

## SHORT COMMUNICATION

MOLECULAR CHARACTERIZATION OF *CITRUS TRISTEZA VIRUS* ISOLATES FROM CYPRUS ON THE BASIS OF THE COAT PROTEIN GENEL.C. Papayiannis<sup>1</sup>, C. Santos<sup>2</sup>, A. Kyriakou<sup>1</sup>, T. Kapari<sup>1</sup>, and G. Nolasco<sup>2</sup><sup>1</sup> Agricultural Research Institute, 1516 Lefkosia, Cyprus<sup>2</sup> CDCTPV, Universidade do Algarve, 8005-139 Faro, Portugal

## SUMMARY

Within the context of a program in Cyprus for the control of *Citrus tristeza virus* (CTV), the coat protein (CP) genes of 12 local isolates of the virus that induced different symptoms on host trees, were compared to those of known isolates. The CP genes were reverse-transcribed (RT) and amplified by polymerase chain reaction (PCR) and the resulting amplicons were cloned and sequenced. Nucleotide sequence analysis revealed no signs of geographic speciation. All the sequences obtained clustered close to those of previously known isolates of worldwide origin that are in five distinct groups. The nucleotide diversity was high compared to that found using a worldwide database of CP gene sequences. These data support the existence of different CTV introductions into Cyprus or an introduction from a location in which CTV is relatively diverse. Some of the isolates induced stem pitting on branches of grapefruit and sweet orange. Such isolates have not been noted often in the Mediterranean basin. They were close in CP sequence to isolate B249 from Venezuela, which induces stem pitting, and are of particular concern for the whole region.

*Key words:* Citrus, nucleotide diversity, SSCP, CTV clustering, Stem pitting.

*Citrus tristeza virus* (CTV, family *Closteroviridae*, genus *Closterovirus*) is the most destructive and economically important virus of citrus worldwide (Bar-Joseph *et al.*, 1989). The virus is transmitted by aphids in a non-circulative, semi-persistent manner (Shepherd, 1977) and the major aphid vectors are *Toxoptera citricida* (Kirkaldy), *Aphis gossypii* (Glover) and *Aphis spiraeicola* Patch. *T. citricida* is the most efficient and most important in virus transmission (Bar-Joseph *et al.*, 1989), but as yet not widespread in the Mediterranean region.

CTV has a positive sense RNA genome that is encapsidated in flexuous particles about 2000 nm long (Bar-Joseph *et al.*, 1989) and is composed of about 19226 nucleotides (Karasev *et al.*, 1995). The virus causes various types of disease, the most important of which is quick decline of trees grafted on sour orange rootstock. Isolates of CTV can adversely affect vigour and tree quality and cause stem pitting of branches of trees grafted on tolerant rootstocks. However, it is not infrequent to find infected trees that do not manifest any noticeable symptoms. In each region, the particular disease syndrome depends on the kind of CTV strain present. Discrimination of prevailing CTV strains is a key element for predicting disease impact and devising appropriate control strategies for particular regions. A number of the existing methods available for strain discrimination, albeit imperfect, target the coat protein (CP) gene (Niblett *et al.*, 2000).

Citrus is one of the most valuable crops in Cyprus. It covers an area of 5000 ha and has an annual production of about 120 000 tons (Markou and Mavrogenis, 2002). After CTV was detected on the island in the 1980's (Kyriakou *et al.*, 1992), a project was undertaken in 1992 for the control of the disease (Kyriakou *et al.*, 1995; Kapari *et al.*, 1998) through programs of eradication and certification. In Cyprus, CTV is presently transmitted in nature by *A. gossypii* in a non-epidemic form (Kapari *et al.*, 1998).

This paper reports results of molecular characterization and strain differentiation among several CTV isolates that were selected from the main citrus-growing areas of the island on the basis of differences in symptomatology on host trees and on the Mexican lime indicators. The CP gene of the virus was analyzed and characterized by SSCP and by the determination of its nucleotide sequence.

