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Characterization and occurrence of squash chlorotic leaf spot virus, a tentative new torradovirus species infecting cucurbits in Sudan

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Abstract

During a survey conducted in Sudan in 2012, a virus with spherical particles was isolated from a squash plant showing chlorotic leaf spots. The virus was transmitted mechanically and by two whitefly species, but not by aphids. RT-PCR with generic torradovirus primers yielded a band of expected size from total RNA of a symptomatic plant. Next-generation sequencing confirmed that this is tentatively a new torradovirus species, for which we propose the name ‘squash chlorotic leaf spot virus’. Using specific RT-PCR primers, the virus was detected in cucurbit samples collected since 1992 at different locations in Sudan.

Keywords SCLSV, melon, squash, whitefly, next-generation sequencing

The complete nucleotide sequences of SCLSV RNA1 and RNA2 were deposited in GenBank under accession numbers KU052530 and KU052531.

GenBank accession numbers for partial RNA2 sequences are: KU052532-KU052562.

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29 In Sudan, cucurbit crops are severely affected by a number of viruses transmitted by aphids,
30 whiteflies or beetles [1, 2, 4, 5]. During a survey conducted in 2012, severe chlorotic leaf
31 spots were observed on a squash (*Cucurbita moschata*) plant in a farm located at Fadasi, near
32 Wad Medani (Gezira State, Sudan) (Fig. 1a). A leaf sample (Su12-10) was tested by DAS-
33 ELISA, and no positive reaction was observed using IgGs and alkaline phosphatase
34 conjugated IgGs prepared in our laboratory for some of the most common cucurbit viruses
35 occurring in Sudan (cucumber mosaic virus, cucumber vein yellowing virus (CVYV),
36 cucurbit aphid-borne yellows virus, melon rugose mosaic virus, Moroccan watermelon
37 mosaic virus, papaya ringspot virus, snake melon asteroid mosaic virus, squash mosaic virus
38 (SqMV), watermelon chlorotic stunt virus (WmCSV) and zucchini yellow mosaic virus
39 (ZYMV)) [1-3]. After mechanical inoculation of sap extracts in 0.03 M Na₂HPO₄ containing
40 0.2% diethyldithiocarbamate to zucchini squash (*Cucurbita pepo*) cv Diamant in a quarantine
41 greenhouse, very severe mosaic symptoms, leaf curling and limited necrosis developed within
42 two weeks after inoculation. Leaf-dip observation using a Philips CM10 (Eindhoven, The
43 Netherlands) electron microscope and ammonium molybdate as a contrasting agent showed
44 the presence of a few isometric virus particles circa 30 nm in diameter and a number of
45 slightly flexuous filamentous particles. Extracts from a symptomatic plant were again tested
46 with DAS-ELISA, and a positive reaction was observed only for CVYV. This virus was
47 probably present at a too low concentration in the original sample to be detected by DAS-
48 ELISA in the first test.

49 After two serial mechanical transmissions to zucchini squash, neither CVYV nor flexuous
50 particles were detected, while the severe symptoms and the small spherical particles were still
51 observed (Fig. 1b and c). After mechanical inoculation of the spherical virus alone, squash (*C.*
52 *moschata*) cv. Pleine de Naples and cv. Menina developed chlorotic leaf spot symptoms
53 similar to those observed in the field. In order to further characterize the spherical virus,
54 vector transmission experiments were conducted using aphids and whiteflies. No transmission
55 was observed when 10 *Myzus persicae* or *Aphis gossypii* were deposited on each of 10
56 zucchini squash plants, using non-persistent transmission conditions (3 min acquisition access
57 period (AAP), 2 h inoculation access period (IAP)). Similarly, no transmission was observed
58 when 20 *M. persicae* or *A. gossypii* were deposited on each of 10 or 3 zucchini squash plants,
59 respectively, using persistent transmission conditions (1 day AAP, 3 days IAP). Using circa
60 50 whiteflies per plant, *Bemisia tabaci* biotype Q transmitted the virus to 3 and 4 out of 6

zucchini squash plants after AAPs of 6 and 30 h, respectively. In similar conditions, *Trialeurodes vaporariorum* transmitted the virus to 2 and 1 out of 6 zucchini squash plants after AAPs of 6 and 30 h, respectively. The shape of the particles and transmission properties of the virus suggested the presence of a torradovirus.

