

SHORT COMMUNICATION

PRESENCE OF *CITRUS TRISTEZA VIRUS* IN ANGOLA AND SÃO TOMÉ E PRÍNCIPE: CHARACTERIZATION OF ISOLATES BASED ON COAT PROTEIN GENE ANALYSIS

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SUMMARY

A first report is given of the presence of *Citrus tristeza virus* (CTV) in Angola and São Tomé e Príncipe. Two out of twenty citrus samples from São Tomé e Príncipe and all of the seven samples from Angola were shown by ELISA to contain CTV. The capsid protein gene variants obtained by IC/RT-PCR were characterized by SSCP analysis to test for the presence of different haplotypes in each isolate and nucleotide sequence analysis of each variant leading to a different SSCP pattern, followed by *in silico* comparison with previously characterized CTV groups. The results confirmed that each isolate could contain different variants, which clustered with the different groups. Variants obtained from each country fitted into the same three clusters.

Key words: Citrus, CTV diversity, SSCP, IC/RT-PCR.

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* (family *Closteroviridae*), is the causal agent of serious diseases of citrus. CTV has a single-stranded, positive sense RNA molecule of about 19,296 nucleotides encapsidated in flexuous filamentous particles about 2000 nm long (Bar-Joseph *et al.*, 1989) with two capsid proteins, a major 25 kDa capsid protein (CP) covering about 95% of the particle length and a minor 27 kDa capsid protein that covers only one extremity (Febres *et al.*, 1996).

Several CTV isolates have been described that differ in their biological characteristics, particularly in the symptoms elicited in various citrus hosts (Roistacher and Moreno, 1991). Some strains are responsible for quick decline of trees grafted onto sour orange while others may also induce stem pitting of the branches of sweet orange or grapefruit. These are the most economically damaging strains. Other strains that cause symptoms only on Mexican lime indicator plants are referred to as mild strains. The virus is phloem-limited and is

transmitted in nature in a semi-persistent manner by several aphid species, mainly *Aphis gossypii* and *Toxoptera citricida*. CTV is present in most citrus-growing areas. The presence of CTV and its vector *T. citricida* has also been reported from several African countries, in particular in central and southern regions (reviewed by Michaud, 1998). These findings indicate problems for flourishing citrus industries like that in South Africa and potentially serious problems for developing countries that intend to establish a stable citrus production.

Little is known about the sanitary status of citrus in Angola in spite of its recognized potential for the development of a citrus industry. Although citrus orchards have been traditionally based on seedling trees, expansion and establishment of new orchards in Angola in the past five years have resulted in new production techniques and the use of grafted plant material from Brazil and South Africa. In São Tomé e Príncipe, interest in assessing the presence of CTV is mostly related to its historical links with Angola, the extent of exchange of people and fresh products and the consequent risks of diseases spreading. The aim of the present study was to assess the possible presence of CTV in Angola and São Tomé e Príncipe and obtain CP sequences of different isolates, in order to estimate their relatedness to other isolates found worldwide and thus better estimate the geographical dispersion of CTV strains.

Young twigs were collected in São Tomé e Príncipe from 20 trees in different places and from different citrus varieties. In Angola, comparable samples were from old orange trees of unknown varieties in an orchard at Sumbe, in the province of Kwanza Sul. The plant material was tested by ELISA following standard methods, using anti-CTV polyclonal antibody SP7 for coating (Sequeira and Nolasco, 2002) and a commercial anti-CTV alkaline phosphatase conjugate (Bioreba AG, Reinach, Germany, ref. 151522).

Seven trees from Angola were found to be infected. For São Tomé e Príncipe, one clementine tree of the local variety São Jacinto collected at Mesquita and one orange tree of an undetermined local variety collected at Mesquita, were found to be infected. These positive samples were selected for further molecular characterization.

