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Research Article

First report of citrus virus A in Australia

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Abstract

Citrus virus A (CiVA) was detected for the first time in Australia in a living pathogen collection. Buds were originally collected from a Washington navel field tree prior to 1970 and graft-inoculated onto a Symons sweet orange indicator plant. The virus was detected using conventional and quantitative reverse transcription polymerase chain reactions and high-throughput sequencing. This Australian variant, known as CL223, shares 96.3% (RNA1) and 96.7% (RNA2) nucleotide identity with isolates of CiVA from South Africa and China, respectively. Foliar symptoms of leaf flecking and oak leaf patterns, consistent with detections of CiVA in other regions, were observed on the foliage of the original accession and inoculated indicator plants. Subsequent surveys of an Australian citrus variety collection detected a different CiVA sequence variant in two accessions of Pera sweet orange; this variant had 97% similarity to CL223, the other Australian variant.

Keywords: citrus, graft-transmissible, *Coguvirus*, chlorotic flecking, oak leaf pattern

Introduction

The international community has long reported psorosis-like diseases of citrus including psorosis, ringspot, impietratura, cristacortis and concave gum. These diseases produce similar chlorotic flecking and oak leaf patterns on the leaves of indicator plants, leading to the initial conclusion that they were all caused by the same agent; however, traditional tools were not sufficient to fully characterize the causal agents. The etiology of citrus psorosis disease was eventually determined to be the *Ophiovirus*, citrus psorosis virus (CPsV) (da Graca et al. 1991). Additional knowledge, provided by high-throughput sequencing (HTS), is helping researchers unravel the complexity of other viruses causing psorosis-like diseases of citrus, including citrus virus A (CiVA).

CiVA is a member of the genus *Coguvirus* within the order *Bunyavirales* (Navarro et al. 2018) and is assigned to the species *Coguvirus eburi* (Kuhn et al. 2020). This virus has a bipartite single-stranded RNA genome consisting of RNA1, encoding an RNA-dependent RNA polymerase in the negative strand, and an ambisense RNA2 encoding the nucleoprotein and a putative movement protein (Di Serio et al. 2019; Navarro et al. 2018). CiVA is graft-transmissible to sweet orange (*Citrus* × *aurantium* var. *sinensis* L.), grapefruit (*C.* × *aurantium* var. *paradisi* ined.), rough lemon (*C.* × *limonia* var. *jambhiri* ined.) and Dweet tangor (*C. reticulata* Blanco × *C.* × *aurantium* var. *sinensis* L.) (Navarro et al. 2018). The virus has also been detected in sweet orange, clementine (*C.* × *aurantium* var. *clementina*), mandarin (*C. reticulata* Blanco) and lemon (*C.* × *limon* var. *limon* (L.) Burm. f.) field trees (Navarro et al. 2018), as well as in pear (*Pyrus communis* L.) (Svanella-Dumas et al. 2019). No symptoms were observed in association with CiVA infection in graft-inoculated citrus plants or infected field trees in Italy (Navarro et al. 2018), although more recent studies suggest symptoms may be induced in susceptible varieties (de Bruyn et al. 2022; Beris et al. 2021; Bester et al. 2021; Park et al. 2021). Further work is needed to elucidate the symptoms associated with CiVA infection in different citrus varieties and determine the global distribution of this virus. This paper reports on the detection of CiVA in Australia.

Materials and Methods

A historic accession, code CL223, was used in this study. CL223 was sourced from a citrus pathogen collection maintained in living plants in a controlled environment greenhouse at the New South Wales Department of Primary Industries' Elizabeth Macarthur Agricultural Institute (EMAI) in Sydney, Australia. The origin of the accession was Washington navel budwood collected from a field tree prior to 1970 that was graft-inoculated onto a Symons sweet orange indicator plant. The plant was labelled 'Psorosis B' because flecking and

oak leaf patterns had been observed on the foliage (Figure 1).

Fig. 1. Leaf flecking and oak leaf patterns on the foliage of a Symons sweet orange plant, accession CL223 in a living pathogen collection (a); close-up of symptoms (b) (images taken in late summer – February 2019)

a)  b) 

Bark chips from CL223 were graft-inoculated in autumn (April 2019) onto the following indicator plants: Leng navel (grafted onto rough lemon) and seedlings of Duncan grapefruit and Dweet tangor. The inoculated plants were then maintained in a controlled environment greenhouse at 26 °C day / 22 °C night temperatures with natural daylength and subsequent growth flushes were periodically monitored for symptoms.

