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Research Papers

Characterization of *Citrus tristeza virus* isolates recovered in Syria and Apulia (southern Italy) using different molecular tools

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Summary. *Citrus tristeza virus* (CTV) is the causal agent of the most important virus disease of citrus. CTV isolates differing in biological and molecular characteristics have been reported worldwide. Recently, CTV was detected in Syria in citrus groves from two Governorates (Lattakia and Tartous) and several CTV outbreaks have been reported in Apulia (southern Italy) since 2003. To molecularly characterize the CTV populations spreading in Syria and Italy, a number of isolates from each region was selected and examined by different molecular approaches including: Multiple Molecular Markers analysis (MMM), real time RT-(q)PCR, single strand conformation polymorphism (SSCP) of the major coat protein (CP) gene (P25), and sequence analysis of the CP (P25), P18, P20 and RdRp genes. SSCP analysis of CP25 yielded two distinct simple patterns among the Syrian isolates and three different patterns in the Italian isolates. Based on MMM analysis, all Syrian CTV isolates were categorized as VT-like genotype, whereas the Italian isolates reacted only with the markers specific for the T30 genotype. These findings were also confirmed by RT-qPCR and by sequencing analysis of four genomic regions. The Italian isolates had nucleotide identities which varied: from 99.5 to 99.8 for the CP gene; from 97.4% to 98.3% for the P18 gene; from 98.6% to 99.8% for the P20 and from 97.8% to 99.1% for the partial RdRp sequenced. High sequence identity was found for all genomic regions analyzed between the Syrian isolates (from 98.9% to 99.6%). These results show that the CTV populations spreading in Apulia and Syria are associated with different genotypes, indicating different potential impacts on the citrus trees in the field. Since in both areas the introduction of the virus is relatively recent, infected plants resulted to contain a single and common genotype, suggesting that CTV is spreading from the first outbreaks by aphids or local movement of autochthonous infected plant material.

Key words: Citrus, CTV, molecular markers, strain, sequence analysis.

Introduction

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, family *Closteroviridae* (Martelli *et al.*, 2005), is the causal agent of the most serious viral disease of Citrus *spp.* (Rocha-Pena *et al.*, 1995). The CTV genome consists of a single-stranded, positive sense RNA molecule of about 19,296 nucleotides, encapsulated in flexuous filamentous particles approximately 11 nm wide and 2,000 nm long (Bar-Joseph *et al.*, 1989). The virions contain two capsid proteins, a major 25-kDa coat protein (CP) covering about 95% of the particle length and a minor 27-kDa CP (Febres *et al.*, 1996), which covers only one end of the particles. CTV dissemination occurs by propagation of virus-infected buds and by aphid transmission. Budwood transfer is responsible for most CTV introductions into new areas, whereas aphid transmission is important for local spread (Moreno *et al.*, 2008). Members of the *Aphididae*, *Toxoptera citricida* Kirkaldy, *Aphis gossypii* Glover, and *A. spiraecola* Patch, are the most important vectors of CTV (Roistacher and Bar-Joseph, 1987).

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CTV isolates differ in the type and intensity of symptoms induced in different citrus species and cultivars, and in their aphid transmissibility (Roistacher and Moreno, 1991). Comparison of CTV isolates to provide pathogenicity profiles has been and is still achieved by indexing on different indicator plants (Garnsey *et al*., 2005), which is time-consuming and requires considerable greenhouse space. Isolates can be characterized on a citrus host range as: mild; decline inducing death of scions grafted onto sour orange (SO) rootstock; sweet orange stem pitting (OSP) and grapefruit stem pitting (GSP). In addition to bioindexing, several serological and molecular approaches have been developed for specific identification of severe CTV strains. These include: (i) ELISA tests using monoclonal antibodies (MAbs) for the detection of decline or stem-pitting isolates (Permar *et al*., 1990; Nikolaeva *et al*., 1998); (ii) reverse transcription (RT)-polymerase chain reaction (PCR) assays with selective primers/probes (Cevik *et al*., 1996; Hilf and Garnsey, 2000; Huang *et al*., 2004); (iii) hybridization with specific probes (Cevik, 1995; Genc, 2005); (iv) asymmetric PCR-ELISA (Nolasco *et al*., 2009); (iv) real-time RT-qPCR assays using strain-specific TaqMan probes (Ruiz-Ruiz *et al*., 2009; Yokomi *et al*., 2010). Although none of these strain-specific molecular markers can be strictly associated with the biological characteristics of the isolates, high correlation (>90%) has been shown between the genotypes, as defined by molecular and serological assays, and the biological activities of the isolates (Sieburth *et al*., 2005; Nolasco *et al*., 2009; Ruiz-Ruiz *et al*., 2009). Furthermore, serological and molecular tests can reveal the presence of severe strains hidden in multiple strain infections associated with mild symptoms.

