First Report of Watermelon Crinkle Leaf-Associated Virus 1 (WCLaV-1) and WCLaV-2 in Watermelon in Slovenia

Ana Vučurović[1]†, Irena Bajde[1], Jakob Brodarič[1], Anja Pecman[1], Zala Kogej-Zwitter[1, 2], Veronika Bukvič[1], Nejc Jakoš[1,3], Denis Kutnjak[1], Mojca Rot[4], and Nataša Mehle[1,5]

[1]Department of Biotechnology and Systems Biology, National Institute of Biology, 1000 Ljubljana, Slovenia

[2]Jožef Stefan International Postgraduate School, 1000 Ljubljana, Slovenia

[3]Niba Labs d.o.o., 1000 Ljubljana, Slovenia (current affiliation)

[4]Institute of Agriculture and Forestry Nova Gorica, Chamber of Agriculture and Forestry of Slovenia, Nova Gorica, 5000 Nova Gorica, Slovenia

[5]School for Viticulture and Enology, University of Nova Gorica, 5000 Nova Gorica, Slovenia

†corresponding author e-mail: ana.vucurovic@nib.si

Funding: The study was funded by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, and the Slovenian Research and Innovation Agency (ARIS), Republic of Slovenia core financing (P4-0165).

In July 2024, a pooled leaf sample (D760/24) was collected from several plants of three watermelon cultivars (*Citrullus lanatus* cvs. Crimson Sweet, Asahi Miyako Hybrid F1 and Top Gun) grown in an open field (approx. 0.5ha) in Dombrava, Slovenia. The plants which were included in the pooled sample showed virus-like symptoms, such as leaf mosaic, wilting and

necrosis (eXtra Supplementary material Fig. S1). The disease incidence was estimated at 10%. DNA and RNA were extracted following Mehle et al. (2013) and RNeasy Plant Mini Kit (Qiagen, Germany) protocols, respectively. The sample was tested positive by reverse-transcription (RT)-PCR for watermelon crinkle leaf-associated virus 1 (WCLaV-1) and WCLaV-2 (Hernandez et al. 2021) and negative for other viruses (details on viruses tested and primers used are available in eXtra Table S1). The obtained amplicons of expected sizes of WCLaV-1 and WCLaV-2 movement protein (MP) and RNA-dependent RNA polymerase (RdRp) genes (eXtra Fig S2) were then subjected to Sanger sequencing (Eurofins Genomics, Germany) and BLAST analysis. The MP (PQ570004, PQ570006) and the RdRp (PQ570005, PQ570007) sequences exhibited 100% identity with multiple accessions of WCLaV-1, such as PP792977 and PP792976, and WCLaV-2, such as LC636073 and LC636074. Illumina high-throughput sequencing (HTS, Novogene, Germany, NovaSeq X Plus, PE150) identified WCLaV-1 (PV012703-04) and WCLaV-2 (PV012705-06) reads, along with cucumis melo amalgavirus 1 (CmAV1, PV012707) and solanum nigrum ilarvirus 1 reads (insufficient reads to reconstruct genome segments, it may originate from pollen contamination of nearby infected plants in the field (Rivarez et al. 2023)). HTS data were analyzed in CLC Genomics Workbench v. 24 (Qiagen, USA) using the pipeline by (Pecman et al. 2022). Consensus genome sequences were reconstructed by iterative read mapping to the most similar reference sequence of the virus obtained from NCBI GenBank. To check for WCLaVs in watermelon seeds sold in Slovenia, we tested five seed samples from Sugar Baby, Crimstar F1, and Crimson Sweet (three lots) by RT-PCR. We also tested four leaf samples from plants grown from these seeds at 3-5 true leaves stage. Both viruses were found in all seed and leaf extracts. However, mechanical inoculations with the sap of two samples (plants grown from infected seed sample and sample D760/24) on several commonly used indicator plants including *Chenopodium quinoa, Capsicum annuum, Nicotiana clevelandii,*

Nicotiana glutinosa, Nicotiana benthamiana, Nicotiana tabacum cv. White Burley, *Nicotiana rustica, Datura stramonium, Cucurbita pepo* cv. Bianca di Trieste, and *Cucurbita maxima* did not result in their infection. Retrospective analyses of our HTS data of two watermelon and 84 other cucurbits samples from previous years showed WCLaV-1 and WCLaV-2 reads in two pooled samples (containing equal amount of RNA of each sample): one from 2018 and another from 2019. RT-PCR confirmed the presence of WCLaVs only in watermelons. The pool from 2018 was sequenced at GATC (Germany, NovaSeq 6000 S2, PE 150) and from 2019 in-house using Oxford Nanopore Technologies (UK, MinION Mk1B device, SQK-PCS108, R9 flow cell). All HTS reads are deposited in the NCBI Short Reads Archive (PRJNA1202089). This is the first report of WCLaV-1 and WCLaV-2 in Slovenia and Europe, the two viruses which were included to the Alert list of the European and Mediterranean Plant Protection Organization, due to limited knowledge about their epidemiology (EPPO 2023). Further research is necessary to determine the incidence of these viruses in Europe, elucidate their epidemiology, symptoms association and their potential impact on the production of watermelons in the region.

