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First report of apple stem grooving virus in Lao PDR, detected in citrus

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Abstract
Apple stem grooving virus, the causal agent of citrus tatterleaf disease, was detected for the first time in the Lao People's Democratic Republic. Samples were collected from citrus trees across the southern provinces for testing in Australia. RNA was extracted and tested using conventional and real-time reverse transcription polymerase chain reactions with the virus detected in 1 of 59 samples tested. Viral identity was confirmed by Sanger sequencing and high throughput sequencing.

Keywords Citrus · *Capillovirus* · Graft-transmissible · Mechanically transmissible

Citrus tatterleaf disease is caused by apple stem grooving virus (ASGV), a member of the genus *Capillovirus*, and is typically called citrus tatterleaf virus in reports of the virus infecting citrus. ASGV has a wide host range, including apple, pear, cherry and all citrus species, in which it is mostly asymptomatic. Grafting infected but symptomless budwood onto susceptible rootstock varieties of trifoliate orange (*Citrus* (syn. *Poncirus*) *trifoliata*) and its hybrids, results in leaf chlorosis, bud union crease, tree stunting and decline (Calavan et al. 1963; Garnsey and Jones 1968; Miyakawa and Matsui 1976). Bud union crease is seen as a yellow ring under the bark at the bud union, as is observed when scion and rootstock varieties are incompatible.

The Lao People's Democratic Republic (Lao PDR) is a landlocked, mountainous country in South East Asia. As part of an investigation of the health status of citrus trees in Lao PDR, plants were inspected in nurseries, orchards and backyards in the southern provinces of Champasak and Sekong in 2018 and 2020. Some trees were selected for sampling due to their apparent ill health (chlorotic patterns on leaves, lopsided fruit shape, tree dieback), but others were randomly selected. Samples consisting of 5–10 citrus leaves were collected from 59 trees and kept cool until they were processed. Subsequently, the leaf mid-ribs were removed, preserved in 70% ethanol, and transported under permit to Australia for testing. Total RNA was extracted from the samples using an ISOLATE II Plant RNA Kit (Bioline Meridian Biosciences), following the manufacturer's instructions.

Samples were screened for ASGV by real-time RT-PCR (RT-qPCR) using an in-house assay that was designed using Geneious software (Biomatters, Auckland New Zealand) to detect the majority of ASGV isolates, particularly Australian isolates, with primers 6308F17 5'-GGGTTTTCGAGGCAGGT-3' and 6413R 5'-AAAGGCAGGCATGTCAACCT-3', and probe 5'-TGGAACTGGAGGGTTAGRAGTCGT-3'. The probe was modified to include 5' FAM dye, internal ZEN Quencher after the ninth nucleotide, and 3' Iowa Black® Fluorescent Quencher (Integrated DNA Technologies, Singapore). The RT-qPCR amplicon (104 nt) was sent for bi-directional Sanger sequencing and was determined to be 94.3% similar to numerous accessions of ASGV in GenBank. Confirmatory diagnosis of ASGV was done using one-step RT-PCR with the primers of Su (2008), amplifying a 636 nt region of the coat protein (CP) gene, followed by bi-directional Sanger sequencing of the amplicon.

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ASGV was detected using conventional and real-time RT-PCR in 1 of 59 samples (L57). Sequence alignment analysis determined that the conventional RT-PCR amplicon from sample L57 has a 97.2% similarity to isolate YYG (FJ223209) from China. The sample was collected from a tree identified as a Fremont mandarin (*Citrus reticulata*) from the Paksong district (Table 1). The rootstock variety and tree age are not known, and the bud union was not checked for yellow ring incompatibility. However, ASGV infection is symptomless in mandarin.

Further analysis by high throughput sequencing (HTS) was conducted for sample L57. Total RNA was extracted from 100 mg of leaf midrib stored in ethanol at 4 °C using Invitrogen™ Trizol™ Reagent (ThermoFisher Scientific, Australia), with DNA removed using DNaseI (Bioline, Australia). The RNA was sent to the Ramaciotti Centre for Genomics (Sydney, Australia) for library preparation using the Truseq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, California, United States), and sequencing using an Illumina NovaSeq 6000 sequencing system resulting in 126,725,798 paired-end reads (2 × 150 bp). The data was trimmed using BBduk (Bushnell 2017) (parameters used: ktrim = r, k = 23, mink = 11, and hdist = 1) and then assembled *de novo* with rnaSPAdes v3.15.5 (Bushmanova et al. 2019) with kmer sizes 25, 41, 57, 73. The resulting contigs were then analysed using a local NCBI ref_viruses_rep_genomes BLAST database (downloaded 27th March 2023), where a single viral contig, (OQ718412), 6444 nucleotides in length (coverage 28.4x; 1,346 reads), was identified as having 83.7% similarity to ASGV accession NC001749.2. Further online BLASTn analysis of the contig representing a near full-length ASGV genome, showed a 95.1% nucleotide identity to an isolate from *C. sinensis* from Taiwan identified as citrus tatterleaf virus (FJ355920).