In Syria, CTV was first detected in 2008 by Abou Kubaa *et al*. with an incidence varying from 0% to 3.5%. In the Apulia region of southern Italy, an active eradication program for CTV is ongoing and only few foci were found until 2001–2002. Within the last 10 years, however, the virus became a real threat for the citrus industry (including nursery production), with infection levels rising to 60% in some fields. No in depth investigation has been carried out to determine the virulence of the strains associated with these infections.

The purpose of the present study was to determine the genotype associated with the CTV isolates now spreading in the Apulia region of Italy and in Syria. Selected isolates collected in both areas were tested using different molecular markers, and were also subjected to sequence analyses.

Materials and methods

Virus sources

One hundred infected citrus trees, grafted on sour orange rootstock, recovered between 2006 and 2008 within the annual surveys carried out in Apulia (Italy) and Syria, were preliminarily screened by SSCP analysis of the CP (P25) gene. At least one isolate representative of each different SSCP pattern was then selected for further characterization. Specifically, isolates QS2 (from Lattakia, Syria) and QS4 (from Tartous, Syria) were collected from stunted and declining sweet orange trees in 2006, grafted on sweet orange Madam vinous and maintained at the Mediterranean Agronomic Institute of Bari; isolates 1B, 4B, F361, K51, O262, A211 were collected in 2008 in Massafra municipality (Apulia, southern Italy) and maintained at the University of Bari. Isolates 4B and K51 were from symptomless clementine trees, isolates F361, O262, and A211 were from symptomless sweet orange cv. Navelina trees. Isolate 1B was recovered from a sweet orange tree showing decline. All isolates were from trees grafted on sour orange rootstock.

Seedlings of Madam vinous grown under greenhouse conditions were used as healthy controls.

Asymmetric PCR-ELISA typing (APET)

This assay was carried out according to the procedures described by Nolasco *et al.* (2009). Briefly, the CP (P25) gene was amplified for the selected isolates using standard immunocapture RT-PCR procedures and the primers CTV1 and CTV10 described by Sequeira and Nolasco (2002). One μL of the DNA was then used as template in asymmetric PCR reactions containing the digoxigenin (DIG)-labelled dUTP. An aliquot of the resultant DIG-labelled PCR products was loaded on the microplates previously precoated with 8 CTV–specific capture probes. After 90 min of the incubation at 45°C and three washing steps, the anti-digoxigenin alkaline phosphatase conjugate (diluted 1:1000) and the substrate p-nitrophenyl phosphate (1 mg mL^{-1}) were added following the manufacturer's procedure. The colorimetric reaction was evaluated by measuring the absorbance at 405 nm. Probe reactions were determined by measuring their reaction

rates during the exponential phase, and compared to those obtained from a standard panel of known isolates and strains. Analysis of the results was achieved in two steps using specifically developed software.

Total nucleic acid extraction

Total nucleic acids (TNAs) were extracted from approximately 200 mg of leaf petioles, homogenized using a Mixer Mill MM400 (Retsch, Stadt Haan, Germany) in 1 mL of grinding buffer (4.0 M guanidine isothiocyanate, 0.2M NaOAc pH 5.2, 25mM EDTA, 1.0M KOAc pH 5.0 and 2.5% PVP-40) and purified using silica particles as described by Foissac *et al*. (2001).

Single Strand Conformation Polymorphism (SSCP) analysis of P25 coat protein gene

SSCP analysis was carried out on the CP (P25) gene amplified using the primer T36CP (Hilf and Garnsey, 2000). One μL from each positive RT-PCR product and 9 μL of denaturing solution (95% formamide and 0.05% bromophenol blue), were incubated at 95°C for 10 min, prior to electrophoresis in a 10% non-denaturing polyacrylamide gel. Electrophoresis was performed at room temperature for 15 min at 100 V and the following 3 h at 200V. DNA bands were then visualized after silver staining.

