

SOME PROPERTIES OF FIG LATENT VIRUS 1, A NEW MEMBER OF THE FAMILY *FLEXIVIRIDAE*

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SUMMARY

A virus with filamentous particles *ca.* 700 nm long, denoted Fig latent virus 1 (FLV-1) is widespread in Apulian (southern Italy) fig orchards, in trees showing or not mosaic symptoms and in symptomless seedlings. This virus was transmitted by sap inoculation to a very restricted range of herbaceous hosts without inducing apparent symptoms. It was successfully purified from root tissues of infected figs. A virus-specific antiserum raised in rabbits, proved useful for its detection in fig leaf dips by immunosorbent electron microscopy. The cytology of infected cells was little affected. Bundles of filamentous particles were observed in the cytoplasm of parenchyma cells of infected fig trees and seedlings. The viral genome is a single-stranded positive-sense RNA with an estimated size of *ca.* 8,000 nt, 6,620 of which have been sequenced, starting from the polyadenylated 3' terminus. Genomic RNA consists of four open reading frames encoding, in the 5'→3' direction, the replication-associated proteins (ORF 1), a 43 kDa putative movement protein (ORF 2), the 46 kDa coat protein (ORF 3), and a 12 kDa protein with nucleic acid binding properties. The viral genome structure and organization resembles that of members of the genus *Trichovirus*, family *Flexiviridae* and, indeed, FLV-1 clusters with trichoviruses in phylogenetic trees constructed with coat protein sequences. However, a distinct difference with all members of the genus rests with the size of the coat protein subunits (46 versus 22-27 kDa) and the presence of ORF 4, which is present only in three tentative species of this genus.

Key words: fig latent virus, *Trichovirus*, serology, partial genome sequence, cytopathology.

INTRODUCTION

The presence of viruses with filamentous particles in figs (*Ficus carica* L.) affected by mosaic disease (FMD) has been reported from several countries. Some of these viruses were identified as possible potyviruses (Grbelja and Eric, 1983; Salomon *et al.*, 2003, 2008), or carlaviruses (Namba, 1983; Doi, 1989) or were not assigned taxonomically (Serrano *et al.*, 2004). Two other viruses were identified as tentative members of the family *Closteroviridae* and described under the name of Fig leaf mottle-associated virus 1 (FLMaV-1) and Fig leaf mottle-associated virus 2 (FLMaV-2) (Elbeaino *et al.*, 2006, 2007), respectively. Filamentous virus particles were also observed in the cytoplasm of thin-sectioned mesophyll cells of FMD-affected plants from Italy (Martelli *et al.*, 1993) and, more recently, from Mexico and South Africa, where they coexisted with FLMaV-1 (Castellano *et al.*, 2007).

During a survey for viruses in a fig germplasm collection of the Faculty of Agriculture of the University of Bari (southern Italy), filamentous virus-like particles were very frequently observed in negatively stained leaf dips from a number of fig accessions, regardless of whether they showed mosaic symptoms or not. Similar particles were also found in 1-year-old symptomless seedlings from seeds collected from mosaic-diseased figs and in 1- and 2-year-old symptomless volunteer seedlings, growing on the trunk of palm trees in a private garden in the vicinity of Bari.

As reported in this paper, we have now determined some properties of this virus for which, because of the occurrence in symptomless adult fig trees and seedlings, the provisional name of Fig latent virus 1 (FLV-1) is proposed.

MATERIALS AND METHODS

Virus sources. Materials used in this study were collected from: (i) an adult mosaic-diseased (accession F5P5) and a symptomless fig tree, both of undetermined variety, from the fig germplasm collection of the University of Bari; (ii) symptomless seedlings grown

from F5P5 seeds under screenhouse on the premises of the Faculty of Agriculture of the University of Bari; (iii) symptomless volunteer seedlings.

Mechanical transmission. Attempts to isolate FLV-1 by mechanical inoculation from accession F5P5 were made using extracts from root tissues ground in 0.1 M phosphate buffer pH 7.2 containing 2.5% nicotine. Inocula were rubbed on celite-dusted cotyledons of *Cucumis sativus* and leaves of *Chenopodium quinoa*, *C. amaranticolor*, *Nicotiana benthamiana*, *N. glutinosa*, *N. occidentalis* and *N. cavicola*, grown in a glasshouse at 22–24°C. Plants were kept under observation for over a month for symptom appearance and, starting two weeks after inoculation, dips in 2% uranyl acetate from inoculated and upper leaves were observed under an electron microscope (EM) Philips Morgagni 282D for the presence of virus particles.

