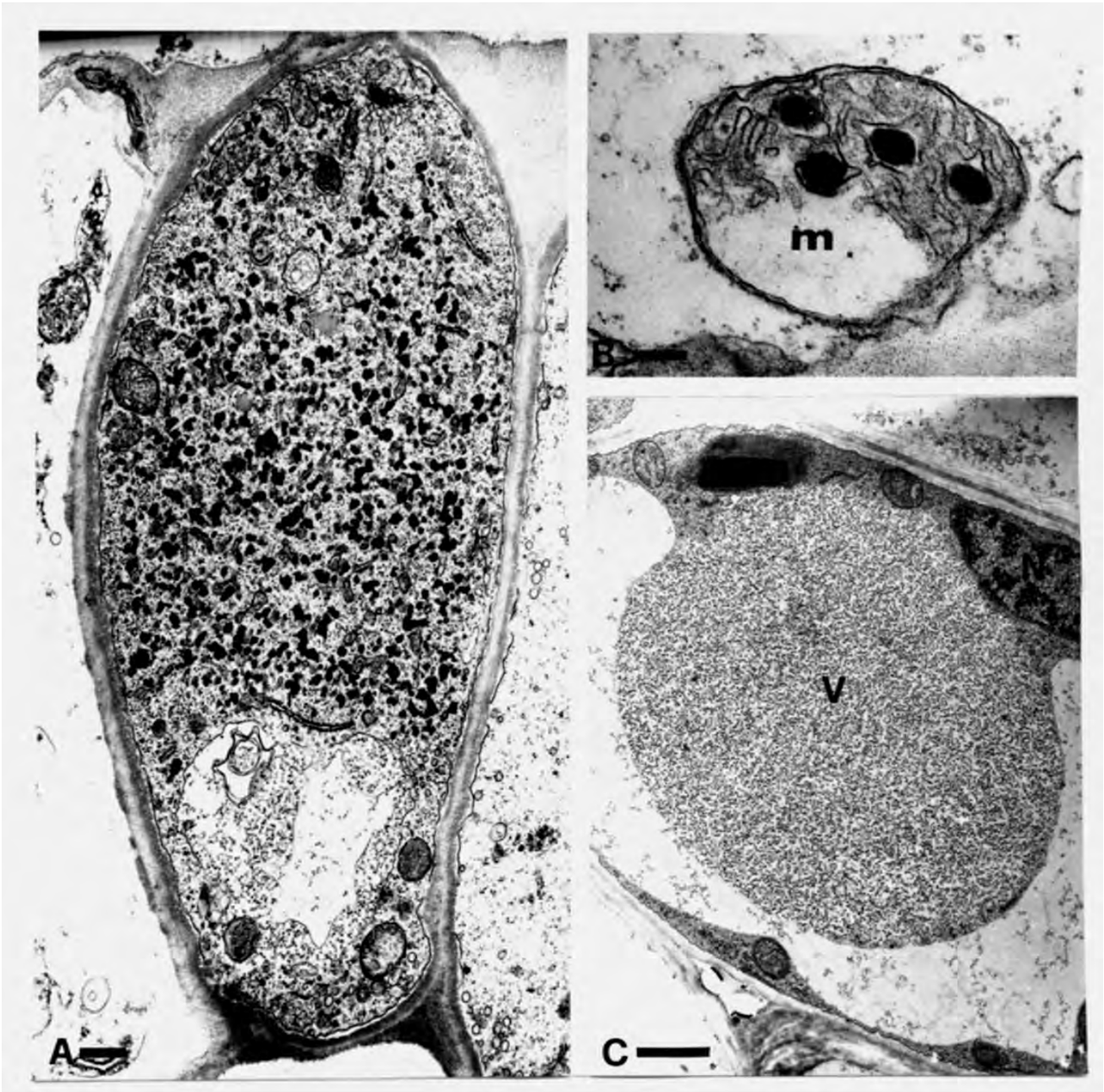

Fig. 2. A. Maize cell 7 d.p.i. showing a compact cytoplasm containing virus particles and a large number of clumps of electron-dense material. B. A mitochondrion (m) with dilated cristae containing an electron-dense material inclusion. C. Extensive accumulation of virus particles (V) in a bleb-like evagination of the tonoplast in the vacuole of a mesophyll cell 50 d.p.i. Bars A and C = 500 nm; B = 250 nm.

ous uranyl acetate for 5 min and lead citrate for 10 min.

