Eur J Plant Pathol (2009) 124:701–706
DOI 10.1007/s10658-009-9444-0

East Adriatic—a reservoir region of severe *Citrus tristeza virus* strains

Silvija Cerni · Dijana Skoric · Jelena Ruscic · Mladen Krajacic · Tatjana Papic · Khaled Djelouah · Gustavo Nolasco

Received: 2 December 2008 / Accepted: 5 February 2009 / Published online: 26 February 2009

Abstract *Citrus tristeza virus* (CTV) represents one of the major threats to citrus production worldwide. In the East Adriatic region, CTV symptoms are mostly absent due to traditional citrus grafting on trifoliate orange (*Poncirus trifoliata*), a CTV-tolerant rootstock. Therefore, the virus has been continuously spreading by the propagation of infected material. The genetic variability of CTV was studied on nineteen citrus samples, collected from orchards in the coastal region of Croatia, Montenegro and Albania, that previously tested positive by ELISA and immunocapture RT-PCR. Single-strand conformation polymorphism of the amplified coat protein gene demonstrated the presence of different CTV variants in each amplicon, while sequence analysis of cloned CP gene variants confirmed their clustering into six out of the seven phylogenetic groups so far delineated. Four of these groups include sequences of severe quick decline, seedling yellows and stem-pitting (SP) isolates, thought to be found only rarely in the Mediterranean region. Regardless of the lack of symptoms in the field, CTV isolates from the East Adriatic displayed high genetic variability and pathogenic potential, additionally confirmed by biological characterisation. The high percentage of mixed infections suggest the potential for further diversification and a greater risk of severe variants spreading into new areas.

Keywords CP gene · CTV · Phylogenetic analysis · SSCP

S. Cerni (✉) · D. Skoric · J. Ruscic · M. Krajacic
Faculty of Science, Department of Biology,
University of Zagreb,
Rooseveltov trg 6,
HR-10000 Zagreb, Croatia
e-mail: scerni@botanic.hr

T. Papic · K. Djelouah
Istituto Agronomico Mediterraneo,
Via Ceglie 09,
I-70010 Valenzano (Ba), Italy

G. Nolasco
Center for Biodiversity Functional and Integrative Genomics, Universidade do Algarve,
Campus de Gambelas,
8005–139 Faro, Portugal

One of the factors adversely affecting the development of modern citrus production worldwide is the high incidence of *Citrus tristeza virus* (CTV), a pathogen that causes the most prevalent and the most destructive virosis of citrus (Moreno et al. 2008). This filamentous (2,000×11 nm) RNA virus from the *Closteroviridae* family (Dolja et al. 1994) is locally transmitted by several aphid species (Hermoso de Mendoza et al. 1984), while its long-distance spread mainly occurs through the propagation of infected material. Depending on virus strains and on scion cultivar-rootstock combinations, CTV may cause three severe syndromes: the decline of different citrus species grafted onto sour orange, seedling yellows characterised by stunting and leaf yellowing, and stem-pitting (SP) syndrome, named after the appearance of elongated pits in the infected

branches, but also characterised by lower yield and diminished fruit quality, independently of the rootstock (Niblett et al. 2000). Due to the traditional use of trifoliate orange (*Poncirus trifoliata*) rootstock, tolerant for decline symptoms, CTV is continuously spreading by the propagation of infected material making the East Adriatic a reservoir region of diverse CTV genotypes.

The basis of current CTV classification is mainly biological indexing on different indicator plants, although many authors support the existence of a relationship between virus phenotype and coat protein (CP) gene sequences (Pappu et al. 1993; Rubio et al. 2001; Zemzami et al. 2002; Nolasco et al. 2009). As a result of error-prone replication, recombination events, and repeated vector-mediated transmission, naturally occurring CTV isolates exist mostly as populations of genetic variants (Kong et al. 2000) that can be distinguished by the analysis of single-strand conformation polymorphism (SSCP) profiles (Rubio et al. 1996). It is often found that a field isolate is a mix of genomic variants displaying different biological characteristics.

Published reports, based mostly on the analysis of few samples, indicate the presence of severe CTV isolates in the East Adriatic region (Cerni et al. 2005; Papic et al. 2005; Stamo et al. 2000). In this study, we determined the population variability of 19 CTV Adriatic isolates. High pathogenic potential of severe CTV variants was confirmed by biological characterisation.

