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Biological and molecular characterization of a recombinant isolate of *Watermelon mosaic virus* associated with a watermelon necrotic disease in Italy

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Abstract The biological and molecular characterization is reported of a *Watermelon mosaic virus* isolate, denoted WMV-Le, associated with a necrotic phenotype of watermelon plants grown in the Provinces of Lecce and Taranto (Apulia, southern Italy). The fully sequenced WMV-Le genome consists of 10,045 nucleotides and is 99.1% similar to that of WMV-C05-270, a French isolate from melon of the WMV molecular group 3. Using recombination detection program RDP3, putative recombination breakpoints were identified close to nucleotide positions 42 to 1892, covering the 5′UTR/P1/HC-Pro region. The event represents the insertion of a sequence fragment of an isolate similar to WMV-FBR04-37 in the background of an isolate similar to WMV-FMF00-LL1. The field symptomatology was reproduced in watermelon plants grown in an experimental greenhouse but the virus induced severe symptoms also in *Cucumis sativus*, *C. melo*, *Cucurbita maxima* and *C. pepo* .

Keywords WMV · Watermelon · Recombination · Cloning · Genome sequence · Phylogenetic analysis · 5′UTR/P1/HC-Pro region

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Watermelon mosaic virus (WMV), genus *Potyvirus*, family *Potyviridae*, has a single messenger-polarity RNA genome of about 10 kb encapsidated in a flexuous filament *c.* 750 nm long. Viral RNA contains a unique open reading frame (ORF) coding for a polyprotein that, on cleavage, yields 10 putative functional proteins with motifs conserved among homologous proteins of other potyviruses (Desbiez and Lecoq 2004). The virus is closely related to *Soybean mosaic virus* (SMV), and it has been proposed might have emerged by interspecific recombination between *Bean common mosaic virus* and an SMV-like potyvirus in the P1 protein coding region (Desbiez and Lecoq 2004). Based on their identities in the 5′ and 3′ half of the genome, WMV isolates were classified into three

phylogenetic groups denoted 1, 2 and 3 (Desbiez et al. 2007). Besides this, sequence analyses from recent surveys in Spain, France and Iran reported important variability in specific coding regions and frequent intraspecific recombination (Moreno et al. 2004; Desbiez and Lecoq 2008; Sharifi et al. 2008; Desbiez et al. 2009). In France, isolates of group 3 have been found often associated with severe symptoms and identified as "emerging" (EM) (Desbiez et al. 2009).

Watermelon (*Citrullus lanatus*) is an important field crop in southern Italy, where it is exposed to infection by WMV, *Zucchini yellow mosaic virus* (ZYMV) and *Cucumber mosaic virus* (CMV) (Gallitelli et al. 1988; Gallitelli 2006). Until recently, the incidence of WMV infections on watermelon was not relevant economically because symptoms were mild and there was no significant impact on fruit yield and quality. However, since 2000, strains inducing various types of necrosis have been detected (Crescenzi et al. 2001) but none of them was characterized molecularly. In 2007, watermelon plants grown in the Provinces of Lecce and Taranto (Apulia region, southern Italy) had fruits with extended necrotic spots on the pericarp (Fig. 1a, b); and necrotic streaks on leaves (Fig. 1c) and occasionally stems. Leaf dips from naturally infected plants revealed the presence of a virus with filamentous particles (not shown), which were recognised by an antiserum to WMV in immunoelectron microscopy (IEM) (Milne and Luisoni 1977).

Here we report the biological and molecular characterization of this WMV isolate, named WMV-Le, providing evidence that it is a putative recombinant in

