Detection of a new variant of *Citrus tristeza virus* in Greek citrus crops

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**Summary.** *Citrus tristeza virus* (CTV), the most destructive virus of citrus, is a quarantine pathogen in Greece. Since 2000, several accidental imports of infected propagation material have been detected in the country, and while eradication measures were applied, a few disease foci still remain. CTV isolates were collected from Chania (Crete) and the “lemonwood” of Poros (Peloponnese), and their genetic variability was studied using single-strand conformation polymorphism (SSCP). One previously characterized isolate from Argolida grafted on a Mexican lime (GR3) and two Italian isolates from Calamondin were also included in the study. ELISA and RT-PCR tests confirmed CTV presence, and SSCP analysis of the virus amplified coat protein (CP) gene was used to separate either distinct virus isolates for cloning the CP gene or variants (haplotypes) for sequencing. Analyses showed that selected variants of four representative isolates clustered into three of the seven defined phylogenetic groups: groups 3b and 5 (severe isolates) and group M (mild isolates). The prevalent haplotypes detected in the CTV from lemonwood of Poros (GR9) were in group 3b, confirming previous results. However, one sequence variant was identified as a recombinant between haplotypes from groups 3b and 5. Variants of these two groups were also detected in the Italian Calamondin isolate. In the grafted Mexican lime isolate (GR3) from Argolida, only one haplotype was found which belonged to group M, while in the field isolate from Chania (GR6) the only haplotype detected was in group 5. This is the first report of variants of group 5 in Greece, suggesting an unknown virus introduction. The prevalence of severe isolates in the area is of particular concern, and implications for the future of the CTV epidemics are discussed.

**Key words:** CP gene, CTV isolates, nucleotide diversity, phylogenetic analysis, SSCP, recombination.

**Introduction**

*Citrus tristeza virus* (CTV) (genus *Closterovirus*, family *Closteroviridae*) is the most economically important and damaging virus of citrus trees (Moreno and Garnsey, 2010). This virus is listed among the largest positive stranded RNA viruses of higher plants; its 19.3 kb positive sense ssRNA genome is encapsidated mainly by the p25 capsid protein (CP) and the p27 proteins, in thread-like filamentous particles of size about 2000 × 11 nm (Moreno et al., 2008).

CTV worldwide dispersal occurred via the movement of infected plant material, often grafted on sour orange rootstocks (*Citrus aurantium* L.) used to control *Phytophthora* root rots. Local spread of the virus is mainly by aphids in a semi-persistent manner (Moreno et al., 2008). Virus spatial and temporal spread depends on the aphid vector species present (Gottwald et al., 1999). In Europe, virus epidemics are associated with the presence of *Aphis gossypii* Glover (Cambra et al., 2000), while the less competent vectors *A. spiraecola* (Patch) and *Toxoptera aurantii* (Boyer de Fonscolombe) also occur (Hermoso de Mendoza et al., 1988; Cambra et al., 2000). *T. citricida* (Kirkaldy), the most efficient vector of CTV (Yokomi et al., 1994) is established in Asia, Australia, sub-Saharan Africa, Central and South America, and different Caribbean countries and it is now in Madeira, northern Portugal and Spain (Ilharco et al., 2005; Hermoso de Mendoza et al., 2008).
Disease syndromes caused by CTV fall into three types depending on virus strain and rootstock-cultivar combination (Moreno et al., 2008). The so-called “severe” CTV strains are associated with (i) quick decline (QD) or tristeza of citrus species grafted onto sour orange or lemon [C. limon (L.) Burm. f.], (ii) the stem pitting (SP) syndrome characterized by the presence of elongated pits on branches of trees grown on tristeza-tolerant rootstocks, which reduces plant vigour and fruit quality, and (iii) seedling yellows (SY), that is observed by biological indexing. Mild CTV strains also occur in field trees but they cause barely detectable symptoms, usually only observed on indicator plants such as Mexican lime [C. aurantifolia (Christm.) Swing.] (Hancevic et al., 2013).

