SHORT COMMUNICATION

RNA-5 AND -6, TWO ADDITIONAL NEGATIVE-SENSE RNA SEGMENTS ASSOCIATED WITH *FIG MOSAIC VIRUS*

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SUMMARY

Two novel RNA molecules, denoted RNA-5 and RNA-6, found in *Fig mosaic virus*-infected plants in addition to the previously identified four RNA segments were completely sequenced. Each segment consisted of a single open reading frame (ORF). In particular, RNA-5 is 1,752 nucleotides in size and expresses a polypeptide of 502 amino acids (*ca.* 59 kDa), whereas RNA-6 comprises 1,212 nucleotides and codes for a polypeptide of 188 amino acids (21.5 kDa). Both RNA segments possess, at their 5'/3' termini, stretches of conserved nucleotides that are common to the other previously identified FMV RNA segments. The proteins encoded by RNA-5- and RNA-6 did not reveal any potential function nor similarities with any known viral protein or conserved motif in database, including those of related Emaravirus-like viruses. The presence of two novel RNA segments in the genome of FMV is in accordance with findings reporting more than four RNA molecules in some of the viruses that, like FMV, have enveloped particles originally denoted "double-membraned bodies".

Key words: Fig mosaic, *Fig mosaic virus*, *Emaravirus*, sequencing.

Fig mosaic virus (FMV), an approved species (Adams and Carstens, 2012) of the recently established genus *Emaravirus* (Muehlbach and Mielke-Ehret, 2011), is a multipartite single-stranded negative-sense RNA virus that has recently been recognised as the causal agent of fig mosaic disease (FMD) (Elbeaino *et al*., 2009a, 2009b). Intracellular enveloped structures described by Bradfute *et al.* (1970) as double-membraned bodies (DMBs) occur in the mesophyll cells of infected figs (Ahn *et al.*, 1996; Appiano *et al.*, 1995; Castellano *et al.*, 2007; Martelli *et al.*, 1993). These structures were identified as particles of the putative causal agents of fig mosaic and of the ringspot disease of European mountain ash (Mielke and Muehlbach, 2007; Elbeaino *et al*., 2009a, 2009b) and were also observed in infected cells of mosaic-diseased plants of pigeonpea (*Cajanus cajan* L.), maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.), thought to be induced by Pigeonpea sterility mosaic virus (PPSMV) and Maize red stripe virus (MRSV) (Kumar *et al.*, 2002; Ahn *et al.*, 1998; Jensen *et al.*, 1996). Besides the *European mountain ash ringspot-associated virus* (EMARaV), FMV, PPSMV and MRSV, three novel viruses with DMBs structures have recently joined the list, i.e. Redbud yellow ringspot virus (RYRV; Laney *et al.*, 2011), Rose rosette virus (RRV; Laney *et al*., 2012) and Raspberry leaf blotch virus (RLBV; McGavin *et al*., 2012). Most of these viruses are vectored in nature by eriophyid mites and have a multipartite RNA genome, with a number of segments differing among species, i.e. four in the case of EMARaV, FMV, RRV and RYRV (Mielke and Muehlbach, 2007; Elbeaino *et al*., 2009b; Laney *et al*., 2011), five for RLBV (McGavin *et al*., 2012), six for PPSMV (Kumar *et al.,* 2002) and up to seven for MRSV (Jensen *et al*., 1996).

As to FMV, the analysis in polyacrylamide gels of double-stranded RNAs (dsRNAs) patterns extracted from symptomatic fig plants revealed often the presence of more than four bands, also in seedlings that had been inoculated via *Aceria ficus* (Elbeaino *et al.,* 2009a), the alleged vector of fig mosaic, and apparently did not contain any virus other than FMV. The nature of these bands was therefore investigated and, as reported in the present paper, found to be of viral origin. In particular, two additional RNA segments of the FMV genome were identified and their complete nucleotide sequence determined.