RT-PCR amplification and multiple molecular markers (MMM) analysis

Five μL of TNA extracts (approximately 0.5 μg of total RNA) were mixed with 1 μL random hexamer primers, (Roche, Mannheim, Germany) (0.5 μg μL^{-1}), denatured at 95°C for 5 min and quickly chilled on ice. Reverse transcription reactions were performed for 1 h at 39°C by adding 4 μL M-MLV buffer 5× (50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$), 2 μL of 10mM DTT, 0.5 μL of 10mM dNTPs, and 200 units of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μL. PCR was carried out following the protocol described by Hilf and Garnsey (2000) using eleven primer pairs specifically designed, by the same authors, to amplify three noncontiguous regions of the genome of T30, T3, VT and T36 strains. Genotype was then assigned based on primer pair(s) which produced amplicons of the expected sizes.

Real time (q) RT-PCR

An aliquot of 1.5 μL of TNAs was subjected to one-step RT-qPCR assays using three strain-specific TaqMan probes, as reported by Yokomi *et al*. (2010). Each sample was also tested in RT-qPCR using the broad-spectrum TaqMan probe CP-CY5 (Saponari *et al*., 2008). The four sets of primer/TaqMan probes were used in a singleplex reaction using the iScript reverse transcription supermix for RT-qPCR (Biorad, Hercules, CA, USA). Assays included healthy and non-template controls; each sample was tested in duplicate wells.

Sequence analysis. Nucleotide sequence analyses were performed for the entire or partial CTV genes encoding the major CP (P25), the RNA dependent RNA polymerase (RdRp), the 18K and 20K proteins. Amplification of the target genes was performed using T36CP primers (Hilf and Garnsey, 2000) for the coat protein, PM44/45 and PM48/49 for P18 and P20 genes, respectively (Sambade *et al.*, 2002) and P56F/P56R, designed in this work, which amplify a DNA fragment of 468 bp (position 9435-9902 on the GenBank acc.n. EU937520) within the RdRp gene (P56F - 5′ CTTCTGTCGTACGAAAGTCGT3′; P56R - 5′ CGCCAACCCAGCGTTCGTCA3′). PCR conditions for all primer sets were those reported by the respective authors.

RT-PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and ligated into pGEM-T easy vector (Promega, Madison, WI, USA), both following the manufacturer's instructions. Ten recombinant clones were PCR-amplified and subjected to SSCP analysis using the conditions described above. Based on the SSCP pattern, at least three recombinant clones, displaying the same pattern for each target amplicon, were sequenced.

Results

Asymmetric PCR-ELISA typing (APET)

The results obtained using the eight strain-specific probes showed that all analyzed isolates from Syria or Apulia reacted with one specific probe, thus presumably each harbouring a single CTV strain. Specifically, Syrian isolates reacted with probe III and B10 and were in the Gp. 3b group, which includes a subset of VT strains (Hilf *et al*., 2005) associated with moderate quick decline symptoms. In the case of the Apulian isolates, reactions were observed

