FURTHER CHARACTERIZATION OF OLIVE LEAF YELLOWING-ASSOCIATED VIRUS

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SUMMARY

RACE-PCR using a denatured dsRNA extract from an olive plant infected by Olive leaf yellowing-associated virus (OLYaV), extended a previously sequenced large fragment of the viral genome by 854 nt downstream in the heat shock protein 90 (HSP90). This sequence showed from 45 to 53% identity at the nucleotide level with that of putative HSP90s of different members of the family *Closteroviridae.* In phylogenetic trees constructed with published HSP90 and RdRp sequences, OLYaV was well separated from other viruses, as was Little cherry virus 1 (LChV-1), another unassigned species in the family *Closteroviridae.* A polyclonal antiserum was raised to a recombinant protein encoded by the HSP70 gene in fusion with sequence encoding the maltose binding protein. In Western blots of extracts from infected olive tissues, IgGs purified from this antiserum reacted with a polypeptide with a M_r of *ca*. 65 kDa, which was identified as the putative viral HSP70. Closterovirus-like flexuous particles with distinct cross banding and a maximum length of *ca*. 2000 nm were seen in extracts from infected roots and leaves of rooted olive cuttings. The cytopathology of infected leaves was similar to that induced by other members of the family *Closteroviridae*. However, the new information is still insufficient for the ultimate classification of OLYaV.

Key words: Olive, closterovirus, sequencing, RACE-PCR, cytopathology, recombinant protein, serology, Western blot, Immunodot blot.

INTRODUCTION

Olive leaf yellowing-associated virus (OLYaV), is currently classified as unassigned species in the family *Closteroviridae*, because the extant biological and molecular information does not allow its assignment to any of the three genera of the family (Martelli *et al.*, 2002). Only part of the viral genome, comprising 4,605 nt from ORFs 1b (RdRp), 2 (21K), 3 (7K), 4 (HSP70), and the 5' end of ORF 5 (HSP90) has been sequenced (Sabanadzovic *et al.*, 1999; Saponari, 2001; Saponari *et al.*, 2004, under GenBank accession n. AJ440010). In this work, genome sequencing was extended towards the 3' terminus of ORF 5 (HSP90), an antiserum was produced to the recombinant protein coded for by ORF 4 (HSP70), and the cytopathology of infected olive leaves was investigated.

MATERIALS AND METHODS

Double-stranded RNA extraction, cloning and sequencing. Cortical shavings from cuttings of an OLYaV-infected olive cv Ogliarola (accession OG9) were used for dsRNA extraction (Sabanadzovic *et al.*, 1999).

The nuclease-treated dsRNA (about 500 ng) was eluted and denaturated by incubation with 20 mM methylmercuric hydroxide for 10 min at room temperature. The reaction was stopped by adding 1 µl β-2-mercaptoethanol (0.5 M) and cooled in ice. cDNA was synthesized by using an OLYaV-specific primer (NES8: 5'-TTCGTTCTTGGGAGGTCTTTACTC-3') designed from the terminal sequence of the HSP70 gene (position: 4517-4540, GenBank accession number AJ440010). For reverse transcription 200 U μl^{-1} of *Avian myeloblastosis virus* (Roche Diagnostic, Indianapolis, USA) was used. At the 3' end of cDNA a poly(A) tail was added by incubating the mixture at 37°C for 10 min with 10 U of Terminal deoxynucleotidyl Transferase (TdT).

A 2.5 ml aliquot of PolyA-cDNA was amplified in a single primer amplification reaction (Lambden *et al.*, 1992) with 1.25 ml (10 pmol μl^{-1}) primer oligo $dT_{(16)}$, 1.25 ml (10 pmol μl^{-1}) of OLYaV-specific primer NES8 and 0.25 µl Taq polymerase (4.5 U) (Takara, Shiga, Japan). An annealing temperature gradient from 42 to 55°C was used.

