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Typing of Egyptian *Citrus tristeza virus* (CTV) isolates based on the capsid protein gene

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Summary. The capsid protein gene of three Egyptian CTV isolates from two locations was amplified by immunocapture RT-PCR and analysed by single stranded conformation polymorphism and sequencing. The CTV isolates studied did not differ significantly in sequence composition and each isolate consisted of very similar haplotypes. Comparison with reference sequences from isolates elsewhere in the world showed that these haplotypes clustered very close to the severe strain T3 from Florida causing quick decline and stem pitting. Analysis of the deduced amino acid sequence showed the epitope characteristic of reactivity with the MCA13 antibody. Sequence comparison with the sequence of an Egyptian isolate (Qaha) available in the Genbank showed a distance of about 8%, suggesting that it had a different origin.

Key words: capsid protein gene, MCA13.

Introduction

Citrus tristeza virus (family: *Closteroviridae*, genus: *closterovirus*, CTV) is one of the most important citrus pathogens worldwide. The virus is phloem-limited and the virions are thread-like filamentous particles, about 2000×11 nm in size (Bar-Joseph *et al.,* 1989). The CTV genome is a single stranded, positive-sense RNA molecule of 19226–19296 nucleotides organized in 12 open reading frames encoding at least 19 proteins (Pappu *et al.*, 1994; Karasev *et al.*, 1995). The virus is encapsidated by at least two proteins, a major capsid protein (CP) with a molecular weight close to 25 kDa which covers most of the particle length, and a diverged copy of this protein, with a molecular weight around 27 kDa and covering only one extremity (Febres *et al.*, 1996).

CTV isolates differ widely in their biological characteristics, particularly in the symptoms they produce in various citrus species or cultivars (Roistacher and Moreno, 1991). Syndromes having different economic consequences can be produced ranging from barely noticeable symptoms to those causing the quick decline of scions grafted on sour orange rootstock, or stem pitting of the branches with poor fruit quality, regardless of scion or rootstock. A number of efforts have been made to develop typing methods for the virus, most of which target the CP or the CP gene (Niblett *et al*., 2000). The capacity of ELISA to distinguish between CTV

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strains is limited to discriminating between mild and quick decline-inducing isolates from Florida based on the use of the MCA13 monoclonal antibody (Permar *et al*., 1990).

The virus is naturally transmitted by several aphid species of which *Toxoptera citricida* (Kirkaldy) and *Aphis gossypii* (Glover) are the most important (Rocha-Pena *et al*., 1995). *T. citricida* though the most efficient and important in virus transmission (Yokomi *et al*., 1994) is not present in the Mediterranean basin.

CTV is not yet a major cause of citrus disease in Egypt and the most efficient aphid vector, *T. citricida*, has not been reported in this country. However, the majority of Egyptian citrus plantations consist of sweet orange (*Citrus sinensis* Osbek) grafted on sour orange (*C. auratium* L.) rootstock, and because CTV and *Aphis gossypii* occur on both, CTV is a major threat to the Egyptian citrus industry.

One accession of the complete sequence of a CTV Egyptian isolate (Qaha) is available at the Genbank (AY340974) but there are no further details regarding its origin. The aim of the present work was to undertake the molecular characterization of some additional Egyptian CTV isolates by CP analysis. One CP gene sequence was deposited at the Genbank with the accession number DQ211658.

Materials and methods

Virus isolates

Three Egyptian CTV isolates were collected from citrus orchards at two locations 60 km apart, El-Kanater (K1 and K2) and Anshas (ANO). Isolates K1 and K2 were obtained from rough lemon trees (*C. jambhiri* Lush.) grafted on sour orange rootstock with typical CTV decline symptoms (decline, scion overgrowth, honey-combing) and maintained on Mexican lime (*C. aurantifolia* [Christm.] Swing.) plants in a greenhouse kept at 28°C. The reactions caused by these two isolates on Mexican lime included chlorotic flecks, vein clearing, leaf cupping and mild stem pitting. Isolate ANO was collected from Washington Navel sweet orange grafted on a sour orange rootstock field tree showing typical CTV decline symptoms but this isolate produced no noticeable symptoms (very slight stunting) on Mexican lime. Isolate ANO was maintained in 'Madam Vinous' sweet orange. In previous assays these isolates had reacted positively with polyclonal antibodies and with the monoclonal antibodies MCA13 and 3CA5 but negatively with the monoclonal antibody 3DF1.