Virus purification. Virus was purified from the roots of accession F5P5. Cortical tissues (50 to 70 g) were frozen in liquid nitrogen and ground in a blender in the presence of 5 ml/g of extraction buffer (0.05 M phosphate pH 6.2, 5 mM β -mercaptoethanol, 5 mM DIECA, 5 g/l PEG 8000). The slurry was squeezed through cheesecloth, centrifuged at 8,000 rpm for 15 min in a refrigerated centrifuge and the supernatant was treated with 10% PEG-8000 and 1% NaCl. After 1 h incubation at 4°C, the suspension was centrifuged again at 8,000 rpm for 15 min. Pellets were resuspended overnight in 15 ml of 0.05 M phosphate buffer pH 6.8, containing 5 mM β -mercaptoethanol, 5 mM EDTA and 0.2 M NaCl. After a centrifugation at 1000 rpm for 10 min, the supernatant was centrifuged at high speed (115,000 g) for 1 h, the pellets were resuspended in 1 ml of 0.02 M Na-citrate buffer pH 6.0 and centrifuged on a 35% sucrose gradient for 1 h at 36,000 rpm in a Beckman SW41 rotor. Two-ml fractions were collected and observed under the EM for the presence of virions.

Fractions with the highest concentration of virus particles were pooled, centrifuged at 115,000 g for 90 min, and the pellets resuspended in 200 μ l citrate buffer.

Antiserum production. Two-ml aliquots of purified virus preparations containing *ca.* 0.9 mg nucleoprotein mixed 1:1 with Freund's incomplete adjuvant were injected subcutaneously to a New Zealand white rabbit. Boost injections were delivered intramuscularly three times, 10 days apart from one another. Bleedings were four, beginning one week after the last boost injection. The antiserum was absorbed twice with extracts from healthy fig roots to eliminate antibodies to normal plant components. The rough titre was determined by decorating virus particles (Milne, 1993) with progressive antiserum dilutions.

Western blot. Leaf tissues (100 mg) from accession F5P5 and virus-free sources were extracted in 1 ml of denaturing buffer (Berger *et al.*, 1989) and aliquots equivalent to 10 mg were fractionated after boiling for 5 min in SDS-polyacrylamide gel electrophoresis. Likewise, partially purified virus preparations were treated in denaturing buffer. After transferring in a semidry apparatus (BioRad, USA) on PVDF Immobilon membranes (Millipore, USA), blots were treated overnight at 4°C with the antiserum diluted 1:500 in TBS-blocking buffer and were developed by NBT-BCIP solution (Sigma-Aldrich, USA).

Electron microscopy. Immunosorbent electron microscopy (ISEM) assays were done as described by Milne (1993), using leaf dips in 2% uranyl acetate. For trapping, microscope grids were floated for 1 h at 38°C on a drop of antiserum diluted 1:150. Decoration was with antisera to FLV-1, *Grapevine berry inner necrosis virus* (GINV), *Apple chlorotic leaf spot virus* (ACLSV), *Cherry mottle leaf virus* (ChMLV), genus *Trichovirus* and *Grapevine virus A* (GVA), genus *Vitivirus*, diluted

Table 1. Primers used for detection and extension of the FLV-1 genome.

Name	Sequence	Target region	nt position
CPtr1	CCATCTTCACCACACAAATGTC	coat protein	5040-5060
CPtr2	CAATCTTCTTGCCCTCCATAAG	"	5398-5419
rep-up	WGCI AARGCIGGICARAC	rep domain	3427-3444
rep-down	RMYTCICCISWR AICKCAT	"	3770-3789
oligo-dT anchor	GGCCACGCGTCGACTAGTACG(T) ₂₀	3'end/5' RACE	
anchor RACE	GGCCACGCGTCGACTAGTAC	"	
ff-up	CGCTTTGCCCAATGTGCAGAT	rep domain	3476-3497
ff-down	TCGAAGGCCAGAGTTGATGCA	rep domain	3649-3669
h1rev	CTCTGCCCAAGGAAGATTTGCC	ORF1	2835-2856
i1 rev	AGCCTCAAATGTTTTGCAGCTCAG	ORF1	2048-2071
m1 rev	TGGGCACATCACGCCTCTGGATCC	ORF1	1236-1259

1:20. For thin sectioning, tissue fragments were excised from leaves of an infected symptomless seedling, a symptomless adult fig tree, and from accession F5P5. All samples were processed according to standard procedures (Martelli and 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 (Agar Scientific, UK). Thin sections were stained with lead citrate prior to viewing under the EM.