Immunolabeling of cells was relatively light. Colloidal gold was distributed in the cytoplasm on virus particles as well as around and on the patches of electron-dense amorphous material (Fig. 3 and inset b), suggesting that this material may consist of virus coat protein. There was no apparent labelling of nuclei or mitochondria containing osmiophilic inclusions (Fig. 3). Some of the plasmodesmata were clearly labelled (Fig. 3, inset a) indicating that they contained virus particles.

Some ultrastructural features of MWLMV-infected cells, e.g. lobate nuclei, localization of virus particles in tonoplast evaginations bulging into the vacuoles, plentiful occurence of virions and of aggregates of virus coat protein in the cytosol, intramitochondrial patches of electron-dense material, were consistent with those reported for a number of members of the family *Tombusviridae* (Martelli *et al*., 1988; De Stradis *et al*., 2005). However, differences were observed with the cytopathology of cells infected by JCSMV (Izadpanah *et*

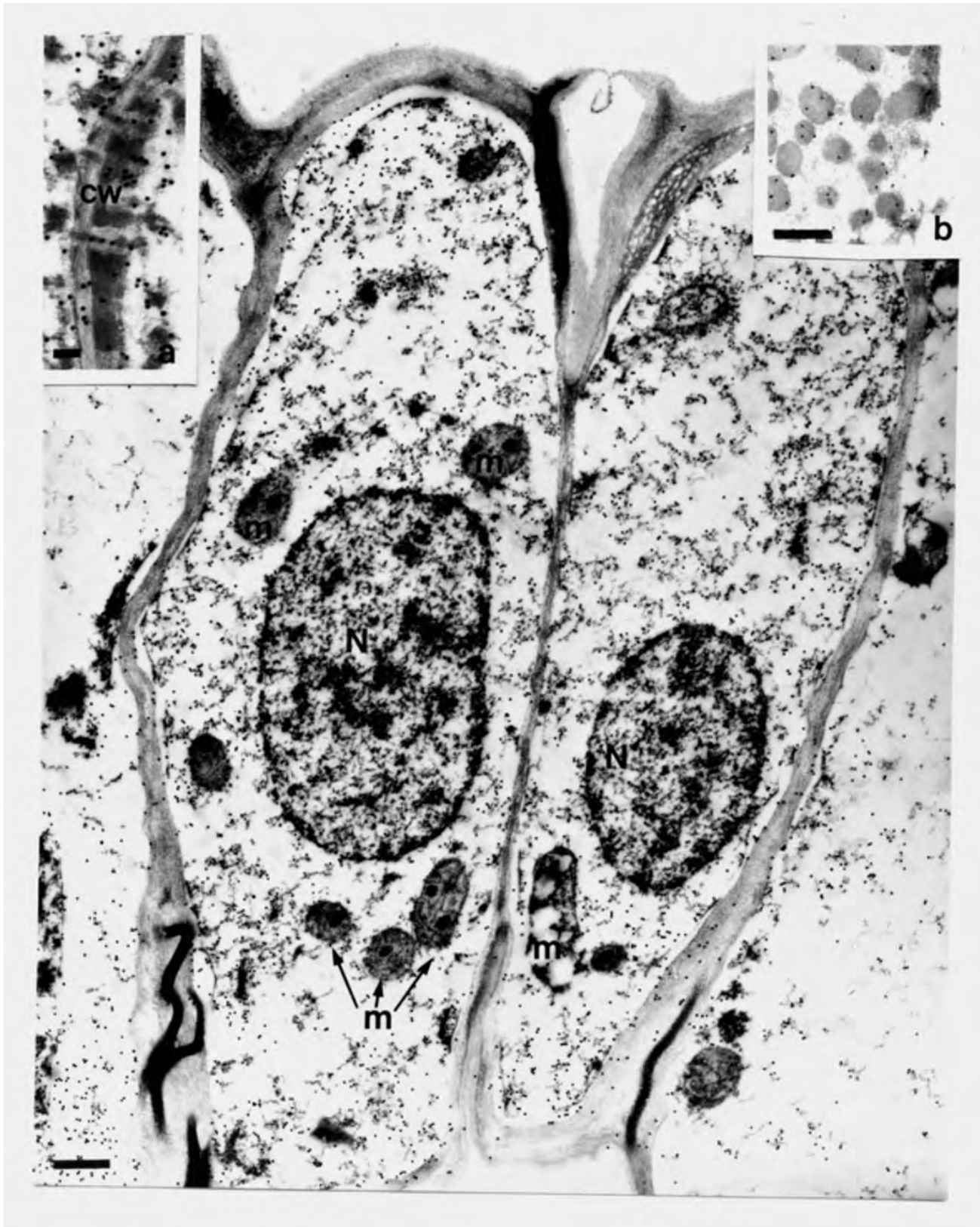

Fig. 3. MWLMV in guard cells 27 d.p.i. The virus was detected immunochemically using a gold-labelled secondary antibody. Gold particles are localized on virions, around and on the cytoplasmic clumps of electron-dense material (inset b) but are not detected in nuclei (N) and mitochondrial inclusions (m), Bars = 250 nm. Tagging of plasmodesmata in inset a. Bar = 50 nm.

al., 1993), which is the closest aureusvirus to MWLMV from the molecular point of view (Table 1, Fig. 5B).

For cDNA cloning and sequencing, RNA was extracted from purified virions by adding 1 vol of extraction buffer (0.1 M glycine-NaOH, pH 9.0, containing 100 mM NaCl, 10 mM EDTA, 2% sodium dodecyl sulfate and 1% sodium lauroyl sarcosine) and 2 vol of water-saturated phenol. The aqueous phase was extracted with phenol and chloroform, RNA was precipitated with ethanol in the presence of 0.3 M sodium acetate and resuspended in sterile water. Approximately 1 µg viral RNA was polyadenylated with poly(A) polymerase (United States Biochemicals, USA) following the manufacturer's instructions. Polyadenylated RNA was extracted with phenol-chloroform, precipitated