The coat protein (CP) gene was amplified by im-

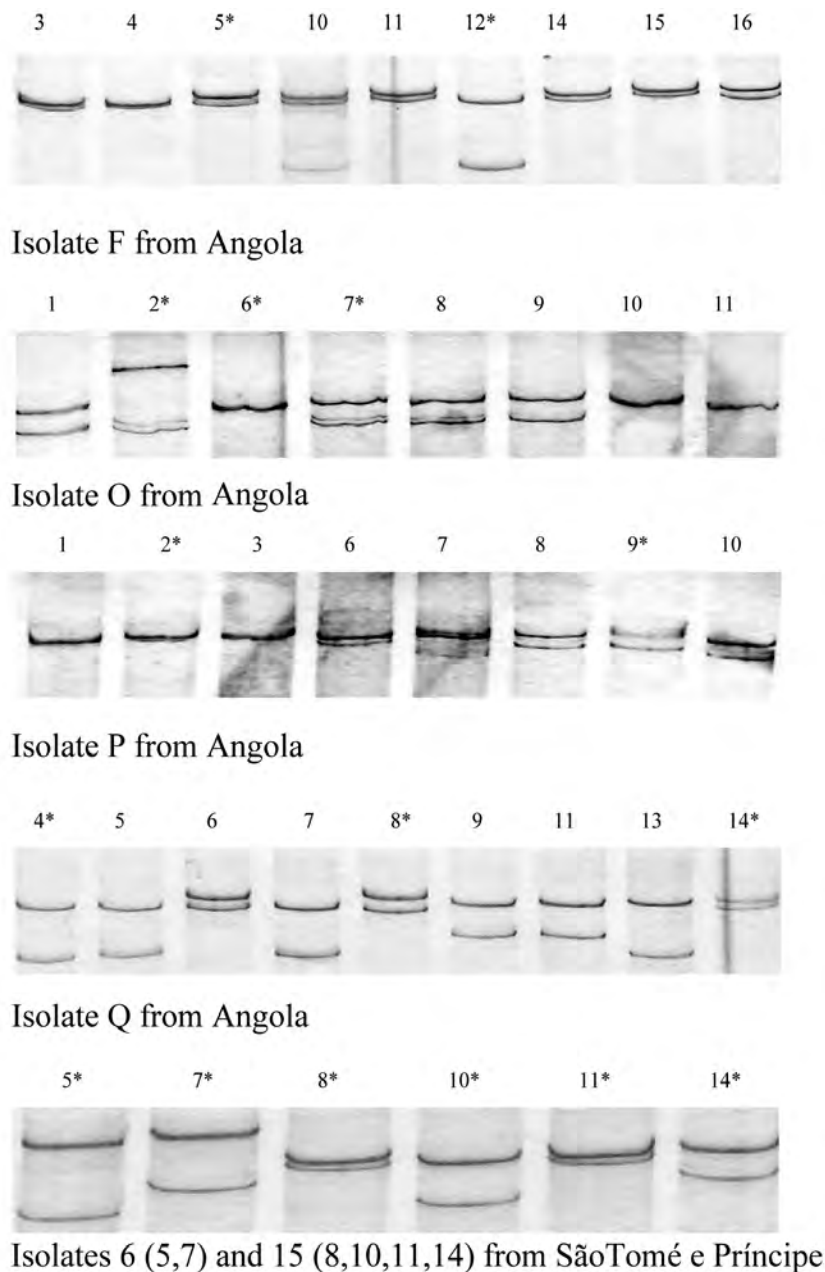


Fig. 1. SSCP patterns obtained from PCR products from the cloned CP gene of isolates F, O, P, Q from Angola and isolates 6 and 15 from São Tomé e Príncipe. Lanes marked with * indicate sequenced haplotypes.

munocapture-reverse transcription-polymerase chain reaction (IC/RT-PCR) according to Nolasco *et al.* (1993) using tubes coated with anti-CTV polyclonal antibody SP7 and primers CTV1 and CTV10 (Sequeira and Nolasco, 2002) that amplified the sequence of the whole CP gene and an additional nucleotide. A single DNA product of the expected size (673 bp) was obtained from all the infected samples. The amplified RT-PCR products were ligated into pGEM-T Easy vector (Promega, Madison, WI, USA), according to the manufacturer's instructions and transformed into competent *E. coli* INV α F' cells (Invitrogen, Carlsbad, CA, USA).

