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Abstract

A mechanically transmissible virus tentatively named melon chlorotic spot virus (MeCSV) was isolated in Southeastern France from a melon plant showing chlorotic spots and yellowing of the older leaves. Its complete sequence was obtained by Illumina and Sanger sequencing. The genome comprises 8 RNAs for a total size of 20,079 nt and is distantly related Ramu stunt virus and maize yellow stunt virus, two tentative tenuiviruses. MeCSV differs from other tenuiviruses by its number of genomic fragments, by being readily mechanically transmissible and infecting only dicotyledonous hosts. MeCSV should thus be considered as a tentative new species related to tenuiviruses.

Keywords : phenuiviridae, melon, tenuivirus, NGS

The complete nucleotide sequences of MeCSV RNAs 1 to 8 were deposited in GenBank under accession numbers MH817469-MH817476.

A melon (*Cucumis melo*) sample showing chlorotic leaf spots merging into yellow areas on older leaves (Figure 1a) was collected in 2011 in a greenhouse near Tarascon (Bouches-du-Rhône, South-eastern France). Sap inoculation of isolate E11-018 was performed to *Nicotiana benthamiana*, *N. tabacum* cv. Xanthi, *Chenopodium quinoa* and melon cv. Védrantais. Four weeks after inoculation, symptoms of vein clearing, yellowing, leaf deformations and stunting were observed on *N. benthamiana*, systemic yellowing and necrosis on *N. tabacum*, chlorotic lesions becoming systemic on *C. quinoa*, and local chlorotic spots similar to those of the original sample occurred on melon suggesting the presence of a mechanically transmissible virus. No virus particles were observed in transmission electron microscopy in sap extracts from symptomatic plants (data not shown). No reactivity was detected in DAS-ELISA with antisera raised against common cucurbit-infecting viruses including zucchini yellow mosaic virus, watermelon mosaic virus, cucumber mosaic virus and cucurbit aphid-borne yellows virus. The original sample was stored as dried leaf material on calcium chloride.

Total RNA was extracted using TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH) from 10 mg of the original sample dried on calcium chloride. Extracted RNAs were pooled with 3 other samples and sent for sequencing to Fasteris SA, Switzerland. Small RNAs (21-24 nt) were purified from gel before Illumina HiSeq sequencing. The sequencing yielded 19,895,479 reads of 21 to 24 nt. The sequences were assembled *de novo* with CLC Genomics Workbench 7 (CLC Bio, Aarhus, Denmark). Blastn and blastx analyses were performed on the assembled fragments. Thirty contigs of 102 nt to 377 nt each had similarities with RNAs and associated putative proteins of two tentative tenuiviruses: Ramu stunt virus (RmSV) -similarity with RNA1 encoding RNA-dependent RNA polymerase (RdRp), RNA2 and RNA5 (nucleoprotein and

nucleocapsid)-, and maize yellow stripe virus (MYSV) RNAs 3 and 4 (unknown products, partial sequences available). Despite the small size of the contigs and the limited similarity (less than 50% protein identity) with the aforementioned viruses, this suggested the presence of a tenui-like virus in the infected sample. In order to obtain the full-length sequence of the virus, primers were designed on the different contigs and used for RT-PCR amplification on RNA from dried or fresh plant tissue extracted with TRI-reagent (see below). Since tenuiviruses as well as phleboviruses within the recently defined family *Phenuiviridae* (https://talk.ictvonline.org/taxonomy/) have highly conserved complementary extremities ACACAAAGTC [4, 6], the same property was expected for the new melon virus, and primer TenuiV [3] was used to amplify the extremities of the viral RNAs.

One-step RT-PCR were performed using Takara One-step RT-PCR kit (Takara Bio Inc., Japan). For the amplification of the large RNA1, specific primers were defined based on the Illumina contigs available. For the other RNAs, RT-PCR was performed with primer TenuiV only. Using primer TenuiV, a major band of 1.8 kb and a minor one of 1.5 kb were obtained, excised from agarose gel and ligated in pGemT easy® (Promega, France) before transformation of electrocompetent *Escherichia coli* DH5alpha, with white/blue selection. White colonies were used for direct PCR amplification with universal primers M13-21 and M13-Rev. PCR fragments of circa 1.6 to 2kb were obtained for most colonies, and they were sent to Genoscreen (Lille, France) for direct Sanger sequencing with primers M13-21 and M13-Rev. Three independent amplifications and cloning processes were performed: one from the original dried material, one from infected *N. benthamiana* and one from *Physalis floridana*, both obtained after mechanical inoculation (see below). A total number of 111 colonies were tested by PCR amplification of plasmid DNA and digestion with the restriction enzymes *Hin*dIII, *Xba*I and *Bam*HI. Among the 111 clones, 37 were sent for sequencing to Genoscreen. Specific primers

