Interactions between Citrus Cachexia Viroid and Closely Related Sequence Variants May Impair Expression of Cachexia Symptoms

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Abstract

We report the sequences of four variants of *Hop stunt viroid* (HSVd) isolated from a naturally infected citrus tree of the variety 'Clementinier' in Tunisia. One of the variants (HSVd Tun/cl3) had the characteristic changes of the cachexia-inducing variant CVd-IIb, whereas the others were more similar to variant CV-IIa not inducing cachexia. However, when material from this tree was bioassayed in the cachexia indicator 'Parson's special', no symptoms were observed for two years after inoculation. This might result from the concurrent presence of non-cachexia inducing variants that could interfere with CVd-IIb. Our data also show the worldwide distribution of this viroid and the presence of mixture of sequence variants in natural infections.

INTRODUCTION

Xyloporosis/cachexia of citrus was considered for some time a virus disease but recent evidence suggests that it is caused by a viroid (Reanwarakron and Semancik, 1998). Although xyloporosis and cachexia were originally described in sweet lime and 'Orlando tangelo', respectively, both diseases are apparently identical. Cachexia is common in old-line trees and is a continuing problem where growers have top-worked these trees with sensitive cultivars such as mandarin hybrids.

Cachexia may remain symptomless in old-line citrus cultivars whereas it is particularly pronounced in some mandarins, tangelos and rootstocks. It causes stunting, chlorotic foliage and decline symptoms that can be similar to those caused by other agents (Semancik et al., 1988). The definitive symptoms are stem pitting and gum impregnation of the bark in sensitive cultivars. Pitting and gum development usually start near the bud union and may spread to the sensitive portion of the tree. Pegs on the bark face usually contain gum. Once these symptoms become well developed, a flat cut about halfway into the bark will reveal gum pockets of varying size. Symptoms usually take 18-48 months to develop in highly susceptible cultivars such as 'Orlando tangelo' and *Citrus macrophylla,* and longer in less sensitive cultivars. Indexing is usually done by inoculating an indicator side graft of 'Parson's Special' mandarin on a vigorous rootstock such as 'Rough lemon'. Cachexia can affect most tangelos, some mandarins, many mandarin hybrids, sweet lime and *Citrus macrophylla*. Infected rough lemon may also show mild symptoms. It is especially severe on *Citrus macrophylla* and 'Orlando tangelo'. Oranges, grapefruit, lemons, acid limes, some mandarins, trifoliate orange and its hybrids are considered as susceptible hosts even if they usually do not show obvious symptoms. The disease is transmitted through infected budwood by budding and pruning equipment.

Cachexia is caused by one variant of *Hop stunt viroid* (HSVd) called CVd-IIb. Other citrus variants of HSVd are CVd-IIa and CVd-IIc. Sequence identity between variants CVd-IIa and CVd-IIb is 95%, with the principal nucleotide differences being located in the variable domain of the viroid (Reanwarakron and Semancik, 1998).

In the present work we have cloned and sequenced HSVd variants from a single infected citrus tree. Sequence analysis showed that one has the characteristic changes of the cachexia inducing variant CV-IIb, whereas the others are more similar to the CVd-IIa variant. Because indexing of the source material did not promote any symptoms in the 'Parson's special' indicator two years after inoculation, this might be interpreted as a result of interferences between CV-IIb and the other HSVd variants.

MATERIALS AND METHODS

Plant Material

The vegetable material consisted on bark and leaves from a 4-years-old tree of the variety 'Clementinier' grafted on *Citrus volkamerina.* Bark was grafted on 'Parson's special' grown on *Citrus volkamerina*. Symptoms of the cachexia disease were not expressed by the indicator two years after inoculation.

RNA Extraction

Total leaf RNA was isolated by phenol extraction and partially purified by chromatography on non-ionic cellulose (Whatman, CF11). The resulting RNAs were fractionated by lithium chloride precipitation, quantified by UV spectroscopy and their quality assessed by electrophoresis in 1% agarose gel.