RNA extraction, cloning and sequence analysis. The possibility that the virus particles observed in leaf dip preparations belonged to a flexivirus-like virus was investigated by RT-PCR amplification of silica-extracted total nucleic acids (TNAs) (Foissac *et al.*, 2000) or double-stranded RNA (dsRNA) from F5P5 leaves (Valverde *et al.*, 1986) denatured in a 20 mM solution of methylmercuric hydroxide for 20 min at room temperature. A degenerate primer set (rep up/rep down; Table 1) designed on a strongly conserved domain of the replicase gene of members of the *Flexiviridae* family (Saldarelli *et al.*, 1999) and containing inosine in the positions of highest variability (Dovas and Katis, 2003) amplified a 363 bp fragment. Genomic RNA was extracted from purified virions according to Diener and Schneider (1968) and fractionated on oligo-dT cellulose. About 500 ng of RNA were reverse transcribed with an oligo-dT-anchored primer by the Transcriptase enzyme (Roche, USA) at 52°C. A PCR fragment of 3200 bp was obtained by amplification of this cDNA with the primer sense ff-up and the reverse anchor-RACE primer (Table 1). For 5' extension of the viral sequence, dsRNA was extracted by batches of 40 g of leaf tissues according to Valverde *et al.* (1986) with a second passage through CC41 cellulose (Whatman, USA) and a final nuclease digestion (Saldarelli *et al.*, 1994). The dsRNA was denatured as described above and transcribed by reverse primers designed on subsequently sequenced viral genome fragments. The 5'/3' RACE kit (Roche, USA) was used for the extensions. Reverse primers were chosen at a distance of about 150-200 bp from the known 5' termini, obtained after any extension step, and the cDNA was purified, polyadenylated by Terminal d-transferase and finally amplified.

All PCR fragments were cloned with the Strataclone kit (Stratagene, USA), plasmids were purified by boiling and PEG precipitation and automatically sequenced (Primm, Italy). Sequence data were assembled using the DNA Strider 1.4 program (Marck, 1988) and aligned by Clustal X (version 1.8; Thompson *et al.*, 1997). Trees were drawn using NJPlot program (neighbor-joining method) after 1000 bootstrapping replicates on the alignments. BLAST analysis and CD-Search for conserved domains were performed at the NCBI web-

site (Altschul *et al.*, 1990).

Virus detection. For diagnostic purposes, besides the virus-specific antiserum, the primers CPtr1 and CPtr2 (Table 1) were used in RT-PCR on silica-extracted TNAs template from tissue of a number of different fig trees from the above mentioned collection. Amplicons (389 bp) were resolved in agarose gels stained by ethidium bromide.

RESULTS AND DISCUSSION

Mechanical transmission. None of the inoculated herbaceous hosts showed visible symptoms except for *N. glutinosa* which was slightly stunted, as compared with non-inoculated controls. Virus presence, however, was checked by ISEM in extracts from inoculated and non inoculated leaves of all hosts at different times after inoculation. Virus particles were detected after 20 days in inoculated leaves of *C. quinoa*, *C. amaranticolor*, *N. occidentalis*, *N. glutinosa* and *N. benthamiana* and after 40 days in some of the upper non-inoculated leaves of *C. quinoa*, *N. occidentalis*, *N. cavicola* and *N. glutinosa*. However, tests made at 50 d.p.i., detected virions only in *N. glutinosa*. These results suggest that FLV-1 can be transmitted by sap inoculation to herbaceous hosts, in which it does not multiply efficiently, at least under our experimental conditions.

Purification and serology. Although final preparations contained a substantial amount of plant contaminants, virus particles showing a distinct cross banding (Fig. 1) were plentiful. Most of the particles were fragmented, so their size was determined from leaf dip preparations. The most frequent length of some 60 particles measured was *ca.* 700 nm.

The antiserum had a titre of 1:160. It clearly decorated homologous virions (Fig. 1, inset). These, however, were not decorated by antisera to ACLSV, GINV, ChMLV and GVA. Western blots of extracts from F5P5 leaf tissue and partially purified virus preparation resolved a major band with mol. wt. of *ca.* 45 kDa, which was recognized by the antiserum to FLV-1 and was absent in leaf extracts from virus-free sources.

Cytopathology. Regardless of the sources (seedlings or adult trees) and of whether they were symptomless or showed mosaic symptoms, the architectural organization of mesophyll cells was apparently well preserved (Fig. 2 and 3) and organelles, like nuclei and mitochondria, had a normal aspect (Fig. 2B). Chloroplasts, however, were misshapen, swollen and exhibited a disarranged membrane system. (Fig. 2A and 3). The ground cytoplasm of cells of all samples examined contained a great number of filamentous virus-like particles