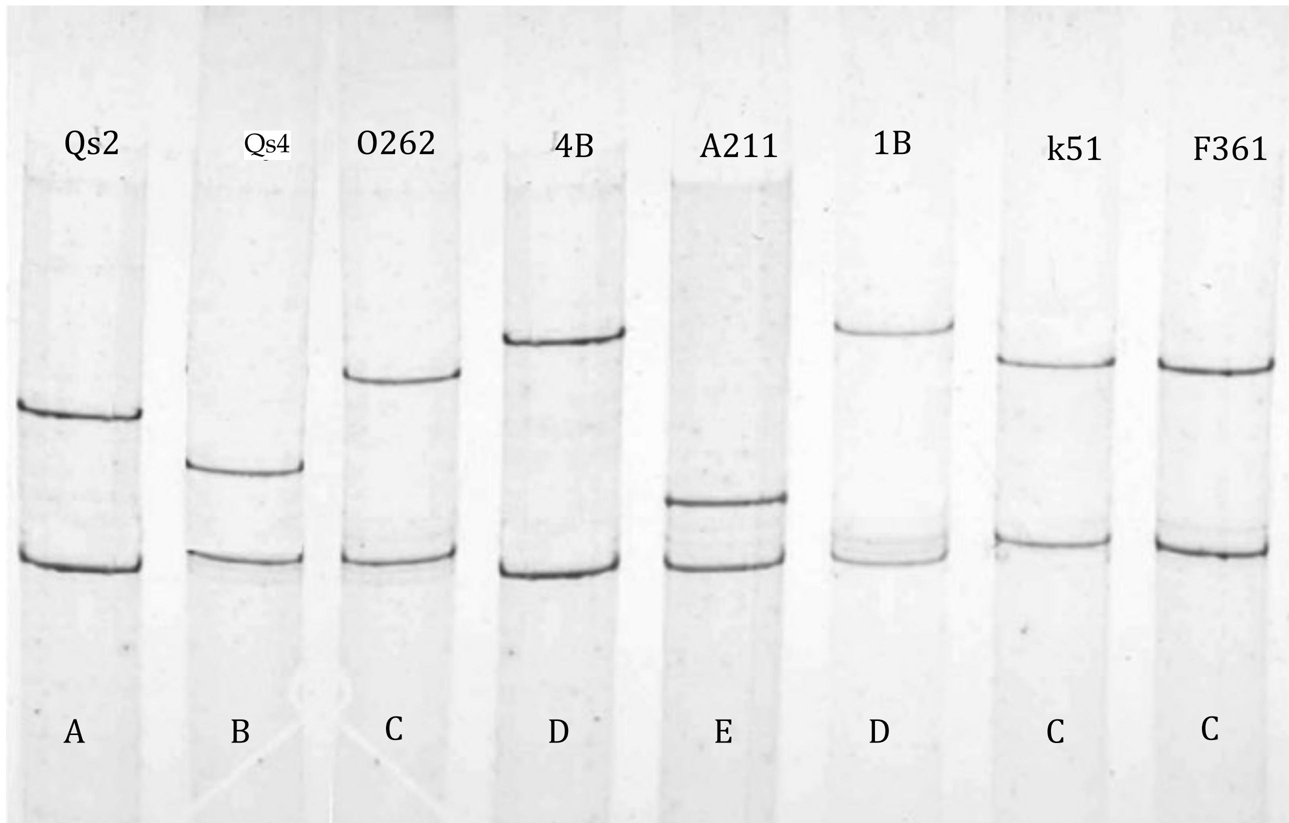

Figure 1. Electrophoretic SSCP patterns of the CP gene RT-PCR amplicons from isolates of *Citrus tristeza virus*. Isolates from left to right: Qs2, Qs4 (from Syria), and O262, 4B, A211, 1B, k51, F361 (from Apulia). Isolates with similar pattern are indicated with the same letter of the alphabet.

only with probe B8, indicating they could be related to the group Gp. M (T30 genotype), which includes strains causing mild symptoms on indicator plants.

Single-Strand Conformation Polymorphism (SSCP)

A total of five different SSCP patterns (Figure 1) were observed for the RT-PCR products of CP (P25) gene obtained from the 100 CTV-infected samples analyzed. In particular, two different SSCP patterns were observed for the Syrian CTV isolates and three different patterns for the Apulian isolates. All SSCP-screened samples showed a simple pattern consisting of two bands.

Multiple Molecular Marker analysis (MMM)

All tested isolates reacted with the broad-spectrum T36CP primers. The six Apulian isolates reacted with the T30POL, T30K17 and T30-5′ primers indicating the presence of a T30–like genotype. Syrian isolates reacted only with the VTPOL, VTK17 and VT-5′primers, indicating they contained a VT-like genotype. None of the analyzed isolates reacted with the T3- or T36-specific primers and with a combination of more than one genotype-specific primer (Table 1).

Real time (q) RT-PCR

Positive reactions with a cycle threshold (Ct) between 18.55 and 22.34 were obtained for all samples tested with the CP-CY5 probe. However, none of the Apulian samples reacted with the CPi-probes, indicating that no T36-, T36NS-, VT- or T3-like genotypes were associated with the selected infected samples. Clear positive reactions with the CPi-VT3 probe were obtained for all isolates from Syria (Table

Table 1. Results obtained after amplification using specific primers in selected *Citrus tristeza virus* isolates with the multiple molecular markers (MMM).

