

A multipartite single-stranded negative-sense RNA virus is the putative agent of fig mosaic disease

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Several dsRNA bands (approx. 0.6–7 kbp in size) were recovered from tissues of mosaic-diseased fig seedlings which contained the enveloped round structures known as double membrane bodies (DMBs). BLAST analysis of a 4353 and a 1120 nt sequence from the two largest RNA segments showed homology with the polymerase and the putative glycoprotein precursor genes of negative-sense single-stranded RNA viruses of the family *Bunyaviridae*. Negative- and positive-sense riboprobes designed from both RNA segments hybridized to two bands of approximately 7 and 2.3 kbp in Northern blots of dsRNAs. Thus, these segments were identified as putative RNA-1 and RNA-2 of a novel virus for which the name fig mosaic virus (FMV) is proposed. Identity levels of predicted amino acids of the protein encoded by FMV RNA-1 with those of species of the family *Bunyaviridae* and European mountain ash ringspot-associated virus (EMERaV) were 28 and 54 %, respectively. RNA-2 showed 38 % identity at the amino acid level only with EMARaV. RNA-1 segment contained five conserved motifs (A–E) and an endonucleolytic centre of comparable genes of L RNA of bunyaviruses and EMARaV RNA-1. In a phylogenetic tree constructed with RdRp sequences, EMARaV grouped with FMV in a clade distinct from those of all bunyavirus genera. The consistent association of DMBs with mosaic symptoms and the results of molecular investigations strongly indicate that DMBs are particles of FMV, the aetiological agent of fig mosaic disease.

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INTRODUCTION

Fig (*Ficus carica*) mosaic disease (FMD), the only currently known infectious disorder of this species, has an extremely variable symptomatology (Condit & Horne, 1933; Martelli *et al.*, 1993). FMD is graft- but not seed-transmissible (Condit & Horne, 1933; Blodgett & Gömeç, 1967) and, in nature, is spread by the eriophyid mite *Aceria ficus* with a semipersistent modality (Flock & Wallace, 1955; Proeseler, 1972; Slykhuis, 1973). Although a number of different isometric and filamentous viruses have been found in infected trees (Quacquarelli, 1971; Grbelja & Eric, 1983; Namba, 1983; Doi, 1989; Serrano *et al.*, 2004; Martelli *et al.*, 1993; Elbeaino *et al.*, 2006, 2007; Castellano *et al.*, 2007), none of them was identified as the possible causal agent of the disease. Thus, the aetiology of FMD is still undetermined.

The GenBank/EMBL/DDBJ accession nos of FMV RNA-1 and FMV RNA-2 determined in this study are AM941711 and FM864225, respectively.

Electron microscope observations of thin-sectioned tissues of symptomatic fig leaves have shown the presence of peculiar membrane-bound, round to ovoid electron-dense structures 90–200 nm in diameter in the cytoplasm of parenchyma cells, known as double membrane bodies (DMBs; Bradfute *et al.*, 1970).

DMBs have an envelope consisting of a unit membrane approximately 12 nm thick, which is apparently acquired from the endoplasmic reticulum (Martelli *et al.*, 1993; Appiano *et al.*, 1995), contain proteinaceous material and fine fibrils, and are often next to aggregates of convoluted electron-dense filamentous elements that contain carbohydrates and are partly digested by pronase (Martelli *et al.*, 1993; Appiano & Conti, 1993). DMBs are insensitive to tetracycline (Martelli *et al.*, 1993).

DMBs are a consistent feature of diseased figs, regardless of the variety and the country of origin of infected samples (Plavsic & Milicic, 1980; Martelli *et al.*, 1993; Appiano *et al.*, 1995; Castellano *et al.*, 2007). Because of this and of their

cytochemical and ultrastructural properties, DMBs were suggested to represent possible particles of an uncharacterized virus (Martelli *et al.*, 1993; Ahn *et al.*, 1996).

As reported in the present paper, multiple dsRNAs were extracted from symptomatic fig seedlings and used for the partial molecular characterization of what seems to be a novel virus with a multipartite, single-stranded, negative-sense RNA genome, herein referred to as fig mosaic virus (FMV).

METHODS

Plant material. In summer 2007, 15 symptomless fig seedlings at the six leaf stage were placed in a glasshouse box at 28–30 °C, which also contained potted symptomatic rooted cuttings from a mosaic-infected fig tree infested naturally by *A. ficus*. Within a couple of weeks, the leaves of eight seedlings began to show chlorotic ringspots which increased in size, coalesced, and evolved into a mosaic pattern (Fig. 1). These seedlings, but not the symptomless ones, hosted small populations of the mite.

Electron microscopy. Pieces of leaf tissue from symptomatic and symptomless seedlings and symptomatic fig rooted cuttings were processed according to standard procedures (Martelli & Russo, 1984), i.e. fixation for 2 h at 4 °C in 4% glutaraldehyde in 0.05 M phosphate buffer, post-fixation in 1% osmium tetroxide, staining overnight in 2% aqueous uranyl acetate, dehydration in graded ethanol dilutions and embedding in TAAB low viscosity resin. Thin sections were stained with lead citrate and viewed with a Philips Morgagni 268 electron microscope.

Extraction of total nucleic acids (TNAs). TNAs were extracted from 100 mg leaf tissue according to Foissac *et al.* (2001), i.e. grinding in liquid nitrogen and homogenization in 1 ml extraction buffer (6 M guanidine isothiocyanate, 0.2 M sodium acetate, 1 M potassium acetate, 0.025 M EDTA, 2.5% PVP-40), addition of 6 M sodium iodide and 0.15 M sodium sulphite, 150 µl ethanol and 25 µl silica particles suspension (1 g ml⁻¹ pH 2.0). After stripping by heat treatment in sterile water (70 °C for 3 min) and centrifugation for 3 min at 16 000 g, TNAs were recovered and stored at -20 °C until use.