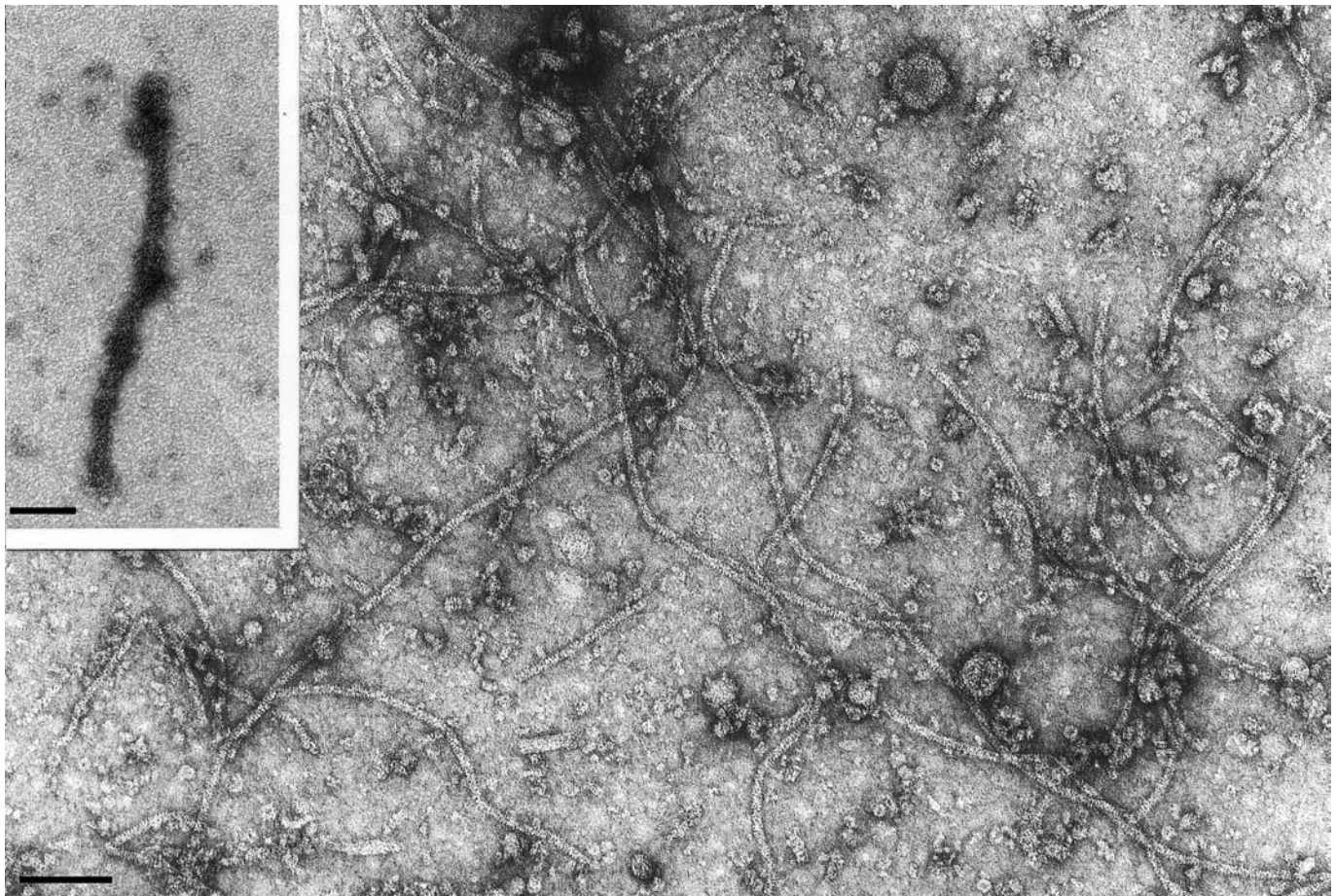


Fig. 1. Partially purified preparation of FLV-1. Particles have variable length, due to fragmentation, and show distinct cross banding. Bar = 100 nm. Inset shows a virus particle decorated by the homologous antiserum. Bar = 100 nm.

scattered in the cytosol (Fig. 2 and 3) or arranged in bundles (Fig. 3, inset). Only this type of particles, interpreted as profiles FLV-1 virions, were seen in the cells of the symptomless field-grown tree (Fig. 2A) and seedlings (Fig. 3). By contrast, cells of the symptomatic tree, in addition to the filamentous virions, contained also groups of electron dense, round to ovoid structures (Fig. 2B) indistinguishable from the double membrane bodies typically associated with fig mosaic disease (Bradford *et al.*, 1970; Appiano, 1982; Martelli *et al.*, 1993), which are thought to be particles of Fig mosaic virus (FMV) (Elbeaino *et al.*, 2009).

Virus detection. Reliable detection of FLV-1 was obtained all year round by RT-PCR of silica-extracted TNAs from leaf tissues or cortical scraping using the CPtr1/CPtr2 primers that amplify a 389 bp segment of the CP gene. A survey for the preliminary assessment of the incidence of FLV-1 infections and the association of this virus with symptoms, showed that FLV-1 infects a high percentage (68%) of 40 different cultivars tested from the fig germplasm collection of the University of Bari and was detected also in fig accessions from Europe, Africa and America (Castellano *et al.*, 2007).

However, its presence does not seem to be associated with mosaic or other symptoms for the virus was detected in more than 40% symptomless trees and in the totality of 10 symptomless seedlings grown from seeds of accession F5P5.

Cloning, sequencing and computer-assisted analysis.