The occurrence of the CP gene insert was confirmed by PCR amplification using the primers described above.

SSCP analysis was done on the PCR amplification products obtained from the selected *E. coli* colonies as described by Rubio *et al.* (2001). Nine clones each were analysed from isolates F and Q and eight from isolates P and O from Angola. Four and two clones from isolates 15 and 6 respectively from São Tomé e Príncipe were also analysed (Fig. 1). Assuming that each SSCP pattern corresponds to a genomic variant (haplotype), SSCP results confirmed that each isolate contained more than one variant.

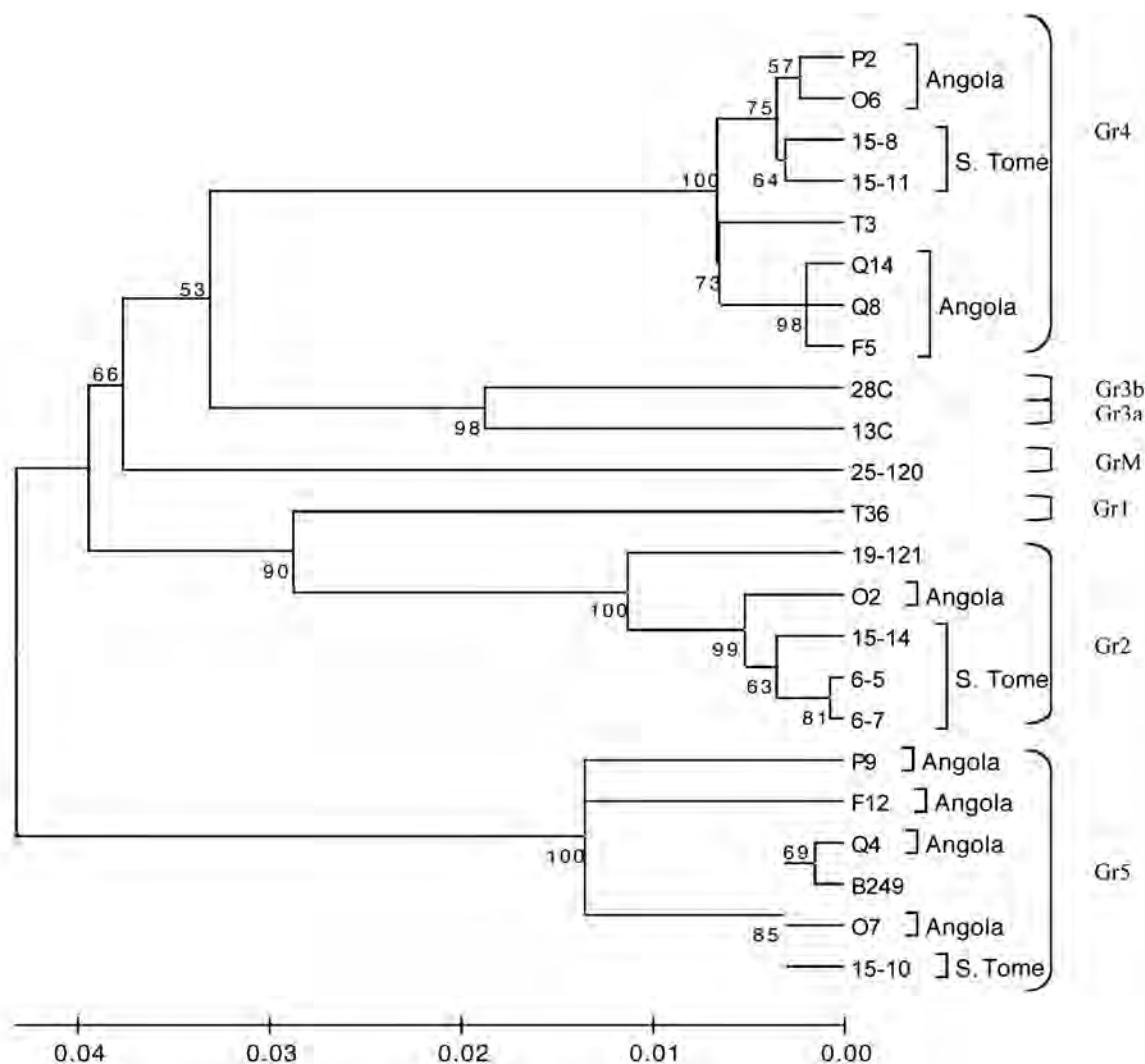


Fig. 2. Dendrogram showing the genetic relationships among the coat protein genes of CTV from Angola, São Tomé e Príncipe and worldwide reference isolates, based on nucleotide sequence distances. Numbers close to branches indicate bootstrap values. Horizontal scale represents the genetic distance (single p-distance).

Minipreps were obtained from colonies corresponding to each clearly different SSCP pattern found for each isolate and sequenced (Macrogen Inc., Korea). The 16 sequences obtained were submitted to the GenBank under the accession numbers DQ660340 to DQ660355.

Using Clustal W software (Thompson *et al.*, 1994), a multiple alignment was done along with the sequences of reference haplotypes representative of the seven group types reported by Zemzami *et al.* (2002): 13C (AF184113), 19-121 (AF184114), 25-120 (AF184115), 28C (AF184118), T36 (M76485). Sequences of B249 and T3 haplotypes were kindly provided by Dr. C.L. Niblett. The sequence zone corresponding to the primers was excised and the pairwise distances (single p-distance) between haplotypes were calculated and used to construct a dendrogram by using the neighbour-joining algorithm implemented in the Mega 3.1 software