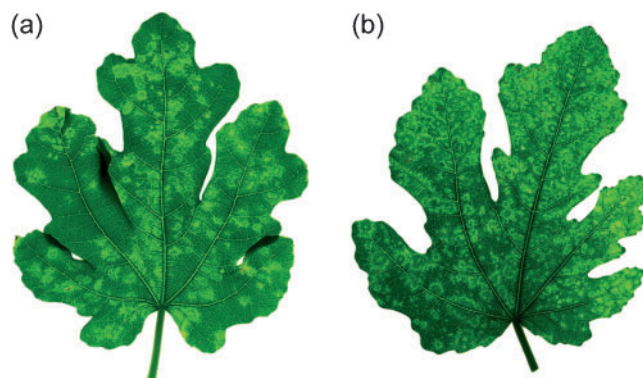


Fig. 1. Leaves from fig seedlings showing FMD symptoms (chlorotic ringspots and mosaic) about three weeks (a) and two months (b) after the appearance of symptoms.

Extraction of double-stranded RNA. Leaf or young root tissues (15–30 g) from symptomatic and symptomless seedlings were used to recover dsRNAs by phenol/chloroform extraction and chromatography through cellulose columns in the presence of 17% ethanol, according to a modified protocol of Dodds (1993). Tissue samples were frozen in liquid nitrogen, ground to a fine powder and homogenized in extraction buffer consisting of 60 ml 1× STE (0.1M Tris/HCl, 0.2 M NaCl, 2 mM EDTA, pH 7.0), 1.6 ml 2b-mercaptoethanol, 30 ml SDS 10% and 50 ml phenol/chloroform (1:1 v/v), respectively, followed by vigorous shaking for 30 min. After centrifugation at 15 300 g for 20 min, 1:5 volume of 96% ethanol and 3 g CF11 cellulose (Sigma) were added to the supernatant and the mixture was again shaken for 30 min. The cellulose was pelleted by centrifugation (10 min at 5500 g) and washed three times in 40 ml washing buffer (1× STE with 17% ethanol). The cellulose was eluted stepwise with 13 ml elution buffer (1× STE). Cellulose CC41 (0.5 g) and 1:5 volume of 96% ethanol were then added to the eluted solution, washed twice and eluted again with 2 ml elution buffer in two aliquots of 1 ml each. DsRNA was then precipitated with 1:10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volume ethanol at -20 °C overnight, and resuspended in double-distilled and DEPC-treated water. DNA and single-stranded RNA were sequentially enzyme-digested as described by Saldarelli *et al.* (1994). A dsRNA quantity roughly equivalent to 5 g of tissues was electrophoresed in 5% TAE polyacrylamide gel and revealed by silver staining.

Strategies for reverse transcription, cloning and sequencing.

Two hundred and fifty nanograms of nuclease-treated dsRNA in 9 µl of water were denatured by boiling at 95 °C for 10 min, chilled on ice and incubated at room temperature for 10 min in the presence of 1 µg random hexamers and 1 µl methyl mercuric hydroxide (100 mM; Saldarelli *et al.*, 1994). The methyl mercuric hydroxide was inactivated by adding 1 µl 2b-mercaptoethanol 0.5 M. Denatured dsRNA were reverse transcribed with the Moloney murine leukemia virus reverse transcriptase enzyme for 1 h at 39 °C according to the manufacturer's instruction (Invitrogen), in a final volume of 20 µl.

Degenerate oligo primed PCR (DOP-PCR), RT-PCR and rapid amplification of cDNA ends PCR (RACE-PCR) were used for the amplification and cloning of FMV sequences.

For DOP-PCR, performed on random-primed cDNA, the primer DOP6 (5'-CCGACTCGAGNNNNNTTCAGG-3') gave a consistent pattern of PCR amplicons. In this protocol, 50 µl PCR mixture contained 4 µl reverse transcription reaction, 0.5 mM each dNTP, 2 µM DOP6 primer, 1× PCR buffer (Roche) and 0.5 µl *Taq* DNA polymerase (5 U µl⁻¹; Roche). PCR was carried out in a Perkin-Elmer 7600 thermal cycler as described by Rott & Jelkmann (2001).

Following a preliminary alignment of some sequenced viral clones, sets of specific primers were used for closing sequence gaps. PCR was performed on a random-primed reverse-transcribed dsRNA template as described by Rott & Jelkmann (2001), but at an annealing temperature of 58 °C. Sequences of some of the viral clones showed high homology with the polymerase gene coded by L RNA of members of the family *Bunyaviridae* (De Haan *et al.*, 1991) and RNA-1 of the bunyavirus-like European mountain ash ringspot-associated virus (EMARaV) (Mielke & Muehlbach, 2007). Therefore, amino acid sequences of the largest RNAs of EMARaV (GenBank accession no. AY563040), tomato spotted wilt virus (TSWV, D10066), groundnut bud necrosis virus (GBNV, AF025538) and watermelon silver mottle virus (WSMoV, NC_003832) were aligned and two degenerate primers were designed on the conserved polymerase motifs A (BUN1-s: 5'-TCNAARTGGTCNGC-3') and C (BUN3-a: 5'-ATCATCAGANTGNACNAT-3'). PCR conditions under which these primers were used were 2.5 mM MgCl₂ (final concentration), 0.5 mM dNTPs, 2 µM each primer, 0.3 µl *Taq* polymerase (5 U µl⁻¹) in a final volume of 25 µl. PCR was carried out at 94 °C for 4 min,

followed by 35 cycles at 94 °C for 30 s, 45 °C for 1 min and 72 °C for 30 s. Final extension was at 72 °C for 7 min.

For 5' RACE-PCR, a poly(A) tail was added at the 3' terminus of total dsRNAs preparations using the poly(A) polymerase enzyme (Promega). A complementary oligo(dT) primer was used to reverse transcribe the termini of the tailed dsRNAs. For amplifying the viral 5' genome terminus, 5 µl cDNA were used in PCR with an antisense-specific primer (E5-a: 5'-AACACTGTTTTGCGATTGG-3') designed on a DOP-PCR clone denoted E5 (see Fig. 4), together with the oligo(dT) primer. PCR consisted of 35 cycles at 94 °C for 30 s, 42 °C for 30 s, 72 °C for 2 min and final extension at 72 °C for 7 min. A similar strategy was used to amplify the 5' terminus of the second virus genome segment obtained by DOP-PCR (clone F1, see Fig. 4), using the antisense-specific primer F1-a (5'-CAGAACCATCACAACTGAAGT-3').