were also defined to complete the sequences of one fragment for which no clone had been obtained. For this fragment, direct sequencing of PCR products yielded unreadable sequences after a specific position, suggesting that the virus population contained at least 2 variants with an insertion/deletion. PCR fragments were then cloned in pGemT easy® and 4 clones were sent for sequencing to Genoscreen.

The genome of isolate E11-018 comprised 8 RNAs, for a total size of 20,079 nt (GenBank accession numbers MH817469-MH817476). For RNA7, two variants were present, one of 1486 nt and one of 1463 nt, with sequence variations starting at position 660. The 21-24 nt Illumina reads were realigned to the full-length sequence obtained. A total of 87820 reads, representing 0.44% of the 21-24 nt reads in the dataset, mapped to the genome of E11-018. Besides, 41 contigs from the initial *de novo* assembly (100 to 291 nt each) that did not yield any hit in blast analyses against GenBank sequences matched with the different RNAs of E11-018. Based on Illumina deep sequencing results, there was no evidence for the presence of any other virus in sample E11-018.

The largest RNA was 9096 nt long, and encoded a putative large ORF of 2943 amino acids with domains similar to RNA-dependent RNA polymerases in the *Phenuiviridae* and *Bunyaviridae* families [10]. The 7 other RNAs ranged in size from 1406 nt to 1847 nt, and encoded one or two tentative proteins in ambisense orientation (Table 1). This genetic organization is consistent with that of tenuiviruses [4, 6] although all tenuiviruses known so far have only 4 to 6 RNAs [6]. As in tenuiviruses and phleboviruses, the 5’ and 3’ termini of each RNA of E11-018 shared not only the 10 highly conserved complementary terminal nucleotides TGTGTTTCAG but also the following 7 to 15 nucleotides, with a looping out of one C residue at position 11 in one of the extremities [7, 14]. Blast analyses revealed limited similarities (25

to 50% amino-acid sequence identity) with several proteins of the tentative tenuiviruses RmSV [9] and MYSV [7] (Table 1). The 341 kDa protein encoded by RNA 1 also shared circa 30% identity with the RdRp of the tenuiviruses rice stripe virus and rice grassy stunt virus, and of several phleboviruses including Rift Valley fever virus and Uukuniemi virus. The 33.3 kDa protein encoded by RNA 4 had motifs conserved in the tenuivirus/phlebovirus nucleocapsid (E-value=4.8 10^{-10}) and a putative 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase motif (E-value=8.8 10^{-3}). It shared 33-35% identity with the two nucleoproteins encoded by RmSV, and less than 25% identity with nucleocapsids of tenuiviruses and phleboviruses.

Phylogenetic trees were built with MEGA6.0 [12] based on the nucleocapsid (CP) (Figure 2) and RdRp (data not shown) amino-acid sequences of tenuiviruses and the related animal-infecting phleboviruses, goukoviruses and phasiviruses in the family *Phenuiviridae*. Although MYSV appears as the virus most closely related to E11-018 in the partial sequences available, it could not be included in the phylogenetic analysis because of lack of CP sequence and full-length RdRp sequence. For both proteins, E11-018 clustered with RmSV with 100% bootstrap support, and was only distantly related to the other tenuiviruses and to the other phenuiviridae (Figure 2 and data not shown).

After 4 years of storage at 4°C, the original dried sample was ground in Na_2HPO_4 0.03M + diethyldithiocarbamate (DIECA) 0.2%. Activated charcoal and carborundum were added and the extract was mechanically inoculated to melon, *N. benthamiana*, *N. tabacum* cv. *Xanthi*, *Chenopodium amaranticolor* and *C. quinoa*. Only a few chlorotic local lesions developed on *C. quinoa* and a mild mottle was observed on a single *N. benthamiana* plant. Using the *C. quinoa* local lesions as inoculum, symptoms similar to those observed after inoculation of the original

sample developed on *N. benthamiana*, *N. tabacum*, *C. quinoa* and melon. In addition to symptom expression, the presence of E11-018 was checked by RT-PCR using specific primers TenCuc1 (5'-ACTGGACTCTGAACTCAGG-3') and TenCuc2 (5'-GAGACATCAGTTTGGGAGCC-3') targeting the nucleocapsid-encoding RNA 4.

