IDENTIFICATION AND PARTIAL CHARACTERIZATION OF AN ISOLATE OF *APRICOT LATENT VIRUS* FROM PALESTINE

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SUMMARY

A filamentous virus was isolated by mechanical sap transmission from GF 305 seedlings showing asteroid ringspots to *Nicotiana occidentalis.* The GF 305 seedlings were graft-inoculated with buds from an apricot tree cv Mistikawi, from Palestine. The electrophoretic dsRNA pattern from symptomatic GF 305 and *N. occidentalis* showed a major band of about 9.5 kbp. Bundles of elongated virus-like particles were present in the cytoplasm of infected *N. occidentalis*. Dissociated coat protein from partially purified virus preparations consisted of a single subunit with an estimated Mr of ca. 50 kDa. RT-PCR using primers specific for *Apricot latent virus* (ApLV) RNA amplified a DNA product of about 200 bp from infected GF 305 and *N. occidentalis* plants. Sequence analysis of this fragment showed 90-93% identity in the encoded amino acid sequence with corresponding sequences from different ApLV isolates. This is the first record of ApLV from the southern Mediterranean. An ApLV-specific digoxygenin-labelled riboprobe was produced and used for molecular hybridisation tests. A survey of apricot orchards in Southern Italy did not show the presence of ApLV.

Keywords: ApLV, foveavirus, diagnosis, molecular hybridisation, RT-PCR, sequence analysis.

INTRODUCTION

Apricot latent virus (ApLV), a definitive species in the genus *Foveavirus*, family *Flexiviridae* (Adams *et al*., 2004), was first described in Moldova in latently infected apricots cv Silistra (Zemtchik and Verderevskaya, 1993), and later reported from France and Italy (Gentit *et al*., 2001a).

Among *Prunus* species experimentally graft-inoculated with ApLV-infected sources, peach is the most susceptible, developing asteroid or sooty ringspot symptoms on the leaves (Zemtchik *et al*., 1998, Grasseau *et al*., 1999; Nemchinov *et al*., 2000; Gentit *et al*., 2001b). The 3' ends of RNAs of several isolates of ApLV have been sequenced, allowing the development of virus-specific nucleic acid-based detection methods (Nemchinov *et al*., 2000; Gentit *et al*., 2001a,b).

In the course of a study for evaluating the sanitary status of stone fruits in Palestine (Jarrar *et al*., 2001), seedlings of the indicator *Prunus persica* GF 305 that had been graft-inoculated with buds from apricot cv Mistikawi, reacted by developing asteroid ringspots on the leaves reminiscent of those typically induced following inoculation with ApLV. Following mechanical inoculation of herbaceous hosts from symptomatic GF 305 seedlings, a virus was isolated and partially characterized, as reported in the present paper.

MATERIALS AND METHODS

Virus source. Asymptomatic apricot leaves of cv Mistikawi were negative for *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *American plum line pattern virus* (APLPV), *Prune dwarf virus* (PDV), *Prunus necrotic ring spot virus* (PNRSV) and *Plum pox virus* (PPV) when tested by DAS-ELISA (Clark and Adams, 1977). Also, simultaneous one-step RT-PCR (Sánchez-Navarro *et al*., 2004) for ACLSV, ApMV, APLPV, PDV, PNRSV, PPV and also for Plum bark necrosis stem-pitting associated virus (PBNSPaV) were negative.

Woody host range. Buds from apricot cv Mistikawi (isolate Apr47) were grafted first onto healthy GF 305 seedlings (GF305-Apr47). These were then used as donors for chip-budding five healthy plants each of the following hosts: *Prunus persica* (cvs Elberta and Springtime), *P. armeniaca* (cvs Luizet, Priana, and Tilton), *P. avium* (cvs Sam, Bing and Canindex) and *P. serrulata* cv Shirofugen. All plants were grown in a glasshouse at 22-24°C, and kept under observation for six months for symptom development. At the end of this period, all grafted plants were analysed by dot-blot hybridisation and then transferred to the field for further observation.

