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Properties of a citrus isolate of olive latent virus 1, a new necrovirus

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Abstract

A virus was recovered by sap transmission from plants of several citrus species exhibiting or not symptoms of chlorotic dwarf (CCD), a disease recently reported from Eastern Mediterranean Turkey. The virus was identified as an isolate of olive latent virus 1 (OLV-1), originally described as a possible sobemovirus. The citrus isolate of OLV-1 (OLV-1/Tk) possesses biological, morphological, physico-chemical, and ultrastructural properties similar, if not identical to those of the OLV-1 type strain and is also serologically indistinguishable from it. In addition, OLV-1/Tk has many properties, especially physico-chemical, in common with serotypes A and D of tobacco necrosis necrovirus (TNV-A and TNV-D). However, OLV-1/Tk is only very distantly related serologically to both TNV-A and D, suggesting that it can be regarded as a distinct species in the genus *Necrovirus*. OLV-1/Tk could not be detected in citrus tissues by ELISA or dot-blot molecular hybridization, probably because of the extremely low virus concentration. By contrast, limited virus recovery was obtained by sap inoculation and fair detection rates were afforded by PCR. OLV-1/Tk was identified in 54 of 92 (59%) citrus plants affected by CCD and in 14 of 49 (28%) symptomless plants. These results do not support the notion that there is a cause-effect relationship between OLV-1/Tk and CCD, even though the more frequent association of this virus with diseased plants remains intriguing.

Introduction

Citrus chlorotic dwarf (CCD) is a disease first reported in the late 1980s from Eastern Mediterranean Turkey (Çinar et al., 1993). CCD affects all economically important citrus species grown in the province of Içel, being especially severe in a number of varieties of mandarin, lemon, grapefruit and sweet orange (Korkmaz et al., 1994a, 1994b). Field symptoms of CCD resemble those induced by citrus crinkly leaf/infectious variegation (Roistacher, 1991), i.e. deformation and puckering of the leaves, chlorotic flecking of interveinal tissues and oak leaf patterns. However, citrus crinkly leaf virus (CCLV), which occurs in the same area as CCD (Sen and Baloglu, 1994), was shown not to be associated with this disease (Korkmaz et al., 1994b).

CCD is reported to be transmitted in nature by the whitefly *Parabemisia myricae* (Korkmaz et al., 1994b; Çinar et al., 1995) and, experimentally, by grafting (Korkmaz et al., 1994a) and cutting tools (S. Baloglu and M. A. Yilmaz, unpublished information), but not by sap inoculation. However, from leaf samples of eight CCD-infected lemon, grapefruit, and Minneola tangelo trees collected in 1994, a virus was transmitted consistently by sap inoculation to herbaceous hosts. The virus was identified serologically as an isolate of olive latent virus 1 (OLV-1), a possible sobemovirus recovered ten years ago in Southern Italy from symptomless olive trees (Gallitelli and Savino, 1985).

Such an intriguing finding appeared to open to the possibility that a close relationship exists between OLV-1 and CCD. Further studies were therefore carried

out, as described in the present paper, which reports the properties of the citrus isolate of OLV-1 and provides evidence that OLV-1 is a newly recognized necrovirus species.

Materials and methods

Mechanical transmission. Symptomatic leaf tissues collected from CCD-infected trees were crushed in a mortar in the presence of 0.1 M phosphate buffer pH 7.2 containing 2.5% nicotine, and the extract was rubbed onto celite dusted primary leaves of *Vigna unguiculata*, a host that, in preliminary trials, had proven satisfactory for virus recovery. For ascertaining the association of OLV-1/Tk with CCD, mechanical transmissions were made with sap from leaf samples collected from both symptomatic and symptomless citrus trees in the Turkish provinces of Adana and Içel. Sap transmissions were also attempted from four symptomless olive trees growing in the immediate vicinity of citrus groves in Adana and Mersin (Içel).

Virus sources. The following virus isolates were studied: (i) a virus (OLV-1/Tk) recovered from a Mexican lime seedling that had been graft-inoculated with buds from a naturally infected Minneola tangelo tree from Mersin (Içel); (ii) an authentic OLV-1 isolate from Italy (OLV-1/I) (Gallitelli and Savino, 1985); (iii) authentic isolates of tobacco necrosis necrovirus, serotype A (TNV-A) and D (TNV-D), from Rothamsted Experimental Station, Harpenden, UK, through the courtesy of Drs. V. Lisa and P. Roggero, Istituto di Fitovirologia Applicata del CNR, Torino, Italy. Virus cultures were maintained in a climatized glasshouse at 22–24 °C in *Nicotiana benthamiana* (OLV-1) and *N. clevelandii* (TNV).