dsRNAs were recovered from leaf vein samples (30 g) of FMV-infected plants by phenol/chloroform extraction and chromatography through cellulose CF-11 column in the presence of 17% ethanol (Elbeaino *et al*., 2009a). Further purification was by chromatography through cellulose CC41 (Whatman, USA) followed by selective enzymatic digestion (DNase and RNase), as described by Saldarelli *et al.* (1994). Extracts were passed through Micro Bio-Spin 30 Columns in RNase-free Tris buffer (Bio-Rad Laboratories, USA) for the elimination of contaminants, then analyzed in 6% PAGE and silver-stained. Two hundred ng of nuclease-

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treated dsRNA extracts were denatured by treatment with 20 mM methyl mercuric hydroxide, and the 3' end of each RNA strand was ligated to 0.5 µg of the primer Adapt-Link [5'-$(UUU)_{RNA}$(AACCGCATCCTTCTC-3')$_{DNA}$] using T4 RNA ligase, according to the manufacturer's instructions (Ambion, USA). Primer-ligated denatured dsRNAs were ethanol-precipitated and re-suspended in distilled sterile water, then mixed with the primer Adapt/comp (5'-GAGAAGGATGCGGTT-3') for cDNA synthesis, using 20 U of reverse transcriptase (Roche, USA) at 52°C for 1 h. Full-length PCR amplifications were done using the downstream primer Adapt/comp and the conditions reported by Elbeaino *et al.* (2009b). All amplicons were transformed in StrataCloneTM PCR Cloning vector pSC-A (Stratagene, USA), subcloned into *Escherichia coli* DH5α or SoloPACK cells, and custom sequenced (Primm, Italy). Nucleotide and protein sequences were analysed with the assistance of the DNA Strider 1.1 program (Marck, 1988). Multiple alignments of nucleotide and amino acid sequences were obtained using the default options of CLUSTALX 1.8 (Pearson and Lipman, 1988). Search for homologies with known proteins from the protein information resources database (PIR, release 47.0) was done with the FASTA (Pearson and Lipman, 1988), BlastX and BlastP programs (Altshul *et al.*, 1990).

The amplification procedure yielded six RNA segments, four of which had the same size of those previously reported (Elbeaino *et al.*, 200b). For determining if the two additional RNA molecules (putative RNA-5 and RNA-6) had a viral origin and were part of FMV genome, their sequence was determined and two couples of sense and antisense primers specific to both RNA molecules, denoted R5s: 5'-TGCTTGTAGGC-GATGAAACA-3', R5a: 5'-TTGTCACATCACAGAA-GATTGC-3' and R6s: 5'-ATTCAACGCAGTTGCA-