3' RACE-PCR was used to further extend the sequence of the putative RNA-1 beyond motif C (see Fig. 4). In this case, the denatured dsRNAs were reverse-transcribed with a sense-specific primer (MotC-s: 5'-TGAACCCATATCTCCATCCTG-3'), designed on the sequence close to the end of motif C. The primer P1 (5'-GATCCACTAGTTCTAGAGCGGC-3') was ligated to the 3' end of the synthesized cDNA using the terminal deoxynucleotidyl transferase enzyme (TdT; Promega). P2 primer, complementary to P1 (Coutts & Livieratos, 2003, modified), was used together with primer MotC-s for amplification. PCR conditions consisted of 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min and final extension at 72 °C for 7 min.

All PCR products were directly cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions and used to transform *Escherichia coli* DH5 α competent cells. Selected clones were subjected to automated sequencing (Primm) and sequences were assembled with the Strider 1.1 program (Marck, 1988). Alignment for nucleotide and amino acid homology was done with the CLUSTAL_X program (Thompson *et al.*, 1997). Homology with known proteins from the Protein Information Resources (PIR, release 47.0) was determined using the FASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs. Phylogenetic trees were constructed and bootstrap analysis made with the NEIGHBOR, SEQBOOT, PROTDIST and CONSENSE programs of the PHYLIP package (Felsenstein, 1989).

Molecular detection of FMV in tissue extracts. TNA extracts from four symptomatic and two symptomless seedlings which had been observed under the electron microscope for the presence of DMBs were assayed by RT-PCR and molecular hybridization.

Primers E5 s (5'-CGGTAGCAAATGGAATGAAA-3') and E5-a (5'-AACACTGTTTTGCGATTGG-3'), which amplify a 302 bp DNA fragment from viral RNA-1, were used for virus detection. Randomly primed cDNA (from either TNA or dsRNA) was added to 2.5 µl *Taq* polymerase buffer 10 \times (Promega) containing a final concentration of 1 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM each specific primer and 0.2 µl *Taq* polymerase (5 U µl⁻¹) in a final volume of 25 µl. PCR consisted of an initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Final extension was at 72 °C for 7 min. Amplification products were visualized in 6% polyacrylamide slab gels run in TBE buffer.

Hybridization was performed on Northern blots of dsRNA extracts and dot blots of TNAs, again from symptomatic and symptomless seedlings. For Northern blots, approximately 50 ng dsRNA were boiled for 5 min, chilled on ice, separated in a denaturing 1% agarose/formaldehyde gel, and transferred onto a Hybond-N+ nylon membrane (GE Healthcare, USA) as described by Chomczynski (1992). TNA extracts (2 µg) were denatured at 65 °C for 5 min in a 50 mM NaOH solution before spotting onto nylon membranes. Hybridization was performed overnight at 56 °C, with digoxigenin-

labelled positive- and negative-sense RNA probes, designed on the sequences of viral clones E5 and F1, as described by Astruc *et al.* (1996). Blots were washed twice for 15 min each in 0.1 \times saline sodium citrate (SSC) and 0.1% SDS at 65 °C. Hybridized RNA bands and spots were visualized using the DIG chemiluminescent detection protocol (Roche), following the manufacturer's instructions. Films were exposed for 20–30 min, and resulting images were digitized.

RESULTS

Electron microscopy

Leaves of the naturally infected glasshouse-grown rooted cuttings and of all symptomatic seedlings exposed to *A. ficus* infestation contained DMBs (Fig. 2a, b). In contrast, no such structures were found in the leaves of symptomless seedlings (not shown). This not only confirmed the alleged ability of *A. ficus* to transmit fig mosaic disease, but was taken as evidence that DMBs are mite-transmissible.

dsRNAs extraction and analysis

dsRNA extraction from symptomatic leaf tissues was unsatisfactory, probably because of the high content of contaminants (polyphenols and polysaccharides) in the preparations. The procedure, however, gave better results when starting materials were young succulent root tissues from symptomatic seedlings. From this material different dsRNA bands, with size ranging from 0.6 kbp to approximately 7 kbp, were obtained (Fig. 3). A consistent pattern of four major bands was observed in repeated extractions from different mosaic-diseased seedlings and from greenhouse-grown mosaic-infected rooted cuttings. Additional faint bands, with a size smaller than that of the main RNAs, were also observed. The nature of these bands, i.e. whether they represent FMV subgenomic RNAs or RNAs of a contaminant latent virus, was not ascertained. The point remains that hybridization assays with probes designed on FMV RNA-1 and RNA-2 sequences gave a

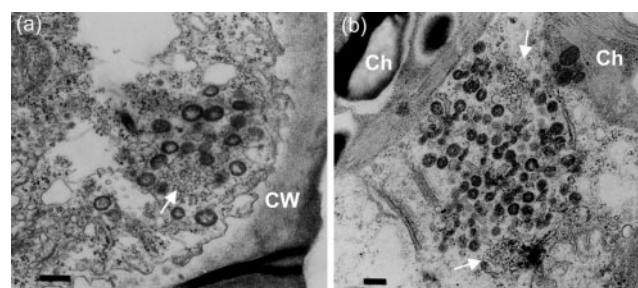


Fig. 2. Groups of DMBs, i.e. FMV particles, in the cytoplasm of cells from a naturally infected fig (a) and a seedling exposed to *A. ficus* (b). Arrows point to the aggregates of convoluted electron-dense filamentous elements that usually accompany DMBs. CW, Cell wall; Ch, chloroplast. Bar, 200 nm.

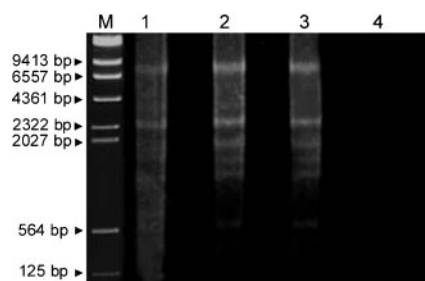


Fig. 3. Electrophoretic pattern of dsRNA extracted from young roots of symptomatic fig seedlings containing DMBs (lanes 1, 2 and 3). The profile shows several dsRNA bands ranging from approximately 0.6 to approximately 7 kbp. No dsRNAs were recovered from symptomless DMB-free fig seedlings (lane 4). DNA molecular marker (λ /HindIII) in lane M.

positive signal only with the largest dsRNAs (see Fig. 6). No dsRNA bands were found in preparations from symptomless seedlings which, as shown by electron microscope observations of thin-sectioned leaf tissues, did not contain DMBs.