Host range. The following herbaceous hosts were inoculated with the four virus isolates and their reactions recorded: *N. benthamiana, N. clevelandii, N. glutinosa, N. occidentalis, N. tabacum* cv. Samsun and White Burley, *Chenopodium quinoa, C. amaranticolor, Gomphrena globosa, V. unguiculata, Phaseolus vulgaris, Cucumis sativus.*

Virus purification. OLV-1 isolates were purified from systemically infected *N. benthamiana* and TNV isolates from locally infected *N. clevelandii* using the following procedure. Infected leaf tissues were homogenized in the presence of an equal volume of 0.1 M phosphate buffer pH 6.0 containing 1% sodium ascorbate and 10% of a 1:1 mixture of chloroform-butanol. The homogenate was centrifuged at 5,500 × g for 10 min, the supernatant recovered and treated with 10% polyethylene glycol MW 8,000 and 1% NaCl. The precipitate was resuspended in 0.02 M phosphate buffer pH 6.0 and centrifuged at 116,000 × g for 1 h. Resuspended pellets were layered on sucrose density gradient columns prepared by freezing and thawing a 25% sucrose solution in 0.02 M phosphate buffer, pH 6.0 and centrifuged at 25,000 rpm in a Beckman SW 27.1 rotor for 2 h. Gradient tubes were scanned with an ISCO ultraviolet absorbance monitor and peaks corresponding to virus fractions collected and concentrated by centrifugation at 120,000 × g for 2 h.

Properties of purified virus. Viral nucleic acids were extracted from gradient-purified particles with a standard SDS-phenol method (Diener and Schneider, 1968) and electrophoresed in 1.2% agarose gels in TBE buffer (89 mM tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) after denaturation with 50% formamide at 65 °C for 5 min. Reference markers were genomic RNAs of cymbidium ringspot tombusvirus (CymRSV, 4733 nt) (Grieco et al., 1989) and cucumber mosaic cucumovirus (CMV, 3410, 3035, 2193 and 1027 nt) (Rybicki, 1995).

Coat protein subunits were obtained by boiling for 5 min purified virus preparations in the presence of 1.5% SDS and 3.5% 2-mercaptoethanol in 0.035 M tris-HCl buffer pH 7.0. Dissociated coat proteins were electrophoresed in 12.5% polyacrylamide slab gels in Laemmli's (1970) discontinuous buffer system, and stained with Coomassie brilliant blue. A MW-DSD-70 L kit (Sigma Chemical Co. St. Louis) with markers from 14,200 (α-lactoalbumin) and 66,000 daltons (bovine serum albumin) was used as reference.

Double-stranded RNAs. dsRNAs were recovered from infected *N. benthamiana* (OLV-1/I and OLV-1/Tk) and *N. clevelandii* (TNV-A and TNV-D) plants by phenol extraction and differential purification through cellulose CF11 columns (Dodds, 1993). After digestion with RNase-free DNase (60 μg/ml) in 10 mM magnesium chloride for 30 min at 37 °C and DNase-free pancreatic RNase (25 ng/ml) in 2 × SSC (1 × SSC buffer: 0.15 M sodium chloride and 0.015 M trisodium citrate) for 30 min at 37 °C, dsRNAs were separated by electrophoresis in 1% agarose gels and stained with ethidium bromide.

Serology. The following reagents were used for serological tests: (i) an antiserum to the authentic isolate of OLV-1/I, with a titre of 1:512 (Gallitelli and Savino, 1985), (ii) three antisera to different isolates of TNV-D available at Bari, with titres ranging from 1:128 to 1:256, one of which had been used in previous OLV-1 studies (Gallitelli and Savino, 1985); (iii) an antiserum to OLV-1/Tk; (iv) antisera and to both authentic TNV serotypes. The latter three antisera were raised in rabbits immunized with one intramuscular and two intraveinal injections of purified virus (1 mg/ml nucleoprotein per injection) at weekly intervals. Antisera collection began one week after the last injection and the titres were estimated in gel double diffusion tests. Immunodiffusion and immunoelectron microscopy (IEM) (Milne and Luisoni, 1977), were used for determining serological relationships. For IEM tests undiluted antisera were used.

Molecular cloning. A 3′ terminal 1200 nt cDNA probe was synthesized as described (Gubler and Hoffman, 1983), using artificially polyadenylated OLV-1/Tk RNA (Sippel, 1973) primed by oligo(dT), and inserted into a *Sma I*-cut pGEM-4Z plasmid with which competent *Escherichia coli* DH5α cells were transformed (Sambrook et al., 1989). Colony hybridization and Southern blot assays were performed according to Sambrook et al. (1989). One microgram of plasmid was linearized by digestion with *HindIII* restriction enzyme and *in vitro*-transcribed with SP6 RNA polymerase. Generation of radioactive riboprobes labelled with ^{32}P-UTP (800 Ci/mM, Amersham International, UK) was performed with the SP6/T7 transcription kit (Boehringer, Mannheim, GmbH) following the manufacturer's instructions. Dot blot hybridization was done as described by Grieco et al. (1992).