RT-PCR Amplification

Template RNAs were denatured at 95°C for 5 min and chilled on ice for 2 min. First-strand HSVd-cDNA was synthesized using the antisense primer VP19 (5'GCCCCGGGGCTCCTTTCTCAGGTAAG3') and avian myeloblastosis virus reverse transcriptase (AMV-RT) according to manufacturer's instructions (Roche). The resulting cDNA was then amplified by the polymerase chain reaction (PCR) using the same anti-sense primer coupled with the sense adjacent primer VP20 (5'CCCGGGGCAACTCTTCTCAGAATCC3') (Kofalvi et al., 1997). These primers correspond to the upper strand of the central conserved region (CCR) and its flanking sequence. The amplifications were performed with *Pwo* DNA polymerase (Roche) in a thermal cycler (Perkin-Elmer) according to the following program (30 cycles): 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C. PCR products were analyzed by electrophoresis on a 2.0% agarose gel.

cDNA Purification, Cloning and Sequencing

Full-size HSVd-cDNA was eluted from gel slices, precipitated with ethanol and resuspended. Using the *Taq* DNA polymerase, a 3' adenylate residue was then added to the 3' ends of the PCR-amplified products so that they could be ligated into the linearized pCR 2.1 vector with overhanging 3'deoxythymidilates (T) (TA cloning kit, Invitrogen). Recombinant clones were identified by restriction analysis. Four clones were manually sequenced in both directions by the dideoxynucleotide chain termination method using the M13 universal and reverse primers (T7 DNA sequencing kit, United State Biochemical).

Sequence Analysis

Sequence analysis was carried out using Clustal X program (Thompson et al., 1994). The RNA secondary structure was predicted with the aid of the mfold program (http://mfold2.wustl.edu/mfold/rna/form).

RESULTS AND DISCUSSION

Four full-length HSVd variants, ranging in size between 295 and 300 nt, were sequenced: HSVd tun/cl1, HSVd tun/cl2, HSV tun/cl3 and HSVd tun/cl4 (Fig. 1.), and deposited in Genbank with the accession numbers AY143167, AY143168, AY143169 and AY143170, respectively. These variants showed more than 90% identity with those reported previously. One (HSVd tun/cl3) had the characteristic changes of the cachexia-inducing variant CVd-IIb (Reanwarakorn and Semancik, 1999), while the others were more similar to the CVd-IIa variant. Differences between the four characterized variants, (including transitions, transversions, insertions, rearrangements and deletions), were observed at 25 positions, giving a total variability of 9.1% (Table 1.). The changes were clustered in the left terminal loop, the lower strand of the central and pathogenicity domains, and within both strands of the variable domain (Fig. 2), and had the potential to alter the rod-like conformation leading to different branched structures for each variant. When a more detailed comparison was made with natural HSVd variants deposited in databases, only the deletion of the two Cs at positions 295-296 in variants HSVd tun/cl3 and HSVd tun/cl4, was found to be previously unreported. Since these positions form part of the terminal conserved hairpin (TCH), which is known for its genetic stability, this deletion may be an artefact of the RT-PCR amplification.

The observed sequence heterogeneity may result from repeated infections of the same tree with different HSVd variants or from the accumulation of mutants appearing de novo during the replication of a single parental genome. This latter possibility is consistent with the error-prone nature of RNA replication (Aranda et al., 1997; Domingo and Holland, 1994), although genetic diversity of viroid population is limited by the need to maintain functional secondary and tertiary structures. The existence of constraints limiting sequence heterogeneity has been advanced for PLMVd and HSVd (Ambrós et al., 1998; Amari et al., 2001).

Because the presence of a cachexia-inducing variant (HSVd Tun/cl3) in the initial source did not induce onset of the typical symptoms in the indicator host two years later inoculation, our results can be interpreted by assuming that the effects of the cachexia-inducing variant can be moderated by the other accompanying HSVd variants. Therefore, the presence of a cachexia-inducing variant, by itself, may not necessarily lead to symptom expression, although we can not exclude the possibility that eventually these symptoms may appear later.

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Tables

Table 1. Mutations found between HSVd variants and their positions in the genome. Specific cachexia-associated changes are indicated in grey and deletions with (-). Viroid domains are: central (C), pathogenic (P), variable (V) and terminal left and right (TL and TR, respectively).