PCR fragments isolated from the agarose gel and ligated in the pGEM-T Easy vector (Promega Corp, Madison, WI, USA) were used to transform *E. coli* DH5-cells. Double-stranded DNA templates were analyzed

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after enzymatic digestion and subjected to automated sequencing (MWG, Biotech, Ebersberg, Germany). The sequence data were assembled using the DNA Strider Program (Marck, 1988). CLUSTAL W (Pearson and Lipman, 1988) was used to align the obtained sequence with comparable sequences of members of the family *Closteroviridae* and phylogenetic analysis was made with programs from the PHYLIP package (version 3.4, Felsenstein, 1989).

Production of recombinant HSP70 protein and immunization. Total nucleic acid (TNA) was extracted as described by Foissac *et al.* (2001) from accession OG9 and reverse transcribed with random primers. A set of primers, based on known OLYaV sequence, was synthesized to amplify and clone the whole HSP70 gene. The set was HSP I (GTTCTAGAATGACGGTTTTGGGT CTTGACTTTGG) and HSP II (ACGTCTGCAGT-CATCTAGTATAGACTGAACATTCCGG). The restriction sites (*XbaI* and *PstI*, respectively) introduced to facilitate directional cloning of the PCR product are underlined. The targeted HSP70 coding region consisted of 1,800 nt. The resultant PCR product was ligated into the protein expression vector pMAL-c2x in fusion with maltose binding protein (MBP) (New England Biolabs, Beverly, MA, USA).

The recombinant protein was synthesized in *E. coli* DH5-α cells by adding isopropyl-β-thiogalactoside (IPTG) to a final concentration 0.3 mM. After incubation at 37°C for 3 h cells were harvested, resuspended in lysis buffer (10 mM sodium phosphate, pH 7.0 buffer containing 30 mM NaCl, 0.25% Tween20, 10 mM 2-mercapthoethanol and 10 mM EDTA) and frozen overnight at -20°C. Then, cells were sonicated and NaCl was added to a final concentration 0.5 M. After centrifugation the resulting supernatant fraction was retained for purification of the fusion protein using chromatography over an amylose resin column (New England Biolabs, Beverly, MA, USA).

Recombinant protein was analyzed by using electrophoresis in the discontinuous SDS-PAGE system (Laemmli, 1970) and transferred to PVDF membrane (Millipore, Bedford, MA, USA) in semi-dry BioRad apparatus (BioRad Laboratories, Hercules, CA, USA). A rabbit antiserum specific to the MBP was used at a dilution of 1:5,000 to detect the fusion products. Five hundred mg of each purified recombinant protein in 0.5 ml of 20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA, pH 7.4, was emulsified with an equal amount of Freund's adjuvant and injected subcutaneously and intramuscularly into a rabbit. Two additional injections were given at weekly intervals. A bleeding was made 2 weeks after the final injection followed by the final bleeding 2 weeks later. Following pre-absorption of antiserum with healthy olive sap, IgGs were purified by passage through a Sepharose-protein A column, designated as IgG-HSP70, and stored at -20°C.

Protein detection in olive tissue extracts. Immunodetection of the homologous antigen using IgG-HSP70 antiserum was done by Western blotting. For this, 100 mg of tissue from OLYaV-infected olive explants grown *in*

Table 1. Percentage nucleotide identity among three genomic regions (a: replicase domain; b: HSP90 whole gene; c: HSP70, phosphate motifs 1 and 2 of eight viruses in the family *Closteroviridae.*

