ISBN 978-85-8084-603-4

CITRUS TRISTEZA VIRUS: STUDY OF THE SEQUENCE OF THREE GENES FROM SELECTED ISOLATES

Carlos Alexandre Zanutto[1], Larissa Siqueira Soares[2], Aline Maria Orbolato Gonçalves-Zuliani[3], Paula Thaís Requena Nocchi[2], Diego Henrique Pereira Catani[2], William Mário de Carvalho Nunes[4]

ABSTRACT: *Citrus tristeza virus* (CTV) is the causal agent of devastating epidemics that changed the course of the citrus industry. CTV are composed of two capsid proteins and a single-stranded, positive sense genomic RNA of 19,3 kb, containing 12 open reading frames. Beyond being required in the correct assembly of the viral particle, the encoding genes of proteins p25 and p27, are also required for the movement, in the suppression of post-transcriptional gene silencing, phenomenon in which p23 also is involved as well as in asymmetrical accumulation of positive and negative strands during RNA replication. To advance itself in the knowledge on CTV isolates present in the Paraná State, p23, p25 and p27 genes of two CTV isolates were considered to determine the sequences of these three genes. The mild Isolate pointed as promising with respect to one program of preimmunization selected in the Paraná State, and the severe isolate called Rolândia, that has caused severe symptoms in many orchards in the North of the State, composes the two isolates of CTV. These isolates were submitted to the cloning of the product of PCR (Polimerase Chain reaction) amplification of the genes in question and had its sequences of the genes p23, p25 and p27 of each haplotype present in each one of the isolates determined. The nucleotide sequence data obtained in this work, were deposited in the data base GenBank and the access numbers are available. The sequences were aligned and compared, genetic distance between haplotypes used for the phylogenetic analysis.

KEYWORDS: cloning; coding genes; cross-protection; preimmunization program; sequencing.

1. INTRODUCTION

Citrus tristeza virus (CTV) is the causal agent of viral disease responsible for the most dramatic effects which the global citrus industry has suffered. Its importance, even today, is huge, since it was responsible for the elimination of approximately 100 million plants in the world that were destroyed or became unproductive (MORENO et al., 2008).

CTV is not a single virus, but a complex of virus; in other words, the virus is composed of two or more haplotypes which are different genetically, but related immunologically (POWELL, 1992), it may or not develop symptoms depending of the varieties and rootstock of citrus.

In this research we aimed a further study of two CTV isolates which had the nucleotide sequences of three genes (coding for the proteins p23, p25 and p27) determined and compared among themselves and with other known isolates and available on sequences databases (GenBank).

[1] Eng. Agrônomo, Doutor, Universidade Estadual de Maringá-UEM, Maringá – PR, cazanutto@uem.br .
[2] Mestrandos Pós Graduação em Agronomia da Universidade Estadual de Maringá, Maringá-PR, paizinhos_larissa@hotmail.com .
[3] Doutoranda Pós Graduação em Agronomia da Universidade Estadual de Maringá, Maringá-PR, alineorb@hotmail.com .
[4] Orientador, Prof. Dr. Pós Graduação em Agronomia da Universidade Estadual de Maringá, Maringá-PR, ,Núcleo de Pesquisa em Biotecnologia Aplicada, Av. Colombo, 5790, CEP 87020-900, Maringá, PR, william.nunes@pq.cnpq.br

ISBN 978-85-8084-603-4

2. MATERIAL AND METHODS

The research was conducted at the Universidade Estadual de Maringá (UEM) Paraná State, Brazil. Both CTV isolates which were studied are: a) The mild protective isolate that showed better results about its behavior upon challenge with the Rolândia severe isolated in a preimmunization program, both by SSCP analysis and by evaluation of the performance and symptoms of infection by CTV, the protective isolate named "CS-1", and b) The severe isolate Rolândia, which has been described for causing severe symptoms of Tristeza in northern Paraná State (CARRARO et al., 2003; CORAZZA et al., 2012; ZANUTTO et al., 2013).

The mild protective isolate of CTV was selected in field from elite plants, as described by Zanutto et al. (2013). It was subjected to protection tests, which was challenged by severe isolate Rolândia through inoculations by tissue union (graft) or with the help of the vector Brown Citrus Aphid (*Toxoptera citricida* Kirk), assessed by SSCP analysis and the infection symptomatology of CTV, having been shown to be the best isolate among the evaluated, been therefore, suitable to be used in programs for preimmunization against CTV severe isolates (ZANUTTO et al., 2013). The severe isolate Rolândia, collected from plants with severe symptoms of Tristeza, was inoculated and monitored in plants of 'Pêra' Orange and kept in a greenhouse with aphid proof screen.