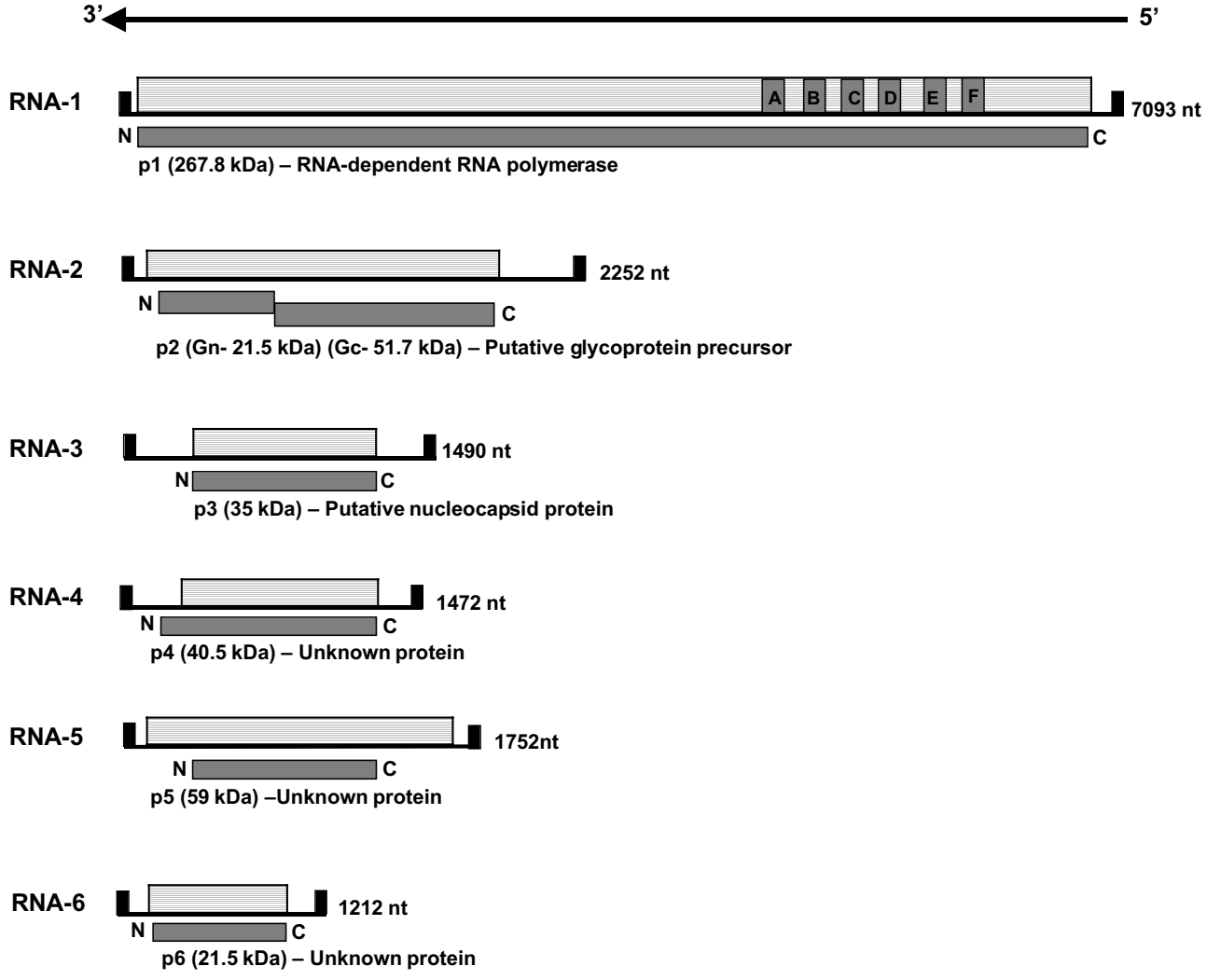

Fig. 1. Schematic representation of the organisation of the six RNA segments constituting the putatively complete genome of FMV. The terminal 13 nucleotides conserved at the 5' and 3' termini are indicated as black boxes on each segment. Letters (A-F) represents the conserved motifs of the RdRp (RNA-1) gene. Expression products of each RNA (p1 to p6) are represented as dark grey boxes. The function and estimated molecular weight of each protein are reported. Figure not drawn to scale.