		OLYaV	LIYV	GLRaV-1	GLRaV-2	GLRaV-3	LChV-1	CTV	BYSV
BYV	a	51	47	47	60	50	43	58	61
	b	46	45	46	53	46	46	49	53
	c	54	46	51	61	51	50	44	61
BYSV	a	48	41	49	63	51	44	53	
	b	47	46	46	53	46	46	49	
	c	53	48	51	59	50	50	43	
CTV	a	51	45	44	53	47	45		
	b	47	46	46	49	46	46		
	c	43	46	43	45	44	44		
LChV-1	a	46	54	47	43	44			
	b	46	48	45	44	45			
	c	51	44	53	50	55			
GLRaV-3	a	46	38	55	53				
	b	46	44	49	46				
	c	52	44	57	51				
GLRaV-2	a	49	40	49					
	b	45	45	46					
	c	53	46	49					
GLRaV-1	a	48	42						
	b	44	46						
	c	51	45						
LIYV	a	43							
	b	45							
	c	46							

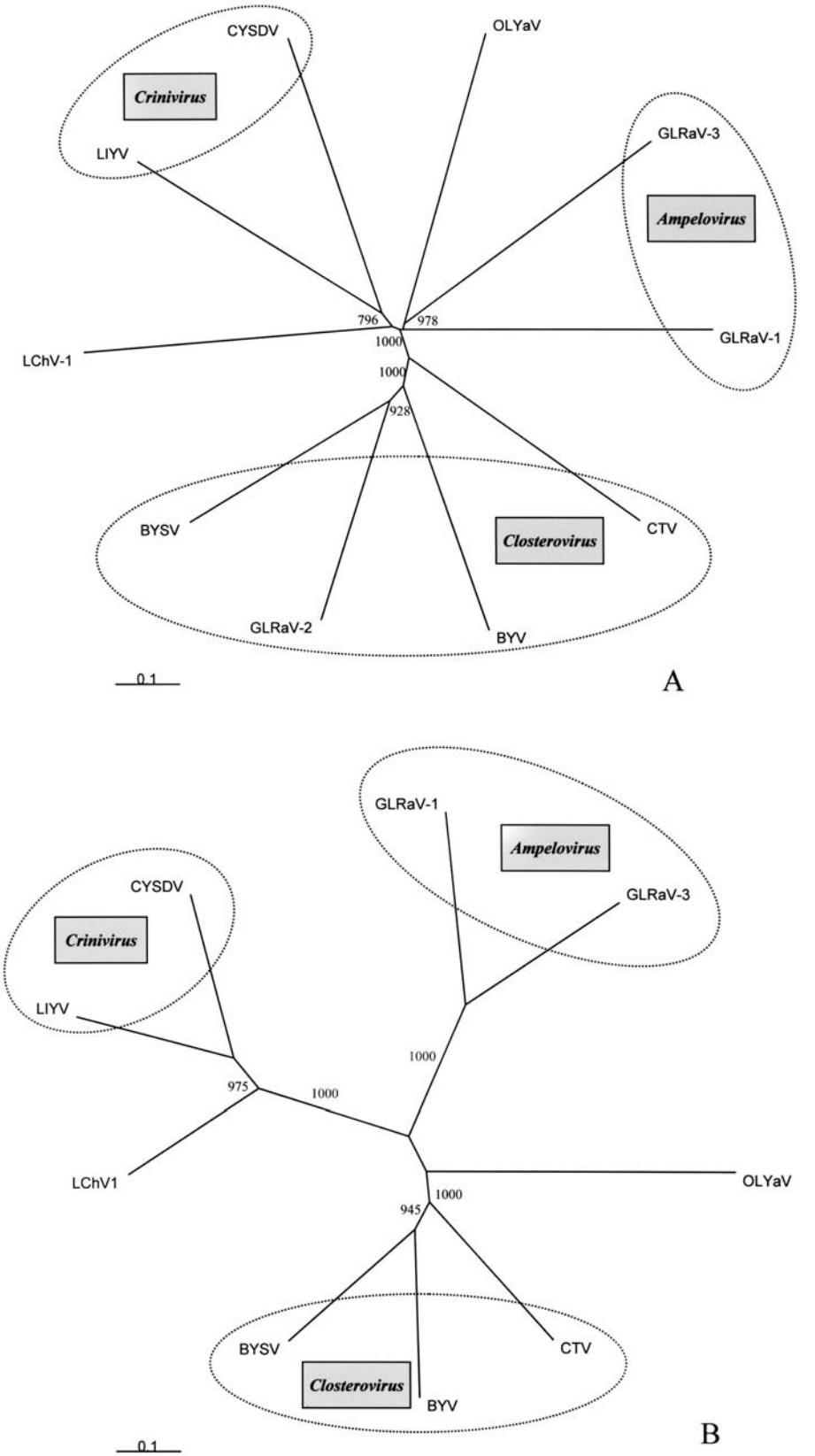

Fig. 1. Phylogenetic trees constructed with available HSP90 (**A**) and replicase domain (**B**) sequences of some members of the family *Closteroviridae*. *Beet yellows virus* (BYV, PMID12368343), *Citrus tristeza virus* (CTV, NC001661), *Beet yellow stunt virus* (BYSV, PMID8207405), *Grapevine leafroll-associated virus 2* (GLRaV-2, PMID 9603345), *Grapevine leafroll-associated virus 1* (GLRaV-1, AF195822), *Grapevine leafroll-associated virus 3* (GLRaV-3, NC004667), *Lettuce infectious yellows virus* (LIYV, U15440, NC003618), *Cucumber yellow stunting disorder virus* (CYSDV, AJ439690, NC004809), Little cherry virus 1 (LChV-1, NC001836), OLYaV (AJ440010). LChV-1 and OLYaV are unassigned species in the family.

vitro were frozen in liquid nitrogen, crushed to a fine powder and extracted in 5 vol. of extraction buffer [Tris-HCl 0.5mM, pH 8.8, 4% (w/v) SDS, 40% (w/v) glucose and 4% (w/v) 2-mercapthoethanol (Berger *et al*., 1989)]. Extracts were boiled for 5 min, briefly centrifuged, and samples of 30 µl aliquots were loaded onto SDS polyacrylamide slab gels, and electro-blotted onto PVDF membrane. The membranes were blocked with a solution of 3.5% non-fat dry milk and 0.5% bovine serum albumin and incubated overnight at 4°C followed by