with ethanol, resuspended in 10 µl sterile water and used as template for oligo(dT)-primed cDNA synthesis using cDNA Synthesis Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol. The 5' and 3' terminal regions were cloned using the 5'/3' RACE kit (Roche Diagnostics, Germany). Double-stranded DNA was ligated to *Sma* I-digested, dephosphorylated pUC18 and cloned in *Escherichia coli* strain XL-Blue. Plasmid DNA was purified using the NucleoSpin kit (Macherey-Nagel, Germany) and sequenced in both directions (MWG Biotech, Germany).

Sequence data were assembled using the DNA Strider software (Marck, 1988). A homology search with known nucleotide and protein sequences was done with the BLAST program (Altschul *et al*., 1990) and pairwise comparisons were made using the EMBOSS Pairwise Alignment Algorithm in the EMBL-EBI package (Rice *et al*., 2000). Sequences were aligned and phylogenetic trees were constructed using the Clustal W package (Thompson *et al*., 1994).

The full genome sequence of MWLMV (accession number NC_00533) was 4293 nt long and encoded five ORFs flanked by non-coding regions at the 5' and 3' ends. The 5' non-coding region (40 nts) is followed by ORF 1 starting from AUG at position 41-43 and terminating with an amber stop codon at nt 863-865. Readthrough of this stop codon would extend the frame up to the stop codon 2432-2434 (ORF 2). ORF 3 starts from nt 2489 and terminates with a stop codon at position 3485-3487. ORF 4 begins at position 3524 and terminates at nt 4205-4207. ORF 5 is nested in ORF4 in a different frame, beginning at position 3652 and terminating with a stop codon at position 4066-4068. The 3' untranslated region is 225 nt long. The predicted Mr of the proteins encoded by the five ORFs are, in the order, 30,009 (p30), 89,140 (p89), 35,080 (p35), 24,644 (p25), and 15,155 (p15).

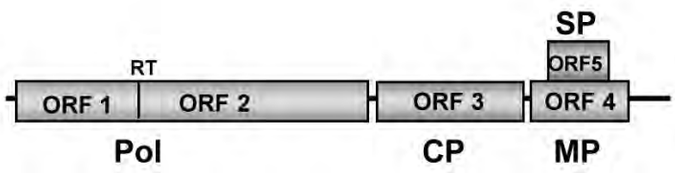

Fig. 4. Genomic organization of MWLMV. In the schematic diagram, boxes represent predicted ORFs encoding, in the order, the replication associated proteins (Pol), coat protein (CP), movement protein (MP) and RNA silencing suppressor protein (SP). RT = amber termination codon that can be read through.