Origin	Isolate ID	MMM primers pairs										
		T36CP	T30POL	T30K17	T30-5′	VTPOL	VT-5′	VTK17	T36-5′	T36POL	T36K17	T3K17
Syria	QS2	1 [a]	0 [a]	0	0	1	1	1	0	0	0	0
	QS4	1	0	0	0	1	1	1	0	0	0	0
Apulia	K51	1	1	1	1	0	0	0	0	0	0	0
	F361	1	1	1	1	0	0	0	0	0	0	0
	1B	1	1	1	1	0	0	0	0	0	0	0
	4B	1	1	1	1	0	0	0	0	0	0	0
	O262	1	1	1	1	0	0	0	0	0	0	0
	A211	1	1	1	1	0	0	0	0	0	0	0

[a] (1), Marker amplification; (0), no amplification.

Table 2. Results obtained with the RT-(q)PCR.

Origin	Isolate ID	Cycle threshold obtained with the different CTV-specific TaqMan probes (Ct values X+SD) [a]			
		CP-CY5	CPi-T36	CPi-T36NS	CPi-VT3
Syria	QS2	19.45±0.18	N/A [b]	N/A	20.45±0.21
	QS4	24.62±0.14	N/A	N/A	23.62±0.16
Apulia	K51	18.55±0.21	N/A	N/A	N/A
	F361	23.32±0.16	N/A	N/A	N/A
	1B	20.48±0.12	N/A	N/A	N/A
	4B	21.64±0.17	N/A	N/A	N/A
	O262	22.34±0.21	N/A	N/A	N/A
	A211	20.11±0.08	N/A	N/A	N/A

[a] (X) Average of two wells; (SD) Standard deviation.
[b] (N/A) No fluorescence detected.

2). No fluorescence was detected in the healthy and non-template controls.

Sequence analysis and phylogenetic relationships

For each isolate, a simple and conserved SSCP pattern was obtained for the ten recombinant clones selected for each target amplicon. The nucleotide chosed sequence identity between the three clones chose for Sanger sequencing ranged from 99.7 to 99.9%. The sequence of one of the three clone was then submitted to GenBank under the accession numbers reported in Table 3.

Phylogenetic relationships were inferred using the following reference isolates: SY568 (AB046398), VT (EU937519), T30 (EU937520) and T36 (EU937521), and for CP gene, the following Apulian CTV isolates 0032 (AJ518842), 0036 (AM407894) and 0038

Table 3. GenBank accession numbers of nucleotide sequences of Syrian and Apulian (Italy) isolates of *Citrus tristeza virus.*

Origin	Isolate ID	Host	Accession numbers for each genomic region sequenced			
			CP (25kDa)	P18	P20	RdRp
Syria	QS2	Sweet orange	FN552118	FN662702	FN662710	FN555140
	QS4	Sweet orange	FN552119	FN662703	FN662711	FN555139
Apulia	K51	Sweet orange	FN661494	FN662700	FN662709	FN662714
	F361	Clementine	FN661495	FN662699	FN662708	FN662713
	1B	Sweet orange	FN661496	FN662696	FN662704	FN662716
	4B	Clementine	FN661497	FN662697	FN662705	FN662717
	O262	Sweet orange	FN661498	FN662701	FN662706	FN662715
	A211	Sweet orange	FN661499	FN662698	FN662707	FN662712

(AM407895) previously characterized in Apulia (Barbarossa and Savino, 2006). The neighbour-joining unrooted dendrograms were generated from the ClustalW alignments (Thompson *et al*., 1994) and phylogenetic tree displayed using MEGA4 software package (Tamura *et al*., 2007).

Phylogenetic analysis showed that for the four genes sequenced, the Apulian and Syrian isolates consistently clustered in two distinct phylogenetic groups. In particular, the Apulian isolates all clustered in the same phylogenetic group with the T30 isolate from Florida, whereas the Syrian isolates were in the same clade as the VT reference isolate (Figure 2).