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Herbaceous host range. Young leaf tissues from a symptomatic GF 305-Apr47 seedling were ground in 0.1M phosphate buffer pH 7.2 containing 2.5% nicotine and the extract was manually inoculated to celite-dusted leaves of *Chenopodium quinoa*, *Chenopodium bushianum*, *Chenopodium amaranticolor*, *Chenopodium foetidum*, *Nicotiana occidentalis*, *Nicotiana clevelandii*, *Nicotiana benthamiana*, *Nicotiana cavicola*, *Cucumis sativus* and *Ocimum basilicum*. Asymptomatic herbaceous hosts were checked for latent infection by back-inoculation to *N. occidentalis*.

Double stranded RNA (dsRNA) analysis. dsRNAs were extracted from symptomatic *N. occidentalis* and GF 305 leaves, three weeks or six months after inoculation respectively, according to Dodds (1993), by phenol-chloroform extraction followed by chromatography through cellulose CF-11 columns. After enzymatic digestion with RNase-free DNase (60 mg ml^{-1}) and DNase-free pancreatic RNase (0.5 mg ml^{-1}) (Saldarelli *et al*., 1994), extracts were assayed by electrophoresis in 6% polyacrylamide slab gels (PAGE) in TAE buffer followed by staining with silver nitrate.

Virus purification. The virus was partially purified from symptomatic *N. occidentalis* according to Zemtchik *et al*. (1998).

Coat protein analysis. Coat protein was extracted from purified virus preparations by boiling for 3-5 min in the presence of 1.5% sodium dodecyl sulphate (SDS) and 3.5% 2-mercaptoethanol in 0.035 M Tris-HCl buffer pH 7. Its relative molecular mass was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

Cytopathology. For ultrastructural studies, tissue fragments consisting of mesophyll cells were excised from symptomatic *N. occidentalis* leaves and processed according to Martelli and Russo (1984). Thin sections were stained with lead citrate before viewing with a Philips Morgani electron microscope.

RT-PCR. RT-PCR tests were first done on denatured dsRNAs as template using H-ALV1 and C-ALV1 primers that amplify a fragment of the virus coat protein of about 200 bp. Cycling parameters were: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension 72°C for 45 sec totalling 35 cycles with a final extension at 72°C for 7 min (Nemchinov and Hadidi, 1998). Total nucleic acid (TNA) extraction using the silica-capture method, as described by Foissac *et al*. (2001), was also used prior to RT-PCR.

cDNA cloning and sequence analysis. Samples of 3 µl of PCR products were ligated to pGEM-T Easy vector following the manufacturer's instructions (pGEM-T Easy Vector System, Promega Corp., Madison, CA, USA). Selected recombinant plasmids containing cDNA inserts of the expected size were used for determining the nucleotide sequences of the PCR products amplified from isolate Apr47. Sequencing was done by the MWG-Biotech (Ebersberg, Germany) custom service. Nucleotide and amino acid sequences were analysed by using the Strider 1.1 program (Marck, 1988). Protein sequences were aligned with Clustal W 1.8 (Thompson *et al*., 1994). Searches for homologies with proteins from the DataBank (Protein Information Resources) were performed using the FASTA (Pearson and Lipman, 1988) and BLASTA (Altschul *et al*., 1990) programs. Tentative phylogenetic trees were constructed using ClustalW and viewed with TreeView 1.6 software (Page, 1996).

Riboprobe synthesis and molecular hybridisation. A non radioactive, DIG-labelled cRNA was transcribed by SP6 RNA polymerase from plasmid pApr47-200 that contains the appropriate DNA insert following linearization with *Aat*II. Transcription was performed according to the manufacturer's instructions [DIG RNA Labelling Kit (SP6/T7); Roche Molecular Biochemicals, Indianapolis, USA]. The specificity of the synthesised riboprobe was checked by hybridizing TNAs from GF 305 seedlings infected by ApLV-Apr47, *Apple stem pitting virus* (ASPV), Cherry green ring mottle virus (CGRMV), Cherry necrotic rusty mottle virus (CNRMV) or from uninfected control plants. Samples of 25 µl of TNAs from virus-infected plants or healthy controls were spotted onto nylon membranes (Hybond N+, Amersham Biosciences, Little Chalfont, England) after denaturation with 50 mM NaOH and 2.5 mM EDTA for 5 min at room temperature. Prehybridizations and hybridizations were carried out according to Pallás *et al*. (1998). The membranes were hybridised using the labelled cRNA probe (pApr47) and hybridization signals were detected by chemiluminescent reaction using commercial kit (DIG Luminescent Detection Kit; Roche Molecular Biochemical, Indianapolis, USA). Films were exposed for 10-30 min.