Citrus samples of different varieties were randomly collected from the commercial orchards scattered throughout the East Adriatic coast (Table 1). Most of these were grafted onto *P. trifoliata* and displayed no tristeza symptoms in the field, although all tested positive by ELISA (Loewe). Using ELISA extracts prepared from the bark tissue of field samples, Immunocapture/Reverse Transcription-Polymerase Chain Reaction (IC/RT-PCR) using primers corresponding to

Table 1 Description of *Citrus tristeza virus* (CTV) samples and the incidence of coat protein (CP) gene sequence types

CTV isolate	Origin	Plant variety	Symptoms on Madam Vinous indicator plant	Phylogenetic grouping of sequenced CP genes[a]					
				1	2	3a	4	5	M
437	Croatia (the island of Vis)	*C. sinensis* 'W. navel'	SP			•			
438	Croatia (the island of Vis)	*C. unshiu* 'Kuno'	0						•
440	Croatia (the island of Vis)	*C. unshiu* 'Kuno'	0		•				
435	Croatia (Kastela region)	*C. unshiu* 'Zorica Rana'	0		•				
13	Croatia (Kastela region)	*C. unshiu* 'Zorica Rana'	0		•				
444	Croatia (Kastela region)	*C. unshiu* 'Chahara'	0					•	
445	Croatia (Kastela region)	*C. unshiu* 'Chahara'	0		•				
446	Croatia (Kastela region)	*C. unshiu* 'Ichimaru'	SP		•	•			
447	Croatia (Kastela region)	*C. unshiu* 'Ichimaru'	SP			•			
FN	Croatia (Kastela region)	*C. sinensis* 'Fukumoto'	SP			•	•	•	
42J	Croatia (Kastela region)	*C. unshiu* 'Ichimaru'	NT		•				
43J	Croatia (Kastela region)	*C. unshiu* 'Seto'	NT						•
44J	Croatia (Kastela region)	*C. unshiu* 'Aoshima'	SP		•	•			
45J	Croatia (Kastela region)	*C. unshiu* 'Ootsu'	SP		•	•			
6J	Croatia (Neretva Valley)	*C. unshiu* 'Kawano Wase'	NT						•
16J	Croatia (Neretva Valley)	*C. limon* 'Lisbon'	NT			•			
T3M	Montenegro (coastal region)	*C. unshiu*	NT		•	•			
T6M	Montenegro (coastal region)	*C. unshiu*	NT						•
Q8	Albania	*C. limon* 'Meyer'	0	•					

0 no symptoms were observed, *SP* stem-pitting symptoms, *NT* not tested

[a] Phylogenetic groups are defined according to classification published by Nolasco et al. (2009)

both ends of the CP gene was performed according to the procedure described by Nolasco et al. (2002). To separate different virus variants presumably present in sample populations, amplicons were cloned into pTZ57R/T vector (Fermentas) according to the manufacturer's instructions. The transformation of competent *Escherichia coli* INVαF′ cells (Invitrogen) was done according to the standard procedure (Sambrook et al. 1989). Transformed colonies were selected by α-complementation, and the presence of the insert was confirmed by PCR using the same primers and PCR reaction conditions as above.

Different CTV variants were identified by SSCP analysis as described by Rubio et al. (1996) using PCR products from approximately ten clones per sample. All products displaying different SSCP patterns were considered different genomic variants (Kong et al. 2000) and plasmids harbouring selected variants were purified using Wizard™ Plus Minipreps DNA Purification System (Promega). Sequencing was performed in both directions (Macrogen Inc.) using the M13-pUC universal primer pair. CP reference gene sequences of biologically well-characterised isolates were retrieved from GenBank: 13C (AF184113), NUaga (AB046398), T318A (DQ151548), 28C (AF184118), VT (U56902), T385 (Y18420), T30 (AF260651), T36 (AY170468), 19–121 (AF184114), Qaha (AY340974), SY568 (AF001623), B165 (EU076703), and Bangalore (AF501867), or were kindly provided by C.L. Niblett (B249, T3). Sequences were aligned using ClustalX 1.8 (Thompson et al. 1994) and analysed by MEGA 3.1 (Kumar et al. 2004) using the neighbour-joining method and applying the Kimura 2-parameter evolutionary model. The tree topology was evaluated by bootstrap analysis based on 1,000 repetitions. All sequences obtained in this work were submitted to GenBank under accession numbers: EU66095–EU660932, EU579412–EU579428, EU288060–EU288064, and AY764154. With the aim of biological confirmation of the obtained results, bark patches taken from 13 samples were graft-inoculated onto Madam Vinous sweet orange indicator and symptoms were evaluated throughout a 2-year period.