Total RNA was extracted from 50 mg of a zucchini squash plant showing severe symptoms using TRI-reagent (Molecular Research Center Inc., Cincinnati, OH), resuspended in 20 µl of RNase-free H₂O, and heated for 5 min at 65°C before RT-PCR reaction. Polyvalent primers Torrado-1F/Torrado-1R designed to amplify torradoviruses [8] were used in an RT-PCR reaction. A PCR signal of the expected size (371 nt) was obtained with sample Su12-10 and was directly sequenced. A BLASTn search in GenBank revealed 76 %-nt identity with Carrot torradovirus 1 RNA1 (KF533719). Total RNA (1µg) of Su12-10 was used for preparation of small RNAs suitable for Illumina HiSeq sequencing (Fasteris SA, Switzerland). A total number of 14,252,180 reads of 21-24 nt was obtained. *De novo* assembly using the CLC Genomics Workbench 7 (CLC Bio, Aarhus, Denmark) generated two large viral contigs of 5690 and 4020 nt, respectively. A BLASTn search performed with these two contigs against GenBank yielded 67%-nt identity of a 843 nt segment with Carrot torradovirus 1 RNA1 (KF533719), and 65%-nt identity of a 246 nt segment with Cassava torrado-like virus RNA2 (KC505251), respectively.

The 3' extremities were sequenced using an oligo(dT) primer and a 5' primer specific for each RNA based on the sequence obtained by NGS. The 5' extremities were sequenced by 5'RACE using an Invitrogen (Paisley, UK) kit according to the manufacturer's manual. The genome is constituted of two RNAs of 7046 and 4730 bases, respectively, excluding the 3'-terminal polyA tail. Mapping of the Illumina reads on the complete sequence with the CLC workbench, indicated a depth of coverage of 3199x for RNA1 and 4216x for RNA2. RNA1 putatively encodes a large protein of 2207 amino-acids with an expected molecular weight of 246 kDa, containing conserved helicase, protease and RNA-dependent RNA-polymerase motifs present in all torradoviruses so far [7]. As for other torradoviruses, RNA2 contained two ORFs corresponding to proteins of 233 aa (26 kDa) and 1054 aa (115 kDa), with an overlap of 116 nt; nt positions of ORF 1 and 2 are 84-783 and 667-3229 respectively (Fig. 2). Low-stringency protein BLAST analysis on the two major ORFs revealed a limited similarity (35- 40% identity) with known torradoviruses. In addition, two putative small ORFs of 432 nt and 306 nt respectively were detected *in silico* in the 3' parts of RNA1 and RNA2. These two small ORFs were not reported in other torradoviruses and had no identity in Blast analysis

with any sequence in the databases. More studies are required to determine if these putative ORFs are biologically significant. If the small putative ORFs are not considered, the 5' and 3' untranslated regions (UTRs) of RNA1 are 231 and 437 nt long, respectively. The putative 5' and 3'UTRs of RNA2 are 83 nt and 904 nt long, respectively. This is within the range of lengths observed for other torradoviruses, which display a great variability in UTR lengths, with often very long 3'UTRs, up to 1.2kb [7].