Isolating, cloning and sequencing of the CP gene

The CP gene was isolated from the bark of infected twigs by the immunocapture reverse transcriptional polymerase chain reaction (IC/RT-PCR) (Nolasco *et al*., 1993). Amplified DNA fragments of the expected size (672 bp) covering the CP gene, the stop codon and one additional base were directly TA cloned into a linearized and thymidylated pTZ57R/T plasmid vector (Fermentas GmbH, St. Leon Rot, Germany). Details concerning the amplification of the coat protein gene, single stranded conformation polymorphism (SSCP) analysis, cloning and sequencing can be found in Lbida *et al*. (2004).

Results and discussion

IC/RT-PCR was used to amplify the CP gene from Egyptian CTV isolates. The amplified products showed a single DNA band of the expected size (672 bp).

SSCP analysis of the IC/RT-PCR products did not reveal differences in haplotype conformations among the three isolates (Fig. 1). The patterns obtained had a single conspicuous band suggesting the absence of mixtures of haplotypes. To avoid cloning similar sequences, only the amplified CP gene product of K1 and ANO isolates was ligated into the pTZ57R/T and cloned.

Analysis of colonies was done by PCR with the same pair of primers as that used in the initial amplification. Twenty-one colonies produced a DNA band corresponding to the correct size for an insert containing the whole CP gene. SSCP analysis was done on these amplified products prior to sequencing (Fig. 2). Low heterozygocity (a measure of intra-isolate variability) was evident since almost all the patterns obtained from the clones that came from the same isolate were similar.

Clones K1-7, K1-76 and ANO-1 were sequenced, revealing 672 base fragments as expected. The two terminal parts, 20 bases long and corresponding to the primers, were excised and a multiple alignment was done along with sequences previously

obtained, which have already been used as reference sequences in other studies (Zemzami *et al.*, 2002; Lbida *et al.*, 2004). The following sequences were used in the comparisons: AF184118 (haplotype 28C), AF184114 (haplotype 19-121), AF184113 (haplotype 13C), M76485 (haplotype T36). The CP gene sequences from the Florida isolates T30 and T3 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). The CP sequence part from accession No. AY340974 (Qaha, Egypt) was also included in the comparisons.

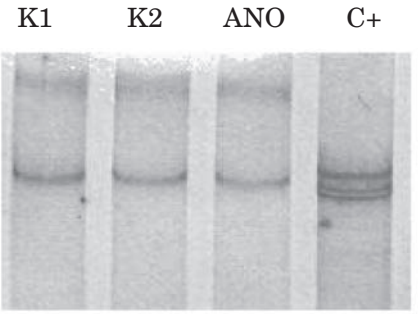

Fig. 1. SSCP patterns of IC/RT-PCR products of the Egyptian isolates (K1, K2 and ANO) and a positive control isolate (C+).

The three haplotypes sequenced were almost identical: K1-7 and K1-76 differed in only one nucleotide and ANO-1 differed from K1-7 and K1-76 in 2 and 3 nucleotides, respectively. This confirmed the low heterozygocity obtained by SSCP analysis and supported the conclusion that only 3 sequences were already enough to display the genomic variability of these isolates.

