04 Sabanadzovic 21-09-2004 9:56 Pagina 37

Journal of Plant Pathology (1999), **81** (1), 37-45 *Edizioni ETS Pisa, 1999*

PARTIAL MOLECULAR CHARACTERIZATION AND RT-PCR DETECTION OF A PUTATIVE CLOSTEROVIRUS ASSOCIATED WITH OLIVE LEAF YELLOWING

S. Sabanadzovic[1], N. Abou-Ghanem[2], P. La Notte[2], V. Savino[2], G. Scarito[3] and G.P. Martelli[2]

[1]*Istituto Agronomico Mediterraneo, Via Ceglie 9, I-70010 Valenzano, Bari, Italy*
[2]*Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via G. Amendola 165/a, I-70126 Bari, Italy*
[3]*Istituto di Patologia Vegetale, Universtà degli Studi di Palermo, Viale delle Scienze, I-90133 Palermo, Italy*

SUMMARY

Electrophoretic analysis of extracts from cortical tissues of olive trees of cvs 'Biancolilla' and 'Nostrana' from Sicily (southern Italy) showing bright chrome yellow discolourations of the leaves, consistently revealed a number of double-stranded RNA (dsRNA) bands, the largest of which had a size (*ca* 15 kbp) similar to that of the full genomic dsRNA of some species of the genus *Closterovirus*. A segment of 611 nucleotides, showing sequence homology with the HSP70 homologue gene of *Closteroviridae* was amplified by RT-PCR from symptomatic trees of both cultivars, using degenerated primers designed on the conserved phosphate 1 and 2 motifs of the HSP70 homologue gene sequence. Computer-assisted phylogenetic analysis showed that the 611 nt HSP70 homologue sequence from olive differed from that of other members of the family *Closteroviridae*, suggesting that it probably belonged to an undescribed closterovirus, for which the tentative name olive leaf yellowing-associated virus (OLYaV) is proposed. A set of OLYaV-specific primers was designed which amplified a 383 nt fragment of the HSP70 homologue. RT-PCR assays with these primers detected OLYaV sequences in olive trees with leaf yellowing from some Italian regions (Calabria and Latium) and Jerusalem, and in individuals of an unidentified pseudococcid mealybug species and of the psyllid *Euphyllura olivina* that had fed on symptomatic cv. 'Biancolilla' trees. No amplification was obtained with extracts from controls (symptomless olives and apparently healthy olive seedlings) or from olive trees from other Italian regions (Sardinia, Tuscany) that showed somewhat differing yellowing symptoms.

RIASSUNTO

PARZIALE CARATTERIZZAZIONE MOLECOLARE ED IDENTIFICAZIONE CON RT-PCR DI UN POSSIBILE CLOSTEROVIRUS ASSOCIATO AD INGIALLIMENTI FOGLIARI DELL'OLIVO. L'analisi elettroforetica di estratti da tessuti corticali di cv. "Biancolilla" e "Nostrana" di origine siciliana con vivaci ingiallimenti fogliari ha rivelato la costante presenza di bande di RNA bicatenari (dsRNA), la più grande delle quali aveva dimensioni (*ca* 15 kbp) analoghe a quelle dei dsRNA dell'intero genoma di alcune specie del genere *Closterovirus*. L'uso di primers degenerati comprendenti i motivi fosfatici 1 e 2 della sequenza del gene HSP70 dei closterovirus ha consentito l'amplificazione mediante RT-PCR di un frammento di 611 nucleotidi da estratti di piante con sintomi di entrambe le cultivars. Il sequenziamento del frammento amplificato e l'analisi filogenetica comparativa con le sequenze note dei geni HSP70 di altri closterovirus ha dimostrato che la sequenza identificata in olivo differisce dalle altre, tanto da far concludere che essa appartenga ad un possibile nuovo membro del genere *Closterovirus,* per il quale si propone il nome di virus associato all'ingiallimento fogliare dell'olivo (OLYaV). La RT-PCR con primers OLYaV-specifici che amplificano un frammento di 383 nt, ha consentito l'identificazione della sequenza virale in olivi con vivaci ingiallimenti fogliari presenti in Calabria e Lazio ed a Gerusalemme, nonché in individui di una specie non identificata di cocciniglia pseucoccide e dello psillide *Euphyllura olivina* presenti su di una pianta infetta di cv. "Biancolilla". La sequenza virale non è stata identificata né nei testimoni (olivi asintomatici e semenzali apparentemente sani) né in piante di origine sarda e toscana con ingiallimenti fogliari che differivano da quelli delle altre regioni.

Key words: olive, leaf yellowing, closterovirus, diagnosis, degenerate primers, RT-PCR, HSP70-like protein, sequencing.

INTRODUCTION

Two disorders denoted 'leaf yellowing' (OLY) and 'yellow mottling and decline' (OYMD) were recently recorded from two different localities of Sicily (southern Italy) in the local olive (*Olea europaea* L.) varieties

Corresponding author: G.P. Martelli
Fax: +39.080. 544-2911
E-mail: martelli@agr.uniba.it

04 Sabanadzovic 21-09-2004 9:56 Pagina 38

'Biancolilla' and 'Nostrana', respectively (Savino *et al*., 1996). Both diseases were characterized by a bright yellow discolouration of the foliage which, in the case of OYMD, was accompanied by leaf necrosis, extensive defoliation, and dieback. The OLY syndrome was reproduced by grafting to healthy cv. 'Biancolilla' olives but no virus could be recovered from naturally or artificially infected symptomatic plants by mechanical transmission to herbaceous hosts. By contrast, a still uncharacterized virus with flexuous filamentous particles *ca* 800 nm long, denoted olive yellow mottle and decline-associated virus (OYMDaV) was transmitted by sap inoculation to a limited range of herbaceous hosts from plants with OYMD symptoms (Savino *et al.,* 1996). Repeated attempts to reproduce OYMD by grafting to a range of olive cultivars failed because there was no graft take (unpublished information).