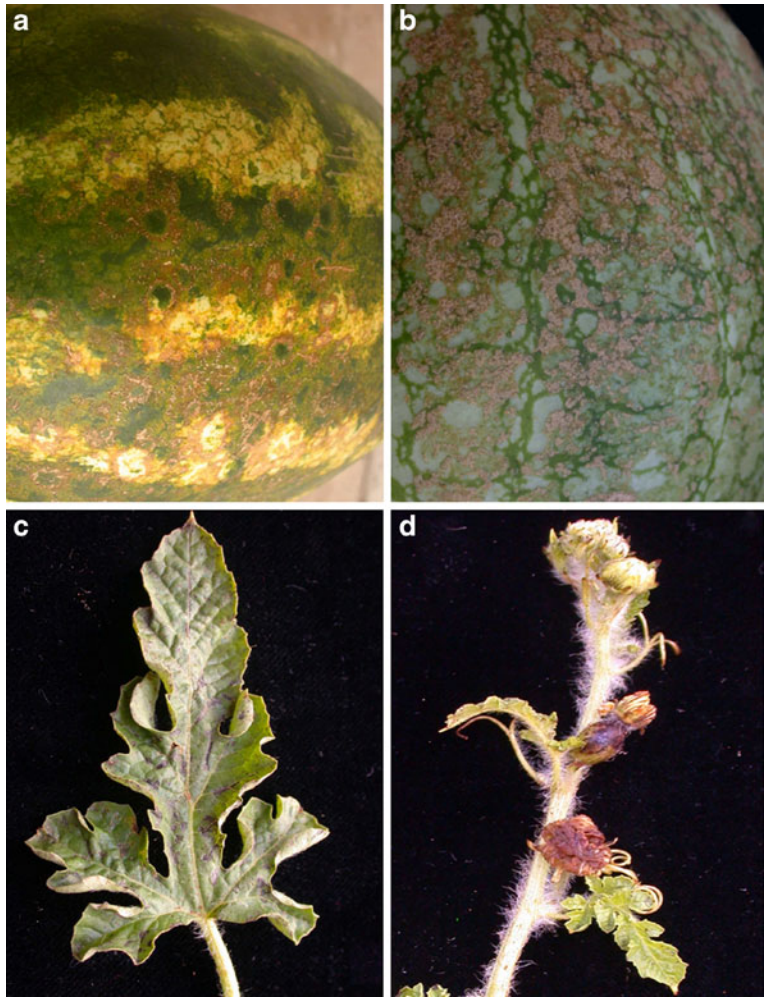

Fig. 1 Symptoms induced by WMV-Le in watermelon. In (**a**) and (**b**), are extended necrotic areas and ringspots observed on the pericarp of watermelon fruits grown in the Apulian Provinces of Taranto and Lecce (southern Italy). In (**c**) and (**d**), reproduction of the disease pattern observed in the field by mechanical inoculation with a purified preparation of WMV-Le onto watermelon cv Eureka. After the inoculation plants were moved to an insect-proof greenhouse where they were kept until fruit set for symptom observation

the 5′UTR-P1-HC-Pro coding region with potential parents from two EM French isolates, denoted FBR04-37 and FMF00-LL1 (Desbiez et al. 2009). We propose to allocate WMV-Le to the molecular group 3 (Desbiez et al. 2007) of WMV.

WMV was the only virus detected in five different watermelon plants showing the necrotic symptoms described. Tests based on molecular hybridization excluded the presence of CMV, ZYMV and *Cucumber vein yellowing virus* (not shown). The virus was transmitted to *Nicotiana tabacum* 'Samsun' at the four leaf stage by mechanical inoculation using extracts from tiny slices of the fruit epidermis, cut near the necrotic areas from one of the field plants. A purified virus preparation was obtained from systemically infected tobacco leaves according to the method described by Thompson et al. (1988) and used at a concentration of 100 ng μl^{-1} in 30 mM Na_2HPO_4 to inoculate six plants each of watermelon 'Eureka', zucchini squash (*Cucurbita pepo*) 'Greyzini', squash (*C. maxima*) 'Butternut', melon (*Cucumis melo*) 'Amarillo oro rugose' and cucumber (*C. sativus*) 'Marketer' and on the leaves of two to four plants of *N. tabacum* 'Xanthi', *N. benthamiana*, *N. glutinosa*, *Chenopodium amaranticolor* and *C. quinoa*. The watermelon plants were transplanted to large pots and moved to an insect-proof screenhouse to follow symptom development on leaves, stems and fruits. Zucchini squash, squash, cucumber, melon and watermelon reacted to WMV-Le infection with symptoms of severe systemic mosaic and leaf blade deformation (Table 1). The disease phenotype seen in the field was reproduced in the six plants of watermelon 'Eureka' as plants developed mosaic, severe distortion on the leaves and necrotic streaks along the stem and flower stalks. Fruits showed necrotic spots soon after setting, which prevented further development (Fig. 1d). WMV-Le was recovered from these plants; while dot blot hybridization with CMV and ZYMV probes were negative (not shown).