Polymerase chain reaction (PCR) assay. For detecting OLV-1 in naturally infected tissue, two deoxyprimers were designed complementary to nt 3448 to 3466 (primer A = 5′ CTCACCCATCGTTGTGTGG3′) and to nt 2720 to 2730 (primer B = 5′ TTTCACCCCAC-CAAATGGC 3′) of OLV1 RNA sequence, respectively (Grieco et al., 1996). One microliter of total nucleic acid (TNA) extracted according to White and Kaper (1989) from citrus tissue, was mixed with 150 ng of random hexamers (Boehringer Mannheim, GbmH), denatured for 10 min at 70 °C then incubated at room temperature for 15 min. Reverse transcription reaction was performed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, 200 units Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) for 1 h at 42 °C (final volume 20 μl). Three μl of the mixture were subjected to PCR amplification in 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM primer A, 0.25 μM primer B and 2.5 units of Taq polymerase (Promega Corporation, USA), in a final volume of 50 μl. cDNAs were amplified in a Perkin Elmer Cetus Thermal Cycler apparatus with 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min. In the last cycle, time extension at 72 °C was 10 min. Amplification products were analysed in 1.2% agarose gel electrophoresis (Sambrook et al., 1989).

Electron microscopy. Purified virus preparations were stained with 2% aqueous uranyl acetate before observation with a Philips 201C electron microscope. For thin sectioning, tissue fragments were excised from OLV-1/Tk-infected *N. benthamiana* plants, were double fixed in 4% gluraldehyde and 1% osmium tetroxide, dehydrated in graded ethanol dilutions and embedded in Spurr's resin. Thin section were observed after staining with uranyl acetate and lead citrate (Martelli and Russo, 1984). Tissues from healthy plants were similarly processed to serve as controls.

Transmission through soil. Surface-sterilized *V. unguiculata* seed were sown in autoclaved river sand in disposable plastic pots. When the seedling had reached the primary leaf stage, sap expressed from OLIV-1/Tk-infected *N. benthamiana* plants or purified preparations of the same virus were poured into small holes made in the vicinity of the seedlings and the pots were watered immediately with distilled sterilized water. Ten to 30 days afterwards, the seedlings were gently removed from the pots, their root system was washed free from sand, immersed for 10 min in a 1% SDS solution in water, then left in running tap water overnight. Roots were collected, crushed in 0.1 M phosphate buffer and the extract, undiluted or after serial dilutions up to 10^{-4}, was inoculated to *V. unguiculata* seedlings. Inoculations were also made with sap expressed from stems and leaves. These experiments were repeated four times. Rootlets were examined under the light microscope for the presence of chytrid fungal structures.

Transmission to citrus. Eight 4-month-old Mexican lime seedlings were inoculated by subcortical injections of purified OLV-1/Tk preparations. Mexican lime

and sour orange seedlings (10 each) at the 2 leaf stage were also inoculated by rubbing their leaves with purified virus preparations of OLV-1/Tk and OLV-1/I.

Results

Host range and symptomatology. All virus isolates under study induced necrotic local lesions in the inoculated leaves of all hosts assayed. Unlike the two TNV serotypes, OLV-1/I and OLV-1/Tk systemically infected *N. benthamiana*, eliciting stunting, withering and severe chlorotic mottling. Both OLV-1 isolates induced in *Petunia hybrida* chlorotic lesions that turned necrotic in a few days, whereas the lesions elicited by TNV serotypes were necrotic from the very beginning.

Properties of purified viruses. All four viruses were readily purified with yields ranging from 3 (TNV serotypes) to 10 (OLV-1 isolates) mg/100 g of plant tissue.

After a 2 h centrifugation in sucrose density gradient columns, purified preparations of both OLV-1 isolates sedimented as a single component banding at 2 cm from the meniscus. The band contained isometric particles with somewhat irregular outline, rounded to angular, and poorly resolved surface structure (Figure 1A).

Comparable sedimentation profiles were exhibited by preparations of TNV-A and TNV-D, both of which also yielded a major band 2.1 cm below the meniscus of the tube. The band contained virions c. 30 nm in diameter, exhibiting a regular, mostly angular outline (Figure 1C). Smaller particles c. 17 nm in diameter, likely belonging to the associated TNV satellite virus were occasionally present (Figure 1C). Most of the satellite virus sedimented in a band at 1.2 cm from the meniscus.

Dissociated coat protein preparations of both OLV-1 isolates contained a single protein species with an estimated M_r of c. 30 kDa, with slightly reduced mobility compared to the protein coat subunits of both TNV serotypes, for which a M_r of c. 29 kDa was calculated (Figure 2A).

No difference was found in the size of the four viral RNAs, all of which migrated at the same rate in PAGE electrophoresis, and had an estimated M_r of c. 1.4×10^6 Da (c. 3 900 nucleotides) (Figure 2B).

Double-stranded RNAs. Electrophoresis of dsRNAs extracts of all viruses produced the same electrophoretic pattern, i.e., three bands with estimated M_r of 2.6×10^6; 1.05×10^6 and 0.94×10^6 Da, respectively (Figure 2C). The dsRNA bands of the two OLV-1 isolates, by analogy with the necrovirus species (Lommel, 1995), were interpreted as the replicative form of the full-length genomic and two sub-genomic RNAs, respectively.

Serological relationships. The antiserum to OLV-1/Tk (titre of 1:512) produced a single precipitin line in immunodiffusion and had no apparent reaction with healthy plant antigens.