exposure for 1 h to anti-HSP70 IgGs, at a dilution of 1:1,500. ECL-plus Western blotting detection kit (Amersham Bioscences, Little Chalfont, England) was used after 30 min incubation with horseradish peroxidase-labelled antibodies (1:10,000) (Amersham Bioscences, Little Chalfont, England). The same denatured plant extracts were spotted on PVDF membranes and processed for detection as above.

Electron microscopy. Dips in 2% aqueous uranyl acetate were made from leaf petioles of several OLYaV-infected olive accessions, including OG9, and from leaf petioles and young roots of a rooted olive explant that had been tissue cultured for the elimination of OLYaV but had remained infected. For thin sectioning, tissue fragments from leaves of the explant were processed according to standard procedures (Martelli and Russo, 1984), i.e. fixation in 4% glutaraldehyde in 0.05M phosphate buffer, post-fixation in 1% osmium tetroxide for 2h at 4°C, staining overnight in 2% aqueous uranyl acetate at 4°C, dehydration in ethanol, and embedding in Spurr's medium**.** Thin sections were stained with lead citrate and viewed with a Philips Morgagni 282D electron microscope. Controls consisted of leaf tissues from a healthy olive seedling processed as above.

RESULTS

Identification of the HSP90 gene in OLYaV and phylogenetical analysis. An excellent source of viral dsRNA was extracts from cortical shavings. These yielded about 25 ng from 100 g of starting material.

A product of *ca*. 854 bp was amplified by RACE-PCR and recognized as a fragment of the putative HSP90 homologue protein. In pairwise comparisons among nucleotide sequences of OLYaV RdRP, HSP70 and HSP90 genes and those of other members of the family *Closteroviridae,* identity values for OLYaV sequences were between 43 and 51% for RdRp, between 44 and 47% for HSP90, and between 43 and 54% for HSP70 (Table 1).

