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Research Papers

First characterization of infectious cDNA clones of *Olive mild mosaic virus*

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Summary. Full-length cDNA clones of an *Olive mild mosaic virus* (OMMV) isolate were constructed in order to find infectious cDNA clones. The sequencing of three individual full-length clones revealed some differences between them. *In vitro* transcription of these clones was performed and the effect of spontaneous mutations in the biological behaviour of the *in vitro* transcripts was evaluated by symptomatology, RNA accumulation and virus replication in inoculated plants. *In vitro* synthesized RNA from one of these clones was found to mimic the wild-type OMMV, making it useful in future studies on protein structure and function by site directed mutagenesis of individual genes. This is the first report on constructing full-length cDNA clones of OMMV from which infectious RNAs can be transcribed *in vitro*.

Key words: full-length cDNA clones; infectious *in vitro* transcripts.

Introduction

The genome of *Olive mild mosaic virus* (OMMV), within the genus *Necrovirus*, family *Tombusviridae*, consists of a single stranded positive-sense RNA molecule of about 3.7 kb. Its genome organization, with five predicted translation products, resembles that of other necroviruses, especially that of *Olive latent virus 1* (OLV-1) (Grieco *et al.*, 1996; Félix *et al.*, 2005) and the Chinese isolate of *Tobacco necrosis virus A* (TNV-A^{C}) (Xi *et al.*, 2008). This organization differs only in the size of the capsid protein (CP), which is similar to that of *Tobacco necrosis virus D* (TNV-D) isolates (Molnar *et al.*, 1997; Coutts *et al.*, 1991). Moreover, OMMV shares a high aa sequence identity of its pre-readthrough, RNA-dependent RNA polymerase (RdRp) and two small proteins (p8 and p6) with the corresponding proteins of OLV-1 and TNV-A isolates, and the CP of OMMV shares high aa sequence identity with that of TNV-D isolates. It is the homology of OMMV 's most relevant proteins (RdRp and CP) with those of different necroviruses which has led to its classification as a new species in the genus *Necrovirus* (Cardoso *et al.*, 2005).

The main role of each translated protein has been experimentally demonstrated for OLV-1, TNV-A and TNV-D, which are closely related to OMMV. Both pre-readthrough and RdRp proteins are essential for RNA replication of OLV-1 and TNV-D (Pantaleo *et al.*, 1999). The involvement of small proteins of necroviruses in cell-to-cell movement was demonstrated for each of the three p7 proteins of TNV-D^{H} (Molnar *et al.*, 1997). This has also been demonstrated for the p8 and p6 proteins of OLV-1 (Pantaleo *et al.*, 1999), for p7a, p7b and p5' of *Beet black scorch virus* (BBSV) (Yuan *et al.*, 2006), and the p8 and p6 proteins of TNV-A^{C} (Li *et al.*, 2008). Preventing the expression of each of these proteins blocked the spreading of

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these necroviruses from infected cells but not virus replication (Molnar *et al.*, 1997, Pantaleo *et al.*, 1999, Yuan *et al.*, 2006; Li *et al.*, 2008). The structural role of the TNV-D CP in encapsidation, and interaction with the zoospores of the fungus vector *Olpidium brassicae*, have been reported (Campbell, 1996). Additionally, studies involving CP mutants of TNV-D (Molnar *et al.*, 1997), OLV-1 (Pantaleo *et al.*, 2006) and BBSV (Yuan *et al.*, 2006) revealed that their CP is not essential for cell-to-cell movement but is probably involved in systemic movement. However, some of these and other possible associated functions of gene products, such as involvement in vector transmission and elicitation of symptoms or suppression of RNA silencing, still require further analysis, characterization and experimentation within the genus *Necrovirus*.

The present study contributes further annotation and genomic sequences of OMMV and describes the construction of infectious clones that facilitated the structure-function analysis of individual genes, which is essential to study the molecular biology of plant viruses (Palukaitis *et al.*, 2008). In this study, we assess the molecular variability of full length cDNA clones of OMMV and the infectivity of their transcripts.

