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Journal of Citrus Pathology

Recently Accepted

BRIEF REPORT

Citrus Bent Leaf Viroid Present in Citrus in South Africa

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Citation: Steyn, C., Breytenbach, J. H, & Cook, G. (2023). Citrus Bent Leaf Viroid Present in Citrus in South Africa. *Journal of Citrus Pathology*, 10. http://dx.doi.org/10.5070/C410160582 Retrieved from https://escholarship.org/uc/item/14h9q8rk.

Abstract

Currently six viroid species are recognised which infect the genera *Citrus* and *Poncirus*, with an additional tentative new species reported. Citrus bent leaf viroid (CBLVd) has been reported from various citrus growing regions world-wide, but has not been formally documented from South Africa. CBLVd was detected in field samples in various citrus growing regions in South Africa during routine diagnostic analyses conducted since 2011. The detection and sequence verification of CBLVd from field samples is reported in this study. Biological confirmation of CBLVd presence was done for one sample that was shown to contain a single viroid infection. Bent-leaf symptom expression was observed after slash inoculation of sample RNA to the 'Etrog' citron indicator host. This study was a retrospective analysis, of previously identified CBLVd-positive samples, to document the long-standing presence of CBLVd in South Africa.

Keywords

Pospiviroidae, Apscaviroid, detection, RT-PCR

Introduction

Citrus viroids are single-stranded, circular RNA species that can infect all citrus types and rootstocks of citrus. Citrus viroid species belong to the family *Pospiviroidae* and four genera including *Pospiviroid* (*Citrus exocortis viroid*), *Cocadviroid* (*Citrus bark cracking viroid*), *Hostuviroid* (*Hop stunt viroid*) and *Apscaviroid* (*Citrus bent leaf viroid*, *Citrus dwarfing viroid*, *Citrus viroid* V, *Citrus viroid* VI) (Di Serio et al. 2021). A further tentative species of the genus *Apscaviroid*, *Citrus viroid* VII, was reported from Australia (Chambers et al. 2018).

Citrus viroids are mechanically and graft transmissible but are not transmitted by seed in citrus. Infected budwood, and not fruit, is how citrus viroids are widely transmitted (Duran-Vila and Semancik 2003).

The natural host range of citrus bent leaf viroid (CBLVd) is rutaceous hosts (Duran-Vila and Semancik 2003). CBLVd, previously designated CVd-Ia and CVd-Ib, was shown to induce a leaf bend on the indicator, 'Etrog' citron Arizona 861-S-1 (*Citrus medica* L.) (Duran-Vila et al. 1988; Ashulin et al. 1991). As a single infection this viroid appears to be latent and its most significant effect on the citrus host is through synergistic or antagonistic interactions with other citrus viroid species. The predominant, commercial impact has been the reduction in tree canopy volume, but only in combination with other citrus viroids (Vernière et al. 2006; Vidalakis et al. 2010). Significant nucleotide variability is reported for this viroid species in addition to genome size differences ranging from 318 to 330 nucleotides (Ashulin et al. 1991; Semancik et al. 1997).

Although CBLVd has been reported in numerous citrus producing countries, its distribution is likely wider but under-reported due to the mild and synergistic disease association. CBLVd was detected using RT-PCR in South Africa, but the presence of this viroid was not verified or formally documented. Therefore, this study was done to confirm the presence of CBLVd in South Africa.

Materials and Methods

Molecular diagnostics of field samples, submitted for pathogen screening, have been conducted at Citrus Research International, Nelspruit since 2011. Analysis of field samples included RT-PCR testing for citrus viroids including CBLVd, citrus dwarfing viroid (CDVd), citrus exocortis viroid (CEVd), hop stunt viroid (HSVd), citrus bark cracking viroid (CBCVd) and citrus viroid V (CVd-V). Samples were commonly obtained as budwood and total RNA was extracted from green bark using an acid-phenol method previously described (Cook et al. 2016). Random primed reverse transcription was done as detailed in Cook et al. (2019) and

viroid PCRs were done as in Cook et al. (2016). For CBLVd PCR detection the CBLVd-F2 and CBLVd-R2 primer pair was used (Wang et al. 2008). Three samples that had tested positive for CBLVd and for which RNA, stored at -20°C, was still available included samples R130821-3, R130902-1 and R160707-4. Sample R130821-3 was obtained in 2013 from a navel orange tree (*Citrus sinensis* cultivar 'Rustenburg' Navel) in the Harry Gwala district of KwaZulu-Natal Province. Sample R130902-1 (*C. sinensis* cultivar 'Bennie' Valencia) was collected in the Mopani district of Limpopo Province in 2013 and sample R160707-41 (*C. sinensis* cultivar 'Turkey' Valencia) was collected in 2016 in the Vhembe district, a northern region of the Limpopo Province. Sample R160707-41 was the only sample that tested positive for a single viroid species, namely CBLVd.

