Arch Virol (2018) 163:215–218
https://doi.org/10.1007/s00705-017-3591-y

BRIEF REPORT

A novel citrus viroid found in Australia, tentatively named citrus viroid VII

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Received: 6 December 2016 / Accepted: 16 September 2017 / Published online: 13 October 2017

Abstract A novel citrus viroid was discovered in a non-symptomatic Lisbon lemon (*Citrus x limon* L. Burm.f.) tree in New South Wales, Australia. Bioindexing, molecular detection and characterization involving sequencing combined with *in silico* analysis for the identification of the viroid-RNA hallmark properties of transmissibility and autonomous replication as well as specific sequence and structural motifs suggest that this viroid is a member of a new species in the genus *Apscaviroid*, family *Pospiviroidae*, which we have tentatively named "citrus viroid VII" (CVd-VII).

Citrus cultivars in Australia have been found to be infected with a number of viroids, including the pathogenic citrus exocortis viroid (CEVd) and the cachexia-inducing citrus variants of hop stunt viroid (HSVd), citrus viroid IIb (CVd-IIb) and citrus viroid IIc (CVd-IIc), as well as the non-cachexia variant CVd-IIa, citrus bent leaf viroid (CBLVd), and citrus dwarfing viroid (CDVd) [7]. Citrus viroids that have not been reported in Australia include citrus bark cracking viroid (CBCVd), CVd-V and CVd-VI. Programmes are in place to test imported and local citrus germplasm for graft-transmissible pathogens, including viroids, to prevent nursery and orchard infections. Routine biological indexing of a Lisbon lemon (*Citrus x limon* L. Burm.f.) field tree located in Dareton, New South Wales, Australia, was initiated in February 2015. Bark pieces from the symptomless Lisbon lemon were grafted onto four 'Etrog' citron Arizona 861-S-1 (*C. medica* L.) indicator plants grown from cuttings and maintained in a temperature-controlled greenhouse at 32 °C. Subsequent growth flushes on all of the indicator plants over a six-month period expressed epinasty symptoms (leaf bending and curling) indicative of citrus viroid infection (Fig. 1).

Total RNA was extracted from fresh bark tissue samples from one uninoculated plant and the four symptomatic 'Etrog' citron indicator plants using a VioTotal Plant RNA extraction miniprep system (Viogene, Taiwan) following the manufacturer's instructions. All samples tested negative by conventional reverse transcription polymerase chain reaction (RT-PCR) with primers designed to detect CEVd, HSVd, and CDVd [1], CBLVd [9], CVd-I-low sequence similarity (CVd-I-LSS) [8, 9], CBCVd [1, 2], CVd-V [12] and CVd-VI [9] (Table S1). A SYBR Green RT-qPCR assay using a degenerate primer pair Apsca-F-3-25/Apsca-R-232-212 (Table S1), which was designed for the universal detection of citrus apscaviroids (CBLVd, CDVd, CVd-V and CVd-VI) [16], produced an amplicon with a larger size and a different melting temperature from the known apscaviroid controls, but only in extracts from the symptomatic 'Etrog' citron plants. The 279-bp PCR product was purified using an Isolate II PCR and Gel Kit (Bioline, Australia) and directly sequenced in both directions (Australian Genome Research Facility-AGRF, Sydney) using the degenerate apscaviroid primers. BLASTn analysis of the 279-bp sequence showed a

Handling Editor: F. Murilo Zerbini.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-017-3591-y) contains supplementary material, which is available to authorized users.

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Fig. 1 Symptoms of leaf epinasty in new flush (left) and mature leaves (right) of 'Etrog' citron (*Citrus medica* L.) Arizona 861-S-1 plants inoculated with bark pieces from a field-grown non-symptomatic Lisbon lemon tree (*C. x limon* L. Burm.f.) are shown

low level similarity to the apscaviroid, Australian grapevine viroid (AGVd).