Tissue extracts from OLY-infected plants were shown to contain a rather complex pattern of double-stranded RNAs (dsRNA) (Savino *et al*., 1996). The largest of these dsRNAs (*ca* 15 kbp) had a size similar to that reported for dsRNAs of some members of the genus *Closterovirus* (Agranovsky, 1996), and was larger than the dsRNAs previously recovered from olives in Italy and Jordan (Martelli *et al*., 1995a, 1995b). This prompted a more detailed study of these dsRNAs extending the investigations to OYMD-infected olives and other trees of different geographical origin that showed yellow leaf symptoms.

MATERIALS AND METHODS

Virus source. Plant material used in the present work was collected from: (*i*) eight plants of cv. 'Biancolilla' and six of cv. 'Nostrana' from Sicily; (*ii*) one plant of cv. 'Leccino' with vein yellowing from Tuscany infected by tobacco mosaic virus (TMV) (Triolo *et al*., 1996); (*iii*) one plant of an undetermined cultivar with bright leaf yellowing from Latium (Faggioli and Barba, 1995); (*iv*) seven plants from Calabria, four from Sardinia and one from Jerusalem all of which showed more or less intense leaf yellowing. In addition, three potted, graft-inoculated 5 year-old trees of cv. 'Biancolilla' showing OLY symptoms were used as virus sources for RT-PCR assays and for transmission trials by vectors.

Mechanical transmission. A number of attempts to isolate viruses by mechanical inoculation from cv. 'Biancolilla' trees were carried out using extracts from different tissues (flowers, leaves and cortex) and buffers (0.1 M phosphate buffer pH 7.2 containing 2% nicotine and 0.5 M Tris-HCl pH 8.2 containing 0.01 M $MgSO_4$), and concentrated partially purified leaf tissue extracts. The herbaceous host range consisted of eight species belonging to the *Solanaceae, Chenopodiaceae, Amaranthaceae* and *Leguminosae.*

Double-stranded RNAs. dsRNAs were recovered by phenol extraction and chromatography through cellulose CF-11 columns (Dodds, 1993) from five plants of cv. 'Biancolilla', six plants of cv. 'Nostrana' from Sicily, and from the undetermined cultivar from Latium. After digestion with RNase-free DNase (60 µg ml^{-1}) and DNase-free pancreatic RNase (0.5 µg ml^{-1}) (Saldarelli *et al*., 1994), preparations were analyzed in 6% PAGE in comparison with dsRNAs from grapevine leafroll-associated virus 2 (GLRaV-2) -infected *Nicotiana benthamiana* (Abou-Ghanem *et al*., 1998), and silver stained. Controls consisted of extracts from healthy olive and *N. benthamiana* seedlings processed in the same way.

Reverse transcription polymerase chain reaction (RT-PCR). The degenerate oligonucleotide primers used (HSP1 and HSP2) were those designed by Tian *et al.* (1996) on the conserved phosphate 1 and 2 motifs of the HSP70 homologue gene of members of the family *Closteroviridae.* Reverse transcription was performed on dsRNA preparations extracted from six Sicilian samples and from the single sample from Latium after denaturation by 20 mM methyl mercuric hydroxide, and priming with 1 µg of random DNA hexanucleotide mixture (Boehringer Mannheim). 200 units M-MLV reverse transcriptase were used in a 50 µl reaction for 1 h at 42°C following the manufacturer's instructions (GIBCO-BRL). dsRNA extracts from GLRaV-2-infected *N. benthamiana* were used as positive controls. Negative controls were extracts from healthy olive seedlings and dsRNAs form eggplant mosaic (EMV), and southern bean mosaic (SBMV) viruses, neither of which is a closterovirus. Degenerate primers were also used for amplifying dsRNAs of OYMDaV, extracted from a declining cv. 'Nostrana' plant.

Five µl (of a total of 50 µl) of cDNA were mixed with 95 µl of the amplification mixture [1x Taq Promega buffer, 3 mM $MgCl_2$, 2 mM of each dNTP, 200 ng of each primer and one unit of Taq DNA Polymerase (Promega Corporation, Madison USA)]. After denaturation at 94°C for 2 min, cycling was as follows: denaturation for 30 s at 94°C, annealing for 30 s at 40°C, extension for 1 min at 72°C for 35 cycles, and final extension for 7 min at 72°C. Amplified products were analyzed by electrophoresis in 1.2% agarose gel in 1x TBE buffer and stained with ethidium bromide (Sambrook *et al*., 1989).