RNA was extracted from leaves of CL223 using an Isolate II Plant RNA kit (Meridian Bioscience, Ohio, United States). RNA extracts were tested for CPsV using a conventional reverse transcription polymerase chain reaction (RT-PCR) assay as per Vaira et al. (2003) using primers specific for ophioviruses (OP-1/OP-2). Extracts were also tested for CPsV using a quantitative RT-PCR (RT-qPCR) assay as per Osman et al. (2015) using primers and probes targeting the coat protein region of different sequence variants of CPsV (CPsV-792 F1 / CPsV-791 F2 / CPsV-851p-Vic / CPsV-946 R1 / CPsV946 R2).

CL223 was sampled for further analysis by high throughput sequencing (HTS). Total RNA was extracted from 200 mg of green bark tissue using a mirVana miRNA Isolation Kit (Life Technologies, Australia) without completing the small RNA enrichment step. DNA was then removed using DNaseI (Bioline, Australia). Following subsequent rRNA depletion, a cDNA library was prepared using the Truseq RNA Sample Prep Library (Illumina, California, United States) and sequenced using an Illumina NextSeq500 sequencing platform. The data was trimmed using BBduk (parameters used: ktrim=r, k=23, mink=11, and hdist=1) and then assembled *de novo* using SPAdes v3.13 (Bankevich et al. 2012) using kmer sizes 21, 41, 71, 101, 127 and the --careful option. The contigs were then analysed with a local BLASTn using the NCBI virus database (txid 10239) (downloaded 11th October 2021).

CL223 and trees (n= 429) in a citrus collection held at EMAI were screened for CiVA. Leaf samples were collected from each quadrant of each tree in the collection and pooled to form one sample per tree. RNA was extracted using a MagMAX Plant RNA extraction kit (Applied Biosystems, Massachusetts, United States) according to the manufacturer's instructions with the exception that the DNase I digestion step was omitted. RNA extracts from the trees in the collection, and from CL223, were tested using RT-PCR as per Navarro et al. (2018) with primers designed to amplify a 620 nt region of CiVA RNA 2 (Ka-1/Ka-3). All CiVA-positive samples were confirmed using bi-directional Sanger sequencing of the RT-PCR products, and by SYBR RT-qPCR using primers RdRp-qF$_{3581}$ and RdRp-qR$_{3688}$ (Beris et al. 2021).

Partial sequences of the nucleocapsid protein gene of RNA2 produced in this study (Navarro et al. 2018) and other CiVA isolates available in GenBank were aligned using MUSCLE (Version 3.8.425) (Edgar 2004). A maximum likelihood phylogenetic tree was constructed using the neighbor-joining method and the genetic distances were estimated according to the Jukes-Cantor model, with 1000 bootstrap replicates. Branches had a bootstrap support threshold of 50%. Phylogenetic analysis was conducted with Geneious Prime (Biomatters, New Zealand).

Results

Symptoms of leaf flecking and oak leaf patterns were first observed in the new growth of inoculated Leng navel (Figure 2) and Duncan grapefruit indicator plants three months after inoculation and in subsequent flush growth. Leaf flecking was also observed in the new growth of Dweet tangor indicator plants. CPsV was not detected in CL223 using conventional RT-PCR and RT-qPCR assays.

Fig. 2. Leaf flecking and oak leaf patterns on foliage of Leng navel indicator plants infected with citrus virus A (a); close up of symptoms (b) and (c) (images taken five months after inoculation)

a) 

b)  c) 

HTS generated 26,607,508 × 75 base paired-end reads from isolate CL223, which were subsequently trimmed of adaptor sequences and analyzed. *De novo* assembly of reads using SPAdes yielded 41,904 contigs >200 nt and two contigs of 6,673 (OM272943) and 2,734 (OM272944) nucleotides in length were identified as CiVA RNA1 and RNA2, respectively. BLASTn analysis of the 6,673 nt contig showed a 96.3% nucleotide identity to RNA1 (MT720885) of a CiVA isolate from South Africa, and BLASTn analysis of the 2,734 nt showed 96.7% nucleotide identity to RNA2 (MZ436806) of a CiVA isolate from China. BLASTp analysis of the contigs showed that the RNA-dependent RNA polymerase has 97.4% identity to isolate 1.8 (QUX80215), the movement protein showed 98.4% identity to isolate Nkw (QWE91515) and the nucleocapsid protein had 98.1% identity to CiVA isolate Tsp (QWE91522); these isolates were all reported from South Africa (de Bruyn et al. 2022). No other pathogens were detected using HTS.