Full genome nucleotide sequencing of CBLVd from the three selected samples was done from the stored total RNA extracts by RT-PCR and using two overlapping primer pairs as previously detailed (Steyn et al. 2016), but using GoTaq G2 Hot Start Green Master Mix (Promega Corp., Madison, WI, USA). PCR amplicons were gel-purified using the Zymoclean gel DNA Recovery kit (Zymo Research, CA, USA) and direct Sanger sequencing was performed in both orientations. Overlapping sequences were aligned and low-quality bases removed using BioEdit (Hall 1999). BLAST was used to determine closest sequence identity (Altschul et al. 1990).

In order to biologically confirm the presence of CBLVd in the singly infected viroid sample, R160707-41, a droplet of total RNA extract was slash inoculated to six 'Etrog' citron plants as previously described (Steyn et al. 2016). Plants were maintained in a temperature-controlled glasshouse with temperatures ranging between 28°C and 32°C. Transmission success was confirmed with RT-PCR, seven months post inoculation (pi). A full genome consensus sequence was obtained from a CBLVd-positive 'Etrog' host plant as described above. The

'Etrog' plants were cut back and regrowth was monitored for symptom development for a period of five months.

Results and Discussion

CBLVd has been detected since 2011 in South Africa with the implementation of RT-PCR screening for citrus viroids. Various disease investigations indicated the presence of CBLVd, in orchards as mixed infections with other citrus viroids and citrus tristeza virus which is endemic in southern Africa. These detections were primarily in older Valencia and Navel orchards which were established prior to the use of shoot-tip grafted budwood supply through the South African Citrus Improvement Scheme. The purpose of this study was not to document each detection over a prolonged period, but to retrospectively verify detection of CBLVd in a few samples.

CBLVd was detected in combination with other citrus viroids in sample R130821-3 (HSVd, CDVd, CBCVd) and sample R130902-1 (HSVd, CDVd), but was found as a single viroid infection in sample R160707-41.

Transmission to one of six 'Etrog' indicator plants, after slash inoculation of sample R160707-41 RNA, was confirmed with RT-PCR seven months post inoculation (pi) and designated as sample R220811-8. The 'Etrog' plants were cut back and regrowth was monitored for symptom development. The single CBLVd positive plant first showed leaf bend ten months pi and no symptoms were noted on the five plants that tested negative for CBLVd. The bent leaf symptom was observed on further growth flushes of this plant (Figure 1).

Figure 1. 'Etrog' plant (R220811-8) showing leaf bend symptoms typical for citrus bent leaf viroid infection (indicated by black arrows), post slash-inoculation with total RNA extract of sample R160707-41.

Full genome consensus sequences for CBLVd were obtained for the three field samples and for the positive 'Etrog' seedling. These nucleotide sequences were deposited in GenBank under accession numbers OP616802, OP616803, OP616804 and OP616805 for samples R130821-3, R130902-1, R160707-41 and R220811-8, respectively. The genomes of the field samples differed. Sequence OP616802 showed 100% sequence identity to Genbank accession AF428053 from Uruguay. Sequence OP616803 showed closest sequence identity (99.06%) to accessions GQ260200, AB006736, AF428056 from Iran, Japan and Uruguay, respectively and sequence OP616804 showed closest identity (99,69%) to accession AF428057 from Uruguay. The consensus sequence, OP616805, of sample R220811-8 from 'Etrog' citron showed two base pair changes compared to sequence OP616804 of sample R160707-41, from which it was derived. These changes may have been induced by the host change as previously reported for CEVd (Bernard et al. 2009). Alternately, a CBLVd variant within the original sample was transmitted by the slash inoculation, rather than the sequence variant OP616804.

The detection and sequence verification of CBLVd from field samples from South African orchards are reported in this study. Biological confirmation of CBLVd presence was demonstrated by symptom expression in 'Etrog' citron for a single viroid infected sample. This study was a retrospective analysis of previously identified CBLVd-positive samples to document the long-standing presence of this citrus viroid in South Africa.

Acknowledgments

This work was funded by Citrus Research International (Pty) Ltd.

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