Phytopathol. Mediterr. (2004) 43, 00–00

Genomic variability of *Citrus tristeza virus* (CTV) isolates introduced into Morocco.

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Summary. Genomic variability of the coat protein gene of *Citrus tristeza virus* isolates obtained from old Meyer lemon introductions in Morocco and more recent budwood introductions from Spain were studied. The coat protein gene of the virus was amplified directly from infected tissue by immunocapture RT-PCR and analysed by single stranded conformation polymorphism (SSCP) and sequencing. Each isolate consisted of several related genomic variants, typical of a quasi-species. Although SSCP analysis has only limited typing ability it could be used in an initial screening to discriminate between isolates of different origin and to analyse the genomic structure of each isolate. Sequence analysis showed that the isolates of Spanish origin were closely related to mild isolates characterised in Florida and in Portugal. The Meyer lemon isolate on the other hand was related to severe strains of Meyer lemon characterised in Florida some years ago and to other severe strains from Brasil. A knowledge of the coat protein gene sequence is useful to trace the origin of the isolates.

Key words: Closterovirus, *KEY WORDS DIFFERENT FROM TITLE*, coat protein gene.

Introduction

Citrus tristeza virus (genus *Closterovirus*, family *Closteroviridae*, CTV) is the causal agent of the most serious disease of *Citrus* spp. (Rocha-Pena *et al.,* 1995). The virus causes various disease types, the most important of which are the quick decline of trees grafted on sour orange rootstock; reduced vigour; reduced tree quality and stem pitting of branches of trees grafted on tolerant rootstocks; leaf yellowing and stop of growth of sour orange and lemon seedlings. In each region affected the particular disease syndrome depends on the kind of CTV strains present. Strains may occur singly or mixed (Grand and Higgins, 1957). A knowledge of which strains are present in an area is fundamental to define a strategy to control the disease. The virus is naturally transmitted in a semi-persistent manner by several species of aphids, of which *Toxoptera citricida* is the most effective; this aphid does not yet occur in the Mediterranean basin. Budwood movement is responsible for the long distance virus dissemination.

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The CTV genome consists of a single-stranded, positive sense RNA molecule of 19,296 nucleotides, encapsulated in flexuous filamentous particles about 11 nm wide × 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 capsid protein (Febres *et al.*, 1996), which covers only one end of the particle.

In Morocco CTV has been detected and infected trees have been eradicated on several occasions. Some of the isolates were biologically and serologically characterised (Zemzami *et al.*, 1999). A comparison between the biological data and hybridisation patterns obtained with a panel of strain-differentiating probes has been reported for some of these isolates (Zemzami *et al.,* 2002). In this work we report on the further characterisation of CTV by SSCP analysis and on the sequencing of the major coat protein gene of additional isolates introduced to Morocco a long time ago, and of others collected recently at the Moroccan border.

Materials and methods

Virus isolates

The CTV isolates P2 and R1 were intercepted by the Moroccan quarantine system as germplasm coming from Spain, in 1998 and 2000 respectively. Virus isolate P_1 was detected in Lemon Meyer in a field near Marrakesh in 1983. Isolates were propagated by graft transmission to Mexican Lime (*Citrus aurantifolia*) and maintained in an insect proof greenhouse at 21–33°C. Additional biological characterisation of these isolates was done on grapefruit, sweet orange, and mandarin grafted on sour orange. Isolate MZ3 was obtained from Troyer citrange and corresponded to isolate number 3 previously characterised (Zemzami *et al.,* 1999, 2002)

PCR amplification of the coat protein gene

Fresh bark and leaf tissue (0.2 g) were used as the source of the virus. Amplification of the coat protein gene was done by immunocapture reverse transcriptional polymerase chain reaction (IC/RT-PCR), using a one-step combined reverse transcription and amplification based on methods previously described (Nolasco *et al.*, 1993). Polyclonal antibodies against an *E. coli* expressed CP gene (Sequeira and Nolasco, 2002) were used to trap the virions. The whole CP gene and the stop codon were amplified with primers CTV1 (5'-ATGGACGACGAAACAAAGAA-3') and CTV10 (5'-ATCAACGTGTGTTGAATTTCC-3'). The PCR-products were separated by migration on 1% agarose gel electrophoresis, and visualized under UV light after staining with ethidium bromide.