04 Sabanadzovic 21-09-2004 9:56 Pagina 39

cDNA cloning and sequencing. PCR-generated cDNA from one of the symptomatic, graft-inoculated cv. 'Biancolilla' plants was cloned into a pGEM-T plasmid (Promega Corporation, Madison), mixed with T-tailed pGEM-T in a 1:1 molar ratio, and ligated at 16°C for 16 h. The resulting recombinant plasmids were cloned in *Escherichia coli* DH5α and transformed cells were plated on Luria-Bertani medium containing 75 mg ml^{-1} ampicillin. Recombinant colonies were selected, plasmids were purified, digested with *PstI* and *NcoI*, and analyzed by agarose gel electrophoresis (Sambrook *et al.*, 1989). Plasmids containing cDNAs of the predicted size (*ca* 600 bp) were used to determine the nucleotide sequence of the amplified product in both cDNA strands. Sequencing was by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using 35S-ATP and the Thermo Sequenase cycle sequencing kit (Amersham).

Computer-assisted analysis of nucleotide and protein sequences. Nucleotide and protein sequences were analysed with the assistance of the Strider 1.1 programs (Marck, 1988). Protein sequences were aligned with CLUSTAL V (Higgins and Sharp, 1988). Search for homologies with proteins from the Protein Information Resources (PIR, release 47.0), was done with the FASTA (Pearson and Lipman, 1988) and BLASTA (Altschul *et al.*, 1990) programs. Tentative phylogenetic trees were constructed and bootstrap analysis made with the NEIGHBOR, SEQBOOT, PROTDIST, and CONSENSE programs of the PHYLIP package (Felsenstein, 1989).

Association of the cloned cDNA sequence with leaf yellowing symptoms. One to 2 year-old shoots were collected from field-grown olive trees with and without leaf yellowing symptoms, and from grafted symptomatic cv. 'Biancolilla' plants. The presence of the cloned sequence was ascertained by RT-PCR. Specific oligonucleotides CAL-1 (5'CGAAGAGAGCGGCTGAAGGCTC3' sense primer) and CAL-2 (5'GGGACGGTTACGGTCGAGAGG3' antisense primer), synthesized by GENENCO-Life Science (Florence, Italy), were designed by computer analysis (Primer Selection Program, Henry M. Jackson Foundation, Bethesda) on the nucleotide sequence of the cDNA clone for amplifying a 383 nt fragment. About 100 mg of cortical scrapings were extracted and processed as described by Wetzel *et al.* (1991) and cDNA synthesis performed according to Minafra and Hadidi (1994). The RT-PCR protocol was as described above, except that annealing was at higher stringency (55°C instead of 40°C) and $MgCl_2$ concentration was 1 mM instead of 3 mM.

Search for possible vectors and transmission trials. An unidentified species of pseudococcid mealybug and the psyllid *Euphyllura olivina* that were infesting a symptomatic graft-inoculated cv. 'Biancolilla' plant were analyzed for the presence of the cloned cDNA sequence. Total nucleic acids (TNA) extracted according to Singh *et al.* (1995) from nine adult mealybugs and ten *E. olivina* nymphs, were resuspended in 50 µl of sterile water, 5 µl of which were used for cDNA synthesis as described above. Five µl of synthesized cDNA were mixed with 95 µl of PCR reaction mixture and cycled as described above. PCR products were analyzed in 1.2% TBE agarose. TNA extracted from the same insect species collected from apparently healthy olives served as control.

One year-old olive seedlings that indexed negative following PCR with primers CAL-1 and CAL-2 were used for transmission trials. Second and third stage instars and adults of *Pseudococcus longispinus* reared under greenhouse conditions on potato sprouts, were individually transferred into small plastic cages on symptomatic shoots of the source olive plant. After an acquisition access time (AAT) of three weeks, 15 adults were moved to each of five olive seedlings. After an inoculation access period (IAT) of 10 days the seedlings were sprayed with an insecticide.

Field-collected nymphs of an *E. olivina* population that indexed negative following PCR with primers CAL-1 and CAL-2 were individually transferred onto infected cv. 'Biancolilla' plants, to be transferred, after an AAT of three weeks, onto three healthy olive seedlings. The insects were killed by insecticide after 10 days.

Controls consisted of olive seedlings artificially infested with insects that had not been feeding on infected olives and indexed negative following PCR with primers CAL-1 and CAL-2.

Tissues from seedlings exposed to insects that had fed on infected olives were collected three months after the end of the experiment and PCR-analyzed for the presence of the cloned cDNA sequence.

Molecular hybridization. The specificity of PCR products amplified from olive plants and mealybugs was checked by Southern blot analysis. Amplified DNAs were electrophoresed in agarose gels and transferred to a Nylon N+ membrane by capillarity in 400 mM NaOH for 4 h. Hybridization was at 42°C with a DNA Digoxigenin-labelled probe synthesized from an excised fragment of 611 bp from a selected recombinant plasmid (pOY3) containing the specific amplified DNA of the HSP70 gene (DigDNA labelling Kit, Boehringer Mannheim). Hybridization signals were

04 Sabanadzovic 21-09-2004 9:56 Pagina 40

detected by chemiluminescence according to the manufacturer's instructions (Dig-chemiluminescent detection Kit, Boehringer Mannheim).