Materials and methods

Cloning of full-length cDNA

For cloning the full-length cDNA of the OMMV genome, an OMMV isolate (GP isolate) recovered from olive trees (*Olea europaea* L.) was used. OMMV-GP was propagated in plants of *Nicotiana benthamiana* and purified from symptomatic mechanically inoculated plants as described (Cardoso *et al.*, 2004). Viral RNA was extracted from purified virus particles using RNeasy Plant Mini Kit (Qiagen, Germany) excluding the QIA shredder spin column step. The 3′ end of viral RNA was polyadenylated using poly (A) polymerase (Takara) and used as template for oligo(dT) primed cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen, Spain). Amplification of full-length OMMV genome was performed using the high-fidelity, proofreading FideliTaq DNA Polymerase (USB Corporation, United Kingdom) and primers FLGP-5′ and 2FLGP-3′. Primer FLGP-5′ (5′-*AACTGCAG*TAATACGACTCACTATA**GG**AGTATACATACCAAGTATACGG-3′) contains the first 22 nt of OMMV genome at the 3′ terminus followed by 17 nt from bacteriophage T7 RNA polymerase promotor consensus sequence (underlined), plus two extra G residues (bold), to enhance promoter's activity (Dunn and Studier, 1983), and eight residues at the 5′terminus to form a *Pst*I restriction site. Primer 2FLGP-3′ (5′-*GTACCC*GGGGTGGGGCAAAGGCC-3′) contains the last 17 nt of OMMV genome at the 3′ terminus (underlined) followed by six residues at the 5′ terminus that, with the last 3 G of OMMV genome, constitute a *Sma*I restriction site. The full-length RT-PCR product of the OMMV genome (FLOMMV fragment) was digested with the restriction enzymes *Pst*I and *Sma*I, and subsequently ligated with de-phosphorylated *Pst*I-*Sma*I digested pUC18 vector (Figure 1) and cloned in Top10 *E.coli* competent cells.

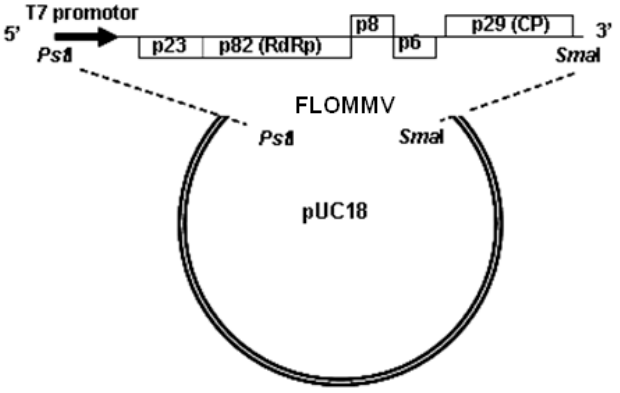

Figure 1. Schematic representation of pUFLOMMV clones. The FLOMMV RT-PCR product contains the *Pst*I and *Sma*I restriction sites for cloning in pUC18 vector and the T7 promotor sequence for *in vitro* transcription purposes.

DNA sequencing and sequence analysis

The DNA sequencing was performed by Macrogen (Seoul, Korea) using universal primers M13Fwd and M13Rev and viral specific primers. Sequence analyses were carried out using BioEdit (Hall, 1999) and Clustal W (Thompson *et al.*, 1994) was used for nucleotide and amino acid sequences alignments. Molecular variability was evaluated using MEGA 3.1 (Kumar *et al.*, 2004) and DnaSP 5.10 (Librado and Rozas, 2009). The nucleotide diversity (Pi), which is the mean number of nucleotide substitutions per site

+/- standard deviation, was estimated using software DnaSP 5.10 using the Jukes-Cantor model as substitution model.

In vitro transcription

Constructed full-length cDNA clones were used for *in vitro* transcription. Plasmidic DNA containing viral full-length cDNA under control of the promoter of bacteriophage T7 was linearized after digestion with *Sma*I restriction endonuclease and used for *in vitro* transcription using RiboMAX™ Large Scale RNA Production System –T7 (Promega, Madisson, WI USA), according to manufacturer's instructions.