Twelve CTV isolates were collected during surveys in the main citrus-growing areas of Cyprus (Kyriakou *et al.*, 2002). The origin and the host trees of the 12 isolates are presented in Table 1. Field symptoms ranged from inconspicuous to stem pitting, tree chlorosis and decline. All isolates were transmitted by grafting to Mexican lime (*Citrus aurantifolia* L.) seedlings and maintained in the Virology glasshouses of the Agricul-

**Table 1.** Origin, host and biological features of CTV isolates on field and Mexican lime (*Citrus aurantifolia*).

Isolate	Origin and relative location in Cyprus	Host plant (on sour orange rootstock)	Field Symptoms	Symptoms on Mexican lime
Cy 89-197	Xylolympou - SE	Clementine	St, LC, Cl	VC, VCo, LC, St, SP
Cy 89-60	Xylolympou - SE	Clementine	St, LC	VC, VCo, LC, St, SP
Cy 89-507	Xylolympou - SE	Eureka lemon	Symptomless	mild VC, mild St
Cy 95-14	Avgorou - SE	Marsh seedless grapefruit	D	D, SP, St, LC, VC
Cy 94-39	Avgorou - SE	Marsh seedless grapefruit	D, SP	D, SP, St
Cy 94-37	Avgorou - SE	Valencia sweet orange	D	SP, St
Cy 92-67	Vrysoulles - SE	Marsh seedless grapefruit	D	VC, LC, St, SP
Cy 92-365	Vrysoulles - SE	Marsh seedless grapefruit	D	VC, LC, St, SP
Cy 98-33	Fassouri - S	Valencia sweet orange	D, Cl	VC, St
Cy 98-30	Fassouri - S	Valencia sweet orange	St, Cl, mild SP	VC, St
Cy 96-18	Polis - NW	Ortanique	Symptomless	Mild VC
Cy 93-10	Polis - NW	Sweetie grapefruit	Symptomless	Mild VC, mild SP

D: Decline; SP: Stem Pitting; St: Stunting; VC: Vein Clearing; VCo: Vein Corking; LC: Leaf cupping; Cl: Chlorosis.

tural Research Institute at Athalassa. Symptoms typical of CTV infection, including vein clearing, stem pitting, vein corking and leaf cupping, were observed in this indicator. In general, the severity of field symptoms related well to the intensity of symptoms on Mexican lime in the glasshouse (14°C-33°C).

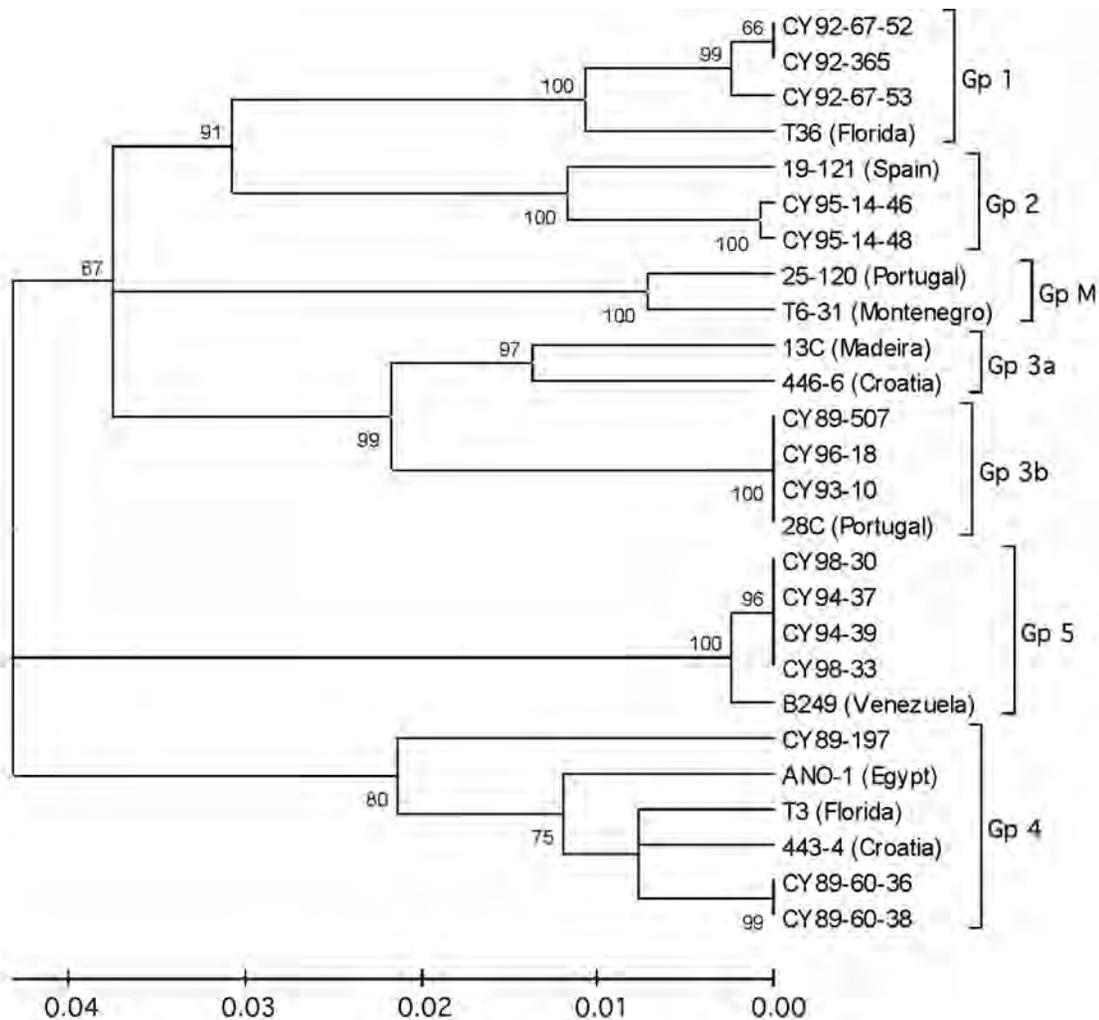
The CP gene was amplified from infected material by one step Immunocapture-Reverse-transcription (RT) Polymerase Chain Reaction (PCR) according to procedures and primers presented by Nolasco *et al.* (2002). By using primers CTV1 and CTV10, a 673 bp product was amplified that covers the whole CP gene and an additional base. Agarose gel electrophoresis showed that a single band of the expected size was obtained. To help choosing characteristic isolates for sequencing, the amplified products were analysed by SSCP as described by Rubio *et al.* (1996). Eleven different patterns were obtained, as two isolates, namely Cy 98-30 and Cy 98-33, created exactly the same pattern (results not shown). In view of the high diversity shown by the SSCP analysis, the CP genes of all isolates were sequenced.

