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Short communication

Complete sequence of Fig fleck-associated virus, a novel member of the family *Tymoviridae*

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ABSTRACT

The complete nucleotide sequence and the genome organization were determined of a novel virus, tentatively named Fig fleck-associated virus (FFkaV). The viral genome is a positive-sense, single-stranded RNA 7046 nucleotides in size excluding the 3′-terminal poly(A) tract, and comprising two open reading frames. ORF1 encodes a polypeptide of 2161 amino acids (p240), which contains the signatures of replication-associated proteins and the coat protein cistron (p24) at its 3′ end. ORF2 codes for a 461 amino acid protein (p50) identified as a putative movement proteins (MP). In phylogenetic trees constructed with sequences of the putative polymerase and CP proteins FFkaV consistently groups with members of the genus *Maculavirus*, family *Tymoviridae*. However, the genome organization diverges from that of the two completely sequenced maculaviruses, *Grapevine fleck virus* (GFkV) and *Bombix mori Macula-like virus* (BmMLV), as it exhibits a structure resembling that of *Maize rayado fino virus* (MRFV), the type species of the genus *Marafivirus* and of Olive latent virus 3 (OLV-3), an unclassified virus in the family *Tymoviridae*. FFkaV was found in field-grown figs from six Mediterranean countries with an incidence ranging from 15% to 25%.

To date, short of 15 different viruses have been identified in fig (*Ficus carica*), the genomes of six of which, classified as definitive or tentative species of the genera *Closterovirus*, *Ampelovirus*, *Trichovirus*, *Alphacryptovirus* and *Emaravirus* (Elbeaino et al., 2006, 2007, 2009, 2010, 2011; Gattoni et al., 2009) have been sequenced completely, or in part. In the course of a recent survey of fig stands in Apulia (southern Italy), a plant of cv. Canestrelle (accession F4) was found, whose younger leaves exhibited a discrete clearing (flecking) of some of the veinlets. This plant was RT-PCR negative for all viruses of the above listed genera studied in our laboratory, but contained a dsRNA of *ca.* 7 kbp in size belonging to a hitherto undescribed member of the family *Tymoviridae*, tentatively called Fig fleck-associated virus (FFkaV). As reported in the present paper, this virus was characterized molecularly and a survey for its presence was carried out in several Mediterranean countries.

The family *Tymoviridae* is currently composed of three genera (*Tymovirus*, *Marafivirus* and *Maculavirus*), comprising viruses infecting cultivated and wild monocotyledonous and dicotyledonous plants as well as one entomovirus (Dreher et al., 2005; Katsuma et al., 2005). Genomes of viruses in the family *Tymoviridae* differ widely in the number (from 1 to 4) and distribution of genes, even within the same genus, but all encode a large polyprotein essential for viral replication (Dreher et al., 2005).

In the attempt to recover the mechanically transmissibile virus, inoculations to herbaceous plants including species of Solanaceae (*Nicotiana benthamiana*, *N. occidentalis*, *N. tabacum* cv. Samsun), Chenopodiaceae (*Chenopodium quinoa*, *C. amaranticolor*), Amaranthaceae (*Gomphrena globosa*) and Fabaceae (*Vigna unguiculata*, *Phaseolus vulgaris* cv. La Victoire) were repeatedly made using extracts from young leaves of accession F4 ground in 0.1 M phosphate buffer pH 7.2 containing 2.5% nicotine. Inoculated plants were kept in a climatized glasshouse at 22–24 °C but in no case symptoms developed, nor or any virus could be transmitted by subculturing from inoculated to a new set of hosts.

For virus sequencing and molecular characterization, dsRNA was extracted from leaf vein tissues (30 g) of accession F4 by phenol/chloroform and purified by chromatography through cellulose CF-11 column (Elbeaino et al., 2009), denatured with 0.1 M hydroxide methyl mercure and reverse-transcribed using random hexamers and/or an oligo(dT_{20}) primer according to Gubler and Hoffman (1983). Degenerate oligonucleotide primed (DOP)-PCR was first used to generate internal virus sequence fragments with primer DOP4 (5′-CCGACTCGAGNNNNNNTTTACG-3′) (Rott and Jelkmann, 2001) (Fig. 1A). Three couples of degenerate primers, which specifically amplify the tymoviral conserved motifs of methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA

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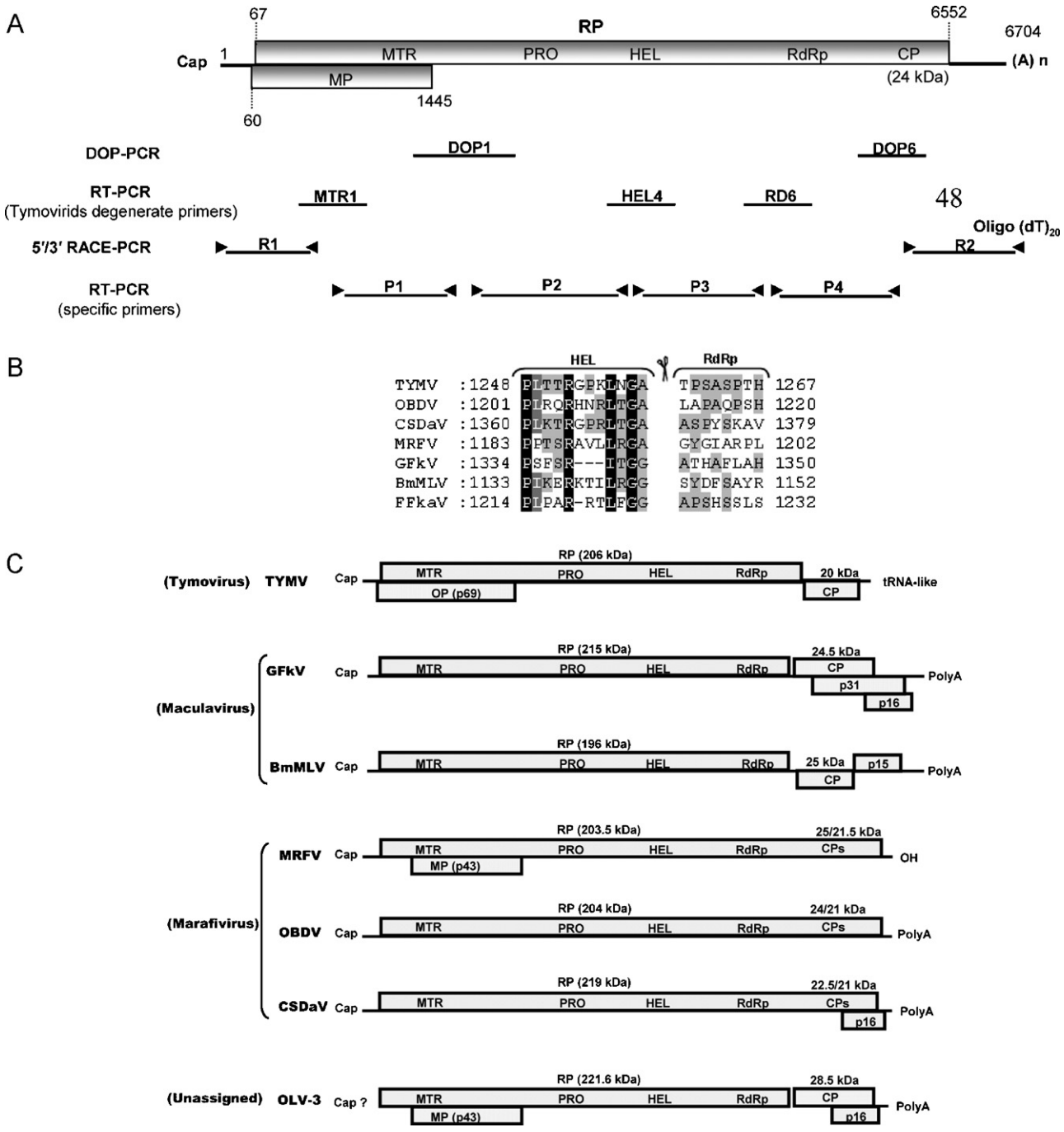

Fig. 1. A cDNA cloning and sequencing strategy of the FFkaV genome and schematic representation of its organization. ORF1 (upper box) codes for the replication-associated polyprotein RP containing the signatures of methyltransferase (MTR); papain-like protease (PRO); helicase (HEL); RNA-dependent RNA polymerase (POL) and the coat protein (CP) cistron. ORF2 (lower box) encodes the putative movement protein (MP). Bars represent the cDNA clones positioned sequentially according to Blast analysis and obtained as follows: (i) DOP1 and DOP6, clones generated from random reverse-transcribed dsRNA of FFkaV; (ii) MTR1, HEL4 and RD6, clones obtained from degenerate tymovirid-specific primers. (iii) R1 and R2, clones generated from RACE-PCR with the use of an oligo(dT_{20}) primer together with sense and antisense virus-specific primers flanking the 5′ and 3′ termini of the viral genome. (iv) P1 to P4, clones generated from multiple RT-PCR of missing sequences between gaps. (B) Prediction of putative papain-like protease cleavage sites (scissor) between the HEL and the RdRp domains of FFkaV and six tymoviruses. Numbers indicate the position of amino acids with respect to the RP polyproteins. Identical or similar residues are shadowed in black or grey, respectively. (C) Schematic representation of the divergent genome organisation of different members of the family *Tymoviridae*.

polymerase (RdRp) domains, were used in RT-PCR as described by Sabanadzovic et al. (2000) (Fig. 1A). The 3′ terminus of the viral RNA was amplified using a FFkaV-specific sense primer designed on the first DOP-PCR-generated clones in conjunction with an oligo(dT) primer, whereas the 5′ sequence was determined using the 5′/3′ RACE kit protocol (Roche Diagnostics, Milan, Italy) and a FFkaV-specific antisense primer designed on the sequence of the 5′ proximal end (Fig. 1A). Additional sense and antisense FFkaV-specific primers were then designed to generate products for filling sequence gaps.

PCR reactions were conducted using a mixture containing 1 mM $MgCl_2$, 2.5 mM dNTPs, 1.25 μM sense and antisense specific primers and 1U Taq polymerase (Promega, Madison, USA). PCR runs consisted of 35 cycles, with an initial denaturing temperature of 94 °C