CiVA was detected in isolate CL223 and two trees of the 429 tested in the EMAI collection using conventional RT-PCR (Navarro et al. 2018); the results were confirmed using RT-qPCR (Beris et al. 2021). Both positive trees in the collection were different accessions of Pera sweet orange. Sanger sequencing of the RT-PCR products from the positive samples produced identical sequences (OM638045 and OM638046) with 97% similarity to the sequence of isolate CL223. Phylogenetic analysis of the partial nucleocapsid protein gene (Figure 3) showed a close relationship between the isolates in the EMAI citrus collection and the South African isolate, 1.8 (MT720886).

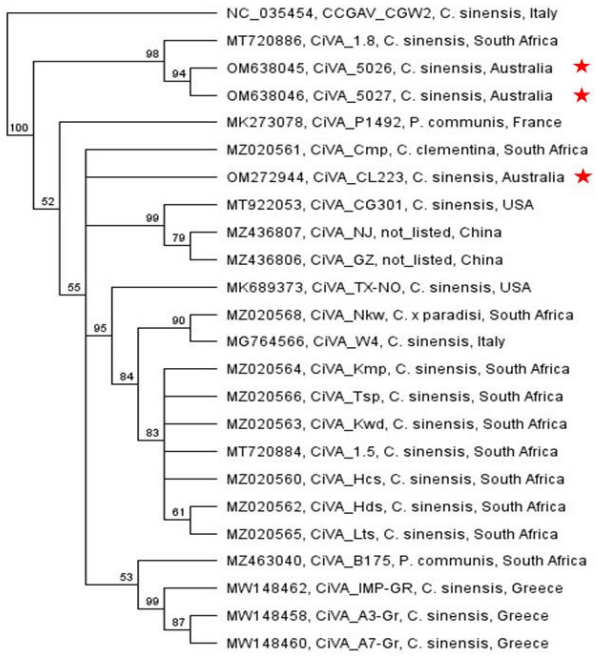

Fig. 3. Phylogenetic analysis of a partial sequence from the nucleocapsid protein gene of CiVA isolates, including Australian isolates 5026 and 5027 from the EMAI citrus collection, and CL223 (highlighted by stars). The neighbor-joining tree was constructed with 1000 bootstrap replicates and citrus concave gum associated virus (CCGaV) (NC_035454), another member of the genus *Coguvirus*, was used as an outgroup.

Discussion

The discovery and characterisation of CiVA in citrus in Italy (Navarro et al. 2018) provided valuable insight into the complex of psorosis-like diseases in citrus and allowed the detection of viral variants of CiVA in citrus in other regions, including the United States (Park et al. 2021), South Africa (Bester et al. 2021), Greece (Beris et al. 2021) and Australia. CiVA was not reported to be associated with symptoms in inoculated citrus indicator plants or infected field trees in Italy. However, studies in South Africa and the United States reported flecking and/or oak leaf patterns on young leaves of infected indicator plants (de Bruyn et al. 2022; Bester et al. 2021; Park et al. 2021), de Bruyn et al. (2022) also observed rind symptoms on fruit from infected field trees in South Africa, and Beris et al. (2021) reported symptoms consistent with impietratura in fruit from an infected field tree and leaf flecking on inoculated indicator plants in Greece. These findings are consistent with our observations of leaf flecking and oak leaf patterns on inoculated Leng navel and Duncan grapefruit indicator plants, and leaf flecking on Dweet tangor. Further work is needed to clearly define the symptoms associated with CiVA infection in citrus.

In this study, we have found at least two sequence variants of CiVA in Australia. One in a plant in a living pathogen collection and another in two accessions of Pera sweet orange. Only a portion of the genome was sequenced of the variants isolated from the two infected Pera sweet orange trees and, whilst initial analyses found those to be identical, further characterisation of those variants by HTS may discover differences.