The amplified products were ligated into a pGEM-T Easy Vector System using the original TA Cloning Kit (Promega Corp, Madison, WI, USA) according to the manufacturer's instructions and used to transform competent Inv  $\alpha$ F' *E. coli* cells. Portions of the white colonies were picked and used directly in PCR reactions with the same primers used for the RT-PCR step to confirm the presence of the specific insert. PCR products were again analysed by SSCP in order to choose clones for sequencing. One clone was chosen for each of the 15 different patterns obtained. Minipreps were done and the inserts were sequenced in both directions by the dideoxynucleotide termination cycle Terminators v3.0

Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) using T7 and SP6 primers. Sequences obtained were submitted to the GenBank with the accession numbers: EF491667 - EF491681.

The 20 terminal nucleotides on both sides of the sequences corresponding to the primers zone were excised and a multiple alignment was done along with the sequences of obtained by other authors, using Clustal W (Thompson *et al.*, 1994). These included worldwide haplotypes representative of the seven group types reported by Zenzami *et al.* (2002): 13C (AF184113), 19-121 (AF184114), 25-120 (AF184115), 28C (AF184118), T36 (M76485), B249, T3 (kindly provided by Dr. C.L. Niblett), as well as other Mediterranean haplotypes obtained from Croatia, 443-4 (AY791844) and 446-6 (AY791842) by Cerni *et al.* (2005), from the Montenegro region T6-31 (AY764154) by Papic *et al.* (2005) and from Egypt ANO-1 (DQ211658) by Amin *et al.* (2006). The pairwise distances (Kimura 2 parameters) between haplotypes were calculated and used to construct a dendrogram (Fig. 1) using the neighbour-joining algorithm implemented in the Mega 3.1 software package (Kumar *et al.*, 2004) and to compute the nucleotide diversity per site ( $\pi$ ).

The haplotypes obtained from the Cypriot isolates were distributed in five of the previously reported seven CP gene groups (Fig. 1). In most instances, isolates of similar origin clustered together, suggesting a local spread of the virus. Except for isolate Cy 95-14, which clustered in group 2, isolates clustered in groups known to contain worldwide isolates of similar severity, e.g. group 1 with decline inducing isolates, group 3b not inducing any particular symptom and group 5 with sweet orange or grapefruit stem pitting inducing isolates.



**Fig. 1.** Dendrogram showing the genetic relationships among the coat protein genes of Cypriot CTV isolates (with the prefix Cy) and CTV worldwide reference isolates and eastern Mediterranean isolates. Numbers close to branches indicate bootstrap values. The genetic distance scale is represented by the horizontal bar.

From a plant protection point of view, particular attention should be paid to isolates of this group which includes the B249 sweet orange and grapefruit stem pitting-inducing isolate previously characterized at USDA - BARC, Beltsville, USA (Febres *et al.*, 2003). None of the above groups is specific for Cypriot isolates, ruling out geographic speciation. These results suggest that introduction of CTV in Cyprus has occurred from a location at which diverse CTV isolates occur or has occurred more than once from different locations. A possible origin is budwood imported from South Africa in the 1930's when little was known about viruses (Papasolomontos and Economides, 1968), or from other Mediterranean places. In the same dendrogram were included sequences from additional haplotypes obtained in the field in the eastern Mediterranean basin. Taking these into account, all the seven CP gene clusters are represented in the region.