Both antisera to TNV-A and TNV-B had a titre of 1:256 to the homologous viral nucleoproteins and a titre of 1:8 (TNV-A) and 1:4 (TNV-D) to the satellite virus. Thus, when used undiluted, they gave a double precipitin line with the respective mixed antigen preparations. In immunodiffusion and ISEM, all antisera reacted with the homologous and heterologous antigens as specified below. However, the three antisera to TNV-D from the Bari collection reacted strongly with the authentic TNV-D antigen, weakly with TNV-A preparations and not at all with OLV-1/I or OLV-1/Tk (not shown).

The precipitin lines produced by antisera to OLV-1 isolates and to TNV serotypes with homologous antigens were always unambiguous, whereas those formed against heterologous antigens varied according to the virus/antiserum combination. For instance, the antisera to either OLV-1 isolate recognized homologous and heterologous antigens equally well, producing a reaction of identity, with precipitin lines merging at the junction (Figure 3A, B). The reaction of the same antisera was weaker with TNV-A antigens, with precipitin lines forming spurs at the junction (Figure 3A, B), but with TNV-D antigens it was barely, if at all, visible, and only if undiluted antisera were used (Figure 3A, B). TNV-A and TNV-D antisera recognized both OLV-1 antigens with very weak, barely visible precipitin lines that formed spurs at the junction (Figure 3C, D).

These results indicate serological identity between OLV-1/I and OLV-1/TK and a distant relationship of both these viruses with TNV serotypes. This was confirmed when the dilution end points of antisera with homologous and heterologous antigens were estimated by immunodiffusion (Table 1) and the serological differentiation index (SDI) calculated. In particular SDI between OLV-1 isolates and TNV-A was 6, where-