Survey for the presence of ApLV. Surveys were carried out in six commercial apricot orchards of Apulia (southern Italy) and in a collection of 87 apricot cultivars in the neighbouring Basilicata region. Leaves were collected randomly from one tree per cultivar in the varietal collection and from a representative number of plants in each commercial orchard, totalling 198 samples. All samples were tested by molecular hybridisation for the presence of ApLV.

RESULTS AND DISCUSSION

Woody host range. Within 4 to 6 months from graft-inoculation, clear-cut symptoms developed in peach

plants cvs GF 305, Springtime and Elberta in the form of yellow asteroid spots and in cherry plants cvs Bing, Canindex and Sam in the form of red to purple rings and mottling. Apricot cvs Luizet, Priana and Tilton and *P. serrulata* Shirofugen remained symptomless. TNAs from all grafted plants, regardless of whether they were symptomatic or not, gave positive hybridisation responses with the riboprobe to isolate Apr47. The symptoms induced by this isolate closely resembled those induced by ApLV in peach trees (Nemchinov *et al*., 2000; Gentit *et al*., 2001b) and myrobalan (Nemchinov *et al*., 2000). Contrary to our findings, Gentit *et al*. (2001b) did not observe symptoms in cherry trees cvs Bing, Sam, or Lambert that were shown to be infected by back-indexing to GF 305. The only striking difference between

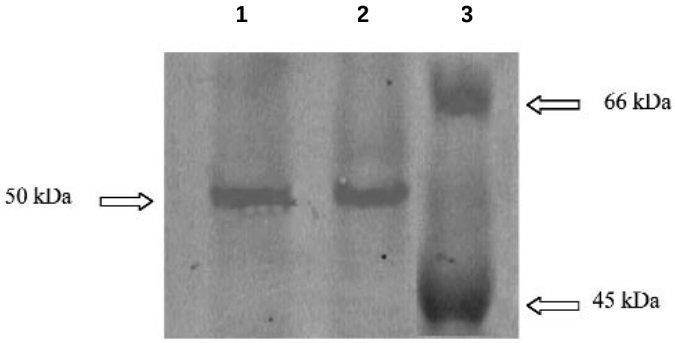

Fig. 1. Electropheretic pattern of dissociated coat protein preparations from ApLV-Apr47 (lanes 1 and 2). Molecular weight markers are shown in lane 3. Albumin egg (Mol. wt. 45,000) and albumine bovine (Mol. wt. 66,000), both from Sigma.