The sequence-specific overlapping primers VIIF1 (5′-CTTGCCTTGAGAAGCGAAGC-3′) and VIIR3 (5′-AAG CAGTTCCAGTTACAG-3′) were designed based on the 279-bp PCR product sequence for the amplification of the full circular genome of the potentially novel viroid. One-step RT-PCR was carried out using a SuperScript™ III One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Australia) according to the manufacturer's instructions. RT-PCR cycling conditions comprised reverse transcription at 55 °C for 30 minutes followed by 2 minutes at 94 °C and 35 cycles of denaturation (20 s at 94 °C), annealing (15 s at 56 °C) and extension (20 s at 72 °C), followed by a final extension step of 5 minutes at 72 °C. The resulting RT-PCR product was purified using an Isolate II PCR and Gel Kit (Bioline, Australia) and cloned using the pGEM-T Easy Vector System (Promega, Australia). Sequencing of selected clones in both directions using SP6 and T7 primers (AGRF, Sydney) revealed a 368-nucleotide insert, thus supporting the circular nature of the amplified RNA.

BLASTn analysis of three variants of these circular RNAs that were deposited in the GenBank database, LD2 (KX013549), LD3 (KX013550) and LD4 (KX013551), revealed sequences almost identical to each other and significantly different from all other known viroids. The highest sequence identity (59-59.5%) was observed with AGVd (FJ746829). The LD4 variant, selected here as the type variant, consisted of 89 A (24.2%), 87 U (23.6%), 96 C (26.1%), and 96 G (26.1%) nucleotides and had a G + C content of 52.2%. The other two variants displayed nucleotide substitutions at position 77, A to G (LD2 - KX013549 and LD3 - KX013550) and at position 231, U to A (LD3 - KX013550) (Fig. S1). The secondary structure of minimal free energy of LD4, as predicted by MFOLD software [17], was of a rod-like conformation with 69% paired nucleotides (Fig. 2a). More importantly, however, the secondary structure contained a terminal conserved region (TCR) and upper and lower central conserved regions (CCR) characteristic of members of the genus *Apscaviroid*. The TCR was found to have two mismatches in comparison to the core sequence (CNNGNGGUUCCUGUGG) of the apscaviroid type reference sequence (NC_001340) of apple scar skin viroid (ASSVd) [3]. Such mismatches in the TCR are observed in other proposed apscaviroids such as apple fruit crinkle viroid (NC_003777) and persimmon viroid 2 (NC_021720) [10]. Both the upper and lower central conserved regions were identical to the apscaviroid type reference sequence (Fig. S1). A thermodynamically stable hairpin I (HPI) structure similar to that described in other apscaviroids including citrus dwarfing viroid IIIa variant [13] and citrus viroid V [12], was present between nucleotides 88 and 120 (Fig. 2b), flanking the upper CCR, with a terminal tetraloop with a 3-bp stem followed by an extended stem at the base. On the lower strand, a hairpin II (HPII)-like metastable structure, with a 7-bp stem and a 71-bp loop containing the lower CCR was present, resembling that described in the apscaviroid CVd-V [12]. The stem of the HPII-like structure in viroid-like RNA did not have a high GC content, unlike the HPII structures present in other viroids [14] (Fig. 2b).

In addition to RT-PCR and sequence analysis, the total RNA extracted from the symptomatic graft-inoculated 'Etrog' citrons was slash inoculated into four new 'Etrog' citrons. The plants were maintained at 32 °C, and the viroid-like RNA was first detected six weeks post-inoculation in new growth above the inoculation site by RT-PCR using the primers VIIF1 and VIIR3, which are homologous and complementary to the nucleotide positions 233-252 and 218-235, respectively, of the type variant LD4 (KX013551). The PCR amplicons were sequenced, verifying their identity to

