Molecular Characterization of a *Citrus viroid III* (CVd-III) Associated with Citrus Dwarfing in Italy

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ABSTRACT. A small circular RNA associated with dwarfing of citrus trees was isolated from a Clementine on *Citrus macrophylla* rootstock (designated CMC) in Italy many years ago and studied by our research group in different rootstocks and conditions in order to evaluate its potential application in high-density plantings. Preliminary studies suggested a close relation of the CMC stunting-associated RNA with the *Citrus viroid III* (CVd-III) group, but its nucleotide sequence was never determined. In order to further characterize the nature of this RNA, two independent cloning experiments with two sets of primers designed in different regions of CVd-III, were carried out. The viroid source was a *Poncirus trifoliata* tree graft-inoculated 18 yr before with bark chips from the original CMC source. In accordance with results of preliminary studies, sequencing of nineteen full-length cDNA clones demonstrated that the CMC-associated RNA had the expected size of 294-295 nucleotides. Most of the sequences were identical to CVd-IIIb, whereas others showed limited mutations. New polymorphic positions, not described in previous studies on CVd-III variability, were detected.

The possibility of employing certain graft transmissible dwarfing agents to increase density planting and facilitate fruit collection in citrus crops was proposed several decades ago (2, 6). It is well known that several citrus viroids in single or mixed infections may cause citrus dwarfing without inducing specific symptoms (4), thus the potentiality of viroids as citrus dwarfing agents in high-density citrus orchards has been tested in several countries (4). However, extensive use of viroids in high density planting needs careful risk assessment, including evaluation of potential for genome mutation and induction of new diseases. In this contest a substantial contribution may be given by studies on the determination of viroid sequence variability. Among citrus viroids, those belonging to the *Citrus viroid III* (CVd-III) group are of particular interest as growth modulating factors (10). An extensive study on CVd-III variability has been recently carried out using several citrus viroid field isolates from Israel and Costa Rica (7).

In 1983, a dwarfing agent from a Clementine tree on *Citrus macrophylla* rootstock (denoted CMC) showing mild stunting, was found in Italy and studied as a potential inoculum to dwarf citrus plants. A single small circular RNA was isolated from the CMC source and from citrus plants graft-inoculated with bark chips from the CMC source (8). Although electrophoretic mobility, hybridization assays and RT-PCR amplifications suggested a close similarity between the CMC dwarfing associated RNA and CVd-III (1, 12), conclusive evidence of its nature was not obtained. To solve this question sequencing of full-length cDNAs clones of the CMC-associated small RNA was carried out.

A trifoliate orange tree graft-inoculated 18 yr ago with bark chips from the CMC source was used as RNA source. Nucleic acids were extracted as described (3). Taking into account the known sequence similarity between CMC dwarfing associated small RNA and CVd-III, the primer pair previously reported by Owens and coworkers in 2000 (7) and designed in the terminal right region of CVd-III was used in RT-PCR. Full-length cDNAs, amplified with Taq DNA polymerase (Roche Molecular Biochemicals), were cloned in pGEM-T vector and

sequenced. Sequencing of eight clones showed that CMC RNA population is composed by sequence variants with size ranging from 294 to 295 nt. The most prevalent variant, identified in five clones, was identical to the previously described CVd-IIIb (9). The other variants, found only once, showed limited modifications consisting of one nucleotide change and/or insertion with respect to the CVd-IIIb sequence. To explore sequence variability in the region covered by the first primer pair, the same cloning strategy was repeated using another set of primers designed in a different non-variable region of the viroid molecule, corresponding to the upper strand of the central conserved region (CCR). Also in this second cloning experiment the master sequence, recovered in five of the eleven sequenced clones, was identical to CVd-IIIb. The other variants, found only once, showed only limited point mutations (one change and/or insertion) with respect to the CVd-IIIb reference sequence. In total, sequencing of 19 clones allowed detection of nine previously unreported CVd-III sequence variants (Table 1), all having a rod-like conformation when the secondary structure of lowest free energy was calculated by the MFOLD program (13). No mutation was found in the central conserved and variable domains of the secondary structure, regions that were reported as most variable in other CVd-III isolates previously characterized (7, 9, 11, 10). With the CMC isolate, nucleotide mutations were concentrated in the terminal right and left domains of the rod-like structure.

An enzyme lacking proofreading activity (Taq DNA polymerase) has been used for the PCR amplifications carried out in the cloning protocol. Therefore, most of the observed nucleotide changes must be considered uncertain because they were detected only in a single clone. However, at least the variant showing the single substitution of a U with a C in the position 138 of the reference sequence exists in the CVd-III population from the CMC source, because the same polymorphic position was found in four different variants.

Our study conclusively identified the CMC dwarfing-associated small RNA as CVd-III quasi-species whose master sequence variant corresponds to the previously described CV-IIIb. To our knowledge, this is the first conclusive report about the presence of CVd-III in Italy. The results of the present investigation open to future studies on CVd-III stability under natural field conditions that could contribute to assess the risk of using citrus viroid as dwarfing agents.

TABLE 1
VARIANT MUTATIONS DETECTED IN COMPARISON WITH CVd-IIIb

Variants	No. clones	Detected mutants	
		Substitution	Insertion
H19-1 = CVd-IIIb	10	—	—
H19-2	1	$A_8 \rightarrow G$	$+A_{279}$
H19-3	1	$A_{59} \rightarrow C$	$+U_{60}$
H19-4	1		$+G_{13}$
H19-5	1	$G_{137} \rightarrow A$, $U_{138} \rightarrow C$	
H19-6	1	$U_{138} \rightarrow C$	
H19-7	1	$U_{23} \rightarrow C$, $U_{138} \rightarrow C$	
H19-8	1	$G_{284} \rightarrow A$	
H19-9	1	$U_{138} \rightarrow C$, $A_{154} \rightarrow C$, $U_{180} \rightarrow A$	
H19-10	1	$G_{132} \rightarrow A$	

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