The nucleotide diversity of the Cypriot haplotypes,  $\pi = 0.071$ , (S.E. 0.0064) was higher than expected when compared to the nucleotide diversity obtained from a database of 213 worldwide CP gene sequences gathered from the GenBank and from additional data of our laboratory (results not shown),  $\pi = 0.074$ , (S.E. 0.0069). In addition, the nucleotide diversity computed only for the eastern Mediterranean haplotypes reached the same value obtained for the worldwide database,  $p = 0.074$  (S.E. Mediterranean 0.0061; S.E. Worldwide 0.0069). Rubio *et al.* (2001) compared the nucleotide diversity of CTV populations from Spain and California and concluded that, from a genetic point of view, these should be regarded as part of the same population. Although the diversity data obtained by these authors are not directly comparable to ours because the CP fragment analysed in their work was shorter (220 nucleotides), their findings reinforce the conclusion obtained in this work, that

a small sub – population of the worldwide CTV population may have a nucleotide diversity that approaches the global nucleotide diversity. Rubio *et al.* (2001) also suggested that the genetic structure of the CTV population suggested migration of isolates among isolated geographic populations. Similar conclusions were presented by Turturo *et al.* (2005) for *Grapevine leafroll-associated virus 3*, another virus in family *Closteroviridae*. Although there is in the history of citriculture and viticulture an extensive exchange of budwood among regions, which would enable a worldwide mixing of isolates, this alone cannot account for the absence of geographic speciation. A low rate of evolution should also be operating to maintain similar genetic structures among different regions. This contradicts the idea, imported from animal virology, that plant RNA viruses are also rapid evolving entities. At least in the case of CTV there are experimental data suggesting low evolutionary rates (Albiach-Marti *et al.*, 2000; Lbida *et al.*, 2004).

The results obtained from this work complement existing notions regarding CTV diversity in the Mediterranean region and reinforce the idea that this is not a particularly preserved region regarding the absence of destructive CTV strains. Strains that induce sweet orange and grapefruit stem pitting are present in the field in Croatia (Cerni *et al.*, 2005) and are now known to be present also in Cyprus. In general, the CTV diversity of the Mediterranean isolates is similar to the diversity among worldwide isolates. If *T. citricida*, which is already present in Portugal and Spain (Ilharco *et al.*, 2005), were to spread throughout the Mediterranean region, then efforts to eradicate CTV will probably fail and alternative solutions should be readily available for application.

## ACKNOWLEDGEMENTS

Part of this work was supported by the grant POCI AGR/59090/2004 from Fundação para a Ciência e Tecnologia, Portugal.