Cloning and analysis of virus sequences

Denatured reverse-transcribed dsRNA subjected to amplification by DOP-PCR yielded amplicons from 300 to 600 bp in size. Of 35 clones sequenced, only six (E1, E3, E5, E7, E11 and F1) proved to be of viral origin (Fig. 4), as they shared homology with those of bunyavirus sequences

following BLAST analysis. In particular, all E clones aligned with sequences encoded by bunyavirus L RNA and EMARaV RNA-1. Thus it was possible to expand the sequence by closing the gaps between the sequenced clones by specific RT-PCR.

Degenerate primers BUN1-s and BUN3-a amplified a 384 bp product from FMV RNA-1, which comprises three conserved motifs, A, B and C, of the RdRp gene (Fig. 4, fragment 'Deg'). RACE-PCR toward the 5' and 3' termini of RNA-1, using the virus-specific primers E5-a and MotC-s, amplified the 5' terminus, yielding a product of 910 bp, and an amplicon of 550 bp beyond motif C, respectively (Fig. 4).

In summary, the strategy encompassing the use of DOP-PCR, RACE-PCR and RT-PCR yielded a 4353 nt sequence (GenBank accession no. AM941711) in the polymerase gene at the 5' terminus of FMV RNA-1 (Fig. 4).

Comparison of the predicted amino acid sequence of FMV RNA-1 with comparable sequences of different members of the family *Bunyaviridae* revealed the presence of five motifs (A–E), which represent a highly conserved region of the RdRp gene (Fig. 5). Various levels of identity at the amino acid level were found, which never exceeded 28%. However, 54% identity was found with the sequence of the RdRp gene of EMARaV RNA-1. According to computer-assisted analysis and comparable amino acids located after the Motif E of EMARaV and members of family *Bunyaviridae*, an additional highly conserved domain of bunyaviral RdRp, named F (Fig. 4), was also identified (Aquino *et al.*, 2003; Duijsings *et al.*, 2001).

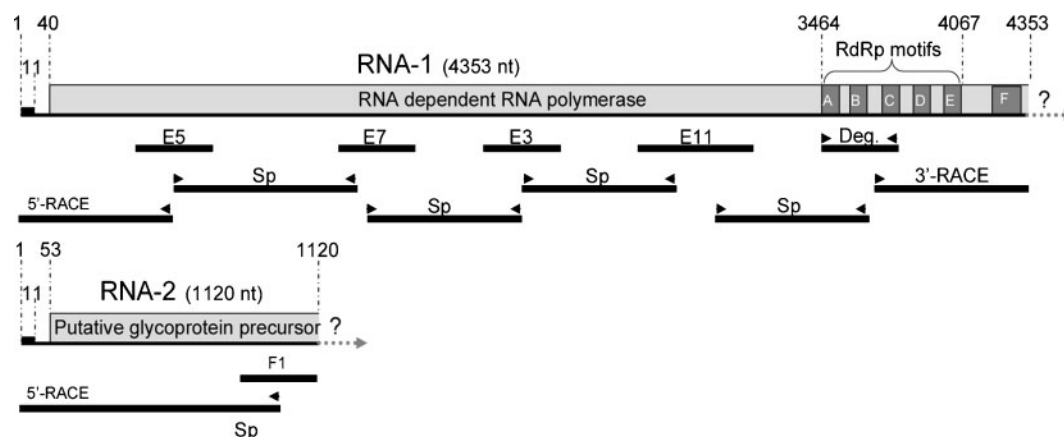


Fig. 4. Schematic representation of the strategy based on the combined use of DOP-PCR, RACE-PCR and RT-PCR, used for cloning FMV RNA-1 and RNA-2. DOP-PCR amplifications are represented by clones denoted E (RNA-1 segment; RdRp gene) and F (RNA-2 segment; putative glycoprotein precursor gene). The *Bunyaviridae*-degenerate primers amplified a sequence (Deg) comprising two conserved motifs, A and C, on RNA-1. Arrows represent the sense- and antisense-specific primers to close the sequence gaps. Sp represents the cloned gaps amplifications using the specific primers. 5'-RACE amplified both 5' termini of RNA-1 and RNA-2, while 3'-RACE extended the sequence beyond Motif C up to F. The protein translation of RNA-1 initiates with a start codon at position 40, and at position 53 in RNA-2.