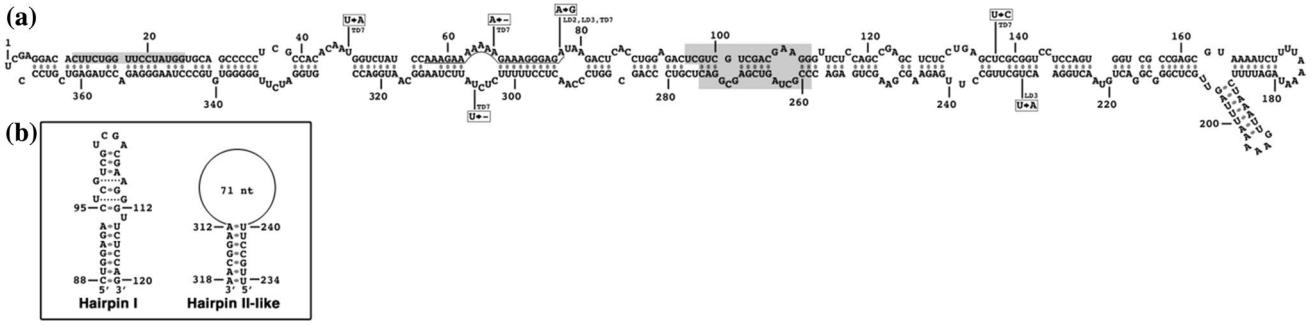

Fig. 2 **a.** Nucleotide sequence and proposed secondary structure of citrus viroid VII (CVd-VII) type variant (LD4). Structures are represented as the minimum free energy form at 24 °C predicted by MFOLD. The changes observed in the four additional variants (LD2, LD3, TD7, and TD8) are shown in marked boxes. The terminal conserved region (TCR) is contained within the shaded box (nucleotides 10-25), and the upper and lower central conserved regions (CCR) are also shaded. The upper CCR is represented by nucleotides 96-111, and the lower CCR is between nucleotides 259 and 275. The oligopurine region (nucleotides 55-77) is underlined. **b.** Schematic representation of the predicted hairpin I and hairpin II-like structures

the LD4 variant. Prominent symptoms of leaf bending and curling developed in all slash-inoculated 'Etrog' citrons at 12 weeks post-inoculation. Sequential polyacrylamide gel electrophoresis (sPAGE) followed by northern blot hybridization analysis [11] of the graft- and slash-inoculated 'Etrog' citrons, using a full-length DIG-labelled LD4 probe, revealed both circular and linear viroid-like RNA forms of the expected size (Fig. 3).

To further investigate the biological hallmarks of the newly identified viroid-like RNA, namely transmissibility and autonomous replication, a recombinant plasmid containing a single copy of the LD4 type variant (positive orientation) was linearized with *Nde*I and transcribed *in vitro* using an Ambion MEGAscript T7 Kit (Life Technologies, Australia). The monomeric RNA transcript, free of DNA, was slash inoculated into six uninfected 'Etrog' citron indicator plants. Four months after inoculation, samples of new leaf growth, above the inoculation site, were tested by RT-PCR with the VIIF1/VIIR3 primers and by RT-qPCR with the universal Apsca-F-3-25/Apsca-R-232-212 primers. The viroid-like RNA was detected by both assays, and the full-length RT-PCR amplicon was cloned and sequenced in both directions, confirming the presence of the full-length LD4 type variant in two of the transcript-inoculated plants. Further evidence of the autonomous replication and biological activity of the viroid-like RNA was obtained by the detection and characterization of the negative-sense RNA in one of the LD4 transcript-inoculated 'Etrog' citrons. More specifically, reverse transcription using the VIIF1 primer, which anneals to the negative RNA viroid strand, followed by amplification with the VIIF1/VIIR3 overlapping primer pair, amplified a product that was directly sequenced and determined to be a negative-sense full-length LD4-type variant. Based on the above biological, molecular, and *in silico* analysis findings,