In a phylogenetic tree constructed with HSP90 gene sequences of members of the family *Closteroviridae*, OLYaV occupied an intermediate position between clades containing viruses transmitted by whiteflies (genus *Crinivirus*) and viruses transmitted by mealybugs (genus *Ampelovirus*) (Fig. 1A). In trees constructed from replicase domains, OLYaV was positioned between the closterovirus and ampelovirus clades (Fig. 1B).

Fusion protein purification and Western and immunoblot assays. Following induction with IPTG, extracts from *E. coli* cells transformed with pMAL/HSP70 and induced with IPTG contained a polypeptide of *ca.* 95 kDa that was absent from control cells transformed with pMAL-c2 (Fig. 2). This size is slightly smaller than that expected for the fusion protein (*ca*. 103 kDa). The yield of the purified recombinant protein was estimated to be *ca.* 0.5 mg ml^{-1}. It reacted with antisera to MBP and to HSP70 polypeptides in immunoblotting assays (not shown). Western blotting with pre-absorbed antiserum diluted 1:1,500 of extracts from *in vitro*-grown olive explants detected a protein of *ca*. 65 kDa that was not present in samples from uninfected olive tissue. This size is about that of the HSP70 gene expression product (Fig. 3). Purified IgGs HSP70 successfully identified the homologous proteins in extracts of *in vitro*-grown infected explants spotted on PVDF membrane and developed with a chemiluminescence substrate (Fig. 4). However, the target protein was not detectable in tissues of field-grown plants collected during active vegetative growth (April to July), presumably because of low concentrations of virus in these tissues.

Electron microscopy. Dips from leaves and roots contained filamentous closterovirus-like particles with a distinct cross banding of mostly variable lengths and a few particles of *ca.* 2,000 nm (Fig. 5A), a length reported for particles of viruses in the family *Closteroviridae* (Martelli *et al*., 2002).

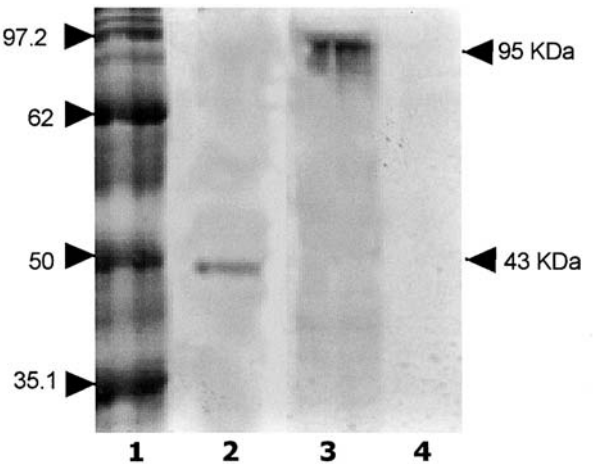

Fig. 2. SDS-PAGE of recombinant HSP70 protein of OLYaV (isolate OG9) fused to maltose binding protein (MBP) after induction in *E. coli* and purification. Lane 1: protein molecular weight marker (Bio-Rad, Richmond, CA, USA). Lane 2: bacterial protein extract of MBP. Lane 3: fusion protein MBP-HSP70. Lane 4: Bacterial protein extract of *E. coli* before IPTG induction. Bands stained with Coomassie blue.

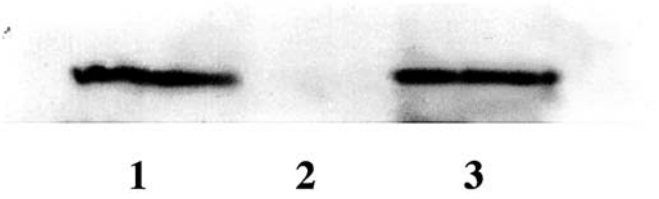

Fig. 3. Detection of OLYaV HSP70 protein in plant extracts by Western blot using purified IgGs to the recombinant protein (lanes 1 and 3). A protein band *ca.* 65 kDa was obtained from OLYaV-infected accessions F4P5 (lane1) and OG9 (lane3). Lane 2 shows a preparation from an uninfected seedling.