		Motif A	Motif B	Motif C	Motif D	Motif E	
FMV	1137-	WAEIYSVSSDASKWSARD	NYFTVRSNWLGQNLNMISSFVHH	MVHSDDSTYDF	ITLNEKITYIS	KEFLSTIIVGN	-1338
EMARaV	1120-	KTEIYSVSSDASKWSARD	NWFNVRSNWLGQNLNMTSSFVHH	MVHSDDSTYDF	ITLNEKITYIS	KEFLSTIIVSN	-1323
TSWV	1358-	KSRLEAFSLADQSKWSASD	NTYPVSMNWLGQNLNLYSSVYHS	IVHSDDNATSL	ITLNPCKSYAS	VEFISERIVNG	-1549
I WSMoV	1361-	ECKMAFLSADQSKWSASD	NTFPVSMNWLGQNLNLYSSVYHS	MVHSDDNATSI	ITLNPCKSYAS	VEFISERIING	-1561
GBNV	1361-	ECKMAFLSADQSKWSASD	NTFPVSMNWLGQNLNLYSSVYHS	MVHSDDNATSI	ITLNPCKSYAS	VEFISERIING	-1561
BUNV	1028-	KALKLEINADMSKWSAQD	NYVQIKRNWLGQNFNYISSVYHS	MVHSDDNQTSI	CQANMKKTYIT	KEFVSLFNLHG	-1123
LACV	1051-	KGLKMEINADMSKWSAQD	NTVLIKRNWLGQNFNYISSVYHS	LHSDDNQTSI	CQANMKKTYIT	KEFVSLFNLHG	-1246
II OROV	1036-	RGLKIEINADMSKWSAQD	NTVEIKRNWLGQNLNLYSSVYHS	MVHSDDNQTSI	NOANMKKTYLT	KEFVSLFNLHG	-1231
DOBV	963-	KRKLMYVSADATKWSPGD	HCGEVRGNWLGQNLNKCSSLFGV	AHSDDALFIY	IKISPCKTTLS	AEFLSTFFESC	-1179
III PUUV	963-	KRKLMYVSADATKWSPGD	VSASIKGNWLGQNLNKCSSLFGV	AHSDDALFIY	IKISPCKTTVS	AEFLSTFFESC	-1179
HTNV	963-	KRKLMYVSADATKWSPGD	HHGEVKGWLGQNLNKCSSLFGV	AHSDDALFIY	IKISPCKTTVS	AEFLSTFFESC	-1179
RVFV	982-	PVWTCATSDARKWNQGH	TYLETTTGMQGIHYTSSLLHT	MQGSDSSMLI	IYPSEKSTANT	MEYNSEFFYHT	-1195
IV TOSV	983-	SVWTCATSDARKWNQGH	TYLKTSTGMQGIHYTSSLLHT	MQGSDSSMLI	IYPSEKSTPNT	MEYNSEFFYHT	-1197
UUKV	985-	HHETVATSDDAKWNQCH	AFVQETGTGMQGIHYTSSLLHT	LQSSDSSGMMI	IYSSVKSTNNT	LEFNSEFFYHT	-1201
V RSV	1479-	EYMTISTSDASKWNQGH	SYIETETGMQGIHYTSSLLFHA	MESSDSSSFI	IYKSPKSTTQT	MEFNSEFFYHT	-1700
RGSV	1510-	SYFTVCTSDASKWNQGH	TYIETESGFMQGIHYTSSLLFHA	MESSDSSSMMI	IYKSIKSTTGT	MEFNSEFFYHT	-1729
VI DUGV	2437-	FFKTVCSIGDNTKWGPIH	MAMNSYNHMGQGIHYTSSLLTS	AGSSDDYAKCI	QMKDSAKTLVG	LEFYSEFFMGN	-2671

Fig. 5. Amino acid alignment between conserved RdRp motifs A–E of FMV (AM941711), EMARaV (AY653040), members of five genera belonging to the family *Bunyaviridae* and two members of the unassigned genus *Tenuivirus*. I, Genus *Tospovirus*: TSWV, tomato spotted wilt virus (GenBank accession no. D10066); WSMoV, watermelon silver mottle virus (NC003832); GBNV, groundnut bud necrosis virus (AF025538). II, Genus *Orthobunyavirus*: LACV, La Crosse virus (GenBank accession no. U12396); BUNV, Bunyamwera virus (X14383); OROV, Oropouche virus (AF484424). III, Genus *Hantavirus*: DOBV, Dobrava-Belgrade virus (GenBank accession no. AJ410619); HTNV, Hantaan virus (X55901); PUUV, Puumala virus (Z66548). IV, Genus *Phlebovirus*: RVFV, Rift Valley fever virus (GenBank accession no. X56464); ToSV, Toscana virus (X68414); UUKV, Uukuniemi virus (D10759). V, Genus *Tenuivirus*: RSV, rice stripe virus (GenBank accession no. AY186787); RGSV, rice grassy stunt virus (AF509470). VI, Genus *Nairovirus*: DUGV, Dugbe virus (GenBank accession no. U15018).

The 5' RACE extension of clone F1 (395 bp) produced an additional PCR fragment 725 bp in size which yielded an overall stretch of 1120 bp at the 5' end of the putative viral RNA-2 (GenBank accession no. FM864225). The expression product of this genomic region was recognized by a BLAST search as the glycoprotein precursor of bunyaviruses showing 38% amino acid identity with the comparable region of EMARaV (Mielke & Muehlbach, 2007).

Alignment of the 5' proximal sequence of RNA-1 and RNA-2 with those of EMARaV and various bunyaviruses showed a conserved stretch of 15 nt (5'-AGUAGUGUUCUCCTC-3') homologous to the 5' terminus of RNA-1 of EMARaV and to the terminal 11 nt of L RNAs of representatives of the genera *Orthobunyavirus* (5'-AGUAGUGUGCU-3') and *Hantavirus* (5'-AGUAGUaUGCU-3'). No such similarity was found with the comparable terminal sequences of L RNA of members of the genera *Tospovirus*, *Nairovirus*, *Phlebovirus* and *Tenuivirus*.

Probe E5 (positions 852–1154 of RNA-1) hybridized to the largest dsRNA band (approx. 7 kbp) (Fig. 6a), thus confirming that it is the replicative form of FMV RNA-1. Likewise, the positive Northern blot hybridization with the F1 probe provided evidence that the smaller dsRNA is the replicative form of FMV RNA-2 (Fig. 6b).

In a phylogenetic tree constructed with polymerase sequences of members of the family *Bunyaviridae* and EMARaV, FMV clustered with EMARaV in a separate clade close to representatives of the genera *Tospovirus*, *Hantavirus* and *Orthobunyavirus* (Fig. 7).