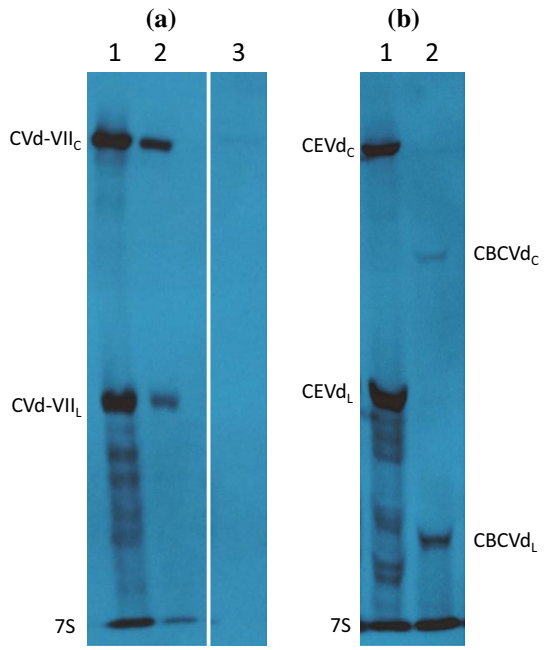

Fig. 3 Sequential polyacrylamide gel electrophoresis in a gel containing 5% acrylamide and 8 M urea, followed by northern-blot hybridization analysis [10] using (a) a full-length DIG-labelled LD4 type variant probe of the putative 368-nt citrus viroid VII (CVd-VII) and (b) full-length DIG-labelled probes of the 371-nt citrus exocortis viroid (CEVd) and the 284-nt citrus bark cracking viroid (CBCVd). Migration of circular (C) and linear (L) forms of viroid RNA and plant 7S RNA is indicated. **a** Lane 1, graft-inoculated 'Etrog' citron with bark pieces from the original Lisbon lemon tree; lane 2, slash-inoculated 'Etrog' citron with total RNA extracts from the graft-inoculated 'Etrog' citron (lane 1); and lane 3, uninoculated 'Etrog' citron. **b** Lanes 1 and 2, CEVd and: CBCVd used as viroid RNA mobility markers

it is proposed that the newly identified viroid-like RNA is a new apscaviroid, which we have tentatively named "citrus viroid VII" (CVd-VII).

CVd-VII was also detected by RT-PCR using the VIIF1/VIIR3 primers in samples collected from the original Lisbon field tree and from 'Etrog' citron indicator plants graft-inoculated with bark chips from a field tree of Taylor Eureka lemon (*C. x limon* L. Burm.f.). The Taylor Eureka tree was in close proximity to the CVd-VII-infected Lisbon lemon tree in the same orchard block. Sequence variants were obtained through cloning (as described previously) from the Taylor-Eureka-lemon-inoculated 'Etrog' indicator; these included TD7, a 366-bp variant (KX013552) that was not detected in the original Lisbon tree, and TD8 (KX013553), which was identical to the type variant LD4.

In summary, it is proposed that CVd-VII is a member of a new species in the genus *Apscaviroid* of the family *Pospiviroidae*, having the closest phylogenetic relationship with AGVd, grapevine yellow speckle viroid 1 and grapevine speckle viroid 2 (Fig. S2). The naturally occurring viroid was found to be a graft- and mechanically transmissible, self-replicating, circular ssRNA molecule of 368 nucleotides in length, showing less than 90% sequence identity to other members of the genus *Apscaviroid*, meeting one of the demarcation criteria for new viroid species [3, 4, 6]. In addition, CVd-VII had a predicted rod-like structure of minimal free energy with TCR and CCR core sequences, an oligopurine region, and HPI and HPII-like elements of secondary structure characteristic of members of the genus *Apscaviroid* [3–5].

Further studies for the identification of distinct biological and molecular properties, such as host range, symptomatology, interaction with other citrus viroids, and genetic and population variability, are required before an official proposal can be made to the International Committee on Taxonomy of Viruses (ICTV) for official recognition of CVd-VII as a member of a novel viroid species.

Acknowledgements The authors would like to gratefully thank Noora Siddiqi for assistance with creating the viroid structure figure (University of California, Riverside), and Allise Fail (NSW Department of Primary Industries) for nursery maintenance. The financial support of Auscitrus and the USDA National Institute of Food and Agriculture, Hatch project 233744, is acknowledged.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This research did not contain studies involving human participants or animals by any of the authors.

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