RESULTS

Mechanical transmission. All attemps to transmit a virus by inoculation of cv. 'Biancollilla' crude or concentrated partially purified sap extracts to herbaceous hosts failed, thus confirming previous results (Savino *et al*., 1996). Virus particles were not seen by electron microscopy of leaf dips from inoculated herbaceous hosts or concentrated preparations from olive tissues.

dsRNA analysis. Electrophoretic analysis of cortical scraping extracts from symptomatic plants showed the presence of complex dsRNA patterns in all samples examined, *i.e.*, six cv. 'Biancolilla' and five cv. 'Nostrana' plants from Sicily and the single plant from Latium (Fig. 1A, B). No dsRNAs bands were seen in any of the controls (Fig. 1A, lane 3). The band with the highest molecular weight, consistently present in all samples, had the same apparent size as that of the replicative form of the entire viral genome of grapevine leafroll-associated virus 2 (GLRaV-2) (Fig. 1B, lane 5), estimated to be *ca* 15 kbp (Zhu *et al*., 1998). dsRNAs profiles from cvs 'Biancolilla' and 'Nostrana' were comparable (Fig. 1A, B). However, the profile of one of the declining cv. 'Nostrana' plants (not shown) contained an additional prominent band comigrating with the full genome dsRNA of OYMDaV (*ca* 6.5 kbp). The sample from Latium (Fig. 1B, lane 4) had a more complex dsRNA profile than the Sicilian samples, conceivably because of the presence of another virus, as reported by Faggioli and Barba (1995).

RT-PCR. Primers HSP1 and HSP2 (Tian *et al*., 1996) amplified a single DNA fragment of about 600 nucleotides from reverse transcribed cDNA, synthesized on dsRNA extracts from infected olives from Sicily and Latium and from GLRaV-2-infected *N. benthamiana* (Fig. 2, lanes 3 to 10). No amplification was obtained from cDNA synthesized on OYMDaV, SBMV and EMV dsRNA templates (not shown) or from healthy olive TNA extracts (Fig. 2, lane 1). When submitted to digestion with different restriction enzymes, amplified products revealed the presence of a *Hind II* site and the same RFLP pattern (Fig. 2, lanes 12-14).

Sequence analysis. The sequence of the cloned cDNA fragment (EMBL accession number Y18128) consisted of 611 nucleotides (Fig. 3) encoding a polypeptide of 203 amino acids. Comparison of the deduced amino acid sequence by the BLAST programme showed that this polypetide had about 30% similarity in average with the HSP70-like proteins of beet yellows (BYV), citrus tristeza (CTV), beet yellow stunt (BYSV), beet pseudoyellows (BPYV), little cherry (LChV), grapevine leafroll associated 1 to 5 and 7 (GLRaV-1 to -5 and GLRaV-7) closteroviruses, lettuce infectious yellows (LIYV), tomato infectious chlorosis (TICV), cucumber yellow stunt disorder (CYSDV) criniviruses, as well as with other proteins of the HSP70 family (*e.g.* HSP70 from carrot). This was taken as evidence that

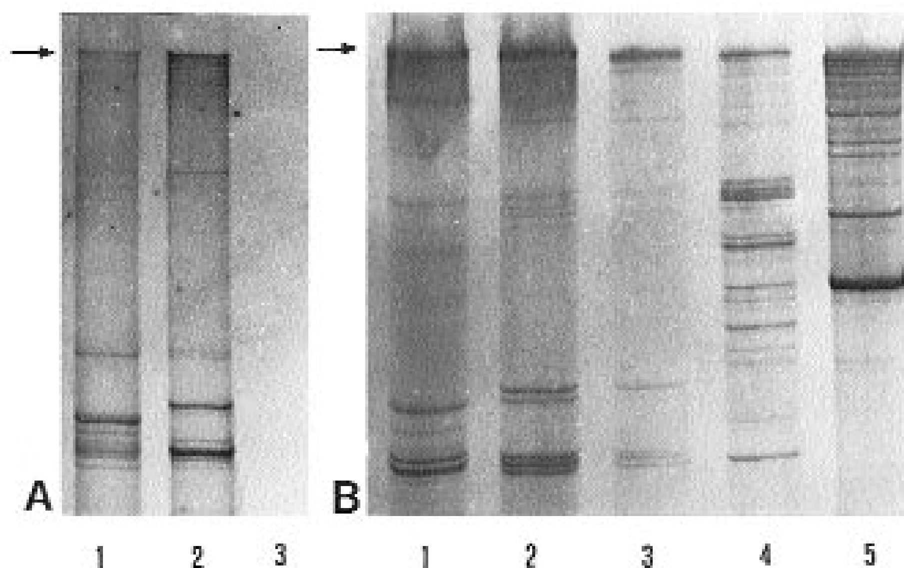
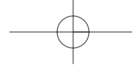


Fig. 1. A. Electrophoretic patterns of dsRNAs extracted from cortical scrapings of two symptomatic cv. 'Biancolilla' olives (lanes 1 and 2) and from a healthy olive seedling (lane 3). **B**. dsRNA profiles form three symptomatic cv. 'Nostrana' olives (lanes 1, 2 and 3) and the undetermined cultivar from Latium (lane 4). dsRNas from GLRaV-2-infected *N. benthamiana* in lane 5. Arrows point to the 15 kbp dsRNA band.