Biological activity of *in vitro* transcripts

The biological activity of *in vitro* transcripts was tested by mechanically inoculating host plants. About 2 μg of synthesized RNAs and 2 μg of viral RNA (positive control), extracted as described, were rubbed on to *N. benthamiana* and *Chenopodium murale* leaves previously dusted with carborundum then lightly scraped. The plants were maintained in a controlled environment chamber with 8 h photoperiod and temperatures that varied from 18ºC (night) to 24ºC (day). Symptoms were recorded during 5 weeks and total RNA was extracted from inoculated and non-inoculated leaves using RNeasy Plant Mini Kit (Qiagen) after 7–8 days post-inoculation (dpi). A total RNA gel electrophoresis was initially carried out to evaluate the quality of the RNA and to normalize the amount of plant RNA to be used in Northern blot analysis. The band corresponding to plant ribosomal 18S RNA (ca. 1.9 kb), was used as internal control. Also, a DNA probe corresponding to a OMMV genomic region with ca. 1.1 kb was obtained by RT-PCR using primers GP_15' and GP_13', previously described (Cardoso *et al.*, 2004). DIG-labelled products were detected using a DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) and were used for the Northern blot analysis.

Results

Genetic differences between full-length cDNA clones

The complete nucleotide sequences of the cDNA inserts of three selected recombinant plasmids, designated (with accession numbers) pUFLOMMV-3 (HQ651832), pUFLOMMV-4 (HQ651833) and pUFLOMMV-5 (HQ651834), were fully determined and submitted to Genbank. Sequence analysis of these three full-length OMMV cDNA clones has indicated 30 nucleotide differences among the cDNA sequences, which correspond to a nucleotide diversity (Pi) of 0.00545+/-0.00172. An interclonal Pi of 0.00554+/-0.00195 was found for the RdRp gene, and 0.00496+/-0.00145 for the CP gene. The nucleotide sequence of the 5′ UTR is conserved among the three sequenced clones. Only one nucleotide variation at the 3′ UTR position 3545 was detected.

Some of the nucleotide differences lead to aa substitutions in the encoded proteins. From a total of 18 nucleotide mutations found at the RdRp, nine were synonymous changes and other nine were replacement changes. Four aa substitutions were detected between FLOMMV-3 and FLOMMV-4 RdRp, and six between FLOMMV-3 and FLOMMV-5 RdRp. Another six aa substitutions were observed between FLOMMV-4 and FLOMMV-5 RdRp sequences. None of these changes are present in conserved motifs previously described (O'Reilly and Kao, 1998). Six nucleotide mutations were found within the CP sequences, two were synonymous changes and four were replacement changes. Three CP aa substitutions were detected between FLOMMV-3 and FLOMMV-4 and only one aa substitution was detected between FLOMMV-3 and FLOMMV-5. Four aa substitutions were found between FLOMMV-4 and FLOMMV-5 CP sequences. Regarding the small proteins, the p6 aa sequences of FLOMMV-3, FLOMMV-4 and FLOMMV-5 were almost identical only differing in one aa (aa 44) in FLOMMV-5. A single aa difference was also detected between each cloned product of p8 analyzed. These variations are located at aa 8 and aa 32.

In vitro transcription and biological activity of *in vitro* transcripts in host plants

In order to find a full-length cDNA clone whose transcript mimics the wild-type OMMV, *in vivo* analysis of the transcripts from all three full-length mutants was performed. *In vivo* analysis of these transcripts was also performed in order to evaluate the viral molecular variability effect in pathogenicity. *In vitro* transcripts of pUFLOMMV-3, pUFLOMMV-4 and pUFLOMMV-5 were analysed by agarose gel electrophoresis and found to be ca. 3.7 kb long, iden-

tical to OMMV viral RNA (not shown). The infectivity of these transcripts was tested in *N. benthamiana* and *C. murale*. Viral RNA extracted from wild-type OMMV was used as reference control. Plants were first assessed for virus infection by examining symptoms in inoculated plants. Five independent assays were performed and the observed symptoms were consistently repeatable but different among inoculated transcript (Figure 2).