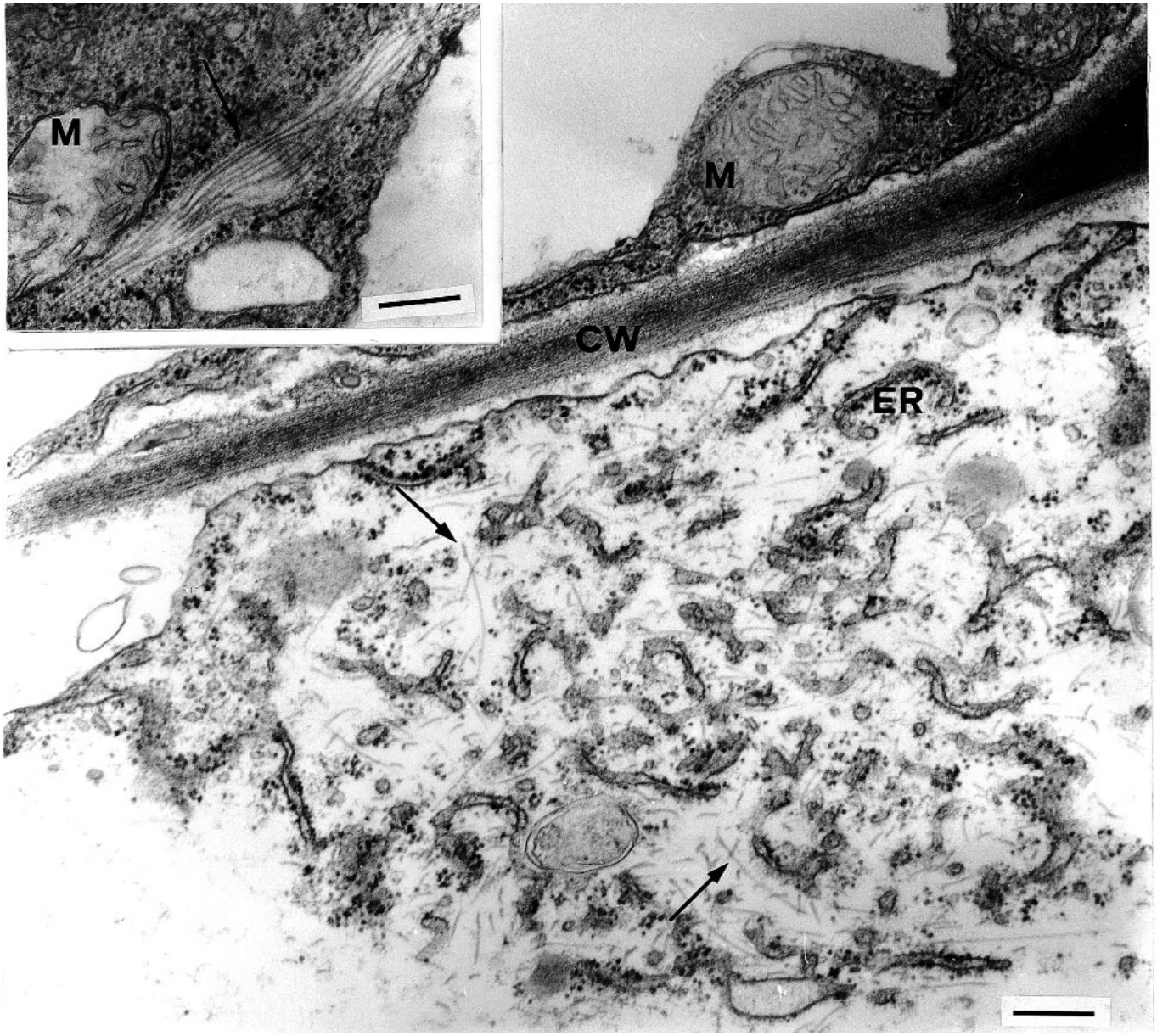

Fig. 2. Filamentous virus particles scattered in the cytoplasm of a *N. occidentalis* cell infected by ApLV-Apr47 and the bundle of virus particles (inset); M = mitochondria; CW = cell wall; ER = endoplasmic reticulum; Bar = 300 nm.

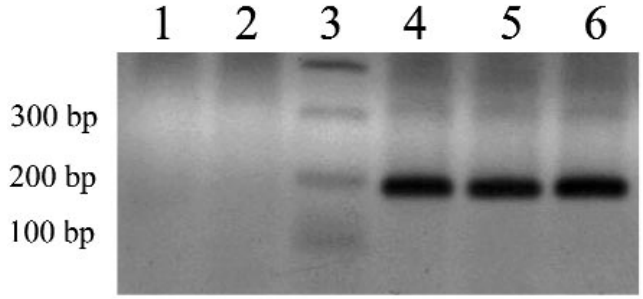

Fig. 3. Agarose gel electrophoresis of RT-PCR amplicons from reverse–transcribed TNA extracts from Apr47-infected GF 305 (lanes 4 and 5), infected *N. occidentalis* (lane 6) and negative controls (lanes 1 and 2). 100 bp Mol. wt. marker in lane 3.

our experiments and those of Gentit *et al*. (2001b) is that our cherry indicators were grafted and grown in a glasshouse, rather than outdoors in a nursery. Whether this differential behaviour depends on the virus isolate or on the environmental conditions under which grafted plants were grown still remains to be investigated.

Herbaceous host range. Of the 10 herbaceous hosts that were mechanically inoculated, *N. occidentalis* showed symptoms consisting of mild vein yellowing and scattered necrotic areas on the leaf blade, 10-15 days after inoculation. None of the other hosts showed any symptoms, and neither were they latently infected as checked by back inoculation to *N. occidentalis*. This extremely restricted host range is consistent with what was reported for ApLV by Zemtchik *et al*. (1998) and Gentit *et al*. (2001b).

dsRNA analysis. PAGE of dsRNAs extracted from GF 305-Apr47 plants revealed multiple bands. The largest band corresponded to an estimated size of about 9.5 kbp (not shown). An RNA of 9.5 kb fits the size range reported for members of the genus *Foveavirus* (8.7-10 kb) (Martelli and Jelkmann, 1998).