## REFERENCES

- Albiach-Marti M.R., Mawassi M., Gowda S., Satyanarayana T., Hilf M.E., Shanker S., Almira E.C., Vives M.C., Lopez C., Guerri J., Flores R., Moreno P., Garnsey S.M., Dawson W.O., 2000. Sequences of *Citrus tristeza virus* separated in time and space are essentially identical. *Journal of Virology* **74**: 6856-6865.
- Amin H.A., Fonseca F., Santos C., Nolasco G., 2006. Typing of *Citrus tristeza virus* (CTV) isolates based on the capsid protein gene. *Phytopathologia Mediterranea* **45**: 10-14.
- Bar-Joseph M., Marcus R., Lee R.F., 1989. The continuous challenge of *Citrus tristeza virus* control. *Annual Review of Phytopathology* **27**: 291-316.
- Cerni S., Skoric D., Krajacic M., Santos C., Martins V., Nolasco G., 2005. Occurrence of stem pitting strains of *Citrus tristeza virus* in Croatia. *Plant Disease* **89**: 342.
- Febres V.J., Niblett C.L., Lee R.F., Moore G.A., 2003. Characterization of grapefruit plants (*Citrus paradisi* Macf.) transformed with citrus tristeza closterovirus genes. *Plant Cell Reports* **21**: 421-428.
- Ilharco F.A., Sousa-Silva C.R., Alvarez A.A., 2005. First report on *Toxoptera citricidus* (Kirkaldy) in Spain and Continental Portugal. *Agronomia Lusitana* **51**: 19-21.
- Kapari T., Kyriakou A., Ioannou N., Gavriel J., Bar-Joseph M., Savva G., Polycarpou D., Loizias N., Iosephidou E., Hadjinicolis A., Papayiannis C., 1998. A six-year fight against *Citrus tristeza virus* in Cyprus. In: *Proceedings of the 14th Conference of the International Organization of Citrus Virologists, Brazil 2000*, IOCV, Riverside, CA, USA: 335-337.
- Karasev A.V., Boyoko V., Gowda N.O.V., Hilf M.E., Koonin E.V., Niblett C.L., Cline K.C., Gumpf J., Lee R.F., Garnsey S.M., Lewandowski D.J., Dawson W.O., 1995. Complete sequence of the *citrus tristeza virus* RNA genome. *Virology* **208**: 511-520.
- Kumar S., Tamura K., Nei M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150-163.
- Kyriakou A., Ioannou N., Gavriel J., Bar-Joseph M., Papayiannis C., Kapari-Isaia T., Savva G., 1995. Management of *Citrus tristeza virus* in Cyprus. In: *Proceedings of the 13th Conference of the International Organization of Citrus Virologists, China 1995*, IOCV, Riverside, CA, USA: 172-178.
- Kyriakou A., Kapari-Isaia T., Ioannou N., 2002. A 10-year programme for the control of citrus tristeza virus (CTV) in Cyprus. Review for 2000-2001, Agricultural Research Institute, Nicosia, Special Edition 40<sup>th</sup> Anniversary: 86-89.
- Kyriakou A., Polycarpou D., Efstathiou A., Hadjinicolis A., 1992. *Citrus tristeza virus* in Cyprus. In: *Proceedings of the 12th Conference of the International Organization of Citrus Virologists, India 1992*, IOCV, Riverside, CA, USA: 69-72.
- Lbida B., Fonseca F., Santos C., Zemzami M., Bennani A., Nolasco G., 2004. Genomic variability of *Citrus tristeza virus* (CTV) isolates introduced into Morocco. *Phytopathologia Mediterranea* **43**: 205-210.
- Markou M., Mavrogenis A.P., 2002. Norm Input-Output Data for the main crop and livestock enterprises of Cyprus, Agricultural Economic Report 41, Agricultural Research Institute, Ministry of Agriculture, Natural Resources and Environment, Lefcosia, Cyprus.
- Niblett C.L., Gench H., Cevik B., Halbert S., Brown L., Nolasco G., Manjunath V.J., Febres H.R., Pappu H.R., Lee R.F., 2000. Progress on strain differentiation and the epidemiology of *Citrus tristeza virus*. *Virus Research* **71**: 97-106.
- Nolasco G., Squeira Z., Soares C., Mansinho A., Bailey A., Niblett C.L., 2002. Asymmetric PCR ELISA: Increased sensitivity and reduced costs for the detection of plant viral and other nucleic acids. *European Journal of Plant Pathology* **108**: 293-298.
- Papic T., Santos C., Nolasco G., 2005. First Report of *Citrus tristeza virus* (CTV) in the State Union of Serbia and Montenegro. *Plant Disease* **89**: 434.

- Papasolomontos A., Economides C.V., 1968. The presence of tristeza virus in certain species of citrus in Cyprus. *FAO Plant Protection Bulletin* **16**: 8-9.
- Rubio L., Ayllon M.A., Guerri J., Pappu H., Niblett C.L., Moreno P., 1996. Differentiation of *Citrus tristeza closterovirus* (CTV) isolates by single strand conformation polymorphism analysis of the coat protein gene. *Annals of Applied Biology* **129**: 479-489.
- Rubio L., Ayllon M.A., Kong P., Fernandez A., Polek M., Guerri J., Moreno P., Falk B.W., 2001. Genetic variation of Citrus tristeza virus isolates from California and Spain: evidence for mixed infections and recombination. *Journal of Virology* **75**: 8054-8062.
- Shepherd R.J., 1977. Intrinsic properties and taxonomy of aphid-borne viruses. In: Harris K.F., Maramorosch K. (eds.). *Aphids as Virus Vectors*, pp. 126-136. Academic Press, New York, NY, USA.
- Turturo C., Saldarelli P., Yafeng D., Digiario M., Minafra A., Savino V., Martelli G.P., 2005. Genetic variability and population structure of *Grapevine leafroll-associated virus 3* isolates. *Journal of General Virology* **86**: 217-224.
- Thompson J.D., Higgins D.G., Gibson T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.
- Zemzami M., Soares C.M., Bailey A.M., Niblett C.L., Nolasco G., 2002. Molecular characterization and classification of Moroccan isolates of Citrus tristeza closterovirus. *Proceedings of the 15th Conference of the International Organization of Citrus Virologists, IOCV, Riverside, CA, USA*: 8-12.

Received January 17, 2007

Accepted April 2, 2007