For sequencing of three encoding genes of the proteins p23, p25 and p27, were collected leaves and branches from infected plants with the respective isolate, after being washed and identified, were removed the new shoots peels and the leaves main veins which were used for extraction of virus RNA by extraction cycles using the TRIZOL reagent. This reagent performs the RNA total extraction. The extraction procedure was conducted according to the manufacturer's instructions (Invitrogen) to TRIZOL Reagent.

The viral RNA present among the total RNA served as a template for the synthesis of complementary strand of DNA of the virus (cDNA) according to the procedure described by Sambrook et al. (1989). The synthesis was performed in a thermocycler apparatus at a temperature of 37˚C for a period of two hours. The genes were isolated and amplified by polymerase chain reaction (PCR - Polymerase Chain Reaction) using specific primers.

The amplification was performed using a thermocycler appropriately programmed for 40 cycles. The PCR product was visualized on agarose gel at 1% according to the procedures described by Beidler et al. (1982). The PCR amplified products of the respective genes were ligated to plasmid and the ligation mix was used to transform competent cells of *Escherichia coli* DH5α. For this purpose, were used the kit for cloning and sequencing TOPO TA Cloning Kit and T1 One Shot Chemically Competent Cell, which were used according to manufacturer's instructions (Invitrogen).

The products obtained of PCR amplification from the genes were subjected to nucleotide sequencing, using a sequencing platform by Applied Biosystems ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 for automated DNA sequencer *ABI Prism* 3100 genetic analyzer. Sequences were aligned and compared using CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and then they were submitted to GenBank [National Center for Biotechnology Information - NCBI] (http://www.ncbi.nlm.nih.gov/) to verify the genetic similarity among the obtained sequences.

3. RESULTS AND DISCUSSION

The alignments of the nucleotide sequences from the three regions of CTV genome indicated that changes in nucleotide level were simple nucleotide exchanges. Were

obtained four different haplotypes (among 10 analyzed colonies) for the p23 protein gene, from Rolândia isolate, and each other had differences ranging from 82.5 to 97.5% of homology. The difference is considered too large due to the fact that these haplotypes are present in the same plant, compounding the severe isolate Rolândia.

Similarly, five different haplotypes were obtained of the p27 protein gene from Rolândia strain. These haplotypes showed still, minor differences among them, ranging from 95.1 to 98.3% of homology. As for the p25 protein gene, seven different haplotypes were obtained and the differences among these ranged from 92.3 to 99.2% of homology.

The isolate CS-1, likewise, had the nucleotide sequences of the three genome regions determined. However, we obtained only a haplotype of the coding gene of p23 protein, four haplotypes of p25 and three haplotypes of p27. This was probably a failed target sequence link to the plasmid or in the transformation of competent cells, where from the ten colonies transformed for each gene in this isolate, few processed colonies were recovered, preventing the full haplotypes scope contained in this isolate.

For the four different haplotypes obtained for the p25 protein gene from the strain CS-1 were observed homologies ranging from 96.2 to 99.3%. As for the p27 protein gene, were obtained only three different haplotypes for the isolate CS-1, and the differences among these ranged from 98.3 to 99.1% of homology.

The obtained nucleotide sequences were deposited on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) and access numbers are distinguished on Table 1.

Table 1. Accession Numbers of nucleotide sequences deposited in GenBank for the three genes from the two isolates.

		ISOLATES	
Genes	Haplotypes	Rolândia	CS-1
p23	1	KC202886	KC202908
	2	KC202887	--
	3	KC202888	--
	4	KC202889	--
p27	1	KC202890	KC202905
	2	KC202891	KC202906
	3	KC202892	KC202907
	4	KC202893	--
	5	KC202894	--
p25	1	KC202895	KC202902
	2	KC202896	KC202903
	3	KC202897	KC202904
	4	KC202898	--
	5	KC202899	--
	6	KC202900	--
	7	KC202901	--

4. CONCLUSION

This research evidenced that the severe isolate of CTV called 'Rolândia' which is causing severe losses in orchards of northern Paraná State, including leading technical assistance to not recommend planting the Pêra sweet orange in these regions due this isolate presence, showed a high homology (P23=98%, P25=98%, P27=99%) with the severe isolate Capão Bonito, that occurs in São Paulo State.

Nevertheless, the major observed homologies for this isolate were with isolates from Argentina, which were observed for up to 99% of homology. This may mean that the

severe isolated Rolândia that nowadays occurs in Paraná, originates from Argentina, since the high homology found.

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