FMV detection in fig seedlings

In dot spot hybridization assays, the digoxigenin-labelled riboprobes synthesized from the positive strand of FMV RNA-1 sequence hybridized TNA extracted from symptomatic DMB-containing fig seedlings (Fig. 8a). No hybridization signal was obtained with extracts from symptomless DMB-free seedlings. The negative strand riboprobe gave a very weak signal resulting from the

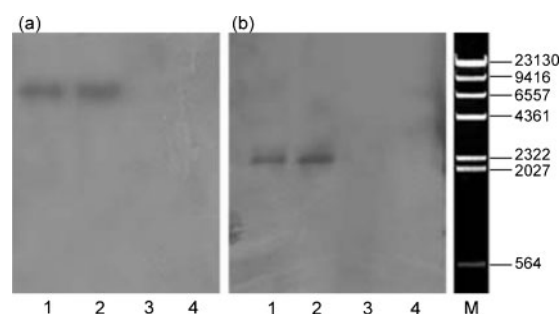


Fig. 6. Northern blot hybridization of symptomatic and symptomless fig accessions using a digoxigenin-labelled RNA probe designed on E5 and F1 clones of RNA-1 and RNA-2, respectively. The riboprobes, constructed on clones E5 and F1, hybridized a dsRNA species of approximately 7 kbp (a) and approximately 2.3 kbp (b) from two symptomatic seedlings (lanes 1 and 2). No hybridization of extracts from symptomless seedlings was obtained (lanes 3 and 4). DNA molecular marker (λ HindIII) in lane M.

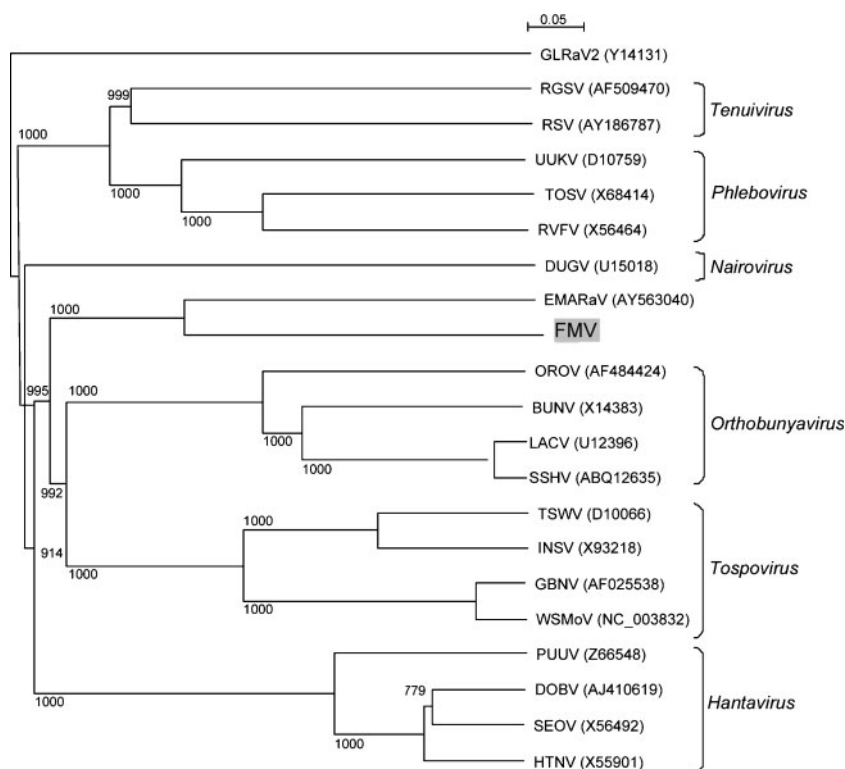


Fig. 7. Phylogenetic tree constructed with predicted aa sequences of FMV RNA-1 RdRp and the L RNA polymerase of members of the family *Bunyaviridae* and the unassigned genus *Tenuivirus*. Predicted aa sequences were aligned, and their phylogenetic relationships determined using the CLUSTAL_x Multiple Sequence Alignment Program (Thompson *et al.*, 1997). Bootstrap values are shown on the branches. BUNV, Bunyamwera virus; DOBV, Dobrava-Belgrade virus; DUGV, Dugbe virus; EMARaV, European mountain ash ringspot-associated virus; GBNV, groundnut bud necrosis virus; GLRaV2, grapevine leafroll associated virus 2; HTNV, Hantaan virus; INSV, *Impatiens* necrotic spot virus; LACV, La Crosse virus; OROV, Oropouche virus; PUUV, Puumala virus; RGSV, rice grassy stunt virus; RSV, rice stripe virus; RVFV, Rift Valley fever virus; SEOV, Seoul virus; SSHV, snowshoe hare virus; TOSV, Toscana virus; TSWV, tomato spotted wilt virus; UUKV, Uukuniemi virus; WSMoV, watermelon silver mottle virus. GLRaV2 was used as an outgroup.

hybridization of this probe to mRNA in the infected cells (Figs 8, 2b and 3b). These findings were taken as further evidence of the strict relationships between DMBs and FMD, and as a strong indication that, most likely, FMV

RNA-1 accumulates in infected tissues as negative strand. Similar results were obtained when the F1 positive- and negative-sense riboprobes were used for dot spot hybridization of TNA (not shown).

The E5 primers used for RT-PCR on nucleic acid extract templates from infected fig seedling proved efficient and reliable as a detection tool.

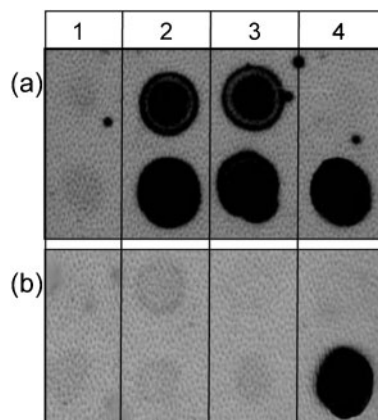


Fig. 8. Dot blot hybridization of TNA extracts from symptomatic and symptomless fig seedlings previously observed under the electron microscope for the presence of DMBs. (a) The positive-sense riboprobe did not give hybridization signals with symptomless DMB-free extracts (lane 1). On the contrary, symptomatic DMB-containing seedlings gave a positive reaction (lanes 2 and 3). (b) Very weak hybridization signals were obtained from the infected samples when the negative-sense riboprobe was used (e.g. lane 2). Plasmid E5 used as a positive control in lane 4.

DISCUSSION

In the present investigation there was a complete agreement between the results of RT-PCR, molecular hybridization tests, dsRNA analysis and electron microscope observations of symptomatic and symptomless fig seedlings. Only symptomatic plants were PCR-positive, gave a hybridization signal, and contained dsRNAs and DMBs. This can be retained as evidence that, as previously suggested (Martelli *et al.*, 1993; Ahn *et al.*, 1996), DMBs are indeed the particles of an enveloped virus eliciting FMD, for which the name FMV seems appropriate. In this context, it is worth mentioning that dsRNAs, with sizes ranging from 0.6 to 6.6 kb, were recovered in Portugal and Turkey from mosaic-diseased figs (Nolasco & de Sequeira, 1991; Açıkğös & Döken, 2003), which further supports our conclusions.