The *N. benthamiana* plants inoculated with viral OMMV RNA (control) developed necrotic spots on inoculated leaves 3–5 dpi and systemic symptoms consisting of leaf curling and necrosis 5–8 dpi. *Nicotiana benthamiana* plants older than four to six leaf stages did not express systemic symptoms. Necrotic spots were observed in leaves of *C. murale* inoculated with OMMV RNA. The necrotic spots were observed 3–5 dpi and no systemic symptoms developed after 5 weeks post-inoculation. Only the FLOMMV-5 transcript caused similar symptoms to the wild-type RNA. Systemic symptom expression of FLOMMV-5 is modulated by the growth stage of inoculated *N. benthamiana*. FLOMMV-4 transcripts did not induce symptoms in *N. benthamiana*, even after 5 weeks post-inoculation. FLOMMV-3 transcripts consistently induced necrotic spots in inoculated leaves of *C. murale* 3–5 dpi and only once induced small necrotic lesions in inoculated leaves of *N. benthamiana* 3–5 dpi. No systemic symptoms were induced in plants inoculated with FLOMMV-3.

Analysis of total RNA extracted from host plants inoculated with transcripts and the wild-type RNA was made by northern blot analysis, seeking to assess the effect of the spontaneous mutations on viral RNA accumulation and virus replication. Total RNA was extracted from symptomatic and/or asymptomatic, inoculated and non-inoculated upper leaves. Total RNA from non-inoculated (healthy) *N. benthamiana* and *C. murale* plants was used as negative control (Figure 3; lane 1 and 2). The detection of RNA ca. 3.7 kb corresponds to genomic RNA (gRNA), and the smaller RNAs, of 1.6 kb and 1.3 kb, correspond to subgenomic RNAs (sgRNAs). Both types of RNA are indicative of virus replication and were detected in the reference control OMMV RNA inoculated *N. benthamiana* plants, indicating that both inoculated

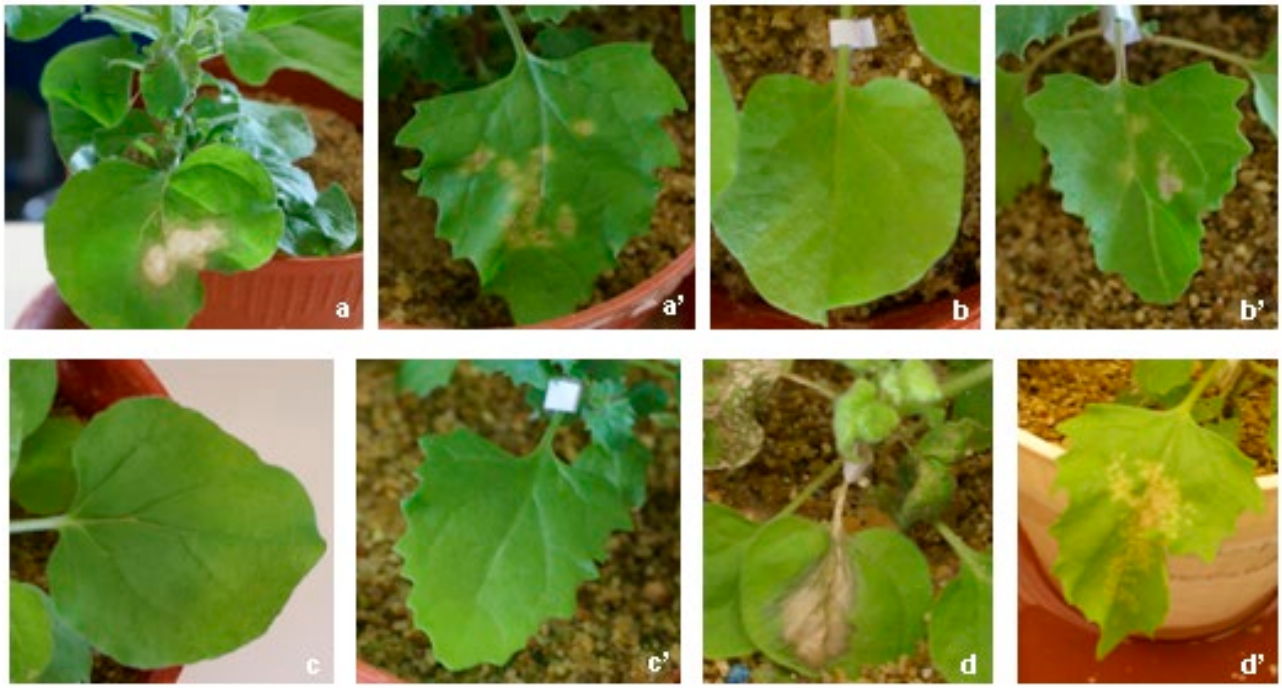

Figure 2. Symptoms induced in *Nicotiana benthamiana* and *Chenopodium murale* after inoculation with viral RNA from wild-type OMMV (a and a'), and viral RNA transcripts from pUFLOMMV-3 (b and b'), pUFLOMMV-4 (c and c') and pUFLOMMV-5 (d and d').