Since the first sequenced viral genome fragment (clone c13) was from the replicase domain and showed a high similarity with the RdRp of trichoviruses, the opportunity to obtain the whole 3' end was sought. Thus, a polyA-terminal PCR fragment of about 3200 bp was cloned (clone sc4) and the virus sequence was further extended by 5' RACE in four subsequent steps (Fig. 4), with an average of 900 bp gained per step. The sequences at the 3' ends of extended clones, overlapping with the 5' terminations used to design extension primers, matched in these short segments with identities higher than 95% (not shown). A final contig of 6,620 bp was then assembled, excluding the polyA tail, while the 5' terminus, estimated to account for *ca.* 1600 nt by aligning the known trichovirus genomes, is still missing. The obtained sequence was deposited in the EMBL database under the accession No. FN377573.

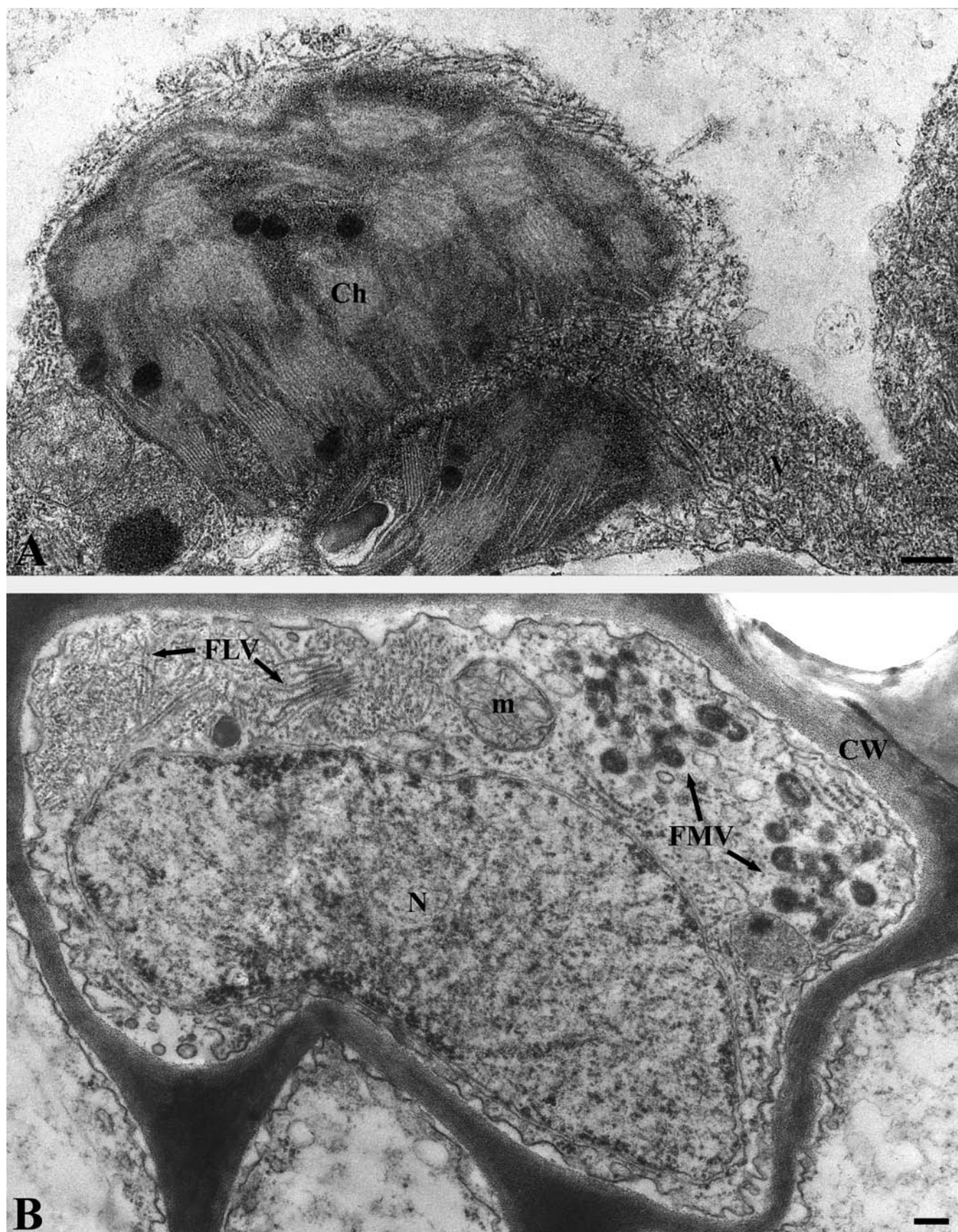


Fig. 2. A. A mesophyll cell of a symptomless adult fig plant showing plenty of filamentous particles, scattered in the cytosol (V) and around a misshapen chloroplast (Ch), interpreted as profiles of FLV-1 virions. Bar = 200 nm. B. A mesophyll cell of a symptomatic adult fig tree (accession F5P5) showing aggregates of Fig mosaic virus (FMV) and Fig latent virus 1 (FLV) particles. Nucleus (N) and mitochondrion (m) are apparently normal. CW = cell wall. Bar = 200 nm.