package (Kumar *et al.*, 2004). In the dendrogram generated, sequences from Angola and São Tomé e Príncipe fell into three clusters (Fig. 2): Group 4, (reference haplotype T3), Group 2 (reference haplotype 19-121) and Group 5 (reference haplotype B249). None of the sequences determined in this work clustered in the other groups.

Worldwide, isolates harbouring haplotypes that cluster in Group 5 can be found in South America, like haplotype B249 from Venezuela or haplotype Capão Bonito from Brazil (Targon *et al.*, 2000), or associated with some Meyer Lemon lines that were distributed in Florida and the Mediterranean Region in the first half of the 20th Century (Lbida *et al.*, 2004).

Additional haplotypes that cluster in Group 4 can be found in isolates from Egypt (Amin *et al.*, 2006), Croatia (Cerni *et al.*, 2005) or Morocco (Lbida *et al.*, 2004). *In silico* analysis shows that, except for haplotype P9,

the haplotypes that clustered in Groups 2, 4 and 5 have a perfect match respectively to probes II, IV and V (presented in the United States patent 6,140,046), which were used by Halbert *et al.* (2004) and by Herron *et al.*, (2005) to characterize CTV isolates from Florida and Texas. The sequence of isolate P9 has one mismatch to probe V and its position in the dendrogram suggests that it is an outlier.

In conclusion, the molecular data presented here show that isolates obtained from Angola and São Tomé e Príncipe harbour mixtures of haplotypes which are closely related to haplotypes obtained from other areas of the world. Haplotypes from Angola and São Tomé e Príncipe fall into the same clusters. In view of the shared history of these countries, the results suggest a strong influence of human activity on the dissemination of CTV.

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