ELISA results were corroborated by IC/RT-PCR confirming the presence of CTV in all tested samples. The SSCP analysis of separated CP variants confirmed the coexistence of different genomic variants in all isolates suggesting the possibility of mixed infections with variants displaying different biological characteristics. Most isolates had a population structure consisting of one predominant SSCP haplotype whose frequency ranged from 42% to 83%. To estimate the virus variability within analysed populations, we applied Nei's formula (Nei 1987) for the heterozygosity calculation. The heterozygosity value can range from 0 (if all clones are represented by the same haplotype) to 1 (if all clones are represented by different haplotypes). The average heterozygosity value for our isolates was 0.63. This is almost twice the value calculated for Californian isolates (Kong et al. 2000) and similar to the heterozygosity level of the Argentinean isolates (Iglesias et al. 2008). High sequence variability among the latter is explained by the high frequency of vector-mediated re-infections and the presence of the most efficient vector *Toxoptera citricida* (Iglesias et al. 2008). Since no significant rate of vector-mediated CTV transmission has been reported in the East Adriatic region, the only plausible explanation of such high sequence variability in this region may be the high variability among the CTV isolates initially introduced with infected plant material from different citrus-growing countries in the last century, mostly from Japan, former USSR, and Turkey (Gatin 1992; Stamo et al. 2000). Based on previous reports from Cyprus (Papayiannis et al. 2007) and Iran (Barzegar et al. 2005), we suggest that a high CTV diversity may also be correlated with the type of citrus growing practice. In countries where many different varieties are grown in smaller scattered plots, a high CTV diversity is usually found, while in regions with a highly developed citrus industry, the prevalence of a few major varieties probably leads to the uniformity of the virus variants present in the field.

The phylogenetic relationship analysis of CTV isolates showed a clear categorisation of analysed CP-CTV sequences, including the reference sequences, into seven well-defined clusters (Fig. 1). The classification obtained supports that published by Nolasco et al. (2009) grouping isolates with similar biological characteristics. All virus groups were supported by high bootstrap values (Fig. 1). The sequences from the East Adriatic clustered into six out of the seven groups. Two of these groups (2 and M) harbour mild CTV isolates, while four of them (1, 3a, 4, and 5) harbour CP sequences of isolates that cause severe CTV symptoms of quick decline (groups 1, 3a, 4 and