SSCP analysis of IC/RT-PCR or PCR product

SSCP analysis was performed directly on the IC/RT-PCR products (from plant tissue) or on the PCR products (from *E. coli* colonies), following confirmation of the specificity of the amplified product. The amplified product (1 µl) was added to 9 µl of denaturing buffer (950 µl of 95% formamide, 40 µl of 500 mM EDTA, [pH 8], and a minimal amount of bromophenol blue), the sample was then heated to 90°C for 5 min and quickly transferred to ice. Denatured products were electrophoresed in the refrigerator (4°C) for 3 h at 200 V in a native 8% polyacrylamide gel (7×8 cm, 0.75 mm, Bio-Rad Mini-Protean II), using TBE as buffer. The gels were stained with silver nitrate using the procedure of Beidler *et al*. (1982) and dried.

Cloning and sequencing

The amplified RT-PCR product was TA-ligated into pGEMT-Easy vector (Promega, Madison, WI, USA), according to manufacturer's instructions, transformed into competent *E. coli* INVaF' cells (Invitrogen, Carlsbad, CA, USA) and plated according to standard procedures (Sambrook *et al*., 1989). Transformed colonies harbouring the recombinant plasmid were selected by α-complementation on the plates supplied with X-gal and ampicilin (Sambrook *et al*., 1989). The occurrence of the coat protein gene insert was confirmed by direct PCR of a portion of selected white colonies, using the same set of primers as above. This PCR product was in some instances analyzed by SSCP.

Selected colonies harbouring the CP gene insert were transferred to liquid medium and the respective plasmids purified using the Wizard™ plus miniprep DNA Purification System (Promega) according to manufacturer's instructions. An additional confirmation of the insert was done by restriction digestion of an aliquot of the plasmid with *Eco* RI. Sequencing reactions were done using the forward and reverse universal primers by Macrogen (Seoul, Korea).

Sequence analysis

Sequence analysis, alignment and clustering were done using the software packages Bioedit (Hall, 1999) and Mega 2 (Kumar *et al.,* 2001). In addition to the sequences obtained in this work, the following sequences of the CP gene were retrieved from the GenBank and used in the comparisons: AF184118 (haplotype 28C), AF184115 (haplotype 25-120), AF184114 (haplotype 19-121), AF184113 (haplotype 13C), M76485 (haplotype T36). The CP gene sequences from Florida isolates T30, T3 and Meyer Lemon FL-7 were kindly provided by C.L. Niblett. The sequence of haplotype CB3-104 from the Capão Bonito isolate from Brasil was reported in Targon *et al*. (2000).

Results and discussion

Fragments of approximately 672 bp corresponding to the expected size of the CP gene were obtained by IC/RT-PCR (results not shown). A preliminary assessment of the variability of the CP gene was done by SSCP analysis of the amplified fragments (Fig. 1). SSCP patterns consisted of two or three conspicuous bands in addition to a faster migrating band corresponding to the non-denatured or partially renatured PCR product. Some of the single-stranded bands were doublets that could be better observed in an amplified picture. Samples of Spanish origin depicted a very similar pattern, which differed from the Meyer lemon isolate P1. This indicated that SSCP of the whole CP gene might be useful in an initial discrimination of CTV isolates without the need for smaller fragments as suggested by Rubio *et al*. (1996), which would have brought lengths closer to a putative optimum of 200 bp (Sheffield *et al*., 1993).

The IC/RT-PCR products were cloned and a number of the transformed *E. coli* colonies were analyzed by PCR using the same pair of primers. Most of these PCR products were of the expected size for the specific amplicon (672 bp) and were analysed by SSCP (Fig. 2). Only two clones were obtained from isolate MZ3; these were not analysed

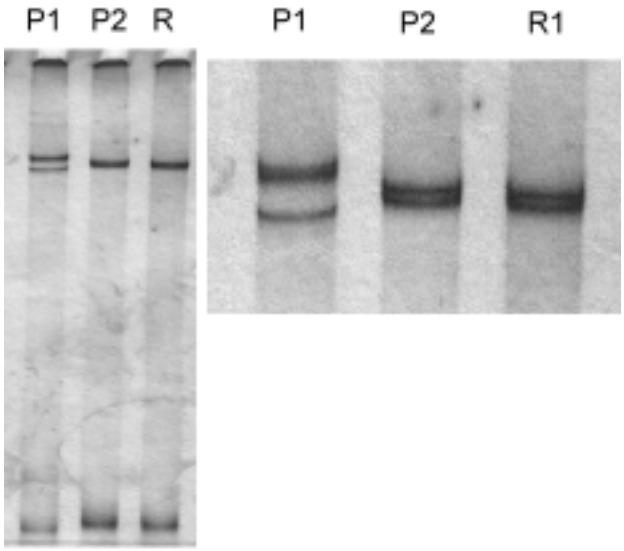

Fig. 1. Single-stranded conformation patterns of IC/RT-PCR fragments containing the coat protein gene. Bottom bands on the left side panel correspond to ds-DNA. Upper bands correspond to ss-DNA. The picture on the right is a magnification of the ss-DNA region.