In nature, CTV exists as a mixture of sequence variants, subisolates or recombinants (Broadbent et al., 1996; Kong et al., 2000; Rubio et al., 2001; Matos et al., 2013), displaying high levels of genetic and phenotypic diversity. Biological data appeared inconsistent when analyzing virus variability, while typing methods which target the coat protein (CP) gene are more reliable (Niblett et al., 2000). Single-strand conformation polymorphism (SSCP) is further used to estimate the genetic diversity within and between isolates (Rubio et al., 1996; Sambade et al., 2002), and worldwide occurring isolates of CTV are now grouped in seven clusters based on the analysis of their CP gene (Nolasco et al., 2009) associated with different symptoms (Hancevic et al., 2013).

In Greece, the citrus industry represents an important branch of the economy, with an annual production of 1.3 million t; citrus crops are cultivated in 58 000 ha consisting mainly of oranges [Citrus sinensis (L.) Osbeck] (70%) and lemons (17%), grafted onto sour orange rootstock (Hellenic Statistical Authority, 2006). CTV is regulated as a quarantine pathogen in the country; all areas of citrus cultivation are surveyed, and eradication measures are applied when the virus is found (Dimou and Coutretsis, 2009). CTV was firstly detected in 2000, in imported sweet orange cv. Lane Late trees grafted on Carrizo citrange, in Argolida (North East Peloponnese) (Dimou et al., 2002) and Chania (Crete) (Dimou and Coutretsis, 2009). Analyses of the nucleotide sequences of the p20 gene of these isolates showed high homology with the Spanish mild T385 isolate (Varveri, 2006; Shegani et al., 2012). A similar isolate was collected in 2005, from sweet orange trees of the cv. Washington navel and Navelina in Arta prefecture (North West Greece) (Barbarossa et al., 2007). In 2007, CTV was detected in an orchard in Scala Laconias (South Peloponnese), and subsequently in 2008, in Attiki and Chalkidiki, in greenhouses producing ornamental citrus Calamondin [Citrofortunella microcarpa (Bunge.) Wijnands]. In 2009, a more virulent strain was obtained from old lemon and mandarin trees from the “lemonwood” (an area with ca. 100-year-old lemon trees) in Poros (North East Peloponnese), and this strain clustered in group 3b (Nolasco et al., 2009) according to its CP and p20 gene sequence analysis (Malandraki et al., 2011). Although quarantine measures were applied, limited dissemination has occurred from the initial foci in Argolida and Chania (Dimou and Coutretsis, 2009).

Typing of CTV strains is considered a key element for predicting disease spread and adopting more efficient control strategies (Moreno et al., 2008). In this study, SSCP analysis of the CP gene of CTV and specific sequencing of selected variants was used to characterize the population structure of CTV isolates occurring in the main disease foci in Greece.

Materials and methods

Virus isolates

Ten CTV isolates were initially used in the study in order to select distinct isolates for further molecular analysis. Most isolates were obtained from field plants; five from sweet orange trees in Chania, Crete (designated GR 1, 4-7) and two from lemon trees of the lemonwood in Poros (GR 8, 9). CTV isolate GR3 was obtained from a Mexican lime seedling grafted with a sweet orange isolate from Argolida that was previously characterized by Varveri (2006), and was maintained in an insect-proof greenhouse at the Benaki Phytopathological Institute (Athens, Greece). Field plants infected with these isolates were originally detected by the Greek quarantine system and sampled before eradication. Two isolates (CTV 10 and 11) from Calamondin plants originating from Italy were also included in the study. The presence of CTV in the infected plant material was confirmed in double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) tests with polyclonal antibodies (Agdia Biofords) according to the manufacture’s procedures.
IC/RT-PCR, RT-PCR or PCR amplification of the coat protein gene

One step Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) and Immunocapture (IC) RT-PCR were performed using the primers CTV1 (5'-ATGGACGACGAAAAACAAAAGAA-3') and CTV10 (5'-ATCAAACGTGTGTTGAATTTCC-3') amplifying a 672 bp product including the whole CP gene (Papayannis et al., 2007).