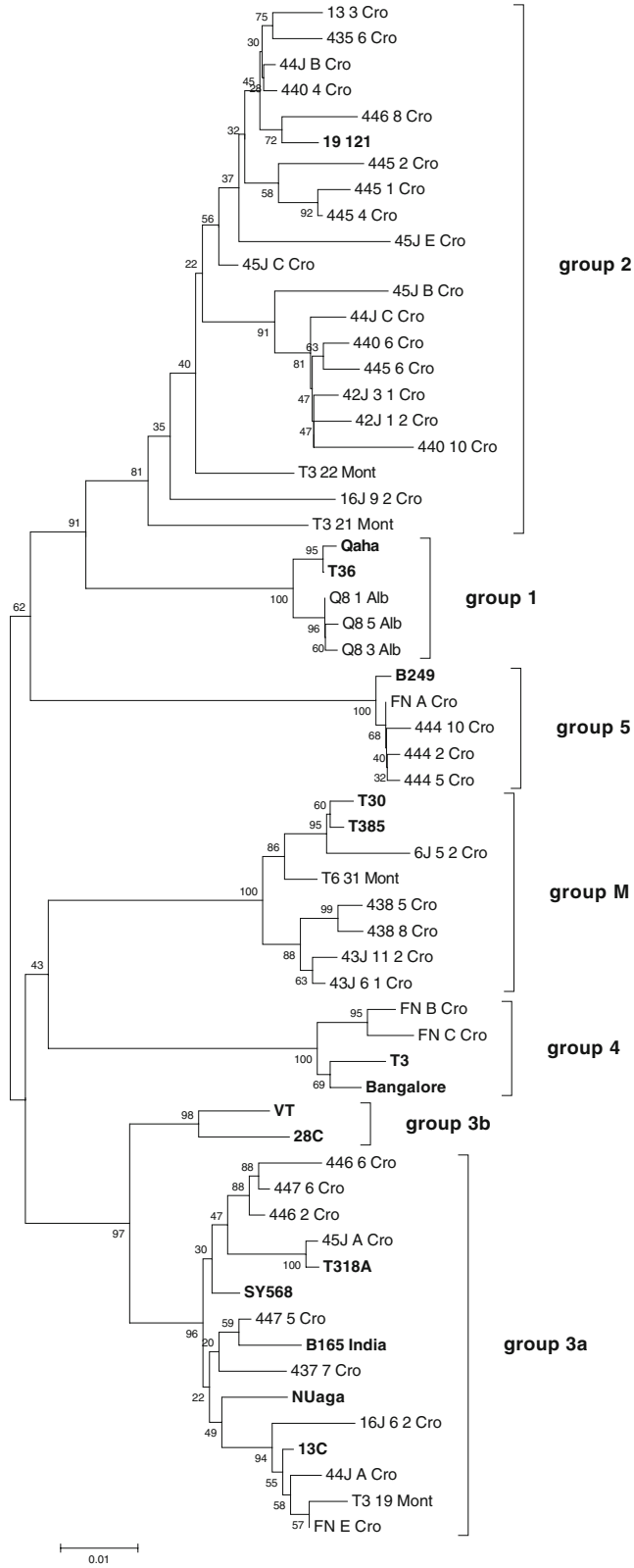

Fig. 1 Neighbour-joining phylogenetic tree obtained by the analysis *of Citrus tristeza virus* coat protein gene sequences. Sequences are named after the CTV isolate followed by the clone number and isolate origin (Alb-Albania, Cro-Croatia, Mont-Montenegro). Reference sequences of isolates with known biological characteristics are included in the analysis (*bold*). Bootstrap values are presented next to tree nodes. The *bar* represents 0.01 nucleotide substitution per site

5), SP (groups 3a and 5), and seedling yellows (Nolasco et al. 2009). Surprisingly, sequences of almost half of our isolates clustered into one of the severe groups (Table 1) with the highest occurrence of sequences in group 3a. Since the appearance of SP symptoms is independent of the rootstock type (Niblett et al. 2000), these variants probably present the major threat for plants grafted onto *P. trifoliata* rootstock. In five samples, we detected a mixture of genomic variants belonging to different phylogenetic groups. The results of phylogenetic analysis were further corroborated biologically. In all Madam Vinous plants inoculated with samples infected with group 3a variants, we found clear symptoms of SP (Table 1). Although Zemzami et al. (2002) reported the appearance of SP symptoms in plants infected with group 5 variants, new results indicate that it is not necessarily the case (Cerni et al. 2008). In accordance with the latter report, we have not found SP symptoms in Madam Vinous inoculated with samples infected with group 5 variants exclusively.

Regardless of the lack of symptoms in field trees, severe CTV variants appear to be widespread and long-present in the East Adriatic region. This genetically heterogeneous population with a high percentage of mixed infections, which also implies a high recombination potential, presents a stumbling-block for the development of a modern citrus industry in this area. Bearing in mind that severe CTV variants show higher fitness levels and become predominant in co-infected plants (Moreno et al. 2008), and that the spread of the most efficient CTV vector *T. citricida* have all been recently reported in the north of Spain and Portugal (Ilharco et al. 2005), we believe that the dispersal of severe variants from the specific niche of the East Adriatic to new regions and scion/rootstock combinations could represent a significant risk to citrus production in the wider region.

Acknowledgments This study was supported by grants POCTI/AGR/59090/2004 (Fundacao para a Ciencia e Tecnologia, Portugal) and 119-1191192-1222 (Ministry of Science, Education and Sports, Croatia).

References

Barzegar, A., Sohi, H. H., & Rahimian, H. (2005). Comparative sequence analysis of coat protein gene of Iranian *Citrus tristeza virus* isolates. *Journal of Phytopathology*, *153*, 457–463. doi:10.1111/j.1439-0434.2005.01001.x.

Cerni, S., Skoric, D., Krajacic, M., Gatin, Z., Santos, C., Martins, V., & Nolasco, G. (2005). Occurrence of stem pitting strains of *Citrus tristeza virus* in Croatia. *Plant Disease*, *89*, 342 doi:10.1094/PD-89-0342B.

Cerni, S., Ruscic, J., Nolasco, G., Gatin, Z., Krajacic, M., & Skoric, D. (2008). Stem pitting and seedling yellows symptoms of *Citrus tristeza virus* infection may be determined by minor sequence variants. *Virus Genes*, *36*, 241–249. doi:10.1007/s11262-007-0183-z.

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. doi:10.1146/annurev.py.32.090194.001401.

Gatin, Z. (1992). Ugrozen unikatni genofond agruma u Hrvatskoj. *Hrvatski vocarski glasnik*, *1–4*, 10–13 (in Croatian).

Hermoso de Mendoza, A., Ballester-Olmos, J. F., & Pina-Lorca, J. A. (1984). Transmission of *Citrus tristeza virus* by aphids (Homoptera, Aphididae) in Spain. In S. M. Garnsey, L. W. Timmer & J. A. Doods (Eds.), *Proceedings of the 9th Conference of the International Organization of Citrus Virologists (IOCV)* (pp. 23–27). Riverside.