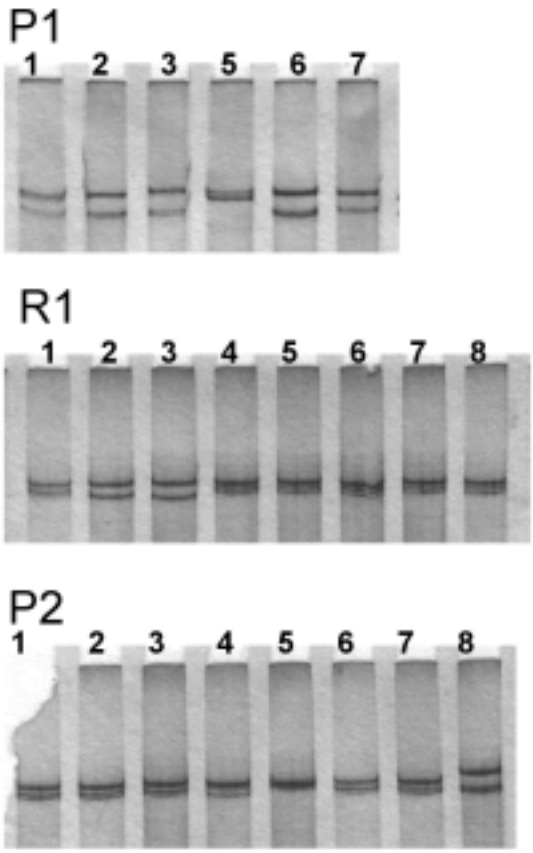

Fig. 2. Single-stranded conformation patterns of the cloned coat protein gene from isolates P1, P2 and R1. The lane number indicates the haplotype.

by SSCP. Six clones were analysed from isolate P1 and eight each from isolates P2 and R1. Two, 2 and 3 SSCP patterns were obtained from P1, P2 and R1 respectively; this polymorphism is typical for a viral *quasi-species*. As in Kong *et al*. (2000), we found a predominant pattern which occurred in 83% of clones from P1 and 75% of clones with a Spanish origin. If each SSCP pattern is assumed to correspond to a genomic variant (haplotype), the distribution of the variants is characterised by heterozygocity (Nei and Kumar, 2000). Heterozygocity may take values ranging from 0, if there is only one haplotype, to 1, if all the haplotypes are different. The heterozygocity of P1 was 0.33, of R1, 0.43, and of P2, 0.46. The Spanish isolates appear related in their composition of variants since their major SSCP pattern was very similar.

The cloned IC/RT-PCR products giving the most representative SSCP patterns of each isolate were sequenced and the portions corresponding to the primers were excised to eliminate any bias introduced by primer sequence. The sequences were aligned and compared with other available accessions of diverse origin (Fig. 3), representative of the seven group types reported by Zemzami *et al*. (2002). The overall genetic diversity of these sequences (mean nucleotide distance between two random selected variants) was 0.072 (SD 0.006). This value increased to 0.081 (SD 0.007) when the variants sequenced in this work were excluded. As these new sequences did not contribute to increase overall diversity, it was concluded that they represented true-to-type CTV CP gene variants.

The CP gene variants clustered very clearly, as inferred from the high bootstrap values, in four distinct groups. The haplotypes of Spanish origin