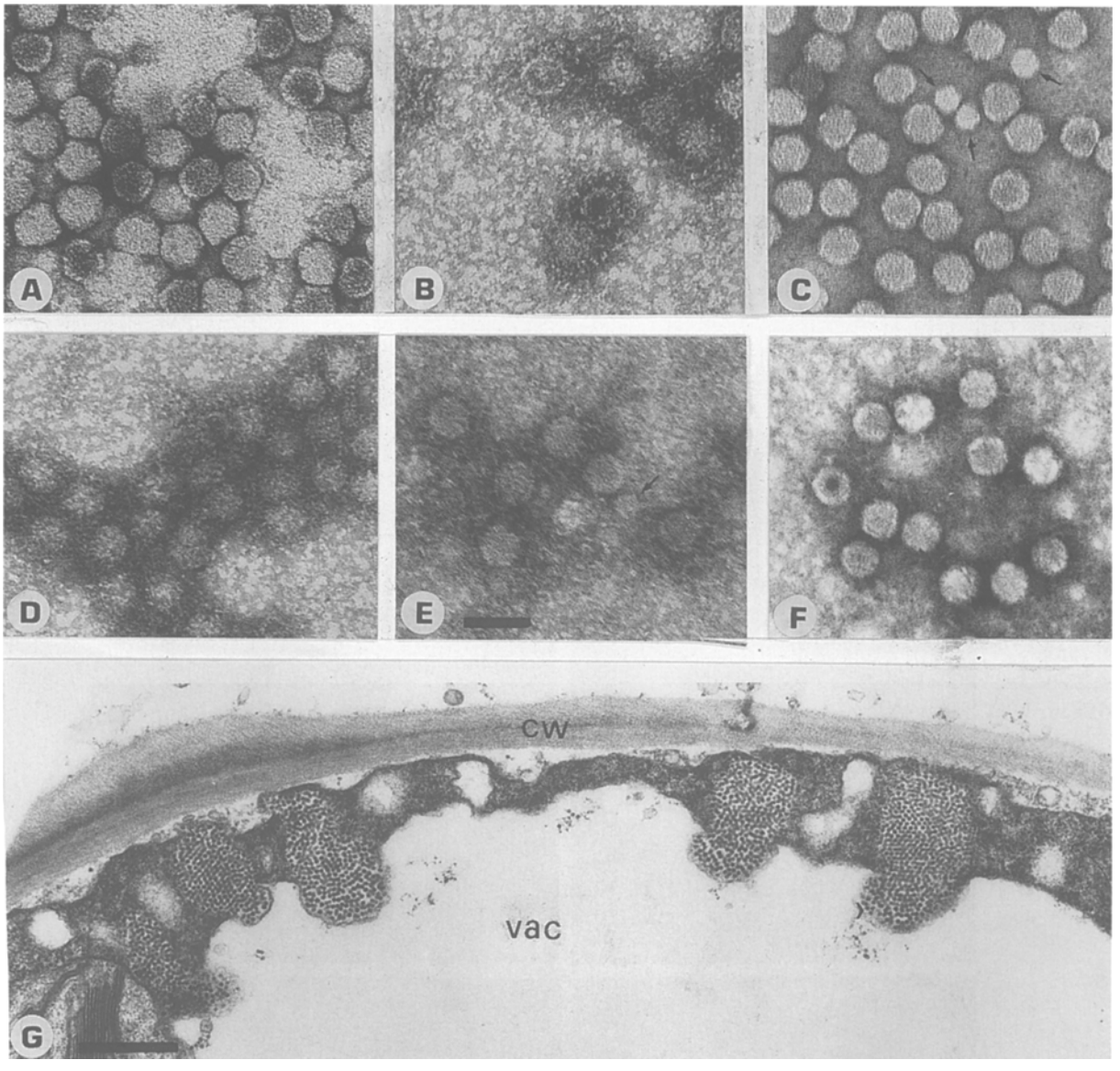

Figure 1. (A) Negatively stained particles of OLV-1/Tk; (B) OLV-1/Tk particles decorated by OLV-1/I antiserum; (C) TNV-A particles. Arrows point to satellite virus particles; (D) TNV-A particles decorated by the homologous antiserum; (E) TNV-A particles exposed to OLV-1/Tk antiserum. The arrow points to a non decorated satellite virus particle; (F) TNV-D particles exposed to OLV-1/Tk antiserum. Note lack of decoration. Bar = 50 nm; (G) Accumulation of OLV-1/Tk virions in bleb-like evaginations of the tonoplast protruding into the vacuole (Vac) of an infected *N. benthamiana* cell. CW = cell wall. Bar = 500 nm.

as SDI between OLV-1 isolates and TNV-D ranged between 7 and 9.

In IEM tests, all viruses particles were always heavily decorated by their homologous antisera (Figure 1D), whereas decoration by heterologous antisera varied according to the antigen/antiserum combination. Decoration was strong when OLV-1 isolates were exposed to heterologous OLV-1 antisera (Figure 1B), but was weak or not detected, when particles of TNV-A and TNV-D were treated with the antiserum to OLV-1/Tk (Figure 1E, F) or OLV-1/I (not shown).

Cytopathology. The structural architecture of both hosts infected with OLV-1/Tk was well preserved and most of the major organelles including mitochondria, chloroplasts and nuclei were apparently unaffected or

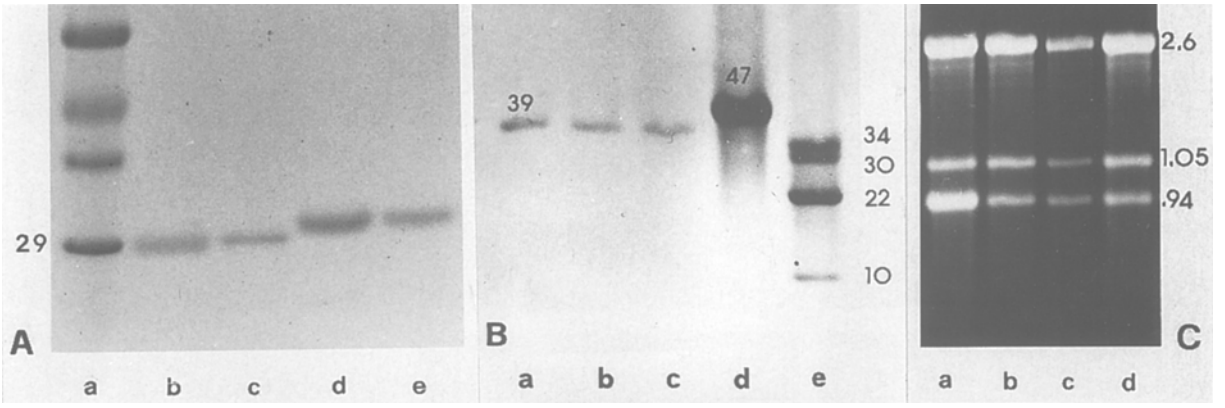

Figure 2. (A) Electrophoretic profiles of dissociated coat protein preparations of TNV-A (lane b), TNV-D (lane c), OLV-1/I (lane d) OLV-1/Tk (lane e). Mol. wt markers in lane a. (B) Electrophoretic profiles of genomic RNAs of TNV-A (lane a), OLV-1/I (lane b) and OLV-1/Tk (lane c) (c. 3,900 nt); CyRSV (4,733 nt) (lane d); RNA-1 (3,410 nt), RNA-2 (3,035 nt), RNA-3 (2,193 nt) and RNA-4 (1,027 nt) of CMV (lane e). (C) Electrophoretic profiles of dsRNAs extracted from *Nicotiana* plants infected by OLV-1/I (lane a), OLV-1/Tk (lane b); TNV-A (lane c) and TNV-D (lane d). Figures are the estimated mol. wt in kDa.