MWLMV has the same genome organization (Fig. 4) of members of the genera *Tombusvirus* and *Aureusvirus.* By analogy to these taxa, the proteins encoded by ORF 1 and 2 constitute the viral replicase, as suggested by the presence of the GDD motif characterizing the RNA-dependent RNA polymerase within the readthrough domain of p89 (ORF 2). As to the pre-readthrough domain of p89 (p30; ORF 1) the presence of three helical transmembrane regions (Rost *et al*., 1995) at positions 52-76, 81-98 and 122-148, respectively, suggests its involvement in targeting to and anchor-

Table 1. Pairwise amino acid sequence comparison of proteins encoded by the genome of MWLMV with corresponding gene products of aureusviruses and selected tombusviruses.

Virus	Amino acid identity (%)				
	ORF 1	ORF 2	ORF 3	ORF 4	ORF 5
Aureusviruses					
CLSV	24	64	31	39	35
JCSMV	57	80	34	62	62
PoLV	24	62	35	42	40
SNMV	21	61	41	41	39
Tombusviruses					
CIRV	16	44	36	23	19
CymRSV	19	44	36	22	16
TBSV	20	44	37	22	28
Unassigned in the family *Tombusviridae*					
MNeSV	20	44	17	16	20

CLSV, *Cucumber leafspot virus*; JCSMV, Johnsongrass chlorotic stripe mosaic virus; PoLV, *Pothos latent virus*; SNMV, Sesame necrotic mosaic virus; CIRV, *Carnation Italian ringspot virus*; CymRSV, *Cymbidium ringspot virus*; TBSV, *Tomato bushy stunt virus*; MNSeV, Maize necrotic streak virus