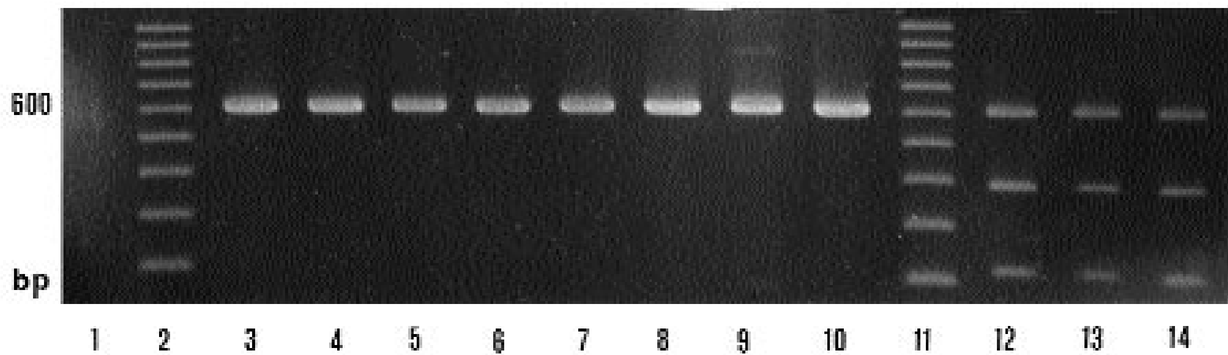


Fig. 2. RT-PCR products amplified from dsRNA extracts using Tian *et al.* (1996) HSP70 degenerate primers HSP1 and HSP2. Extracts are from cv. 'Biancolilla' (lanes 4, 5, 6), cv. 'Nostrana' (lanes 7, 8, 9) and the undetermined cultivar from Latium (lane 10). Lane 3 contains a dsRNA extract from a GLRaV-2-infected *N. benthamiana* (positive control) and lane 1 an extract from a healthy olive seedling (negative control). Markers (Sigma PCR MW ladder) indicating the 600 base pair band are in lanes 2 and 11. RFLP patterns of PCR-amplified products from cv. 'Biancolilla' (lane 12), 'Nostrana' (lane 13), and the undetermined cultivar from Latium (lane 14). The uppermost band is the non-digested PCR product, the two lower bands are the products of digestion with *Hind II* restriction enzyme.

```
1    [illegible]   60
     G F K F G T T F S T V C I A S S N N I L
61   [illegible]   120
     M L N N D D S K F I P V I I G F S V S D
121  [illegible]   180
     D T I V Y G Y D A I T R D G Y G R E G F
181  [illegible]   240
     S V Y R D L K K N I G V M S K T L S E R
241  [illegible]   300
     R A K L K P L Y N V T C P G V H F S L R
301  [illegible]   360
     I D F T F G Q F R L K N L H K I V S F F
361  [illegible]   420
     I A L V V R D F E K L R E F R C S G L V
421  [illegible]   480
     I S V P S Q Y T S V Q R F E M R T V K K
481  [illegible]   540
     R T G L P V F H I K N N P S A A L F A S
541  [illegible]   600
     K L D K K K T S D V D S Y V V Y D F G G
601  [illegible]   611
     G T F
```

Fig. 3. Nucleotide sequence of the cloned 611 nt fragment of the HSP70 homologue gene attributed to the putative closterovirus OLYaV, amplified from dsRNA extracts from a graft-inoculated symptomatic cv. 'Biancolilla' plant by primers HSP1 and HSP2. Capital letters indicate the amino acid sequence of the encoded polypeptide.

the 611 nt cDNA clone was indeed a fragment of the HSP70 homologue coding region. This was further supported by the presence of consensus amino acid residues in multiple alignments of the deduced amino acid sequence encoded by the 611 nt fragment with comparable sequences of the HSP70-like proteins of clostero- and criniviruses (not shown). Comparative phylogenetic analysis of the HSP70 sequence from olive with those of other sequenced members of the family *Closteroviridae,* showed that the sequence in question stands on its own (Fig. 4), differing from the major lineages identified by Tian *et al.* (1996) and Saldarelli *et al.* (1998).

Association of the HSP70-like protein sequence with leaf yellowing symptoms. PCR primers specific for