		HSVd variant			
Position	Domain	tun/cl1	tun/cl2	tun/cl3	tun/cl4
15	TL	(-)	U	(-)	(-)
25	TL	A	A	(-)	A
27	P	G	G	A	G
106	V	G	A	A	G
108	V	(-)	A	A	(-)
114	V	(-)	A	A	(-)
152	TR	A	U	U	A
187	V	(-)	(-)	U	(-)
191	V	C	C	U	C
205	C	C	(-)	C	C
207	C	A	(-)	G	A
208	C	G	C	A	G
215	C	(-)	G	(-)	C
216	C	G	C	G	G
242	P	C	G	C	C
243	P	G	C	G	G
244	P	U	U	(-)	U
245	P	C	C	(-)	C
246	P	U	U	(-)	U
250	P	A	A	G	A
255	P	G	G	C	G
257	P	U	U	G	U
259	P	(-)	G	(-)	(-)
302	TL	C	C	(-)	(-)
303	TL	C	C	(-)	(-)

Figures

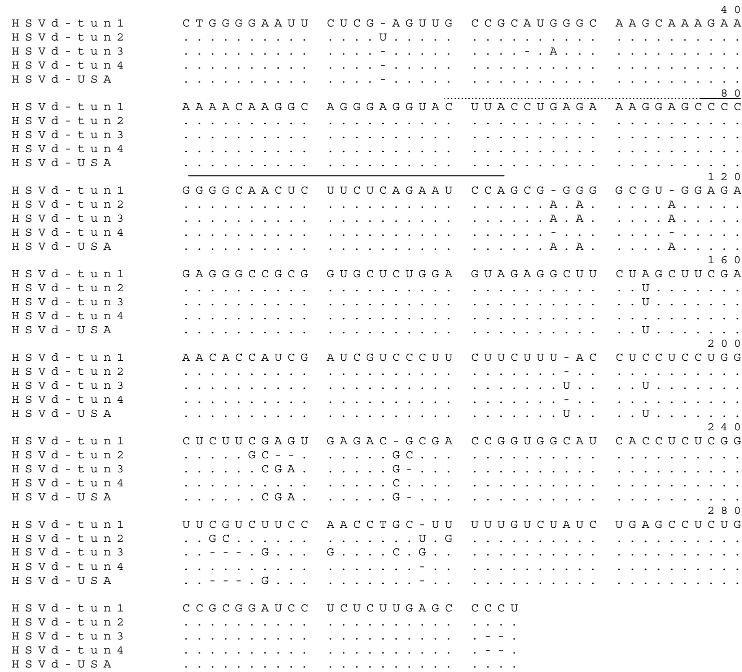

Fig. 1. Alignment of the HSVd variants from the Tunisian cultivar 'clementinier' with a previously reported American citrus variant. Positions covered by the antisense and sense primers are identified by the discontinuous and continuous lines, respectively.

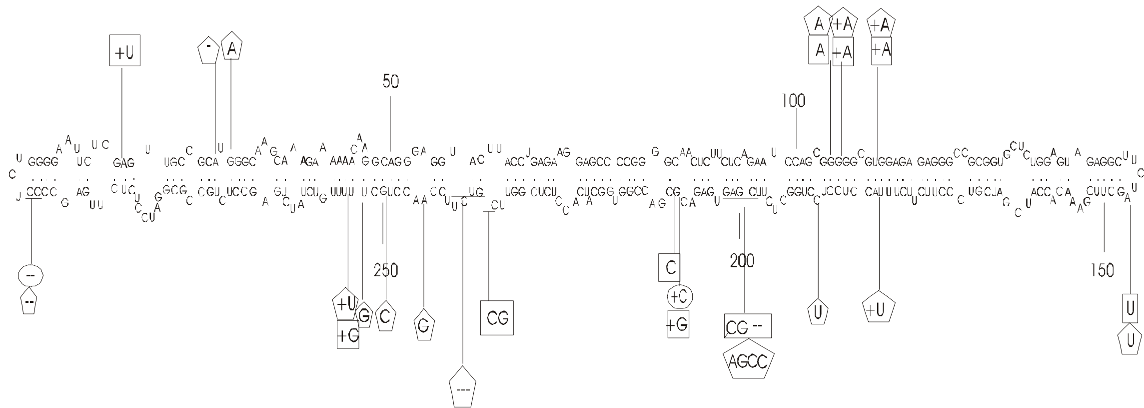

Fig. 2. Predicted rod-like secondary structures for HSVd-tun/cl1 variant. Changes found in the other variants are indicated by: ▭ (HSVd tun/cl2), ⬠ (HSVd tun/cl3) and ⬭ (HSVd tun/cl4). Insertions and deletions are denoted by (+) and (-), respectively.