Nucleotide sequence identity among the Apulian isolates varied from 99.5 to 99.8% for the CP gene, from 97.4 to 98.3% for the P18 gene, from 98.6 to 99.8% for the P20 and from 97.8 to 99.1% for the RdRp gene. Comparison with the previously characterized Apulian isolates showed a greater sequence identity with the isolate 0036 than with the isolates 0032 and 0038. Although, all these three isolates were found to cause mild symptoms in the indicator plants, only the isolates 0036 and 0038 caused mild inverse pitting at the bud union of Madam vinous indicator plants grafted on sour orange (Saponari and Barbarossa, data not published). Similarity among the Apulian isolates and the reference genotype T30 ranged from 99.1% in the CP gene, to 98.9% in the P18, 97.2% for the P20 gene and 98.0% for the RdRp gene. High sequence identity was found for all genes analyzed between the two Syrian isolates (from 98.9% to 99.6%). Nucleotide identity with the reference VT strain varied from 98.6% in the CP (P25) gene, to 98.3% in the P18 gene, 99.8% in the P20 gene and 98.5% in the RdRp.

Discussion

In the last 10 years CTV became widespread in some areas of the Mediterranean basin, and infected citrus trees harboring severe strains of the virus have been reported in different countries (Davino *et al*., 2003; Anfoka *et al*., 2005; Cerni *et al*., 2005; Amin *et al*., 2006; Papayiannis *et al*., 2007; Baloglu and Birisik, 2009; Saponari *et al*., 2009; Malandraki *et al*., 2011). The prevalence of sour orange rootstock in the southern and eastern part of the Mediterranean Basin is thus threatened by the ongoing spread of the virus, and its main vector *Toxoptera citricida* has been identified in Northern Portugal and Spain (Ilharco *et al*., 2005) and is apparently already beyond eradicable stage.

A collaboration was developed with the Department of Pest Management of the Ministry of Agriculture from Damascus (Syria) and with the Apulian Phytosanitary Service, in order to investigate the genotype associated with the CTV isolates that are spreading in Syria and in the Apulia region of Italy. It is known that with mild CTV strains, disease mitigation can be obtained using tolerant rootstocks, whereas, severe strains, such as those that cause sweet orange or grapefruit stem pitting, can affect