References:

EPPO. 2023. EPPO Report. Serv. no. 05. :116: https://gd.eppo.int/reporting/article-7598.

Hernandez, R. N., et al. 2021. Plant Dis. 105:2025. https://doi.org/10.1094/PDIS-02-21-0249-PDN

Mehle, N., et al. 2013. Phytoplasma methods Protoc. 139–145. https://doi.org/10.1007/978-1-62703-089-2_12

Pecman, A., et al. 2022. Front. Microbiol. 13. https://doi.org/10.3389/fmicb.2022.883921

Rivarez, M. P. S., et al. 2023. Phytopathology. 113:1729–1744. https://doi.org/10.1094/PHYTO-12-22-0465-V

eXtra Supplemantary material

Fig. S1. Photographs of watermelons from the surveyed field: watermelon sample infected with watermelon crinkle leaf-associated virus 1 (WCLaV-1) and WCLaV-2 showing necrosis, drying, leaf wilting (A) and leaf mosaic symptoms (B), and leaf of the plant without obvious virus-like symptoms (C).

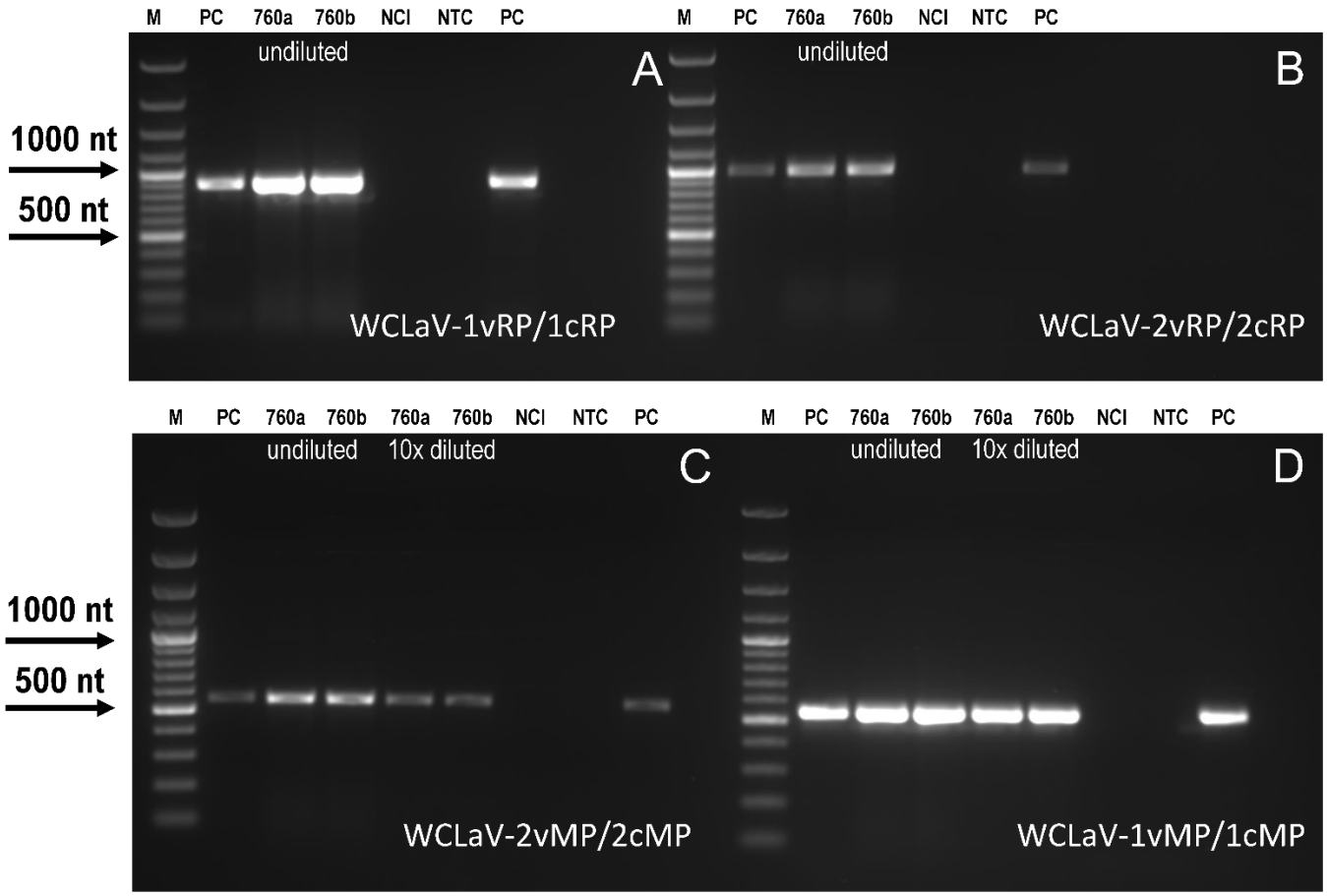

Fig. S2. Detection of watermelon crinkle leaf-associated virus 1 (WCLaV-1) and WCLaV-2 by reverse transcription–polymerase chain reaction (RT-PCR): RNA-dependent RNA polymerase gene sequences A) of WCLaV-1 with primers WCLaV-1vRP/1cRP, B) of WCLaV-2 with primers WCLaV-2vRP/2cRP; movement protein gene sequences C) of WCLaV-2 with primers WCLaV-2vMP/2cMP, and D) of WCLaV-1 with primers WCLaV-1vMP/1cMP. M - GeneRuler 100bp Plus DNA Ladder (Thermo Scientific), PC - positive control, NCI - negative control of extraction, NTC - no template control, 760 – sample D760/24, a and b – technical replicates.

Supplementary Table S1. The PCR, qPCR and RT-PCR conditions, primers, and probes used for the detection of for tomato leaf curl New Delhi virus (ToLCNDV), other begomoviruses and the watermelon crinkle leaf-associated virus 1 (WCLaV-1) and WCLaV-2

Virus tested	Primer	Sequence (5' → 3')[a]	Cycling conditions	NA used for testing	Amplicon size (bp)	Reference