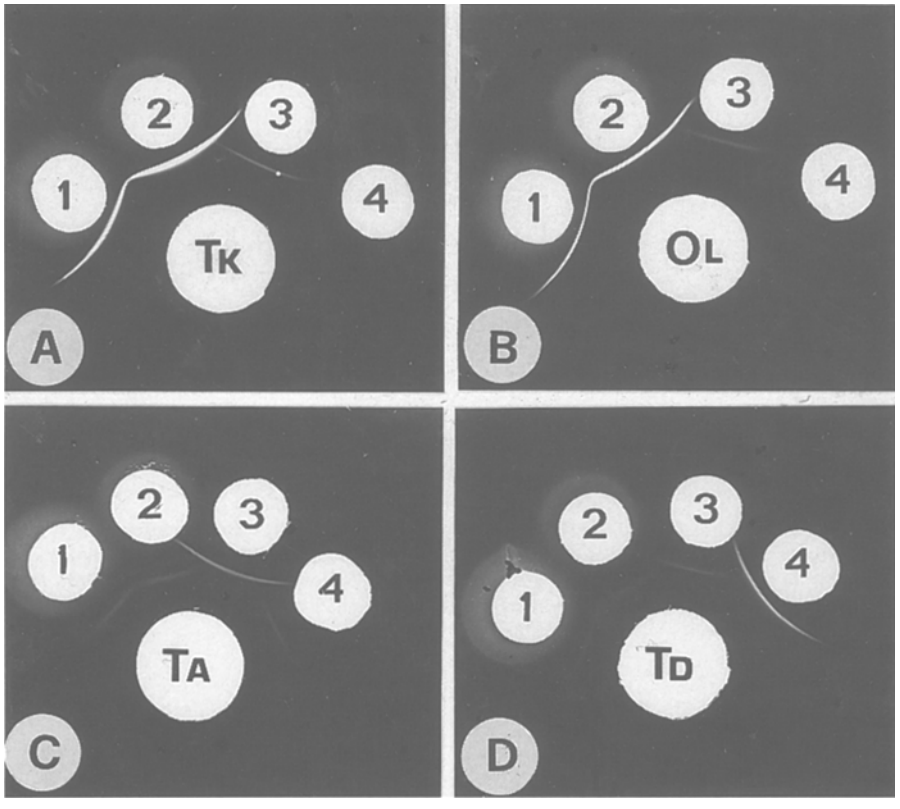

Figure 3. Evidence that OLV-1/I and OLV-1/Tk are serologically identical (A, B) and are very distantly related to TNV-A and TNV-D (A, B, C, D). Note that in all cases homologous reactions are much stronger than heterologous reactions. Undiluted antisera are in the central wells and density gradient-purified antigens in the perimetric wells. Tk = antiserum to OLV-1/Tk; OL-antiserum to OLV-1/I; TA = antiserum to TNV-A; TD = antiserum to TNV-D. Wells 1, 2, 3, and 4 contain the following antigens OLV-1/Tk, OLV-1/I, TNV-A, and TNV-D, respectively.

Table 1. Reciprocal dilution end points of antisera to OLV-1/I, OLV-1/Tk, TNV-A and TNV-D with homologous and heterologous antigens in immunodiffusion tests

Antiserum	Antigen			
	OLV-1/I	OLV-1/Tk	TNV-A	TNV-D
OLV-1/I	512	512	8	4
OLV-1/Tk	512	512	8	4
TNV-A	8	8	256	8
TNV-D	1	1	8	256

showed mild signs of derangement of the internal structure. Proliferation and vesiculation of the endoplasmic reticulum was frequent. Virus particles were plentiful in most cells, dispersed in the cytoplasm, or forming more or less compact aggregates, some displaying a paracrystalline array, or in bleb-like evaginations of the tonoplast protruding into the vacuole (Figure 1G). Except for the latter feature, which is more typical of tombusvirus infections (Russo et al., 1987) and had not been previously observed in OLV-1 infections (Castellano et al., 1987), the cytopathology of OLV-1/Tk did not differ substantially from that elicited by OLV-1/I or TNV serotypes in infected hosts (Castellano et al., 1987; Appiano and Redolfi, 1993).

Presence of OLV-1/Tk in citrus. To assess the occurrence of OLV-1/Tk in citrus, a total of 141 samples from eight different species were assayed. Of these, 92 samples had been collected from plants with incipient to severe CCD symptoms, whereas 49 samples were from symptomless trees. Virus detection was attempted by inoculation of sap, DAS-ELISA, dot blot hybridization, and PCR.

For mechanical transmission, sap expressed from young leaf tissues of each sample was inoculated to the primary leaves of c. 80 seedlings of *V. uniguiculata* grown in a tray. Successful inoculations produced usually 1 or 2 reddish lesions in no more than 1 or 2 seedlings. The lesions turned necrotic in a few days and were followed by invasion of the closest veins (Figure 4A) without systemic spread. Virus recovery by sap inoculation was obtained only from 30 out of 141 samples.

For DAS-ELISA, IgGs from the OLV-1/Tk antiserum were used at a concentration of 1 μg/ml both for plate coating and antigen detection. Clear-cut positive reaction were obtained with sap expressed from infected herbaceous hosts but not from the citrus samples despite repeated attempts using both aged and young leaves.

Dot-blot hybridization assays with the OLV-1/Tk riboprobe were comparable to ELISA tests. Signals developed only with sap expressed from infected herbaceous hosts (Figure 4B).

PCR gave somewhat more reproducible results. In 63 of 141 samples a product of the expected size (747 nt) was amplified and confirmed by Southern blotting to be part of the OLV-1/Tk genome (Figure 4C, D).

The overall outcome of virus detection trials in citrus was that OLV-1/Tk was identified in 6 samples by sap inoculation, in 43 samples by PCR, and in 20 samples by a combination of PCR and mechanical inoculation.