WMV-Le RNA was extracted from purified particles and used for cDNA synthesis, cloning and sequencing as described by Finetti Sialer et al. (1997). The complete WMV-Le genome sequence (GenBank accession no. FJ823122) consists of 10,045 nt, excluding the 3′ poly (A) tail. It contains a single large ORF, starting at nt 132 and ending at nt 9,791 and untranslated regions (UTR) at 5′ and 3′ termini that are 131 and 254 nt-long, respectively. The single ORF has the coding capacity for a polyprotein of 3,219 amino acids (aa). Its putative cleavage sites for the viral-encoded proteinases yield all the ten characteristic potyviral proteins with estimated sizes of 443, 457, 347, 52, 634, 53, 190, 243, 517 and 283 aa for P1, HC-Pro, P3, 6 K1, CI, 6 K2, VPg, NIa-Pro, NIb and CP, respectively. WMV-Le nucleotide sequence showed identity with other WMV isolates ranging from 89.9% (WMV-Pk) to 99.1% (WMV-C05-270). Bioedit (Hall 1999) and Clustal W (Thompson et al. 1994) were used to align the WMV-Le full-length sequence with those of 17 WMV isolates from GenBank (accession no. shown in Fig. 2a) and with that of SMV (accession no. AJ628750) as an outgroup. Phylogenetic relationships among the aligned sequences were inferred with the neighbour-joining method (Saitou and Nei 1987), using the Jukes Cantor distance model (1969) with the distance data matrix bootstrap re-sampled 1,000 times (Felsenstein 1985). The Treecon software (Van de Peer and De Watcher 1997) was used to construct and display the resulting trees. This analysis placed WMV-Le among isolates FMF03-141, FMF00-LL1 and C05-270 (Fig. 2a), all of which belong to the molecular group 3 described by Desbiez and Lecoq (2008). This cluster was distinct from isolates CHN, Korea and FBR04-37, which are isolates tentatively assigned to molecular group 3 (Desbiez and Lecoq 2008). The rest of the isolates, formed a third cluster, which included the molecular group 1 isolate WMV-Fr, the molecular group 2 isolate CHI87-620

Table 1 Symptoms induced by WMV-Le on experimental hosts range

Plant	Symptom strength	Symptom description
Chenopodium amaranticolor	mild	Ch/Nec ll
C. quinoa	severe	Ch/Nec ll, Mo,
Citrullus lanatus 'Eureka'	severe	Mo, Nec.
Cucumis sativus 'Marketer'	severe	Mo, Def.
C. melo 'Amarillo oro rugoso'	severe	Mo, Def.
Cucurbita maxima 'Butternut'	severe	Mo, Def.
Cucurbita pepo 'Greyzini'	severe	Mo, Def.
Nicotiana benthamiana	mild	Mo
N. glutinosa	mild	Mo
Nicotiana tabacum 'Samsun'	mild	Mo
N. tabacum 'Xanthi'	mild	Mo

Mo mosaic; *Def* leaf deformation; *Nec* leaf necrosis; *Ch/Nec ll* chlorotic necrotic local lesions