Iglesias, N. G., Gago-Zachert, S. P., Robledo, G., Costa, N., Plata, M. I., Vera, O., et al. (2008). Population structure of *Citrus tristeza virus* from field Argentinean isolates. *Virus Genes*, *36*, 199–207. doi:10.1007/s11262-007-0169-x.

Ilharco, F. A., Sousa-Silva, C. R., & Alvarez Alvarez, A. (2005). First report of *toxoptera citricidus* (Kirkaldy) in Spain and continental Portugal (Homoptera, Aphidoidea). *Agronomia Lusitana*, *51*, 19–21.

Kong, P., Rubio, L., Polek, M., & Falk, B. W. (2000). Population structure and genetic diversity within California *Citrus tristeza virus* (CTV) Isolates. *Virus Genes*, *21*, 139–145. doi:10.1023/A:1008198311398.

Kumar, S., Tamura, K., & Nei, M. (2004). Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, *5*, 150–163. doi:10.1093/bib/5.2.150.

Moreno, P., Ambros, S., Albiach-Marti, M. R., Guerri, J., & Pena, L. (2008). *Citrus tristeza virus*: a pathogen that changed the course of the citrus industry. *Molecular Plant Pathology*, *9*, 251–268. doi:10.1111/j.1364-3703.2007.00455.x.

Nei, M. (1987). *Molecular evolutionary genetics*. New York: Columbia University Press.

Niblett, C. L., Genc, H., Cevik, B., Halbert, S., Brown, L., Nolasco, G., et al. (2000). Progress on strain differentiation of *Citrus tristeza virus* and its application to the epidemiology of citrus tristeza disease. *Virus Research*, *71*, 97–106. doi:10.1016/S0168-1702(00)00191-X.

Nolasco, G., Sequeira, Z., Soares, C., Mansinho, A., Bailey, A., & Niblett, C. L. (2002). Assymmetric PCR ELISA: Increased sensitivity and reduced cost for the detection of plant viral and other nucleic acids. *European Journal of Plant Pathology*, *108*, 293–298. doi:10.1023/A:1015649429160.

Nolasco, G., Santos, C., Silva, G., & Fonseca, F. (2009). Development of an asymmetric PCR-ELISA typing method for *Citrus tristeza virus* based on the coat protein

gene. *Journal of Virological Methods*, *155*, 97–108. doi:10.1016/j.jviromet.2008.09.030.

Papayiannis, L. C., Santos, C., Kyriakou, A., Kapari, T., & Nolasco, G. (2007). Molecular characterization of *Citrus tristeza virus* isolates from Cyprus on the basis of the coat protein gene. *Journal of Plant Pathology*, *89*, 291–295.

Papic, T., Santos, C., & Nolasco, G. (2005). First report of *Citrus tristeza virus* (CTV) in State Union of Serbia and Montenegro. *Plant Disease*, *89*, 434. doi:10.1094/PD-89-0434B.

Pappu, H. R., Pappu, S. S., Niblett, C. L., Lee, R. F., & Civerolo, E. (1993). Comparative analysis of the coat proteins of biologically distinct *Citrus tristeza closterovirus* isolates. *Virus Genes*, *73*, 255–264. doi:10.1007/BF01702586.

Rubio, L., Ayllon, M. A., Guerri, J., Pappu, H., Niblett, C., & Moreno, P. (1996). Differentiation of *Citrus tristeza closterovirus* (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *The Annals of Applied Biology*, *129*, 479–489. doi:10.1111/j.1744-7348.1996.tb05770.x.

Rubio, L., Ayllon, M. A., Kong, P., Fernandez, A., Polek, M., Guerri, J., et al. (2001). Genetic variation of *Citrus tristeza virus* isolates from California and Spain: evidence for mixed infections and recombination. *Journal of Virology*, *75*, 8054–8062. doi:10.1128/JVI.75.17.8054-8062.2001.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning – a laboratory manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Stamo, B., D'Onghia, A. M., & Savino, V. (2000). Presence of *Citrus tristeza virus* in Albania. In:R. H. Yokomi, J. V. da Graça, & R. F. Lee (Eds.), *Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV)* (pp. 141–143). Riverside.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680. doi:10.1093/nar/22.22.4673.

Zemzami, M., Soares, C. M., Bailey, A. M., Niblett, C. L., & Nolasco, G. (2002). Molecular characterization and classification of Morrocan isolates of *Citrus tristeza closterovirus*. In J. V. da Graça, N. Duran & R. G. Milne (Eds.), *Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV)* (pp. 8–12). Riverside.