As shown in Table 2, OLV-1/Tk was found in all citrus species assayed, except for Satsuma mandarin and clementine, regardless of whether they were affected or not by CCD. However, the infection rate was higher in symptomatic trees (59%) and in the Içel area (62% of all samples) where CCD is more widespread, than in apparently healthy trees (28%) and in Adana (16% of all samples), where CCD incidence is negligible (Çinar et al., 1995).

Two olive samples from Içel were PCR positive, whereas two additional samples, one each from Içel and Adana, were PCR negative.

Transmission through soil. The roots of *V. unguiculata* seedlings grown in autoclaved river sand inoculated with infected plant extracts, or purified virus preparations, did not contain soil-borne fungal structures. However, necrotic streaks were observed in some rootlets of the seedlings grown in pots inoculated with purified virus preparations but not in those of the controls. OLV-1/Tk was recovered from roots showing necrosis, but not from stems or leaves. Virus was isolated after 10 and 30 days, up to the maximum dilution tested (10^{-4}), from extracts of roots that had been treated with SDS and extensively washed in running water. This was taken as an indication that infectivity was not due to mere surface contamination, but rather to virus that had entered root cells and multiplied without spreading systemically.

Transmission to citrus. Of the eight Mexican lime seedlings that were inoculated by subcortical injections, two became systemically infected, as demonstrated by successful virus recovery from upper leaves, but did not show any symptoms. Mexican lime and sour orange seedlings inoculated mechanically with puri-