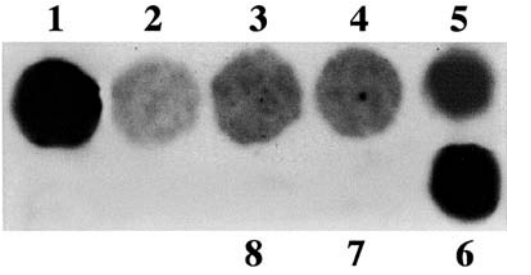

Fig. 4. Detection of HSP70 protein in several OLYaV-infected olive accessions by dot-immunobinding using purified IgG-HSP70: accession F4P5 (1); OG9 (2); Boutellian (3); Cordalez (4); Coratina (5); purified HSP70 protein from bacterial extracts (6); negative controls (7 and 8). Protein extracts were denatured by boiling before spotting.

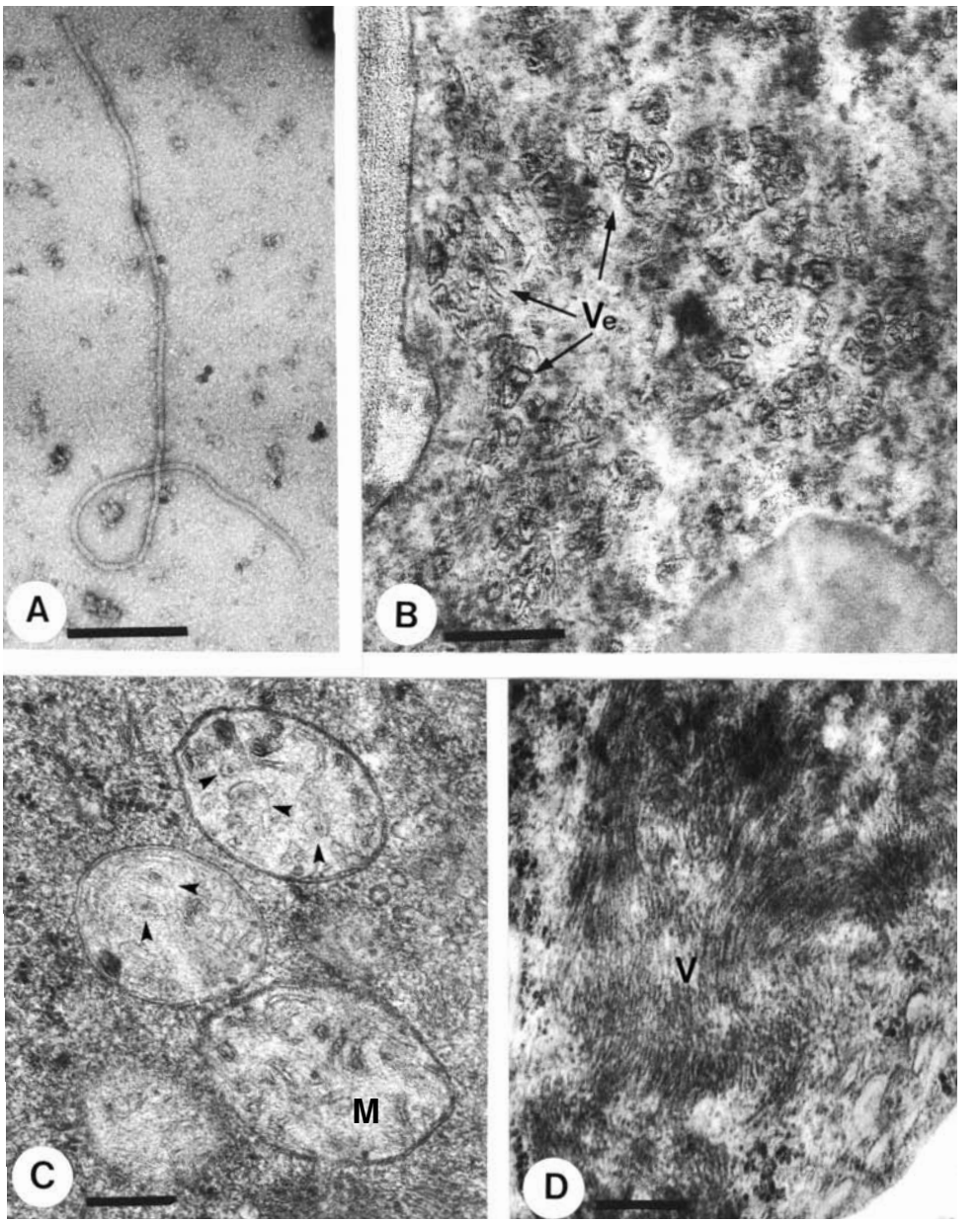

Fig. 5. A) Negatively stained virus particle in a leaf dip from the OLYaV-infected accession OG9; **B**) Thin section of a companion cell from an olive explant infected by OLYaV showing clusters of vesicles (Ve) containing finely fibrillar material; **C**) Vesicular elements (arrow heads) inside mitochondria (m) with a disarranged internal structure; **D**) Bundle of virus particles (V). All bars = 300 nm.

Cytopathological effects were observed in phloem parenchyma and companion cells of infected tissues which, compared to healthy controls, showed accumulations in the cytoplasm of membranous vesicles containing fibrillar electron dense material (Fig. 5B). The origin of these vesicles was not ascertained. However, some mitochondria contained vesicular elements resembling those present in the cytoplasm (Fig. 5C). No evidence was obtained of mitochondrial fragmentation, as reported for some ampeloviruses (Faoro, 1997). Virus particles were either intermingled with cytoplasmic vesicles or, more often, occurred in bundles (Fig. 5D).

DISCUSSION

The results reported here extend towards the 3'-terminus by 854 nt in the HSP90 gene, a previously sequenced large genome fragment that comprised 4,605 nt from ORFs 1b (RdRp), 2 (21K), 3 (7K), 4 (HSP70), and the 5' end (*ca*. 600 nt) of ORF 5 (HSP90) (Sabanadzovic *et al*., 1999; Saponari, 2001).