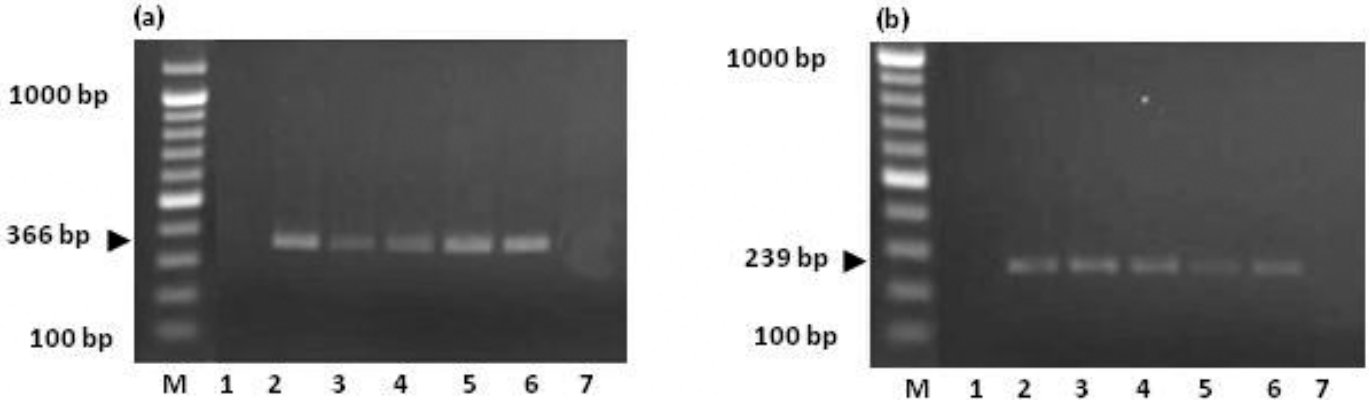

Fig. 2. Electropherogram showing representatives of RT-PCR amplifications obtained by using FMV-RNA-5 (a) and FMV-RNA-6 (b) specific primers on FMV-infected plants. All FMV-infected samples, the positivity of which was previously determined by PCR using different sets of specific primers designed on the four RNAs of FMV, yielded positive reactions with both primers equally (lanes 2-6); while no amplification was obtained from the healthy ones (lanes 1). Lane M: DNA marker XIV (Fermentas, Italy).

GACA-3', R6a: 5'-TGAGACAAGTTGCCATCCAA-3', were designed. In RT-PCR assays, these primers generated two DNA fragments 366 bp and 239 bp in size, respectively.

Reverse-transcribed total nucleic acids (TNAs) templates were extracted from 0.1 g of symptomatic leaf veins, according to Foissac *et al.* (2001). For cDNA synthesis, 10 µl of TNAs were mixed with 1 µl random primers (0.5 µg/µl) and 1.5 µl RNase-free water. The mix was denatured by boiling at 95°C for 5 min, followed by fast cooling, then mixed with 4 µl M-MLV (5X) first strand buffer, 2 µl DTT (0.1 M), 0.5 µl dNTPs (10 mM), 1 µl M-MLV RT enzyme (200 U/µl) (Invitrogen, Italy). The preparation was incubated at 39°C for 60 min, 70°C for 10 min and finally at 4°C before use or stored at -20°C.

For RT-PCR, 2.5 µl cDNA mixture were added to 2.5 µl of 10X Taq polymerase buffer (Promega, USA), 1 µl 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM sense and antisense of each of the above primers and 0.2 µl of Taq polymerase (5 U/µl) in a final volume of 25 µl. PCR conditions consisted of 3 min denaturation at 94°C followed by 35 cycles of 30 sec melting at 94°C, 30 sec annealing at 55°C, and 35 sec elongation at 72°C, with a final extension of 5 min at 72°C. The reaction products were resolved by electrophoresis in 6% polyacrylamide slab gels.

Primers specific for RNA-5 and RNA-6 were used in RT-PCR on 30 fig samples, 15 FMV-positive and 15 negative, in which the presence/absence of FMV had previously been ascertained by RT-PCR using FMV-specific primers designed on the four RNAs previously reported (Elbeaino *et al.*, 2009a, 2009b).

Hybridizations were also performed on Northern blots of dsRNA extracts using two digoxigenin-labelled RNA probes of positive and negative sense, constructed on the sequence of putative FMV RNA-5 and RNA-6. To this aim, about 100 ng of dsRNA extracts were denatured at 95°C for 5 min in the presence of a 50 mM NaOH solution. Once chilled on ice, denatured dsRNAs were separated in a denaturing 1% agarose/formaldehyde gel, followed by alkaline transfer of nucleic acids onto a Hybond N+ nylon membrane (GE Healthcare, USA) as described by Chomczynski (1992). Hybridizations with both RNA-5 and RNA-6 probes were done with overnight incubation at 55°C. Blots were washed twice for 30 min each in SSC 2X and 0.1% SDS at room temperature and twice at 68°C. Hybridized dsRNA bands were visualized using the DIG-chemiluminescent detection protocol following the manufacturer's instructions (Roche Diagnostics, Italy). Films were exposed for 20-30 min and resulting images were digitalized.

The complete sequence of RNA-5 and RNA-6 of FMV were obtained and deposited in GenBank under the accession Nos HE803826 and HE803827.

Like other emaravirus-like viruses (EMARaV, RLBV, RRV and RYRV), RNA-5 and RNA-6 had a single monocistronic ORF.