Total RNA was extracted from fresh bark tissue (0.1 g) using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Subsequently, a single step RT-PCR was performed in 50 µL of a reaction mixture containing 2 mg of RNA, 10 mM Tris (pH8.8), 50 mM KCl, 0.08% Nonidet P-40, 2 mM MgCl2, 200 nM each of the primers, 200 µM of each dNTP, 1U DreamTaq DNA polymerase (Thermo Scientific), 7.5U RNAGuard (Amersham Pharmacia) and 7.5U MuLV reverse transcriptase (Perkin Elmer). RT was performed for 45 min at 38°C followed by PCR amplification with 2 min at 94°C, 30 cycles at 92°C for 30 s, 52°C for 30 s and 72°C for 45 s and finally 5 min at 72°C. PCR-products were separated in agarose gel electrophoresis (1%), stained with ethidium bromide and visualized under UV light. For IC/RT-PCR, RT-PCR tests were performed in tubes previously coated with anti-CTV IgG and ELISA extracts of the CTV infected samples without the addition of total RNA in the RT-PCR reaction mixture (Nolasco et al., 2002). PCR tests were also applied to confirm the transformation of \textit{Escherichia coli} colonies, using the same primers and conditions as described for the IC/RT-PCR and RT-PCR.

SSCP analysis of RT-PCR or PCR products

SSCP analysis was performed on the amplicons obtained by RT-PCR (of infected tissues) and on the PCR products (of recombinant \textit{E. coli} colonies), in order to separate distinct virus variants. The amplified product (1 to 3 µL) was mixed with 9 µL of denaturing buffer [95% formamide, 20 mM EDTA, pH 8, 0.05% bromphenol blue] placed for 5 min at 90°C and then chilled on ice. Denatured products were electrophoresed in a non-denaturing 8% polyacrylamide gel (Bio-Rad Mini-Protean II, Bio Rad Laboratories) for 3 h at 200 V, 4°C, using TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8) as buffer (Rubio et al., 1996). The gels were stained with GelStar (Lonza Bioscience) and visualized under UV light. PCR products displaying different SSCP patterns were considered different genomic variants (haplotypes) (Kong et al., 2000).

Cloning and sequencing

Selected amplicons were TA-ligated into pTZ57R/T vector (InsTAclone PCR Cloning Kit, Thermo Fisher Scientific Inc.) and competent \textit{E. coli} cells (Mach1™-T1R, Invitrogen) were transformed according to manufacturer’s instructions. Transformed colonies were selected by α-complementation on plates supplied with X-gal and ampicillin, according to standard procedures (Sambrook et al., 1989). Transformation was confirmed by PCR amplification of the white colonies using primers CTV1/CTV10, and distinct clones were identified by SSCP analysis of at least 15 colonies per CTV isolate. Selected colonies were transferred to liquid medium and the plasmids harbouring selected variants were purified using the GenJet Plasmid Miniprep Kit (Thermo Fisher Scientific Inc.) according to manufacturer’s instructions.

Sequencing reactions were carried out at CC-MAR, Universidade do Algarve, using the forward and reverse primers. Sequence alignment and clustering were performed using MEGA5 software package (Tamura et al., 2011). Searches for recombination events among the sequences were carried out using the software package RDP (Martin et al., 2010), which implements several algorithms for detecting recombination. The obtained sequences (Table 1) were analysed with the following reference sequences of the CP gene from the GenBank: 19–121, Portugal (AF184114); T36, Florida (M76485); VT, Israel (U56902); SY568, California (AF001623); and T30, Florida (AF260651). Sequences of B249, Venezuela and T3, Florida were provided by C.L. Niblett. These sequences are representative of each of the CTV phylogenetic groups proposed by Nolasco et al. (2009).