The sequence data, virus particle morphology and the cytopathology of infected olive cells when taken together confirm the closteroviral nature of OLYaV. Even so, phylogenetic trees comparing sequences of ORF1b (RdRp) and the large fragment of ORF 5 (HSP90), positioned OLYaV between members of the genera *Closterovirus* (BYV, BYSV, CTV, and GLRaV-2) and *Crinivirus* (LIYV and CYSDV). Furthermore, in no case did two phylogenetically significant OLYaV genes (RdRp and HSP70) have a nucleotide identity higher than 54% with comparable genes of the above viral species, thus substantiating the current taxonomic position of OLYaV as unassigned species to the family *Closterovidae* (Martelli *et al*., 2002).

A polyclonal antiserum was raised against a recombinant fusion protein encoded by the HSP70 gene. Western blotting detected a 65 kDa polypeptide in OLYaV-infected olive explants; this polypeptide is presumably the expression product of the HSP70 gene. This result suggests that a serological assay for OLYaV infection could be developed as an alternative to nucleic acid-based diagnostic reagents. Immuno dot-blot tests gave positive responses when purified and pre-absorbed IgGs were used to probe denatured extracts from infected *in vitro*-grown olive tissues. However, when Western blots or ELISA were tried with samples from field-grown plants, the results were consistently negative. Possible reasons for the failure to detect HSP70 *in vivo* are the low titer of target antigen and the presence of inhibitors in olive sap, which oxidizes very quickly.

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