ToLCNDV	ToLA-up	CATTATTGCACGAATTTCCG	50°C 2 min, 95°C 10 min, 45x (95°C 15 s, 60°C 60 s)	DNA	109	Simon et al. 2018
	ToLA-low	ATCGTAGCCGACTGTGTCTG				
	ToLA-probe	FAM-CATGCACCTTAGACCATGGACGCT-BHQ1				
	B-Rev	CAAGCAGAATTCACAATTCCAATC	50°C 2 min, 95°C 10 min, 45x (95°C 15 s, 60°C 60 s)	DNA	91	Luigi et al. 2020
	B-Fow	TCCAAGGATTCTTATCCTTKAGAGAG				
	B-Probe	HEX-TGAGGAAGAGTAGTAGTGGAGGTTACAGT-BHQ1				
begomoviruses	SPG1	CCCCKGTGCGWRAATCCAT	94°C 2 min, 11x (94°C 15 s, ramping 61°C + 1°C to 72°C 40 s, 72°C 90s), 24x (94°C 40 s, 60°C 40 s, 72°C 90s), 72°C 10 min	DNA	912	Li et al. 2004
	SPG2	ATCCVAAYWTYCAGGGAGCTAA				
	Beg-CP-F	GCCCATGTAYMGRAARCC	94°C 3 min,35x (94°C 30 s, 58°C 35 s, 72°C 30s), 72°C 7 min	DNA	580 or 950	Saison and Gentit 2015
	Beg-580-R	GGRTTAGARGCATGMGTACA				
WCLaV-1	WCLaV-1vRP	GGTGAGTTAGTGTGTCTGAAGG	50°C 30 min, 95°C 15 min, 35x (94°C 60 s, 50°C 30 s, 72°C 60 s), 72°C 10 min	RNA	881	Hernandez et al. 2021
	WCLaV-1cRP	GAGGTTGCCTGAGGTGATAAG				
	WCLaV-1vMP	GAAGGTTTGCTCCCTTGAAATG			538	
	WCLaV-1cMP	GACTGTGGCTGAAGAGTCTATG				
WCLaV-2	WCLaV-2vRP	GTCTCACATTCCTGCACTAACT			968	
	WCLaV-2cRP	ATCGGTCCTGGGTTATTTGTATC				
	WCLaV-2vMP	GACTTCAGAACCTCAACATCCA			562	
	WCLaV-2cMP	CAAGGGAGAGTGCTGACAAA				

[a]Y = C or T; R = A or G; M = A or C; W = A or T, D = A or G or T; H = A or C or T.

References:

Hernandez, R. N., et al. 2021. Plant Dis. 105:2025. https://doi.org/10.1094/PDIS-02-21-0249-PDN

Li, Z. H., et al. 2004. Arch. Virol. 149:1721–1732. https://doi.org/10.1007/s00705-004-0334-7

Luigi, M., et al. 2020. Eur. J. Plant Pathol. 157:969–974. https://doi.org/10.1007/s10658-020-02038-1

Saison, A., and Gentit, P. 2015. TESTA–EPPO Conference on diagnostics for plant pests, Angers, France.

Simón, A., et al. 2018. Plant Dis. 102:165–171. https://doi.org/10.1094/PDIS-06-17-0840-RE