Comparative analysis of the available sequence of FMV RNA-1 and RNA-2 with those of members of the family *Bunyaviridae* showed that it has a low level of similarity with all these viruses (23–28 % identity at the amino acid level). A higher correlation (54 % identity) was found with

EMARaV. Likewise, RNA-2 showed identity only with EMARaV, though at lower level (approx. 38%). Notwithstanding this similarity, FMV and EMARaV are clearly distinct viruses. In fact, EMARaV resembles tospoviruses in the shape and size of the particles and cytopathology of infected cells (Ebrahim-Nesbat & Izadpanah, 1992), but differs enough molecularly from them, to be assigned to a putative new genus denoted *Emaravirus* (Benthack *et al.*, 2005; Mielke & Muehlbach, 2007).

FMV is much closer to a range of different viruses infecting herbaceous and woody hosts, all of which are transmitted by eriophyid mites and have the same type of virions and comparable cytopathological features (Bradfute *et al.*, 1970; Kim & Martin, 1978; Gergerich *et al.*, 1983; Gergerich & Kim, 1983; Ahn *et al.*, 1996; Kumar *et al.*, 2002, 2003; Skare *et al.*, 2006).

Two of these viruses, pigeonpea sterility mosaic virus (PPSMV) and maize red stripe mosaic virus (Skare *et al.*, 2006), renamed by the same authors wheat mosaic virus (WMV), differ biologically from FMV for they can be transmitted artificially either by sap inoculation (PPSMV) or vascular puncture (WMV). However, like FMV, both these viruses have a multipartite, negative-sense, single-stranded RNA genome. In particular, five to seven RNA molecules with size ranging from approximately 1.1 to 6.8 kb were recovered from PPSMV particles (Kumar *et al.*, 2003), whereas WMV has three RNA molecules approximately 8 kb (RNA-1), 2–2.5 kb (RNA-m) and 1.4 kb (RNA-s) in size (Skare *et al.*, 2006). Sequences of a single small genome fragment from each of these viruses are reported in GenBank, i.e. 764 nt from RNA-5 of PPSMV (accession no. AJ439561) and 1329 nt from the nucleocapsid gene of WMV (accession no. DQ324466).

Although neither of these sequences showed any similarity with FMV genome fragments we have sequenced, the point remains that, because of the similarity in genome type and in the epidemiological, morphological and ultrastructural features, FMV, PPSMV and WMV seem to be coherent members of a virus group which, as recently proposed (Kumar *et al.*, 2003; Skare *et al.*, 2006), warrants classification as a novel genus of plant viruses in the family *Bunyaviridae*.

REFERENCES

- Açikgös, S. & Döken, M. T. (2003). The determination of sampling time for dsRNA isolation of the agent of fig mosaic disease prevalent in Aegean region-Turkey. *Acta Horti* **605**, 307–310.
- Ahn, K.-K., Kim, K. S., Gergerich, R. C., Jensen, S. G. & Anderson, E. J. (1996). Comparative ultrastructure of double membrane-bound particles and inclusions associated with eriophyid mite-borne plant disease of unknown etiology: a potentially new group of plant viruses. *J Submicrosc Cytol Pathol* **28**, 345–355.
- Altschul, S. F., Stephen, F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Appiano, A. & Conti, M. (1993). Cytological and cytochemical observations on leaves of fig plants infected by fig mosaic disease. *G Bot Ital (Florence, Italy)* **127**, 300–302.
- Appiano, A., Conti, M. & Zini, N. (1995). Cytopathological study of the double-membrane bodies occurring in fig plants affected by fig mosaic disease. *Acta Horti* **386**, 585–592.
- Aquino, V. H., Morelli, M. & Moraes Figueirdo, L. T. (2003). Analysis of Oropouche virus L protein amino acid sequence showed the presence of an additional conserved region that could harbour an important role for the polymerase activity. *Arch Virol* **148**, 19–28.
- Astruc, N., Marcos, J. F., Macquaire, G., Candresse, T. & Pallás, V. (1996). Studies on the diagnosis of hop stunt viroid in fruit trees: identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. *Eur J Plant Pathol* **102**, 837–846.
- Benthack, W., Mielke, N., Buttner, C. & Muehlbach, H. P. (2005). Double-stranded RNA pattern and partial sequence data indicate plant virus infection associated with ringspot disease of European mountain ash (*Sorbus aucuparia* L.). *Arch Virol* **150**, 37–52.
- Blodgett, E. C. & Gömeç, C. (1967). Fig mosaic. *Plant Disease Reports* **51**, 893–896.
- Bradfute, O. R., Whitmoyer, R. E. & Nault, R. L. (1970). Ultrastructure of plant leaf tissue infected with mite-borne viral-like particles. *Proc Electron Microsc Soc Am* **258**, 178–179.
- Castellano, M. A., Gattoni, G., Minafra, A., Conti, M. & Martelli, G. P. (2007). Fig mosaic in Mexico and South Africa. *J Plant Pathol* **89**, 441–444.
- Chomczynski, P. (1992). One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal Biochem* **201**, 134–139.
- Condit, I. J. & Horne, W. T. (1933). A mosaic of the fig in California. *Phytopathology* **23**, 887–896.
- Coutts, R. H. A. & Livieratos, I. C. (2003). A rapid method for sequencing the 5'- and 3'-termini of double-stranded RNA viral templates using RLM-RACE. *J Phytopathol* **151**, 525–527.
- de Haan, P., Kormelink, R., de Oliveira Resende, R., van Poelwijk, F., Peters, D. & Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *J Gen Virol* **72**, 2207–2216.
- Dodds, J. A. (1993). DsRNA in diagnosis. In *Diagnosis of Plant Virus Diseases*, pp. 273–294. Edited by R. E. F. Matthews. Boca Raton, USA: CRC Press.
- Doi, Y. (1989). Fig virus S. In *Directory and Dictionary of Animal, Bacterial and Plant Viruses*, pp. 76. Edited by R. Hull, F. Brown & C. Payne. London, UK: Nature Publishing Group.
- Duijsings, D., Kormelink, R. & Goldbach, R. (2001). *In vivo* analysis of the TSWV cap-snatching mechanism: single base complementarity and primer length requirements. *EMBO J* **20**, 2545–2552.
- Ebrahim-Nesbat, F. & Izadpanah, K. (1992). Virus-like particles associated with ringfleck mosaic of mountain ash and a mosaic disease of raspberry in the Bavarian forest. *Eur J Forest Pathol* **22**, 1–10.
- Elbeaino, T., Digiario, M., De Stradis, A. & Martelli, G. P. (2006). Partial characterization of a closterovirus associated with a chlorotic mottling of fig. *J Plant Pathol* **88**, 187–192.
- Elbeaino, T., Digiario, M., De Stradis, A. & Martelli, G. P. (2007). Identification of a second member of the family *Closteroviridae* in mosaic-diseased figs. *J Plant Pathol* **89**, 119–124.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Flock, R. A. & Wallace, J. M. (1955). Transmission of fig mosaic by the eriophyid mite *Aceria ficus*. *Phytopathology* **45**, 52–54.