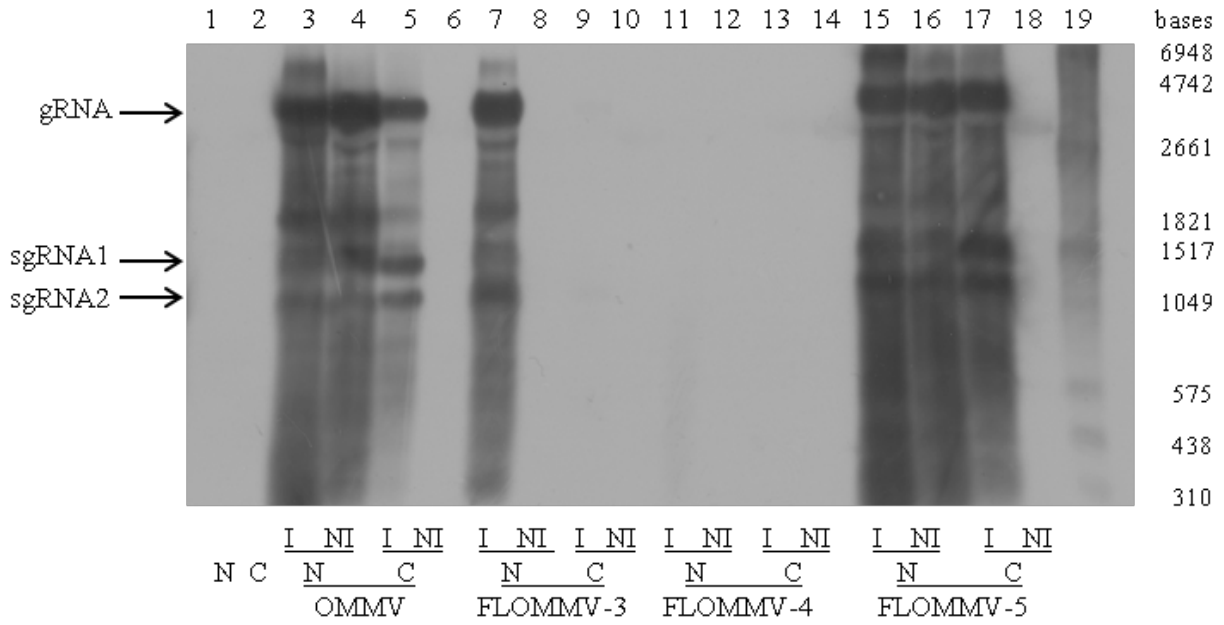

Figure 3. Northern blot analysis of total RNA extracted from *Nicotiana benthamiana* (N) and *Chenopodium murale* (C). Total RNA from non-inoculated plants used as negative control (lanes 1 and 2). Total RNA from inoculated (I) and non-inoculated (NI) leaves is detectable as follow: OMMV RNA (lanes 3, 4, 5 and 6), FLOMMV-3 (lanes 7, 8, 9 and 10); FLOMMV-4 (lanes 11, 12, 13 and 14); FLOMMV-5 (lanes 15, 16, 17 and 18). RNA Molecular Weight Marker I, DIG-labelled, 0.3–6.9 kb (Roche) (lane 19). Arrows indicate the bands corresponding to gRNA (ca. 3.7 kb) and sgRNAs (1.6 and 1.3 kb).

and non-inoculated leaves contain virus specific RNAs (Figure 3; lanes 3 and 4). OMMV RNAs were detected only in inoculated leaves of *C. murale* (Figure 3; lanes 5 and 6).