04 Sabanadzovic 21-09-2004 9:56 Pagina 42

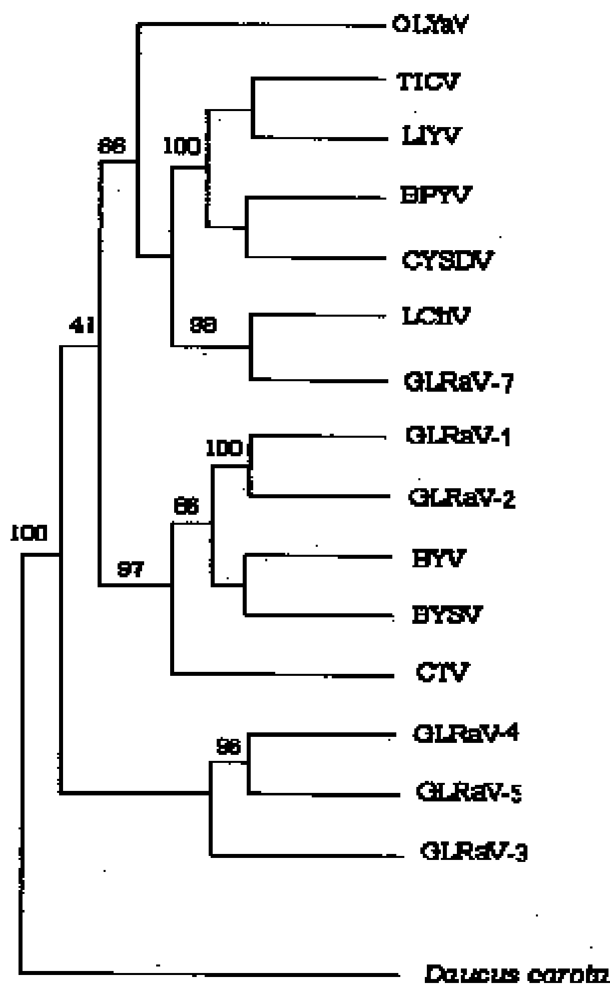


Fig. 4. Comparative phylogenetic analysis of the phosphate 1 and 2 motifs of the OLYaV HSP70 homologue protein with those of members of the family *Closteroviridae*. This tree was generated from an alignment of the amino acid sequences between the motifs A and C. EMBL accession numbers of the sequences used are: Beet yellows virus (BYV) X73476, Citrus tristeza virus (CTV) U02547, Beet yellow stunt virus (BYSV) U51931, Lettuce infectious yellow virus (LIYV) U05242, Little cherry virus (LChV) Y10237, Beet pseudo yellow virus (BPYV) U67447, Tomato infectious chlorosis virus (TICV) U67449, Cucumber yellow stunt disorder virus (CYSDV) U67448, Grapevine leafroll-associated virus 1 (GLRaV-1) Y15890, (GLRaV-2) AF039202, (GLRaV-3) Y1589, (GLRaV-4) AF039553, (GLRaV-5), AF039552, (GLRaV-7) Y15987. The HSP70 sequence from carrot (*Daucus carota*) was used as outgroup.

the HSP70-like protein sequence from olive (CAL-1 and CAL-2) detected this sequence in 19 of 28 tested plants. In particular, positive amplification was obtained from 19 of 23 (83%) plants with bright yellow discolouration of the leaves, *i.e.* all of 14 trees from Sicily, the single trees from Latium and Jerusalem and 3 of 7 trees from Calabria (Fig. 5). The specificity of PCR products was checked by hybridization with a Dig-DNA labelled probe (not shown).

Samples from four olive trees from Sardinia and one from Tuscany that showed somewhat different symptoms, were consistently PCR negative, as were the samples from control olive seedlings (Fig. 5, lanes 13 and 14).

Search for possible vectors and transmission trials. RT-PCR on extracts from the unidentified pseudococcid mealybug and *E. olivina* that had fed on symptomatic cv. 'Biancolilla' plants yielded a single amplified product of the expected size (383 bp) (Fig. 6A, lanes 3 and 5) comigrating with the positive control (Fig. 6A, lane 2). No such band was found in negative controls (Fig. 6A, lanes 4 and 6). Southern hybridization of the same gel as in Fig. 6A with probe pOY3, specifically recognized amplified cDNA from insect extracts and positive controls (Fig. 6B, lanes 2, 3, and 5). No hybridizazion was obtained with negative controls (Fig. 6B, lanes 4 and 6).

Three months after exposure to presumably viruliferous insects (*P. longispinus* and *E. olivina*) olive seedlings remained symptomless and their tissue extracts were PCR negative.

DISCUSSION

Extraction and analysis of dsRNAs from olive trees proved useful not only for determining the presence of virus infections, as previously reported (Martelli *et al.*, 1995a, 1995b; Savino *et al.*, 1996), but gave hints on the possible nature of the infecting virus. For instance, the recovery of an unusually large dsRNA species from several plants with leaf yellowing suggested the presence of a closterovirus. Based on this, degenerate primers designed on a highly conserved closterovirus sequence (Tian *et al.*, 1996) were used, which allowed the identification of a comparable sequence in dsRNA extracts from diseased olive trees. The sequence in question was clearly related to (*ca* 30% homology) but phylogenetically different (Fig. 4) from those of the genes encoding the HSP70-like protein of closteroviruses. This protein, which is involved in cell-to-cell movement (Agranovsky *et al.,* 1998), occurs only in members of the family *Closteroviridae,* of which it represents a veritable hallmark (Dolja *et al.*, 1994).

The HSP70-like protein sequence was consistenly found in all olive plants with bright leaf yellowing from Sicily and Latium which also contained a dsRNA with a size (*ca* 15 kbp) similar to that of several definitive