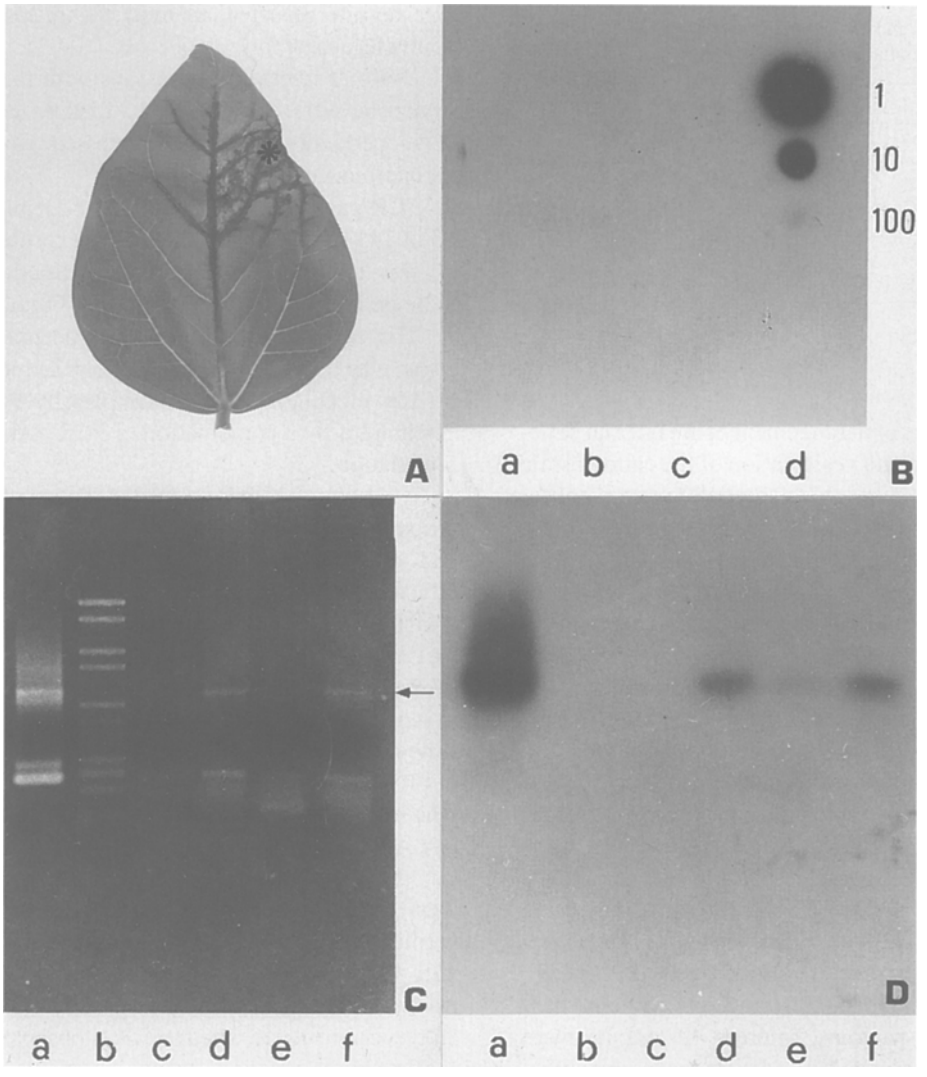

Figure 4. Techniques used for detecting OLV-1/Tk in citrus. (A) Infected cowpea leaf following inoculation with sap expressed from a chlorotic dwarf-infected citrus tree. This photograph illustrates a typical situation in which a single lesion had formed at the margin of the leaf blade (asterisk) from which the virus has spread to the veins. (B) Dot blot hybridization with a OLV-1/Tk RNA riboprobe of different dilutions (1 to 100) of leaf extracts from a healthy citrus (lane a); OLV-1/Tk-infected Mexican lime seedling (lane b); healthy *N. benthamiana* (lane c); infected *N. benthamiana* (lane d). Only the latter extracts gave a positive hybridization signal. (C) Agarose gel electrophoresis of RT-PCR amplified products in extracts from: leaf tissues of a Mexican lime seedling with local lesions induced by mechanically inoculated OLV-1/Tk (lane a); marker (lane b); healthy Mexican lime seedlings (lane c); samples from field-grown citrus plants affected by chlorotic dwarf disease (lanes d–f). The arrow marks the virus-specific amplified product of 747 nt. (D) Southern blot hybridization of the same gel as in (A) showing specific recognition of PCR amplification products only (lanes a, d, e, f).

fied virus preparations all reacted with small necrotic local lesions. These were followed by symptomless systemic invasion in all of the seedlings inoculated with OLV-1/Tk and in about 80% of those inoculated with OLV-1/I.

Discussion

The present results demonstrate that the virus isolated from Turkish citrus species affected or not by CCD is an isolate of OLV-1 indistinguishable from the type strain (Gallitelli and Savino, 1985). However, some differences were found in the physico-chemical prop-

Table 2. OLV-1/Tk detection in field-grown CCD-affected and symptomless citrus species in Turkey

Locality/citrus species	Symptomatic plants	Symptomless plants
ADANA		
Minneola tangelo	5/2*	12/2
Grapefruit	3/1	7/0
Lemon	==	9/1
Orange	==	2/0
IÇEL		
Minneola tangelo	9/4	==
Grapefruit	6/4	2/1
Lemon	52/37	14/10
Sweet orange	4/1	3/2
Sour orange	4/3	==
Clementine	1/0	==
Satsuma mandarin	6/0	==
Mexican lime	2/2	==
TOTAL	92/54 (59%)	49/14 (28%)

* Tested samples/positive samples

erties. OLV-1 was reported to yield upon dissociation of virions a series of RNA molecules smaller than the genomic RNA and three coat protein species. One of these proteins, with M_r of 65 kDa, was interpreted as a possible dimer of the capsid subunits, for which a M_r of 32 kDa was calculated (Gallitelli and Savino, 1985). This latter trait, some aspects of particle stability, and the lack of reaction with an antiserum to TNV-D had prompted Gallitelli and Savino (1985) to hypothesize that OLV-1 was a sobemovirus.

In this study neither OLV-1/Tk nor OLV-1/I, an authentic isolate from olive, showed the heterogeneity in RNA and coat protein previously reported. Rather, both viruses exhibited a single RNA molecule with a size of c. 3 900 nt and a single type of coat protein subunits with a M_r of 30 kDa, consistent with the values reported by Grieco et al. (1996). The same authors also showed that the number and order of genes of OLV-1/Tk genome is the same as in species of the *Necrovirus* genus (Lommel, 1995). This finding supports the results of serological tests that suggest OLV-1 to be a necrovirus. Additional evidence of the above was provided by the outcome of soil transmission tests suggesting the soil-borne nature of OLV-1/Tk, which was acquired by the roots of *V. unguiculata* in the apparent absence of a vector.

The serological relatedness of both OLV-1 isolates to TNV-A was clear-cut but very distant (SDI = 6). The relationship was much less obvious with TNV-D, in which case, precipitin lines in immunodiffusion and particle decoration in IEM tests were barely or not visible, and a SDI of 7–9 was calculated. Based primarily on this very distant serological relationship, it seems plausible to concluded that OLV-1 is a necrovirus species of its own right. This, by analogy with the criteria used for species identification in the related genera *Tombusvirus* and *Carmovirus*, where much closer serological similarities exist between allegedly distinct viruses (Martelli et al., 1988; Morris and Carrington, 1988).

OLV-1 can infect artificially inoculated citrus seedlings, in which it may also spread systemically. Thus, OLV-1 is the second necrovirus known to infect citrus, the first being a TNV-D strain isolated in France from young leaves of Orlando tangelo, sour orange, and Eureka lemon with psorosis symptoms (Yot-Dauthy et al., 1969). Reportedly, this TNV strain infected *V. unguiculata* systemically and could be isolated from citrus only using partially purified leaf extracts, but not by inoculation of crude sap (Dot-Dauty et al., 1969). This further differentiates the French TNV strain from OLV-1/Tk. Its recovery, however, confirms that necroviruses can systemically invade citrus hosts in which, apparently, they do not induce symptoms, nor multiply much.

Yot-Dauthy et al. (1969) could not conclusively link the TNV-D isolated with a specific disease condition. This seems to be also the case of OLV-1, for the results of the survey conducted for investigating its association with CCD were inconclusive. In fact, the data on virus detection in field-grown trees do not seem to support the concept that there may be a cause-effect relationship between OLV-1 and CCD, even though the higher association of the virus with diseased than apparently healthy plants remains intriguing.

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