A neighbour-joining consensus tree was generated from a sequence alignment produced in Geneious Prime (Version 2021.1.1) with 1000 replicates, based on the near full length contig sequence of the L57 isolate and other ASGV accessions from NCBI GenBank database (Fig. 1). This analysis showed that isolate L57 is most likely to be part of cluster III in clade B of ASGV isolates (Tan et al. 2019).

To our knowledge, this is the first report of ASGV in Lao PDR. ASGV is spread by the grafting of infected propagation material or on contaminated cutting tools. An insect vector has not been reported to transmit ASGV to citrus and seed transmission is contentious. A low level of ASGV seed transmission has been reported in Eureka lemon

Table 1 Number of detections of apple stem grooving virus (ASGV) by RT-qPCR in citrus leaf samples collected in Lao PDR.

District	Variety	Number of trees tested	Number of ASGV positive trees
Thateng	pomelo (*Citrus maxima* (Burm.) Merr)	2	0
	West Indian lime (*Citrus* × *aurantiifolia* L.)	2	0
Sanasomboun	West Indian lime	8	0
Bachieng	West Indian lime	1	0
Paksong	kaffir lime (*Citrus hystrix* DC)	1	0
	West Indian lime	8	0
	mandarin (*Citrus reticulata* Blanco)	5	1
	pomelo	10	0
Champasak	kaffir lime	2	0
Phatoumphone	kaffir lime	4	0
	lime	5	0
	mandarin	1	0
	pomelo	2	0
Khong	lime	3	0
	pomelo	5	0
TOTAL		59	1

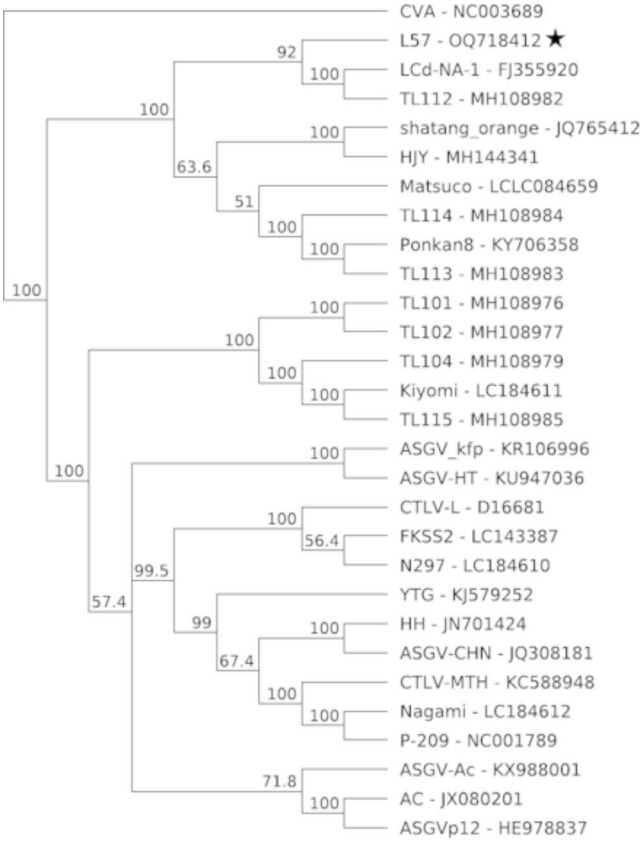

Fig. 1 A neighbour-joining consensus tree was generated in Geneious Prime (Version 2021.1.1) with 1000 bootstrap replicates, consensus support is shown on the branches and cherry virus A (CVA) (NC003689) was used as the outgroup. The tree was constructed based on the near-complete genome of the L57 isolate (OQ718412), marked with a star, and other representative ASGV accessions from NCBI GenBank database

(Tanner et al. 2011) but in a separate study, no evidence of seed transmission was found in Meyer lemon (Cook et al. 2020). Most citrus trees observed during the survey were seedling trees or produced by marcottage (air-layering). Grafted trees were only observed in significant commercial plantings. Therefore, ASGV was likely introduced to Lao PDR through infected plants or budwood. It is not known how widespread this virus is in Lao PDR, given it was only detected in one out of 59 samples collected, but it could potentially be spread further due to the popularity of marcottage to produce new plants.

The management of graft-transmissible citrus viruses relies upon quarantine, sourcing pathogen-free propagation material and growing grafted trees on tolerant rootstocks. It is difficult for small holders in Lao PDR to access high health status propagation material from pathogen tested sources, or grafted nursery trees propagated from pathogen-free material, unless they are linked to large commercial operations or aid projects.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to this article.

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