The detection and characterisation of geographic viral variants of CiVA provides a greater understanding of viral diversity and enhances our confidence in the sensitivity and specificity of the published detection assays (Navarro et al. 2018; Beris et al. 2021). Such diagnostic tests are crucial for maintaining the high health status of citrus germplasm in quarantine and propagation programs. In the absence of a cure, management of graft-transmissible pathogens relies upon detection to avoid propagating plants with infected material, thereby reducing the spread of pathogens, and preventing orchard infections.

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References

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology 19:455–77. doi:10.1089/cmb.2012.0021.

Beris D, Ioanna M, Vassilakos N, Theologidis I, Rampou A, Kektsidou O, Massart S, Varveri C. 2021. Association of Citrus Virus A to Citrus Impietratura Disease Symptoms. Phytopathology 111:1782–1789. doi:10.1094/PHYTO-01-21-0027-R.

Bester R, Karaan M, Cook G, Maree HJ. 2021. First report of citrus virus A in citrus in South Africa. Journal of Citrus Pathology 8(1). doi:10.5070/C481049000.

da Graca JV, Lee RF, Moreno P, Civerolo EL, Derrick KS. 1991. Comparison of isolates of citrus ringspot, psorosis, and other virus-like agents of citrus. Plant Disease 75:613–616. doi:10.1094/PD-75-0613.

de Bruyn R, Bester R, Cook G, Steyn C, Breytenbach JHJ, Maree HJ. 2022. Distribution and genetic diversity of *Coguvirus eburi* in South African citrus and the development of a real-time RT-PCR assay for citrus-infecting Coguviruses. Plant Disease 106:2221–2227. doi.org/10.1094/PDIS-11-21-2409-RE

Di Serio F, Navarro B, Minutolo M, Zicca S, Saponari M, De Stradis, Alioto D. 2019. Two negative-stranded RNA viruses infecting citrus are representative members of the novel genus Coguvirus [abstract]. Journal of Citrus Pathology 6(1):11.

Edgar RC. 2004. Muscle: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792–1797. doi:10.1093/nar/gkh340.

Kuhn JH, Adkins S, Alioto D, Alkhovsky SV, Amarasinghe GK, Anthony SJ, Avšič-Županc T, Ayllón MA, Bahl J, Balkema-Buschmann A, Ballinger MJ. 2020. 2020 taxonomic update for phylum Negarnaviricota (Riboviria: Orthornavirae), including the large orders Bunyavirales and Mononegavirales. Archives of Virology 165:3023–72. doi:10.1007/s00705-309020-04731-2.

Navarro B, Zicca S, Minutolo M, Saponari M, Alioto D, Di Serio F. 2018. A negative-stranded RNA virus infecting citrus trees: the second member of a new genus within the order Bunyavirales. Frontiers of Microbiology 9:2340. doi:10.3389/fmcib.2018.02340.

Osman F, Hodzic E, Kwon S-J, Wang J, Vidalakis G. 2015. Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of *Citrus tristeza virus*, *Citrus psorosis virus*, and *Citrus leaf blotch virus*. Journal of Virological Methods 220:64–75. doi:10.1016/jviromet.2015.04.013.

Park J-W, da Graca J, Gonzalez M, Louzada ES, Alabi OJ, Kunta M. 2021. First Report of citrus virus A in Texas associated with oak leaf patterns in *Citrus sinensis*. Plant Disease PDIS-03. doi:10.1094/PDIS-03-21-0628-PDN.

Svanella-Dumas L, Marais A, Depasse F, Faure C, Lefebre M, Brans Y, Castaing J, Latour F, Bechti A, Candresse T. 2019. First report of Citrus virus A (CiVA) infecting pear (*Pyrus communis*) in France. Plant Disease 103(10):2703. doi:10.1094/PDIS-01-19-0028-PDN.

Vaira AM, Accotto GP, Costantini A, Milne RG. 2003. The partial sequence of RNA 1 of the ophiovirus Ranunculus white mottle virus indicates its relationship to rhabdoviruses and provides candidate primers for an ophiovirus-specific RT-PCR test. Archives of Virology 148:1037–1050. doi:10.1007/s00705-003-0016-x.