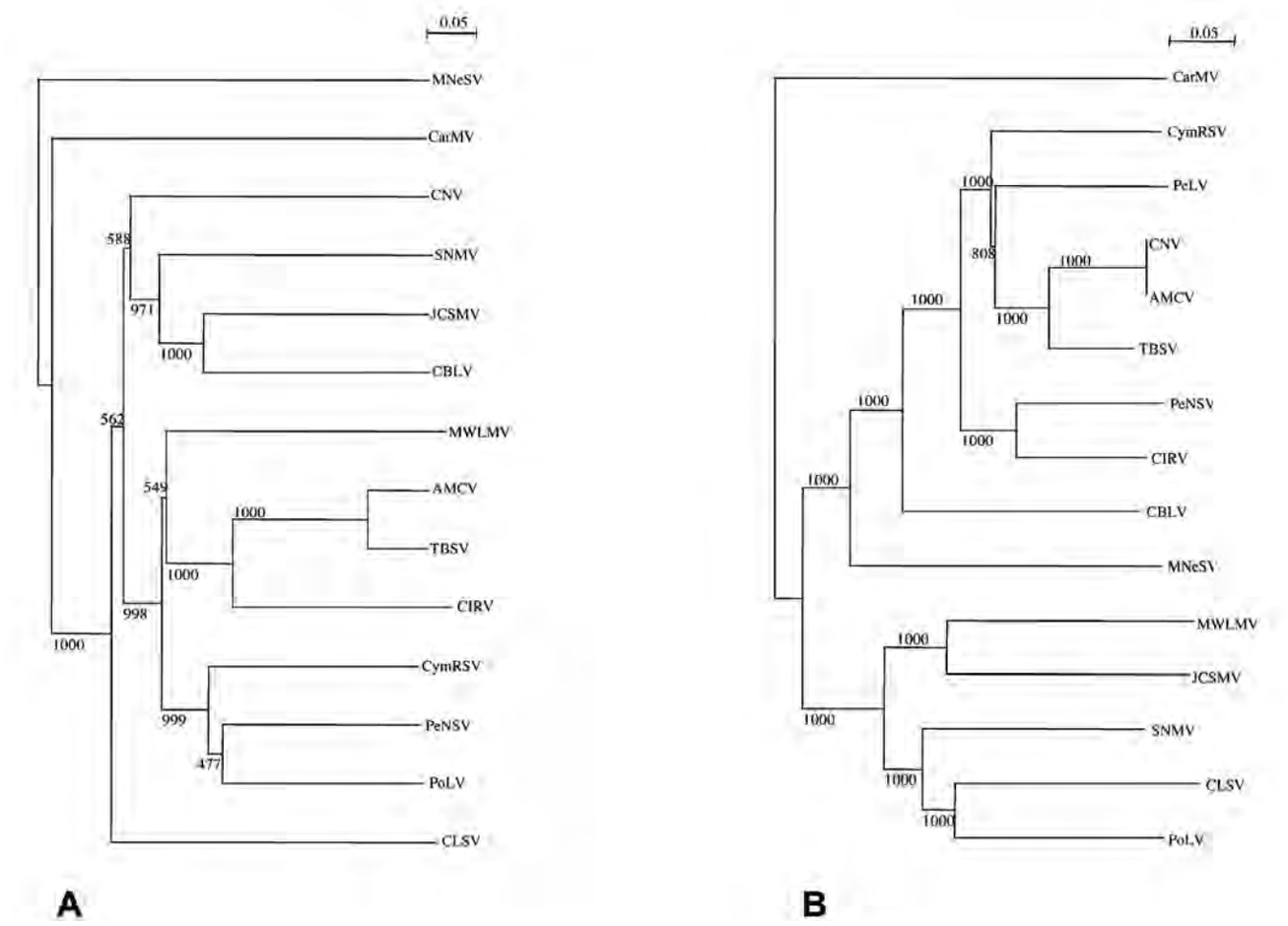

Fig. 5. Phylogenetic analysis of species in the genera *Tombusvirus* and *Aureusvirus*, the two taxa of family *Tombusviridae* sharing a similar genome organization, and Maize necrotic streak vieus (MNeSV) which was recently proposed as unassigned species in the family *Tombusviridae.* A. Deduced coat protein sequences. B. Whole genome RNA sequences. Maize white line mosaic virus (MWLMV, NC_00533); Johnsonsgrass chlorotic stripe mosaic virus (JCSMV,NC_00528), Sesame necrotic mosaic virus (SNMV, DQ367845); *Cucumber leafspot virus* (CLSV, EU127904); *Pothos latent virus* (PoLV, X87115); *Pear latent virus* [(= *Eggplant mottled crinkle virus*), PeLV, AY100482)]; *Cymbidium ringspot virus* (CymRSV, X15511); *Cucumber necrosis virus* (CNV, M25270); *Artichoke mottled crinkle virus* (AMCV, X62493); *Tomato bushy stunt virus* (TBSV, NC_001554); *Pelargonium necrotic spot virus* (PeNSV, AJ607402); *Carnation Italian ringspot virus* (CIRV, X85215); *Cucumber Bulgarian latent virus* (CBLV, AY163842); *Maize necrotic streak virus* (MNeSV, AF266518).

ing in intracellular membranes. The proteins encoded by ORF 3, 4, and 5 were identified as the coat (CP), movement (MP) and silencing suppressor (SP) proteins, respectively, based on their position in the genome (Fig. 4) and the presence of specific conserved motifs (White and Nagy, 2004).

Pairwise amino acid sequence comparison of each protein encoded by the MWLMV genome with the comparable gene products of selected tombusviruses and all sequenced members of the genus *Aureusvirus* showed moderate to low identity with tombusviruses and a somewhat higher identity with aureusviruses (Table 1). In a phylogenetic tree constructed with CP sequences of tombus- and aureusviruses, MWLMV CP did not cluster with that of any of the aureusviruses, which were scattered throughout the tree (Fig. 5A). By contrast, in trees constructed with the whole genome sequence there was a clear-cut clustering of MWLMV with all members of the genus *Aureusvirus* (Fig. 5B). Identical clustering was observed in trees constructed with sequences of the readthrough domain of the replicase protein (p89), the movement protein (p25) and the silencing suppressor (p15) (not shown). Interestingly, in all these trees, MWLMV grouped consistently with JCSMV in a distinct clade (Fig. 5B).

Because of its morphological and molecular properties and phylogenetic relationships MWLMV seems to be a *bona fide* member of the genus *Aureusvirus*. However, since it is molecularly distinct from all sequenced species of this genus, is serologically unrelated to two of them (PoLV and JCSMV), and induces a different cytopathology in infected tissues (Sabanadzovic *et al*., 1995; Izadpanah *et al*., 1993) we suggest it to be regarded as a distinct species in the genus.

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