Virus specific RNAs were also detected in symptomatic *N. benthamiana* and *C. murale* leaves inoculated with transcript from pUFLOMMV-3 (Figure 3; lanes 7 and 9), but the intensity of the signal was less in symptomatic leaves of *C. murale* (Figure 3; lane 9) if compared against the control OMMV RNA in the same host (Figure 3; lane 5). Sometimes these were only visible using longer X-ray exposure time (data not shown). These results show that the accumulation rate of FLOMMV-3 transcripts in *C. murale* is less than that observed in the wild type OMMV RNA. No viral RNAs were detected in asymptomatic leaves revealing lack of systemic invasion by FLOMMV-3 (Figure 3; lane 8) and weak pathogenicity in *N. benthamiana* plants. FLOMMV-3 only induced symptoms in inoculated leaves of *N. benthamiana* once out of the five assays.

No viral RNAs were detected in any of the *N. benthamiana* and *C. murale* plants inoculated with FLOMMV-4 transcript (Figure 3, lanes 11-14). This indicates that RNA transcripts derived from pUFLOMMV-4 are not infectious in *N. benthamiana* and *C. murale* plants.

RNA molecules with the same size and with similar signal intensity to those detected in wild type RNA were identified in inoculated symptomatic and in non-inoculated leaves tested with FLOMMV-5 transcript (Figure 3; lanes 15, 16 and 17). No virus specific RNAs were identified in asymptomatic non-inoculated leaves. RNA transcript of FLOMMV-5 induced systemic infection in *N. benthamiana* in several assays; however, no virus signal was detected in northern blot when no symptoms were observed (data not shown). Similar results were found in all tested non symptomatic leaves. These results indicate that the lack of symptoms is associated to absence of virus infection.

The RNA transcript derived from pUFLOMMV-5 was the only one, among the three clones, whose biological activity is similar to that of wild-type OMMV RNA. For this reason, pUFLOMMV-5 was identified as the OMMV full-length clone from which in vitro synthesized viral RNA mimics the wild-type OMMV.

Discussion

The finding and subsequent sequencing of three individual full-length clones indicate that OMMV exists in nature as a virus population probably made up of several variants. This is explained since RNA viruses have genomes that replicate in the absence of efficient repair mechanisms, and insertion errors often occur (Domingo and Holland, 1997). Yet we found that the nucleotide diversity between the three full length OMMV clones is low. This limited genetic variability (<0.1) is also similar to that reported for other plant viruses (Garcia-Arenal *et al.*, 2001).

The pathogenicity and infectivity of the three natural mutants was analyzed in host plants, revealing that the transcript derived from pUFLOMMV-5 is the one that best mimics the wild type OMMV RNA and all the viral proteins encoded by FLOMMV-5 are functional. This transcript could be used in the future to study structure-function relationships of individual proteins. On the other hand, the lack of infectivity of pUFLOMMV-4 transcript makes it a good candidate to infer mutations affecting infectivity. Failure to replicate could be a result of mutations found in the RdRp aa sequence. Of the six aa substitutions detected between the RdRp sequences of FLOMMV-4 and FLOMMV-5, two were un-favourable changes (C to H and S to P) (Betts and Russell, 2003). These changes could affect the structure/function of the FLOMMV-4 RdRp protein. Alternatively, the lack of infectivity of this transcript may be because the mutations found in the p6 and p8 small proteins affect cell-to-cell movement of viruses. These mutations may be restricting the replication to the initial infection site and are not detectable by our methodology. In the FLOMMV-4 and FLOMMV-5 small proteins, only one unfavourable aa substitution in p6 sequences was detected: T instead of I (Betts and Russell, 2003). The low rate of pUFLOMMV-3 transcript replication in *C. murale* plants and low infectivity in *N. benthamiana* plants could similarly be a result of failure to replicate or inhibited cell-to-cell movement. Among the six aa substitutions detected between the RdRp sequence of FLOMMV-3 and FLOMMV-5, two were also unfavourable changes (C to H and P to L) (Betts and Russell, 2003), one being the same as that found in FLOMMV-4 RdRp. The lack of systemic symptoms in *N. benthamiana* plants could be a result of the mutations detected in pUFLOMMV-3 CP sequence, as the closely related TNV-D requires its CP or intact viral particles for efficient long-distance movement (Molnar *et al.*, 1997).

Our results suggest that small differences in viral proteins such as RdRp, CP or small proteins, may determine variations in the virus infectivity in plant hosts.

We believe this is the first report describing the construction of biologically active, full-length cDNA copies of OMMV genome expressing different phenotypes.

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