04 Sabanadzovic 21-09-2004 9:56 Pagina 43

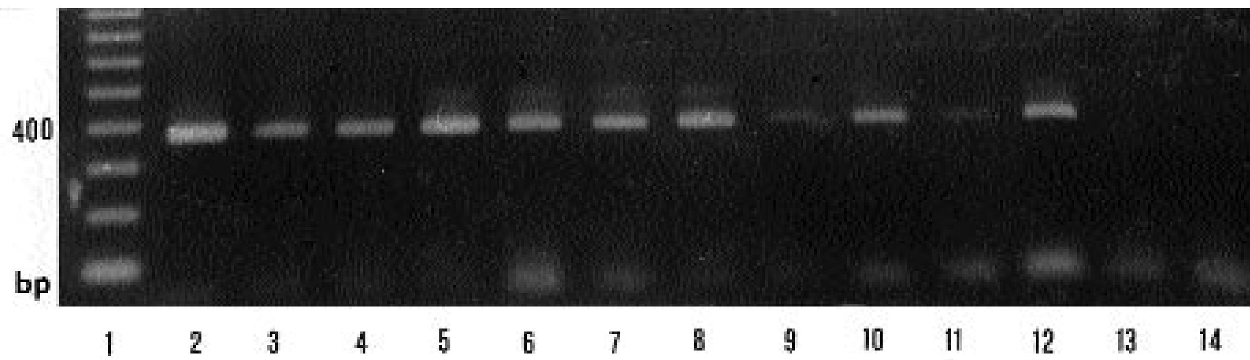


Fig. 5. RT-PCR of crude olive sap samples using OLYaV-specific primers CAL-1 and CAL-2. Amplified DNA products of the expected size (383 bp) are present in samples from cv. 'Biancolilla' (lanes 3 and 4), cv. 'Nostrana' (lanes 5 to 8), undetermined cultivars from Calabria (lanes 9 and 10), Jerusalem (lane 11) and Latium (lane 12). Lane 2 contains the product amplified from a dsRNA extract from a symptomatic, graft-inoculated cv. 'Biancolilla' plant (positive control). No amplification was obtained with samples from healthy olive seedling (negative control, lane 13) and the TMV-infected cv. 'Leccino' from Tuscany (lane 14). Markers are in lane 1; the 400 base pairs band is indicated.

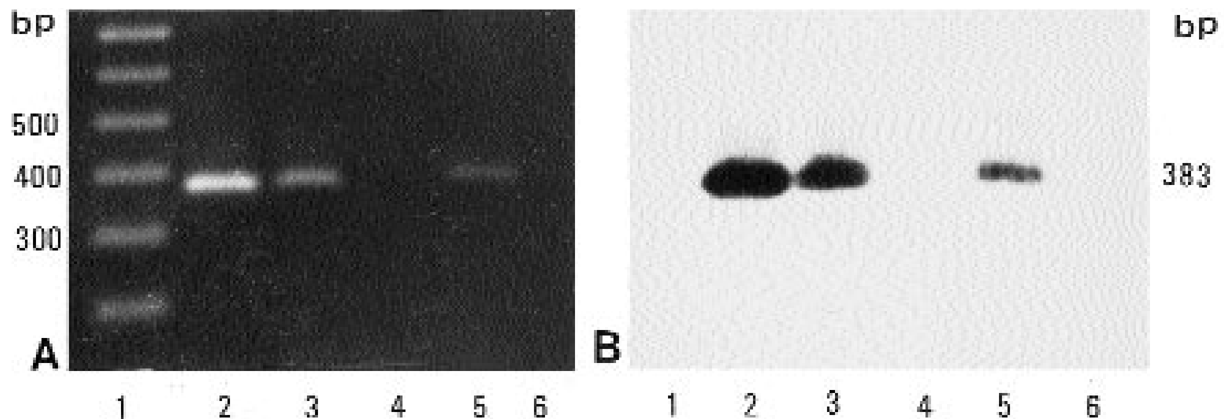


Fig. 6. A. Products of 383 bp amplified from RT-PCR extracts from an undentified pseudococcid mealybug species and *E. olivina* collected from a symptomatic graft-inoculated cv. 'Biancolilla' plant (lanes 3 and 5), using OLYaV-specific primers CAL-1 and CAL-2. Bands comigrate with cDNA amplified from a dsRNA extract from the cv. 'Biancolilla' plant (lane 2, positive control). No amplification was obtained form insects collected from symptomless trees (lanes 4 and 6). Markers in lane 1. **B**. Southern blot of the same gel hybridized with the OLYaV sequence-specific pOY3 probe demostrating the specificity of amplified products.

species of the genus *Closterovirus* (Agranovsky, 1996). Although no virus could be recovered from symptomatic plants by mechanical inoculation and virus particles were not seen under the electron microscope, these findings support the conclusion that the HSP70 sequence from olive belongs to a putative closterovirus differing from those sequenced so far. For this virus, the provisional name of olive leaf yellowing-associated virus (OLYaV) is proposed.

OLYaV is the 12th virus found to infect olive in nature, and the 3rd with filamentous particles (Martelli, 1998). It was readily identified in crude olive tissue extracts by RT-PCR, using 'universal' degenerate primers (Tian *et al.*, 1996) and virus-specific primers, both designed on a conserved region of the HSP70-like protein coding sequence. Thus, RT-PCR confirms its usefulness for the sensitive detection of olive viruses (Bertolini *et al.*, 1998; Martelli, 1998; F. Grieco, personal communication) for which no reliable detection method has existed up to now (Martelli, 1998).

In Sicily and Calabria olive trees with leaf yellowing have a patchy distribution, suggestive of the activity of a slow-moving vector. OYLaV was detected in mealybugs and psyllids (*E. olivina*) feeding on an infected cv. 'Biancolilla' tree. Both these types of insects are phloem feeders, which accounts for the ingestion of a virus like

04 Sabanadzovic 21-09-2004 9:56 Pagina 44

OLYaV, belonging to a family whose members are known to be phloem-limited and sempersistently transmitted (German Retana *et al*., 1998). However, the detection of OLYaV in these insects is not a proof that they are vectors of the virus, as shown also by the negative results of preliminary transmission trials.

ACKNOWLEDGEMENTS

We thank Prof. U. Prota, University of Sassari, Dr. M. Barba, Research Institute for Plant Pathology, Rome, and Dr. A. Materazzi, University of Pisa, for supplying some of the olive samples examined in this study.

