Analysis of the Progeny of an Italian Isolate of *Citrus viroid IIIb*

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ABSTRACT. The use of viroids as tools to obtain tree dwarfing for high density citrus planting needs careful risk assessment, and is a process which should include evaluating dwarfing sources for the potential to generate viroid genome mutations and for the induction of new diseases. In order to study the stability of *Citrus viroid IIIb* (CVd-IIIb) populations in natural field conditions we have analyzed in two different host species the progeny of CVd-IIIb derived from the same original source. Viroid RNA populations from seven trifoliate orange and one Troyer citrange seedling, bark-inoculated in 1983 with the same Italian CVd-IIIb isolate were analyzed. Full-length double-stranded CVd-IIIb cDNAs were synthesized by RT-PCR using primer pairs designed from the upper strand of the central conserved region or the terminal right region. Analysis of about ten full-length cDNA clones obtained from each trifoliate orange tree showed similar RNA populations comprised of a predominant species of 294 nt which was identical to the CVd-IIIb variant previously reported. Similar results were obtained with the CVd-IIIb population derived from Troyer citrange. These results demonstrate the high level of sequence stability of CVd-IIIb in two different hosts grown for a long period in the field.

Citrus trees can harbour single or mixed infections of several viroids belonging to five different groups (1), of which only two are causal agents of important diseases. The use of some citrus viroids as graft transmissible dwarfing agents to increase the density of citrus orchards has been investigated in many citrus growing areas (3, 6) and has allowed the identification of viroids responsible for size reduction without any other detrimental symptoms. To describe this capability, the name Citrus dwarfing viroid (CDVd) was recently proposed for *Citrus viroid IIIb* (CVd-IIIb) (8). At present, specific studies on the genetic stability of this viroid relative to the potential for the induction of new diseases in infected plants grown for a long period in the field have not been done.

In order to characterize the genomic stability of CVd-IIIb populations in natural field conditions we have analyzed, in two different host species, the progeny of CMC-CVd-IIIb, a previously characterized Italian dwarfing agent originally isolated from a Clementine tree on alemow rootstock showing mild stunting (4, 7). Seven trifoliate orange and one Troyer citrange seedling, graft-inoculated about 20 yr before the start of this study with bark chips from a CMC-CVd-IIIb source, were used as viroid RNA sources. Nucleic acids were extracted as described (2), and reverse transcription-polymerase chain reaction (RT-PCR) amplification of viroid cDNA was performed as reported (7) using primer pairs designed from the central conserved region (CCR), which is known to be highly conserved in citrus viroid species.

Full-length cDNAs, amplified with *Taq* DNA polymerase (Promega), were cloned in pGEM-T vector and sequenced. Approximately 10 clones were sequenced for each isolate for a total of 74 clones. All clones were 294 nucleotides (nt) except for variants H6-2 (293 nt) and H17-2 (292 nt). In the viroid population from each plant the most prevalent variant, recovered in 53 out of 74 (73%) of sequenced clones, was identical to the previously

described CVd-IIIb sequence (5, 7). Fifteen previously unreported CVd-IIIb sequence variants were identified, showing limited modifications in one or few positions with respect to the described CVd-IIIb sequence. A total of 22 polymorphic positions distributed throughout the molecule were found in 21 clones, but the real sequence variability could still be lower considering that some of the detected mutations could have been be generated *in vitro* due to the lack of proofreading activity of the *Taq* DNA polymerase.

However, at least two of them, the single substitution of a U with a C at position 138 (10 clones) and substitution of an A with a C (three clones), can be considered real also because the same polymorphic positions were found in our previous study (7). All the progeny variants adopt a rod-like conformation when the secondary structure of lowest free energy is calculated by the MFOLD program (9). No mutations were observed in the region covered by the previous pair of primers when the cloning strategy was repeated with two isolates using a primer pair designed from the right terminal region (7).

Our results demonstrate that the viroid populations from the eight plants inoculated with the same CMC-CVd-IIIb source showed high sequence similarity, including common sequence variants, suggesting that this isolate has a high genetic stability in our experimental hosts. Indeed, no major differences were observed in the Troyer citrange population in comparison to those recovered from trifoliate orange plants, showing that no major selective pressure on viroid evolution was imposed by these host species under field conditions. The results of the present investigation represent the first step of a complex study to evaluate the genetic stability of the dwarfing agent CVd-IIIb, and these results will contribute to the risk assessment analysis necessary before using citrus viroids as dwarfing agents.

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