Coat protein analysis. Dissociated coat protein preparations of isolate Apr47 migrated as a single band in SDS-PAGE with an estimated size of ca. 50 kDa (Fig. 1), in accordance with the data reported for ApLV by Nemchinov *et al*. (2000).

Cytopathology. Cytopathological changes were minor and no inclusion bodies were observed in thin-sectioned cells of *N. occidentalis* infected with isolate Apr47. This agrees with the pattern reported for foveaviruses (Martelli and Jelkmann, 1998). Vesicular structures protruding from the tonoplast and occasional bundles of filamentous structures that were interpreted as profiles of virus particles were seen in the cytoplasm (Fig. 2).

RT-PCR and sequence analysis. RT-PCR using ApLV-specific primers H-ALV1 and C-ALV1 amplified a 200 bp DNA fragment from reverse transcribed dsRNAs isolated from GF 305-Apr47 plants. A DNA fragment of the same size (200 bp) was also amplified using TNA extracts from leaves of infected GF 305 and *N. occidentalis*, but not from healthy controls (Fig. 3). Positive amplifications were obtained also from all woody plants that had been graft-inoculated with Apr47 (not shown).

Sequence analysis of the 200 bp amplicon showed that it represents a fragment of the coat protein cistron of isolate Apr47 that putatively encodes a polypeptide of 66 amino acids. Direct comparison of the deduced polypeptide with available sequences in sequence databases showed high sequence homology (90-93%) with different ApLV isolates: 93% with ApLV-LA2 and ApLV-Caserta12 (Gentit *et al*., 2001a) and 90% with ApLV (Nemchinov *et al*., 2000) (Fig. 4). Phylogenetic relationships were established based on multiple alignments with the corresponding region of viruses belonging to the family *Flexiviridae*. ApLV-Apr47 clustered with ApLV isolates and close to ApLV-Caserta12 (not shown). These results, even though based on limited sequence data, clearly indicate that Apr47 is an isolate of ApLV, and represent the first record of this virus from a southern Mediterranean country.

Molecular hybridisation. In dot-blot assays riboprobe pApr-47 specifically hybridised TNA extracts from infected GF 305 and *N. occidentalis* plants. No hybridisation was obtained with healthy controls (Fig. 5). The probe did not cross-react with TNA extracts from plants infected with ASPV, CGRMV or CNRMV isolates (not shown).

Survey for the presence of ApLV in Southern Italy. ApLV has been recorded from southern Italy in the provinces of Lecce (Grasseau *et al*., 1999) and Caserta (Gentit *et al*., 2001a). However, none of the 198 samples

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                    *        20         *        40         *        60
ApLV-ALV    : .......T.T.........V..R..M..................................... : 66
ApLV-LA2    : .......T.T.........V..................................... : 66
ApLV-Caser : ..........T.............M.........N.T.......................... : 66
ApLV-Apr47 : ..........I.................................................... : 66
             NRAPRRAASSLGGTRRRLDSIGLKSIIYEPQAGVVATDAKMRAIGRALIEMGIREDQLTEVGVYLA
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Fig. 4. Alignment of the predicted amino acid sequences of the Apr47 coat protein fragment with different ApLV isolates. Dots indicate conserved aminoacids.

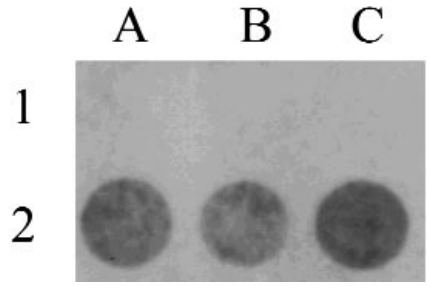

Fig. 5. Dot blot hybridisation with molecular probe to detect Apr47. GF 305 infected plants (row 2, lanes A and B) and *N. occidentalis* (row 2, lane C). Healthy GF 305 (row 1, lanes A and B) and *N. occidentalis* (row 1, lane C).

tested by molecular hybridisation for its presence in our survey gave a positive response. This suggests that ApLV has a limited distribution in southern Italy.

ACKNOWLEDGEMENTS

Grateful thanks are expressed to Prof. S. Sabanadzovic and Prof. G.P. Martelli for helpful discussion and critical reading of the manuscript.

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Received 25 July 2004
Accepted 21 October 2004