REFERENCES

Abou-Ghanem N., Sabanadzovic S., Minafra A., Saldarelli P., Martelli G.P., 1998. Some properties of grapevine leafroll-associated virus 2 and molecular organization of the 3' region of the viral genome. *Journal of Plant Pathology* **80**: 37-46.

Agranovsky A.A., 1996. Principles of molecular organization, expression and evolution of closteroviruses: over the barriers. *Advances in Virus Research* **47**: 119-158.

Agranovsky A.A., Folimonov A.S., Folimonova S., Morozov S., Schieman J., Lesemann D.H., Atabekov J.G., 1998. Beet yellows closterovirus HSP70-like protein mediates the cell-to-cell movement of a potexvirus transport-deficient mutant and a hordeivirus-based chimeric virus. *Journal of General Virology* **79**: 889-895.

Altschul S.F., Stephen F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Bertolini E., Fadda Z., Garcia F., Celada B., Olmos A., Gorris M.T., Del Rio C., Caballero J., Duran-Vila N., Cambra M., 1998. Virisis del olivo detectasa in España. Nuevos metodos de diagnostico. *Phytoma España* **102**: 191-193

Dodds J.A., 1993. DsRNA in diagnosis. In: Matthews, R.E.F. (ed.). Diagnosis of Plant Virus Diseases, pp. 274-294. CRC Press, Boca Raton, USA.

Dolja V.V., Karasev A.V., Koonin E.V., 1994. Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. *Annual Review of Phytopathology* **32**: 261-285.

Faggioli F., Barba M., 1995. An elongated virus isolated from olive (*Olea europaea* L.). *Acta Horticulturae* **386**: 593-597.

Felsenstein J., 1989. PHYLIP-phylogeny inference package (version 3.5). *Cladistics* **5**: 164-166.

German Retana S., Candresse T., Martelli G.P., 1998. Closteroviruses. In: Webster R.G. and Granoff A. (eds.). Encyclopedia of Virology, 2nd edition, Academic Press, New York (in press).

Higgins D.G., Sharp P.M., 1988. Clustal: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**: 237-244.

Marck C., 1988. 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Research* **16**: 1829-1836.

Martelli G.P., 1998. Enfermedades infecciosas y certificacion del olivo: panorama general. *Phytoma España* **102**: 180-186.

Martelli G.P., Savino V., Di Terlizzi B., Catalano L., Sabanadzovic S., 1995a. Viruses and certification of olive in Apulia. *Acta Horticulturae* **386**: 569-573.

Martelli G.P., Sabanadzovic S., Savino V., Abou-Zurayk A.R., Masannat M., 1995b. Virus-like diseases and viruses of olive in Jordan. *Phytopathologia Mediterranea* **34**: 133-136.

Minafra A., Hadidi A., 1994. Sensitive detection of grapevine virus A, B or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. *Journal of Virological Methods* **47**: 175-188.

Pearson W.R., Lipman D.J.,1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences*, *USA* **85**: 2444-2448.

Rothman J.E., 1989. Polypeptide chain binding proteins: catalysis of protein folding and related processes in cells. *Cell* **59**: 591-601.

Saldarelli P., Guglielmi Montano H., Martelli G.P., 1994. Non-radioactive molecular probes for the detection of three filamentous viruses of the grapevine. *Vitis* **33**: 157-170.

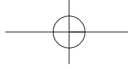
Saldarelli P., Rowhani A., Routh G., Minafra A., Digiaro M., 1998. Use of degenerate primers for the detection of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. *European Journal of Plant Pathology* (in press).

Sambrook J., Fritsch E.E., Maniatis T., 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Harbour Laboratory Press, New York.

Sanger F., Nicklen S., Coulson A.R., 1977. DNA sequencing with chain-termination inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**: 5463.

Savino V., Sabanadzovic S., Scarito G., Laviola C., Martelli G.P., 1996. Due giallumi dell'olivo di possibile origine virale in Sicilia. *Informatore Fitopatologico* **46**(5): 55-59.

Singh R.P., Kurz J., Boiteau G., Bernard G., 1995. Detection of potato leafroll virus in single aphids by the reverse transcription polymerase chain reaction and its potential epidemiological application. *Journal of Virological Methods* **55**: 133-143.



Tian T., Klaassen V.A., Soong J., Wisler G., Duffus J.E., Falk B.W., 1996. Generation of cDNAs specific to lettuce infectious yellows closterovirus and other whitefly-transmitted viruses by RT-PCR and degenerated oligonucleotide primers corresponding to the closterovirus gene encoding the heat shock protein 70 homolog. *Phytopathology* **86**: 1167-1172.

Triolo E., Materazzi, A., Toni S., 1996. An isolate of tobacco mosaic tobamovirus from *Olea europaea* L. *Advances in Horticultural Science* **10**: 39-45.

Received 15 September 1998
Accepted 20 January 1999

Wetzel T., Candresse T., Ravelonandro M., Dunez J., 1991. A model polymerase chain reaction assay adapted to plum pox potyvirus detection. *Journal of Virological Methods* **33**: 355-365.

Zhu H.Y., Ling K.S., Goszczynski D.E., McFerson J.R., Gonsalves D., 1998. Nucleotide sequence and genome organization of grapevine leafroll-associated virus 2 are similar to beet yellows virus, the closterovirus type member. *Journal of General Virology* **79**: 1289-1298.