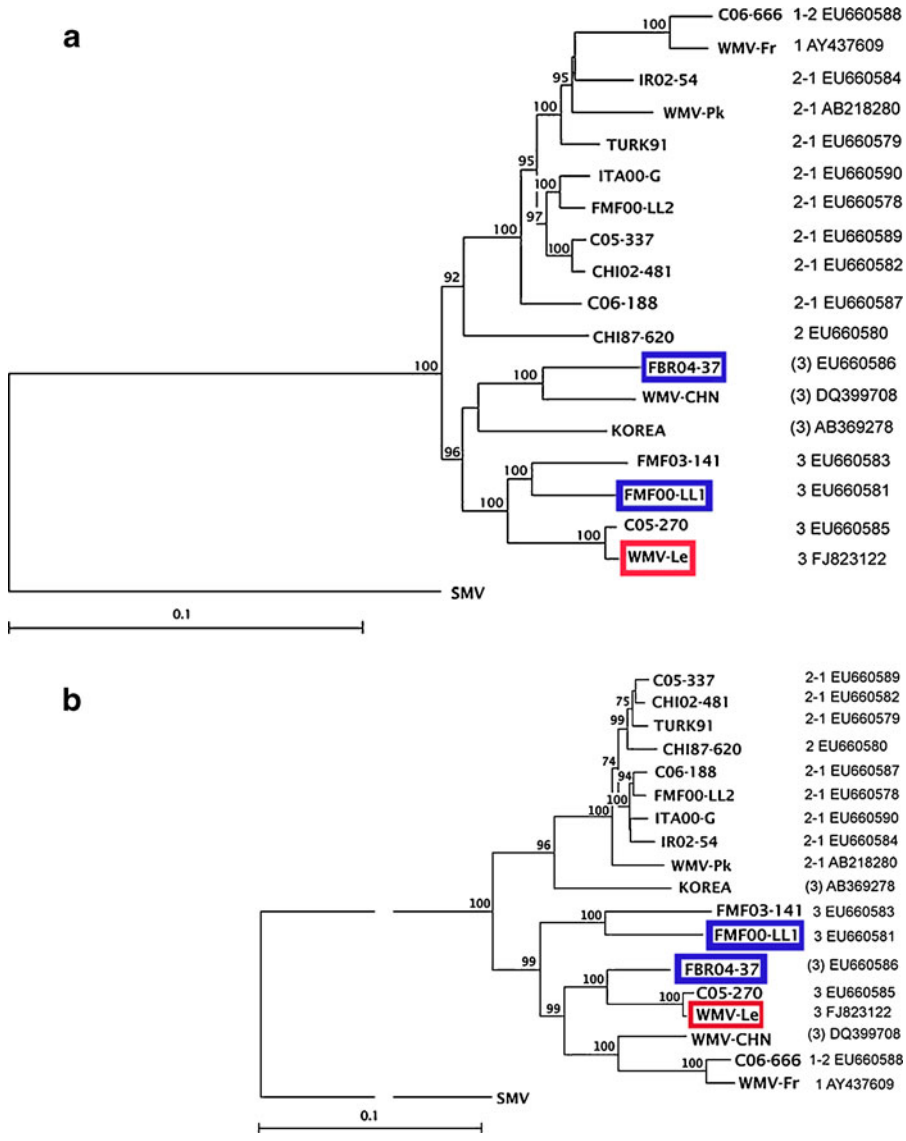

Fig. 2 Neighbour-joining tree between WMV-Le and 17 complete nucleotide sequences (**a**) and 1,850 nt sequence (position 42 to 1892) in the 5′ part of the genome (**b**) of WMV isolates available from database. Molecular group classification of each isolate was deduced from Desbiez and Lecoq (2008) and is reported on the right, followed by the GenBank accession number. Molecular groups were deduced by Desbiez and Lecoq and are as follows: 1, 2 and 3 are non recombinant isolates; 1–2, recombinants between group 1 (for the 5′part of the genome) and group 2 (for the 3′ part); while 2–1 indicates the reverse condition; (3) isolates that can be tentatively assigned to molecular group 3 (Desbiez and Lecoq 2008). Numbers at each node indicate percent bootstrap values. Horizontal branch length is drawn to scale. The bar indicates 0.1 nt replacements per site. The sequence of *Soybean mosaic virus* (SMV) (GenBank accession no. AJ628750) was used as an outgroup. Potential recombinant is boxed in red and potential parents in blue

and other isolates identified as recombinants between isolates of group 1 and 2 (Fig. 2a). In the distance trees based on either the 5′ half of the genome (Fig. 2b) or the region coding for CP (not shown), WMV-Le still clustered among the molecular group 3 isolates.

We screened the WMV-Le genome for any evidence of viral recombination. Putative recombination was identified using the several methods implemented in the recombination detection program version 3.31 (RDP3 Martin et al. 2005). Automated analysis was carried out using the default RDP3 settings with multiple comparison corrected, P-value cut-off of 0.05 and taking into account the linear sequence status. All the different automated programs identified a major recombination breakpoint at nt position 42 to 1892. The 1,850 nt-long putative recombination fragment involved a portion of the 5′ UTR, the entire putative coding region for P1 and about 1/3 of the putative HC-Pro. The recombination breakpoints (Rbps) were identified by RDP ($P=2.544\times10^{-20}$), GENECONV ($P=3.217\times10^{-08}$), Bootscan ($P=1.58\times10^{-21}$) (Fig. 3), MaxChi ($P=6.222\times10^{-10}$), Chimaera ($P=2.265\times10^{-04}$), SiScan ($P=2.860\times10^{-20}$) and 3 Seq ($P=5.854\times10^{-05}$). The same results were also obtained by excluding SMV from the analysis (not