Nucleotide and amino-acid sequences of the new virus were aligned using ClustalW included in MEGA6 [6] with full-length sequences of the genome of acknowledged or putative torradoviruses: *Tomato torrado virus* (GenBank accessions DQ388879 and DQ388880 for RNA1 and RNA2 respectively), *Tomato marchitez virus* (EF681764-765), tomato chocolate virus (FJ560489-490), tomato chocolate spot virus (GQ305131-132), *Lettuce necrotic leaf curl virus* (KC855266-267), tomato necrotic dwarf virus (KC999058-059), carrot torradovirus 1 (KF533719-720) and motherwort yellow mottle virus (KM229700-701). Nucleotide sequence identity between the cucurbit-infecting virus and the 8 acknowledged or tentative torradoviruses were 44-46% and 40-42% for RNA1 and RNA2 respectively, whereas aa sequence identities were 31-34%, 20-27% and 31.7-33.3% for RNA1 ORF1, RNA2 ORF1 and RNA2 ORF2 respectively. Neighbour-joining or maximum likelihood tree reconstruction on different alignments (full-length nucleotide sequences of RNA1 or RNA2, aa sequences of the different ORFs, "Pro-Pol" aa sequence of ORF1 [7]) yielded similar clusterings, with two main branches containing the tomato-infecting torradoviruses vs. the other acknowledged torradoviruses, and the cucurbit virus rather basal within the torradovirus tree (Fig. 2 and data not shown).

Two primers were defined based on the first partial sequence of Su12-10. TorrCuc-5 CGCGGATTGTTGATGATGGC (position 4585-4604) and TorrCuc-3 AGTCGTTTCATGGTGACTCCG (position 4770-4789) were designed for the amplification of a 205 nt fragment in the RdRp-coding region of RNA1. For RT-PCR experiments, total RNA was denatured by heating for 2 min at 80°C and quickly chilling on ice. The RT step was done for 45 min at 42°C using AMV reverse transcriptase (Promega, Madison, WI) and primer TorrCuc2-3. PCR was performed in a second step using Taq polymerase (Promega) with 2µl of RT products with a 3 min denaturation time at 94°C, 40 cycles with a 30 s denaturation time at 94 °C, a 30 s annealing time at 60 °C, a 45 s elongation time at 72 °C, followed by a 10 min final elongation cycle at 72 °C.

A host range study was undertaken that included two plants of four cucurbit species (*C. pepo* (zucchini squash), *Cucumis melo* (melon), *Cucumis sativus* (cucumber) and *Citrullus lanatus* (watermelon)) and of 20 non-cucurbit species (*Brassica rapa*, *Capsella bursa-pastoris*, *Capsicum annuum*, *Chenopodium amaranticolor* , *C. quinoa*, *Lactuca sativa*, *Lavatera trimestris*, *Nicotiana benthamiana*, *N. clevelandii* , *N. glutinosa*, *N. tabacum* cv. Xanthi, *Petunia hybrida*, *Phaseolus vulgaris*, *Physalis floridana*, *Pisum sativum*, *Salvia splendens*, *Solanum lycopersicum*, *Valerianella officinalis*, *Vigna sinensis* and *Zinnia elegans*). Plants were mechanically inoculated with extracts from a zucchini squash showing severe symptoms. This experiment was repeated once. Three weeks after inoculation, local and systemic symptoms were rated, and total RNA extracts were prepared from each plant and tested by RT-PCR using the specific RT-PCR primers. Symptoms were observed on the four cucurbit species, and all RT-PCR tests were positive, producing bands of the expected size. Zucchini squash developed severe mosaic symptoms, leaf curling, stunting and few necrotic spots. In other cucurbits, symptoms were slight yellow mottle in melon, small chlorotic spots mostly along the veins in cucumber, and local and systemic necrotic lesions in watermelon. None of the non-cucurbit species developed symptoms, including tomato and lettuce, which are known hosts of torradoviruses [7], while all RT-PCR tests in these hosts were negative except with extracts from symptomless *N. benthamiana* and *N. clevelandii*, which all yielded a band of the expected size. All RT-PCR tests done with extracts from non-inoculated control plants were negative.

Based on its biological, morphological and molecular properties, we propose that the virus with spherical particles isolated from squash sample Su12-10 is a tentative new member of genus *Torradovirus*, for which we suggest the name squash chlorotic leaf spot virus (SCLSV).