The amplified open reading frame of the three Egyptian isolates were 669 nucleotides long, and the deduced amino acid sequences, which were identical, were 223 amino acid residues long, which is in agreement with previously published CTV CP (Pappu *et al.,* 1993). The sequences were examined for the MCA13 epitope (Pappu *et al.,* 1993). All three sequences had a thymine (T) at position 371 in their nucleotide sequences and this determined the appearance of phenylalanine (F) at position 124 in their amino acid sequences, which was characteristic of the MCA13 epitope, explaining the previous positive results obtained with this antibody. Reactivity with the monoclonal antibody 3DF1 was mapped to a sequencial epitope around the amino acid at position 2 of the coat protein (Pappu *et al.*, 1995); unfortunately this region was covered by the forward primer CTV1 used, which made it impossible to find out why there was no reaction with 3DF1.

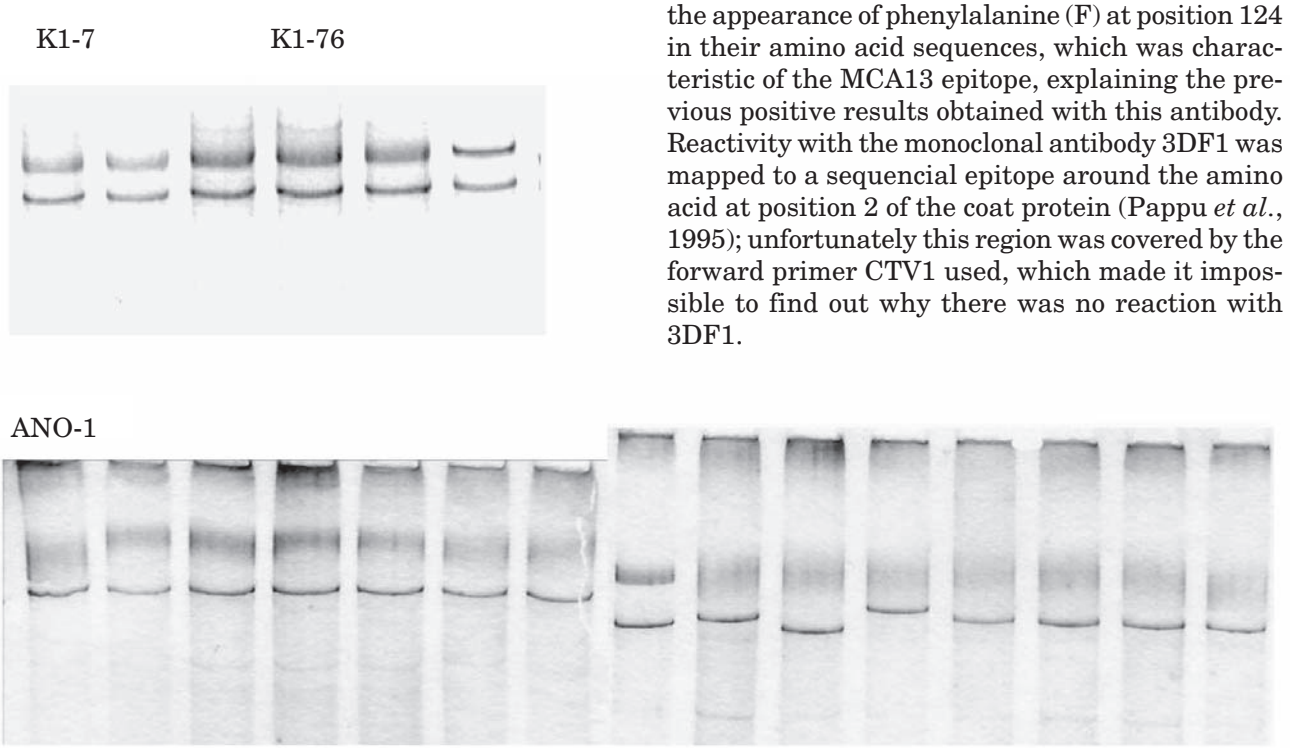

Fig. 2. SSCP patterns obtained from PCR products of the cloned CP gene of isolates K1 (top) and ANO (bottom). Labelled lanes indicate the haplotypes that were sequenced.