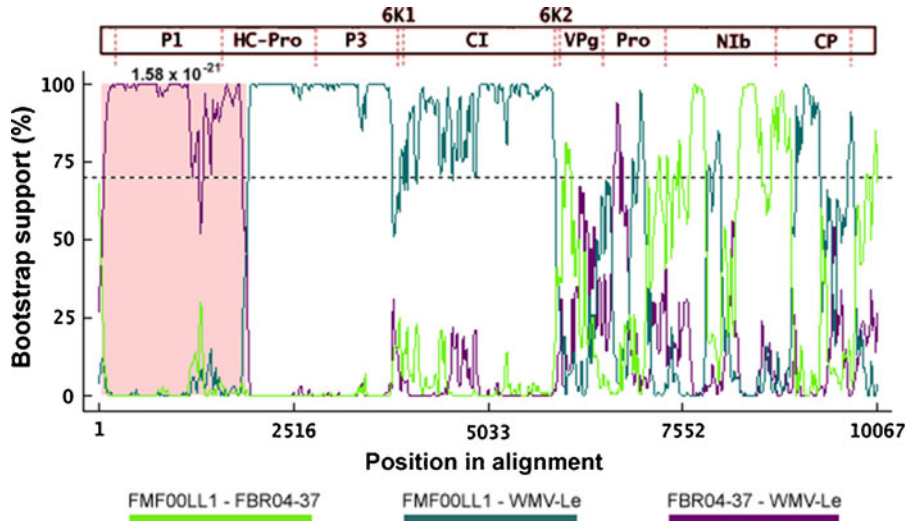

Fig. 3 Evidence of a 1,850 nt-long putative recombinant region in the 5′ part of WMV-Le genome detected by Bootscan ($P=1.58\times10^{-21}$). The sequences of FMF00-LL1 and FBR04-37 represent the likely parental sequences of WMV-Le. Pink box highlights the recombinant region, numbers on the left display the bootstrap values, the horizontal dotted line indicates a bootstrap threshold of 70. On the top of the figure is a map (not drawn to scale) of the virus genome organization. Vertical dotted lines indicate approximate position of WMV-Le ORFs

shown). The event is likely to represent an Rbp of an isolate similar to the FBR04-37 strain into the background of an isolate similar to the FMF00-LL1 strain, identified as putative major parent by all the programs in the RDP3 package. However, by removing the outgroup from the analysis, the strain FMF03-141 can be identified as putative parent as well. A second Rbp was possibly present at ca 6,000 nt, but since the parental genomes were not identified this Rbp was not taken into account.

Our results provide evidence that WMV-Le is the causal agent of the necrotic disease found in watermelon fields and that it is a recombinant isolate within the molecular group 3 of the virus. One of the putative parental strains of WMV-Le, FBR04-37, is a recombinant strain itself, which was tentatively assigned to molecular group 3 because the recombinant breakpoint in the P1-HC-Pro coding region could not be precisely determined and one of the parental sequences was not identified (Desbiez and Lecoq 2008). The other parent FMF00-LL1 (or FMF03-141) is a molecular group 3 isolate that does not contain Rbps (Desbiez and Lecoq 2008). The evidence that WMV-Le belongs to molecular group 3 is also supported by the fact that it potentially codes for the aa motif KEKET at position 3–7 in the N-terminal part of the CP. The KEKET motif has been described by Desbiez et al. (2007) as a characteristic of molecular group 3 isolates while group 1 isolates usually have a KEA and group 2 isolates have a KET.

To our knowledge this is the first complete characterization of a WMV isolate necrotic to watermelon found in Italy. The way by which it was introduced into Italy can only be matter of speculation: in addition to aphid transmission and commercial exchange of infected seedlings, the possibility that infected fruits imported for human consumption and probed by aphids could serve as source for primary virus infection, as demonstrated for ZYMV and *Papaya ringspot virus* in melon (Lecoq et al. 2003), cannot be ruled out.

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