RNA-5 has a 1,752 nt sequence containing an in-frame (AUG) codon at positions 58 and an amber codon (UGA) at position 1,566. The coding region (CR) consists of 1,506 nts and expresses a polypeptide (p5) of 502 amino acid (aa) *ca.* 59 kDa in size, covering 86% of the ORF coding capacity. The 5′ and 3' non-coding regions (5'/3'NCRs) are 57 nts and 186 nts in length, respectively, a size in the range of those of other RNA segments of FMV. Blast analysis conducted with the nucleotide and amino acid sequence of this segment did not show similarity with any known viral product. Furthermore, a Clustal alignment of p5 with proteins expressed by RNA-4 or RNA-5 of the emaravirus-like viruses did not reveal the presence of any conserved motif. Only a predicted potential signal sequence was

identified at the N terminus of p5, suggesting that it may be a membrane-located protein.

RNA-6 (1,212 nts) contains an in-frame AUG codon at position 69 and protein translation ends with an ochre codon (UAA) at position 633. The coding region (CR) consists of 567 nts and expresses a polypeptide (p6) 188 aa in size, with a predicted Mr of *ca.* 21.5 kDa, covering *ca*. 47% of the ORF coding capacity, similarly to other RNA segments of FMV. Following an extensive database search, protein p6 did not show similarity with any known viral protein nor apparently contained any of the motifs conserved in other emaravirus-like viruses.

Both RNA segments had stretches of 13 nts at 5' and 3' ends, which are conserved in the four previously sequenced RNA segments of FMV, and in all other reported emaravirus-like viruses (Mielke *et al*., 2007; Elbeaino *et al*., 2009b; Laney *et al*., 2011; McGavin *et al*., 2012).

Although the nucleotide or amino acid comparisons did not provide any significant information on the putative function of p5 and p6, the presence of conserved nucleotide stretches at the termini of the RNA molecules encoding them and in the four previously identified viral RNAs supports the nature of RNA-5 and -6 as constituents of the FMV genome. Further evidence of this is provided by the results of RT-PCR analysis of 30 fig samples, which identified both novel RNAs exclusively in the 15 FMV-PCR positive samples (Fig. 2). Also the results of Northern blot hybridisation (Fig. 3) support the viral nature and the negative-sense polarity of both segments, similarly to what reported for the FMV genome.

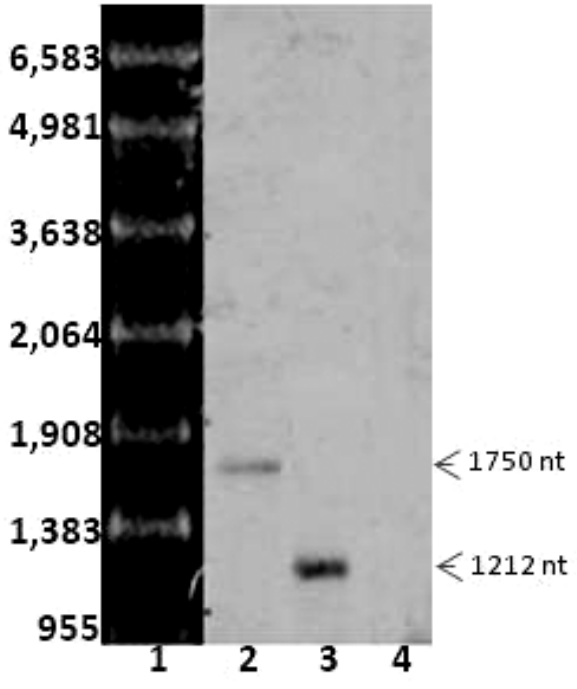

Fig. 3. Northern blot hybridization with positive-sense RNA probes of total nucleic acid extracts from a FMV-infected tree. Two single bands are detected which correspond to full-size RNA-5 (lane 2) and RNA-6 (lane 3). No hybridization signals with extracts from healthy fig tissues (lane 4). Lane 1 contains DIG-labelled DNA Molecular weight marker III (Roche, Italy).

Note added in proofs. The present study had been completed and the relative paper had been submitted and accepted, when we came to know of an ahead of print abstract reporting the discovery of the two additional RNAs in FMV (Ishikawa K., Maejima K., Komatsu K., Kitazawa Y, Hashimoto M., Takata D., Yamaji Y, Namba S., 2012. Identification and characterization of two novel genomic RNA segments of fig mosaic virus, RNA5 and RNA6. *Journal of General Virology* DOI: 10.1099/vir.0.042663-0).

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