The FLV-1 genome sequence comprises four open reading frames (ORFs), a 61 nucleotide (nt) long untranslated region at the 3' terminus, and ends with a

poly(A) tail (Fig. 4). No intergenic regions have been found since all ORFs overlap from both sides.

The partially sequenced ORF 1 ends at position 4261



Fig. 3. Palisade cells from a symptomless seedling infected by FLV-1. Chloroplasts are swollen and show a deranged internal membrane system. FLV-1 particles (V) are plentiful in the ground cytoplasm. Bar = 200 nm. Inset shows a bundle of FLV-1 particles (V). Bar = 200 nm.

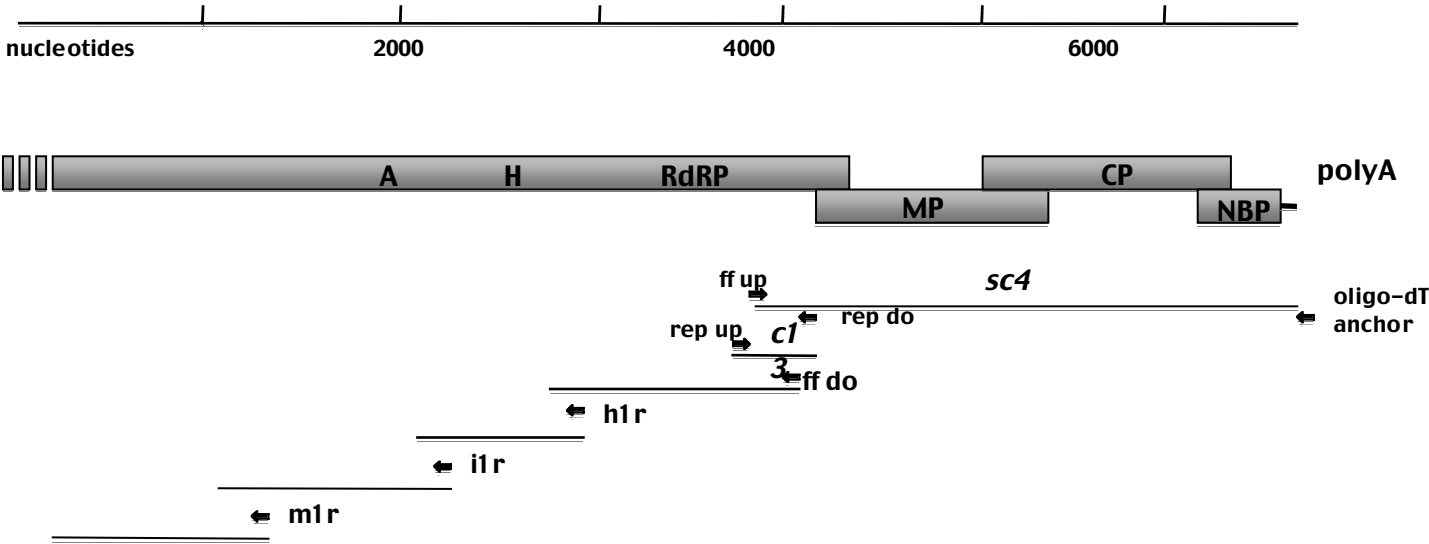


Fig. 4. Diagrammatic representation of the FLV-1 genome and strategy of cloning. A, AlkB domain; H, helicase domain; RpRd, RNA dependent-RNA polymerase domain; MP, movement protein; CP, coat protein; NBP, nucleotide-binding protein. Extension clones obtained by 5' RACE-PCR are shown below the genome map.

and contains, in the 5'→3' direction, the following domains: AlkB (belonging to the 2OG-Fe II oxygenase superfamily, CD-Search class cl01206), RNA helicase-1, and RNA dependent RNA polymerase.

ORF-2, 1143 nt in size, begins with an AUG codon at position 4155 and ends at position 5297. It potentially codes for a polypeptide *ca.* 43 kDa (p43) in size, recognized as the putative movement protein of the TMV 30K-like superfamily (pfam 01107), which shares 25% identity at the amino acid (aa) level with the movement protein of ACLSV. Intriguingly, following BLAST search, p43 showed an amino acid sequence identity of 27% with repeats of several proteins, denoted hypothetical or unnamed, present in the genome of *Vitis vinifera* (Velasco *et al.*, 2007). This may be interpreted as a further example of modular evolution, as reported for some filamentous plant viruses of the family *Flexiviridae*

and *Closteroviridae* (Dolja *et al.*, 2006; Martelli *et al.*, 2007).