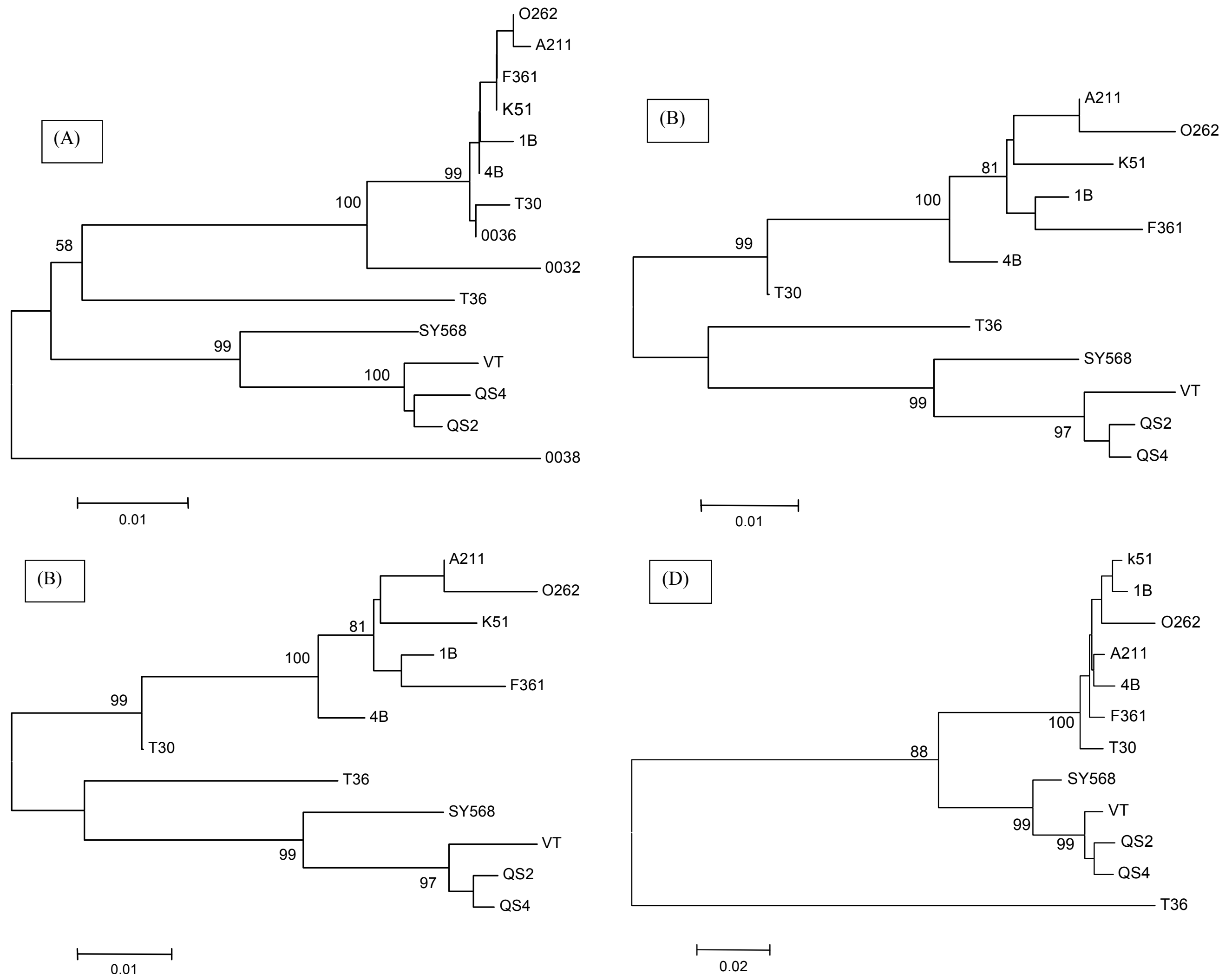

Figure 2. Phylogenetic trees generated by the neighbour-joining method from the alignment of the nucleotide sequences of the major CP (A), P18 gene (B), P20 gene (C) and partial RdRp gene (D) of selected *Citrus tristeza virus* isolates using MEGA (Version 4.1). Bootstrap values (percentage) for 1000 replicates are indicated at the main branches. Branch length is proportional to number of nucleotide changes.

the trees regardless to the rootstock. However, for such severe strains, cross-protection has been adopted to reduce crop losses. Thus, knowledge of the CTV strains causing infections is useful for effective disease management and control.

MMM analysis and APET assay have demonstrated that the Syrian CTV isolates contained a VT-like genotype whereas the Apulian isolates had a T30-like genotype. The results obtained for Syrian isolates were in agreement with those obtained by RT-qPCR, which showed that the Syrian isolates positively reacted with the CPi-VT3 probe specific for VT and T3 genotypes, while the Apulian isolates did not react with any of the CPi-probes indicating that they potentially contained only a T30-like genotype.

This study reveals the presence of VT-like strains of CTV in the coastal region of Syria, where it is also widespread the aphid-vector *Aphis gossypii* (Abou Kubaa *et al.*, 2009a,b). Thus, an effective national CTV management plan must be urgently enforced. Preventive measures, such as monitoring and eradication as well, as the control of the vector popula-

tions, must be initiated as a first step towards the establishment of a certification program for virus-free citrus propagation material.

CTV isolates occurring in the major citrus growing area of Apulia proved to be genetically related to the mild T30 strain, and mixtures of genetically diverse strains were not present in any of the samples tested. Virulent strains have been reported in the neighbourhood areas of Calabria (Ferretti *et al.*, 2009) and Sicily (Rizza *et al.*, 2007), and these represent a real threat to the Apulian citrus industry. Although only the mild T30-like genotype has been detected, several trees were affected by severe symptoms of decline, yellowing, chlorosis and reduction of fruit quality and production. Occurrence of these symptoms could be the result of the combination of several factors (including rootstock and climate), which, even in the presence of mild CTV strains, cause severe effects on the trees grafted on sour orange rootstocks.

In conclusion, the characterizations conducted in this study confirm the reliability of the traditional and newest molecular assays as suitable for determining the virus genotypes associated to the infections caused by CTV. The knowledge of CTV genotypes associated to the infections in Apulia emphasizes: (i) the need to continue the survey program aimed to monitor and intercept severe CTV genotypes and preserve the citrus industry from more devastating infections; and (ii) the possibility of continuing to grow citrus in the contaminated areas by using CTV-tolerant rootstocks. In contrast, because potentially severe strains of the virus have been identified in Syria, research to identify and select cross-protective strains must be supported.

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