Results

IC/RT-PCR, RT-PCR and SSCP analysis of CTV isolates

IC/RT-PCR and RT-PCR of all samples resulted in amplicons of the expected size (672 bp) (results not shown). The preliminary assessment of these amplicons showed four different SSCP patterns (Figure 1a). No variability was observed among field isolates.
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originating from the one area and for each isolate in the patterns of the amplicons produced by either IC/RT-PCR or RT-PCR (results not shown).

The CP genes of the CTV isolates GR3, GR6, GR9 and CTV11, representing different SSCP patterns (Figure 1a), were subsequently cloned. A clear predominance of a single SSCP pattern consisting of two conspicuous bands was obvious in all isolates; for isolate GR3 this was the sole haplotype detected (GR3-11). CTV isolate GR6 consisted of two haplotypes; GR6-30 represented the one observed for the 87% of the analysed clones, while GR6-23 was observed in only 13% of them. Clones of isolate GR9 consisted of 60% of the haplotype GR9-15, 2% of the GR9-28, while one haplotype (6.5%) for each of the GR9-16 and GR9-17 was also present. Isolate CTV11 was difficult to clone; among 40 clones tested only four were successfully transformed showing two haplotypes CTV11-27 (75%) and CTV11-18 (25%) (Figure 1b).

Sequences and comparative analysis of the CTV coat protein

From each isolate one clone representing each haplotype obtained was chosen for sequencing. Nine sequences obtained were submitted to the GenBank with the accession numbers: KF196264, KF196265, KF196266, KF196267, KF196268, KF196269, KF196270, KF196271, KF196272 (Table 1). After excising the two terminal parts 20 bases long that corresponded to the primers, search for evidence of recombination was carried out among the new sequences, and the representative international isolates that were considered for comparisons. A strong recombination signal was obtained with several of the algorithms implemented in the RDP software for sequence GR9-16 in the stretch between positions 440 and 630. According to RDP, the backbone of GR9-16 derives from GR9-17 (group 3b) and the recombination stretch is close to the group 5 Greek sequences. Except for GR9-16, the remaining CTV sequences were aligned, their pairwise distances determined according to the Kimura 2 parameters model, and the resulting distance matrix was used to reconstruct a phylogenetic tree (Figure 2). GR9-16 was not included, as this evolutionary model is not suitable for calculating distances among sequences evolving through recombination.

All sequences matched with the expected sequence of the CTV coat protein. However, they were distributed clearly among three CP gene clusters, with high bootstrap values. The only SSCP variant detected in isolate GR3 (GR3-11) was grouped with the reference sequence from phylogenetic group M, while both variants of isolate GR6 (GR6-23, GR6-30) were clustered in group 5. The CP sequence variants of isolate CTV11 (CTV11-27, CTV11-18) were grouped into two different clusters (groups 5 and 3b), while those of isolate GR9 (GR9-15, GR9-17, GR9-28) were in group 3b.

Figure 1. SSCP patterns obtained from the RT-PCR amplicons of (a) infected plants (GR3, GR6, GR9, CTV11) and (b) their haplotypes subsequently sequenced. Citrus tristeza virus (CTV) isolates: GR3, Mexican lime grafted with the isolate from Argolida (haplotype 11); GR6, Sweet orange from Chania (haplotypes 23, 30); GR9, Lemon from Poros (haplotypes 28, 16, 15, 17); and CTV11, Calamondin from Italy (haplotypes 27, 18).
CTV epidemiology in a region is highly influenced by the susceptibility of the cultivated citrus varieties, the composition and the population dynamic of the aphid vector fauna, and the predominant virus isolates (Moreno et al., 2008). In Greece, the prevalent use of sour orange rootstock provides very many CTV susceptible trees, while the aphid vectors *A. gossypii*, *A. spiraecola* and *T. aurantii* are widespread (Tsitsipis et al., 2007). Therefore, the strain profile of CTV is a key factor for predicting future spread of the disease. In this study, SSCP analysis was employed to assess the population structure and sequence variability of the CP gene of three Greek and one Italian CTV isolates. Due to the quarantine status of CTV in Greece, a limited number of isolates were available; however the genetic diversity of the virus was still obvious.