ORF 3 starts at position 5,056 and ends with an ochre stop codon at position 6,291. It encodes a 412 aa polypeptide 46 kDa (p46) in size, identified as the viral coat protein (CP). p46 shares relevant similarity among aa from position 3 to 135 with the corresponding aa of the CP of GINV, ACLSV, and *Apricot pseudo-chlorotic leafspot virus* (ApCLSV) (60%, 65% and 71% identity, respectively). However, virtually no homology was detected with the CP of any of the above and other trichoviruses from aa 181 to the end, i.e. beyond the conserved salt bridge signature of rod-shaped virus CPs (Dolja *et al.*, 1991), so that the overall identity level dropped to 16% (ACLSV), 17% (GINV) and 16% (ApCLSV) (Table 2). An additional peculiarity of FLV-1 CP resides in its size (46 kDa), which is double than

Table 2. Percent identity/similarity values at the amino acid level of FLV-1 RdRp domain and CP with comparable proteins of representatives of all extant genera in the family *Flexiviridae*.

Genus	Virus	RdRp	CP
<i>Potexvirus</i>	<i>Potato virus X</i> (PVX)	23/36	11/17
<i>Mandarinivirus</i>	<i>Indian citrus ringspot virus</i> (ICRSV)	23/37	9/14
<i>Allexivirus</i>	<i>Garlic virus A</i> (GarV-A)	26/40	8/14
<i>Vitivirus</i>	<i>Grapevine virus A</i> (GVA)	31/44	13/23
<i>Trichovirus</i>	<i>Apple chlorotic leaf spot virus</i> (ACLSV)	34/41	16/27
<i>Trichovirus</i>	<i>Grapevine berry inner necrosis virus</i> (GINV)	40/50	17/28
<i>Capillovirus</i>	<i>Apple stem grooving virus</i> (ASGV)	35/45	16/24
<i>Citriovirus</i>	<i>Citrus leaf blotch virus</i> (CLBV)	38/47	15/27
<i>Foveavirus</i>	<i>Apple stem pitting virus</i> (ASPV)	36/43	7/14
<i>Carlavirus</i>	<i>Potato virus S</i> (PVS)	33/43	12/23

that (22-27 kDa) of members of the genus *Trichovirus* (Adams *et al.*, 2005).

ORF 4 (nt 6215-6559) encodes a protein 12 kDa in size showing an average identity of *ca.* 40% with the C4-carlaviruses-like superfamily (pfam 01623) of nucleic acid-binding proteins (NABp). BLAST search disclosed that,

similarly to MP, which contains stretches of grapevine genomic sequences, FLV-1 NABp has a striking homology (identity up to 44%) with a plant integrase from *Populus trichocarpa*, that bears a conserved domain highly resembling the carlavirus 10 kDa terminal protein.