- Foissac, X., Svanella-Dumas, L., Dulucq, M. J., Candresse, T. & Gentit, P. (2001). Polyvalent detection of fruit tree tricho, capillo and foveavirus by nested RT-PCR using degenerated and inosine containing primers (DOP RT-PCR). *Acta Horti* **550**, 37–44.
- Gergerich, R. C. & Kim, K. S. (1983). A description of the causal agent of rose rosette disease. *Arkansas Farm Res* **3**, 7.
- Gergerich, R. C., Kim, K. S. & Kitajima, E. W. (1983). A particle of unique morphology associated with a disease in rose in Northwest Kansas. *Phytopathology* **73**, 500–501.
- Grbelja, J. & Eric, Z. (1983). Isolation of a potyvirus from *Ficus carica* L. *Acta Bot Croat* **42**, 11–14.
- Kim, K. S. & Martin, E. M. (1978). Virus-like particles associated with yellow ringspot of redbud. *Phytopathology News* **12**, 119.
- Kumar, P. L., Duncan, G. C., Roberts, I. M., Jones, A. T. & Reddy, D. V. R. (2002). Cytopathology of *Pigeonpea sterility mosaic virus* in pigeonpea and *Nicotiana benthamiana*: similarities with those of eriophyid mite-borne agents of undefined aetiology. *Ann Appl Biol* **140**, 87–96.
- Kumar, P. L., Jones, A. T. & Reddy, D. V. R. (2003). A novel mite-transmitted virus with a divided RNA genome closely associated with pigeonpea sterility mosaic disease. *Phytopathology* **93**, 71–81.
- Marck, C. (1988). DNA Strider: a “C” programme for the fast analysis of DNA and protein sequences on the Apple Macintosh family computers. *Nucleic Acids Res* **16**, 1829–1836.
- Martelli, G. P. & Russo, M. (1984). Use of thin sectioning for visualization and identification of plant viruses. *Methods in Virology* **8**, 143–224.
- Martelli, G. P., Castellano, M. A. & Laforteza, R. (1993). An ultrastructural study of fig mosaic. *Phytopathol Mediterr* **32**, 33–43.
- Mielke, N. & Muehlbach, H.-P. (2007). A novel, multipartite, negative-strand RNA virus is associated with the ringspot disease of European mountain ash (*Sorbus aucuparia* L.). *J Gen Virol* **88**, 1337–1346.
- Namba, S. 1983. Fig virus S. In *Handbook of Plant Viruses*, pp. 326–327. Edited by K. Yora, Y. Saito, Y. Doi, T. Inoue & K. Tomaru. Tokyo, Japan: Asakura Shoten.
- Nolasco, G. & de Sequeira, O. A. (1991). Double-stranded RNA (dsRNA) associated with fig mosaic disease. In *Proceedings 4th Portuguese-Spanish Biochemistry Congress, Lisbon 1991*, 5 P2-Mo.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- Plavšić, B. & Milicic, D. (1980). Intracellular changes in trees infected with fig mosaic. *Acta Horti* **110**, 281–286.
- Proeseler, G. (1972). Beziehungen zwischen Virus, Vektor und Wirtspflanze am Beispiel des Feigenmosaik-Virus und *Aceria ficus* Cotte (Eriophyoidea). *Acta Phytopathologica Academiae Scientiarum Hungaricae* **7**, 179–186 (in German).
- Quacquarelli, A. (1971). Il mosaico del fico e il virus latente del *Chenopodium*. *Phytopathol Mediterr* **10**, 283–286 (in Italian).
- Rott, M. E. & Jelkmann, W. (2001). Characterization and detection of several filamentous viruses of cherry: adaptation of an alternative cloning method (DOP-PCR), and modification of RNA extraction protocol. *Eur J Plant Pathol* **107**, 411–420.
- Saldarelli, P., Minafra, A., Martelli, G. P. & Walter, B. (1994). Detection of grapevine leafroll-associated closterovirus III by molecular hybridization. *Plant Pathol* **43**, 91–96.
- Serrano, L., Ramon, J., Segarra, J., Medina, V., Achón, M. A., López, M. & Juárez, M. (2004). New approach in the identification of the causal agent of fig mosaic disease. *Acta Horti* **657**, 559–566.
- Skare, J. M., Wijkamp, I., Rezende, J. A. M., Kitajima, E. W., Park, J. W., Desvoyes, B., Rush, C. M., Michels, G., Scholthof, K. B. G. & Scholthof, H. B. (2006). A new eriophyid mite-borne membrane-enveloped virus-like complex isolated from plants. *Virology* **347**, 343–353.
- Slykhuus, J. T. (1973). Viruses and mites. In *Viruses and Invertebrates*, pp. 391–405. Edited by A. J. Gibbs. Amsterdam, The Netherlands: North Holland Publishing Co.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.