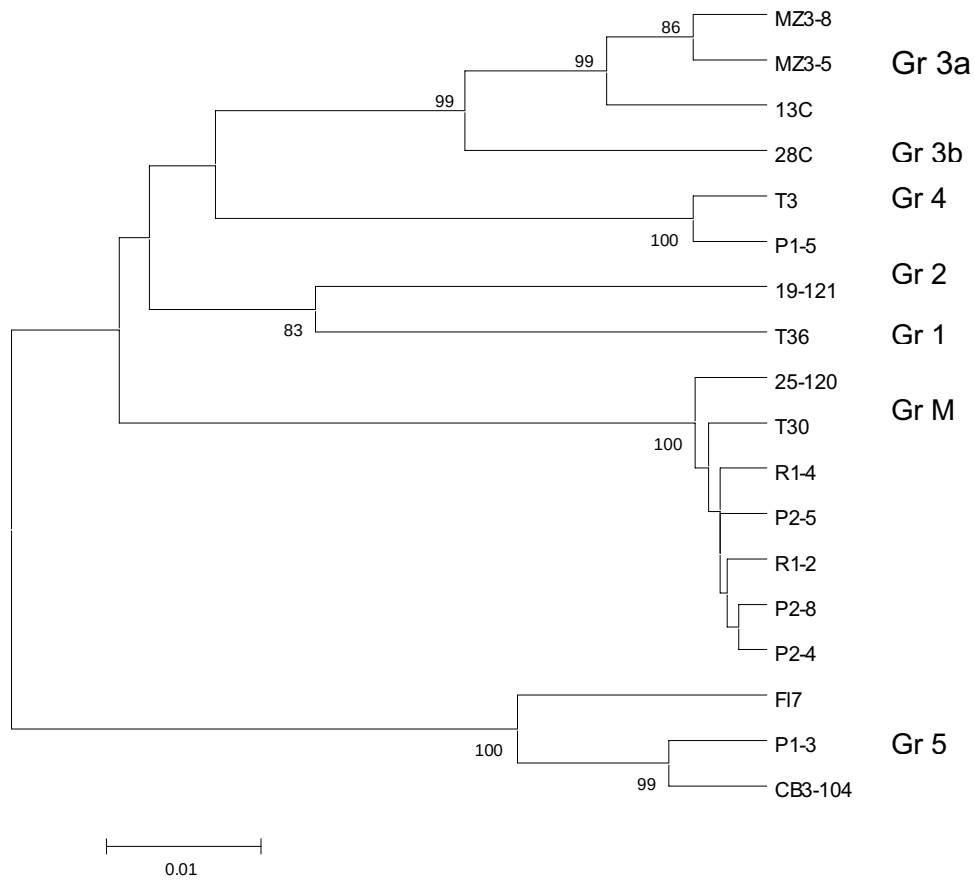

Fig. 3. Dendrogram showing the clustering pattern of the coat protein gene sequences as obtained by UPGMA. Groups mentioned in Zemzami *et al*. (2002) are shown on the right.

differed by less than 1% at nucleotide level from the well characterised isolate T30 (Garnsey *et al*., 1987) originating in Florida, or from isolate 25-120 which, although characterised in Portugal (Bonacalza, 1998), was detected in certified budwood imported from Spain in 1996. These isolates were representative of Group M. Isolates T30 and 25-120 both produced mild vein clearing and mild or no stem-pitting when indexed in Mexican lime; they did not cause stem-pitting on sweet orange or grapefruit, nor induce decline on sweet orange grafted on sour orange. This behaviour coincides with the biological data obtained in this work on isolates P2 and R1.

On the other hand, the predominant genomic variant of isolate P1 (clone P1-3) clustered at less than 4% nucleotide distance to sequences obtained from the severe isolates Capão Bonito from Brasil and Fl-7 (Group 5). It is very interesting that isolate Fl7 was found on Meyer lemon-infected plants from Florida (Cevik *et al*., 1996). The minor variant P1-5 clustered close to the severe isolate T3 (Group 4), also from Florida. The Meyer lemon isolates from Florida produced severe vein clearing and stem pitting on Mexican lime, decline of sweet orange grafted on sour orange, and stem-pitting on grapefruit; all of which are symptoms consistent with the biological properties so far determined for isolate P1. The introduction of the Meyer lemon isolate in Morocco can be traced back to the first half of the 20th century and confirmation that it caused CTV infection came in 1963 (Cassin, 1963). However, twenty years later, infected Meyer lemon trees could still be found in the field, as it was in this study. Isolate P1 was very similar to the isolates from Florida and California, which have a low transmission rate to other plants (Grant and Higgins, 1957), and this may explain why the severe isolate P1 remained hidden for so long.

The two clones from isolate MZ3 clustered in group 3a, with a severe sweet orange stem pitting isolate from Madeira island (Group 3a).

In a previous work (Zemzami *et al.,* 2002) molecular data on the CP gene showed that severe strains occurred in material from old budwood collections in Morocco. These findings strongly suggest the need for continuous monitoring of severe strains of CTV before symptoms appear in the field.

This work also illustrates the importance of the transport of infected budwood as causing a continuous flow of exotic CTV strains to new areas. For a certain period, the newly introduced strains appeared to maintain their original biological and molecular properties, while they remained hidden. During this period the CP gene could be characterised to trace their origins. Changes in cultural practices or in the vector population may induce changes in the spread and mixture of existing strains, leading to disease outbreaks.

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Accepted for publication: June 29, 2004