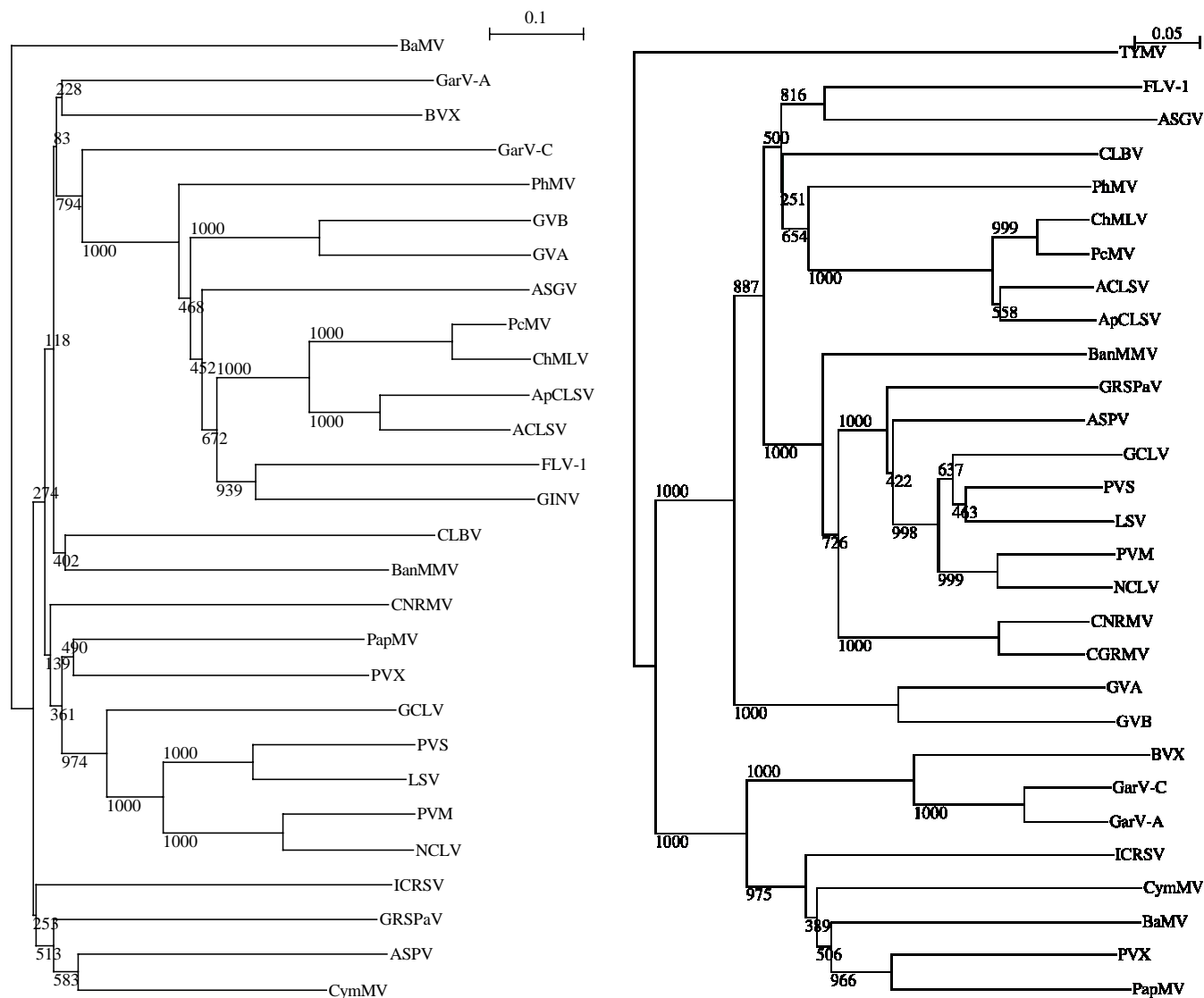


Fig. 5. Phylogenetic trees showing the relationship of FLV-1 with other members of the family *Flexiviridae* in the CP gene (A) and RdRp domain (B). The neighbour-joining tree was produced and bootstrapped 1000 times using CLUSTAL X. Branch lengths are proportional to sequence distances. A- Accession numbers: PVX (*Potato virus X*) NP_040882; PapMV (*Papaya mosaic virus*) NP_044330; ICRSV (*Indian citrus ringspot virus*) f406744; GarV-C (*Garlic virus C*) NP_569132; GarV-A (*Garlic virus A*) BAA61810; CymMV (*Cymbidium mosaic virus*) U62963; CLB (*Citrus leaf blotch virus*) NP_624333; BVX (*Botrytis virus X*) NP_932306; BaMV (*Bamboo mosaic virus*) NP_042582; ASGV (*Apple stem grooving*) AAP80757; GVA (*Grapevine virus A*) NP619662; GVB (*Grapevine virus B*) NP619654; GRSPaV (*Grapevine rupestris stem pitting associated virus*) NP_047281; ASPV (*Apple stem pitting virus*) NP_604464; GINV (*Grapevine berry inner necrosis virus*) D88448; PVS (*Potato virus S*) YP_277428; LSV (*Lily symptomless virus*) CAD92112; PVM (*Potato virus M*) AAP76207; NCLV (*Narcissus common latent virus*) YP_699983; CNRMV (*Cherry necrotic rustle mottle virus*) NP_059937; CGRMV (*Cherry green ring mottle virus*) CAC18739; ACLSV (*Apple chlorotic leafspot virus*) CAA68080; ChMLV (*Cherry mottle leaf virus*) NP062428; PcMV (*Peach mosaic virus*) ABA18636; AP-CLSV (*Apricot pseudo-chlorotic leaf spot virus*) YP224130; GCLV (*Garlic common latent virus*) CAA92815. The scale represents a relative genetic distance of 0.1.

B- The tree was constructed as reported in Fig. 5 A, but the scale represents a genetic distance of 0.05. Additional sequence: TYMV (*Turnip yellow mosaic virus*) AAB92649.

Phylogenetic relationships in the family *Flexiviridae*.

The identity/similarity at the aa level of FLV-1 RdRp and CP with comparable proteins of representatives of all genera of the family *Flexiviridae* did not exceed 40/50% (RdRp) and 17/28% (CP). The highest values in the pairwise alignment were registered for GINV, a trichovirus (Table 2). In accordance with this, in the phylogenetic tree constructed with CP sequences (Fig. 5A), FLV-1 grouped next to GINV in a distinct subcluster of a clade comprising trichovirus species. However, in the tree constructed with RdRp sequences (Fig. 5B), FLV-1 showed a closer association with the capillovirus *Apple stem grooving virus* (ASGV), with which it clustered in a subgroup distinct from that comprising trichoviruses. The presence of ORF 4 has so far been detected in ChMLV, Peach mosaic virus and *Phlomis* mottle virus (James *et al.*, 2000, 2006; Saldarelli *et al.*, 2008), which are the only viruses assigned to the genus *Trichovirus* as tentative species.

All these data taken together, suggest the classification FLV-1 as tentative species in the genus *Trichovirus*. However, some relevant features, like the unusual size of the CP gene and the presence of the fourth terminal ORF coding for a NABp, call for further investigations for a better definition of the taxonomic status of this virus.

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