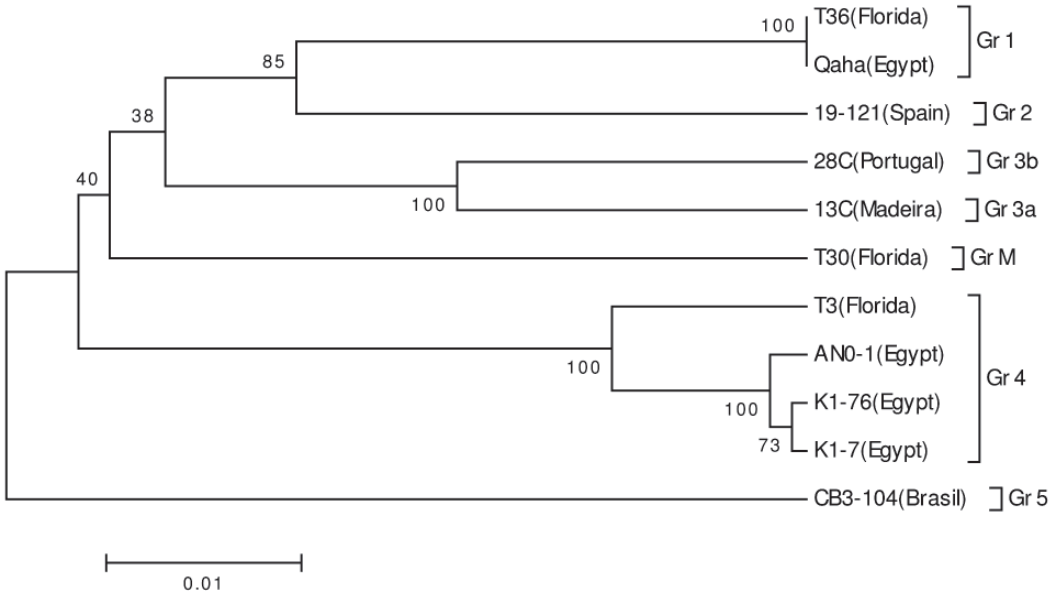

Fig. 3. Dendrogram showing the clustering pattern of the CP gene sequences of Egyptian and worldwide reference isolates as obtained by UPGMA. Numbers close to the branches represent the bootstrap values. The horizontal bar represents the nucleotide distance scale.

The matrix of nucleotide distances (single p-distance) between haplotypes was used to construct the dendrogram shown in Figure 3. The three haplotypes sequenced appear close to the sequence of the reference haplotype T3, which was obtained from an isolate from Florida causing severe quick decline and stem-pitting in sweet orange (Halbert *et al.*, 2004). This is the reference haplotype for the cluster named Gr 4 after Zemzami *et al.* (2002). Haplotypes of this group react with the hybridisation probe IV presented by Halbert *et al.* (2004), as deduced from sequence analysis. As shown in the dendrogram, the CP gene of the Qaha haplotype was far from the Egyptian isolates sequenced in this study (mean distance 0.08). The Qaha haplotype sequence appeared close (identical) to isolate T36 from Florida causing severe quick decline. Judging from the sequence data, the occurrence of CTV in Egypt appeared to be the result of two independent introductions.

The sequencing results and the presence of the MCA13 epitope in the CP suggest that isolates causing severe quick decline are present in Egypt. This appears to be in contradiction with the somewhat attenuated symptoms obtained during the biological indexing of Mexican Lime plants. This was attributed to the higher than optimal greenhouse temperature at which the biological indexing was performed.

Tristeza syndrome epidemics have not yet been reported in this country. Experience in other countries which do not have *T. citricida* as a vector indicates that several years usually pass before natural transmission with other aphid vectors occurs. This delay could be long enough to set up an eradication programme. However, if *T. citricida*, which was recently found in Portugal, should start spreading throughout the Mediterranean region, the CTV eradication effort should be significantly stepped up.

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