Since primers TorrCuc-5 and TorrCuc-3 displayed some cross-reactivity with SqMV in some field samples (data not shown), a new primer pair was designed in the 5' part of RNA2 (TorrCuc2-5 CCGTGGGCCTCCGATCCCCC and TorrCuc2-3 TGCCTCTAGAGGCCAGGCG, positions 696-714 and 1282-1300 respectively in SCLSV RNA2) using sequences generated by NGS. This primer pair allowed the amplification of a 605 bp fragment located in the partially overlapping ORF1 and ORF2 of RNA2. RT-PCR conditions were the same as described above for primers TorrCuc-5 and TorrCuc-3. RT-PCR tests were done with these primers on samples collected in 2012, as well as on samples collected during previous surveys conducted in Sudan in 1992, 1993, 1995, 2002 and 2003. These samples were kept either as crude extracts or as total RNA extracts at -20°C. Of the 380

samples tested, 36 (9.5%) were positive in RT-PCR for SCLSV. This was confirmed by sequencing the RT-PCR products. Positive samples were from snake melon (*C. melo* var *flexuosus*) (23 out of 193 tested; 11.9%), melon (5/44; 11.4%), squash (3/37; 8.1%) watermelon (3/75; 4%) and wild *Cucumis* species (2/16; 12.5%). None of the 15 samples of *Luffa* sp., *Lagenaria* sp. and *Momordica charantia* were positive in RT-PCR. Positive samples were found only from locations along the Nile River (Wad Medani, Khartoum, Shendi, Atbara) covering a 400 km transect from Gezira scheme (Gezira State) in the South to Atbara (Nile State) in the North. Interestingly, positive reactions were obtained for samples originating from the same farm as Su12-10 that were collected in 1992 and 2003, indicating that the virus is well established in the environment.

Sequence analyses revealed up to 6.4% nucleotide divergence between samples in the sequenced region. Two clusters of sequences could be defined (Fig. 3), without obvious correlation to the host plant of the samples. However, a partial correlation with the geographic origin of the samples was noticed. All but 3 samples from Wad Medani region grouped in one cluster, while all samples from the Northern part of the transect (Shendi, Atbara) grouped in the second cluster. Mixed infections with isolates from the same cluster were observed in one sample from Wad Medani (cluster 1) and two samples from Shendi and Atbara (cluster 2). One of the isolates from 1992 originating from Fadasi (Wad Medani area) was divergent from both groups (Fig. 3).

SCLSV probably remained unnoticed until now because it was often found in mixed infections with other severe viruses, such as WmCSV or ZYMV. SCLCV occurrence in wild *Cucumis* sp., potential reservoirs growing all year round in Sudan, and the abundance of whitefly vectors in Sudan are favourable ecological factors for SCLSV maintenance in the environment. To the best of our knowledge, this is the first report of a torradovirus infecting cucurbits, and one of the earliest (1992) reports of a torradovirus infection.

Ethical statement

This research did not involve any human participants and/or animals. The authors declare that they have no conflict of interest.

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Fig. 1 Symptoms of squash chlorotic leaf spot virus (a) in the original squash plant and (b) after mechanical inoculation to zucchini squash. (c) Virus particles observed in a leaf dip preparation of an infected zucchini squash (scale bar = 200 nm)

Fig. 2 (a) Neighbour-joining tree based on aa alignment of the Protease-Polymerase region of ORF1 of SCLSV and eight other acknowledged or proposed torradoviruses. GenBank accession numbers of the isolates are indicated on the tree. Bootstrap values above 70% (500 bootstraps) are indicated for each node. The scale bar represents a genetic distance of 0.2. The comovirus bean pod mottle virus (BPMV) was used as outgroup. (b) Tentative genetic map of SCLSV with the positions of the different ORFs. Relative positions of regions containing conserved helicase (Hel), protease (Pro) and RNA polymerase (RdRp) motifs on RNA1, and movement protein (MP) and coat proteins (CPs) on RNA2, are indicated

Fig. 3 Neighbour-joining tree based on partial nucleotide sequences (519 bases) of RNA2 from 31 SCLCV isolates collected between 1992 and 2012 in Sudan. Bootstrap values (n=500) above 50% are indicated for each node. The host and geographic origin of each isolate is indicated: Csp: wild *Cucumis* sp.; M: melon; SC: snake cucumber; Sq: squash; W: watermelon. WM: Wad Medani; Sh: Shendi; At: Atbara; Kh: Khartoum