Two of the field isolates analysed, Poros (GR9) and the Italian (CTV-11) isolate, had typical quasi-species structures consisting of sequence variants, with the one being predominant (Holland et al.,

### Table 1. The GeneBank accession numbers and geographical locations in Greece and Italy of nine isolates and variants of *Citrus tristeza virus* (CTV).

<table>
<thead>
<tr>
<th>CTV isolate</th>
<th>Host</th>
<th>Geographic origin</th>
<th>Haplotype</th>
<th>Accession No.</th>
</tr>
</thead>
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<tr>
<td>GR3</td>
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<td>Argolida</td>
<td>11</td>
<td>KF196264</td>
</tr>
<tr>
<td>GR6</td>
<td>Orange</td>
<td>Chania</td>
<td>23</td>
<td>KF196269</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>KF196272</td>
</tr>
<tr>
<td>GR9</td>
<td>Lemon</td>
<td>Poros</td>
<td>15</td>
<td>KF196265</td>
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<td></td>
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<td></td>
<td>16</td>
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<td></td>
<td></td>
<td>28</td>
<td>KF196271</td>
</tr>
<tr>
<td>CTV11</td>
<td>Calamondin</td>
<td>Italy</td>
<td>18</td>
<td>KF196268</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27</td>
<td>KF196270</td>
</tr>
</tbody>
</table>

**Figure 2.** Neighbour-joining tree obtained from the matrix of pair-wise distances (nucleotide, Kimura 2-parameter) between the CP gene sequences of nine isolates of *Citrus tristeza virus* (CTV) from Greece and Italy. Bootstrap values obtained from 1000 replications are presented. Nodes with bootstrap values lower than 70% are not individualized. Group names as proposed by Nolasco et al. (2009) are indicated on the right. Nucleotide distance is represented in the horizontal bar.
The prevalent haplotype in the Poros isolate was classified in group 3b, in agreement with previous studies (Malandraki et al., 2011). However, we also detected, at a low concentration, a recombinant that implies the natural presence of variants of group 5, as well. The 100-year-old lemonwood may represent a distinct environment for CTV strain variability and spread. Infected lemon plants grafted on sour orange remain symptomless with low CTV titre and uneven distribution of the virus (Moreno and Garnsey, 2010). Long standing CTV presence within individual trees may therefore remain unnoticed, favouring reinfection and changes such as mutation or recombination (Rubio et al., 2001; Martin et al., 2009; Roy and Bransky, 2009; Melzer et al., 2010; Biswas et al., 2012). On the other hand, the poor colonisation that occurs in the area has been critical for the development of future epidemics, as these strains show greater fitness and become predominant in mixed infected plants (Moreno et al., 2002) or occasionally the major (Sambade et al., 2008) components of the mixture, determine field symptoms. On the other hand, the presence of potentially aggressive strains may be critical for the development of future epidemics, as these strains show greater fitness and become predominant in mixed infected plants (Moreno et al., 2008), while their spread seems to be favoured by T. citricida over the mild strains (Rocha-Rena et al., 1995; Niblett et al., 2000; Halbert et al., 2004; Matos et al., 2013).

In Greece, quarantine measures are constantly applied in order to limit CTV spread. However, the spread of T. citricida will challenge the ability of our agricultural systems to quickly shift to resistant rootstocks, and particularly the efficiency of our quar-
antine and certification programmes against the increasing threats posed by severe CTV strains.

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