The additional host range of E11-018 included several members of the family *Solanaceae*. The virus caused yellowing and a severe stunting on *N. clevelandii*, yellowing, mosaic and leaf deformation on *Physalis floridana* and *Petunia x hybrida* and systemic yellow spots and necrosis on tomato (Figure 1b and c). E11-018 also induced local chlorotic or necrotic lesions on *Vigna unguiculata* (*Fabaceae*) without systemic infection (Figure 1d). On *C. amaranticolor* (*Chenopodiaceae*) E11-018 induced chlorotic local lesions followed occasionally by systemic infection with chlorotic spots and mosaics. No symptoms were observed or systemic infection detected in *Poaceae* including wheat, barley, oat, maize and sorghum (data not shown).

Although the virus was originally observed on melon, showing yellow spotting on several old leaves, it induced only local yellow spots on inoculated cotyledons or leaves and very rarely a few systemic chlorotic spots. This discrepancy in symptom intensity could be due to differences in cultivar susceptibility. Unfortunately the cultivar of the original sample is not known and cv. Védrantais used in our tests may be partially resistant. Also, the mode of inoculation could have an impact on symptom expression. Rice stripe virus (RSV) was shown to cause more severe symptoms when inoculated by its natural planthopper vector *Laodelphax striatellus* than following mechanical inoculation [16], and most tenuiviruses are poorly or not mechanically transmissible in their natural hosts [6].

Tenuiviruses described so far infect only monocotyledonous plants in the family *Poaceae*, in the *Ehrhartoideae* (rice), *Panicoideae* (maize, sugarcane, echinochloa, urochloa) and *Pooideae*

(wheat) subfamilies, even though maize stripe virus and one other uncharacterized tenuivirus were once described on black spruce [2], and rice stripe virus was recently shown to infect *N. benthamiana* and *Arabidopsis thaliana* in laboratory conditions [11]. Criteria for species demarcation in the genus *Tenuivirus* include vector specificity, host range, size/number of RNA components and sequence identity, with a threshold of 85% protein sequence identity or 60% nt identity in the intergenic regions [6]. Even if the vector of E11-018 is not known, this isolate meets unambiguously all the other criteria and obviously belongs to a new species for which the name melon chlorotic spot virus (MeCSV) is proposed. RmSV, MYSV and MeCSV are highly divergent from the other tenuiviruses. MYSV also differs from typical tenuiviruses and from RmSV that are transmitted by planthoppers (*Delphacidae*, *Hemiptera*) in a circulative multiplicative way [15] by its leafhopper vector (*Cicadellidae*, *Hemiptera*). Five of the 8 MeCSV RNAs show some similarity with RmSV. Two nucleoproteins including a putative nucleocapsid and sharing 32% aa identity are encoded by RNAs 2 and 5 of RmSV, whereas only one nucleocapsid-coding region is present on RNA4 of MeCSV. MYSV was described as having 5 RNAs, but there is no evidence of a nucleocapsid coding region among these, suggesting that at least a 6th nucleocapsid-encoding RNA may have been overlooked. The other tenuiviruses characterized so far have 4 to 6 RNAs and a total genome size around 17 kb, except rice grassy stunt virus (RGSV) that presents a total genome size of more than 24 kb for 6 RNAs. Due to the number of RNAs of RGSV and its low similarity with the other tenuiviruses, it has been suggested that RGSV could belong to a distinct genus [14]. The same situation may apply for MeCSV, RmSV and MYSV that present a very low similarity with typical tenuiviruses and with RGSV. This would result in 3 genera of tenui-like viruses within the *Phenuiviridae* family in the recently defined *Bunyavirale* order (https://talk.ictvonline.org/taxonomy/). Members of the *Bunyavirale* contain vertebrate-infecting arboviruses, insect-specific viruses and the plant-

infecting emaraviruses, orthotospoviruses and tenuiviruses, all replicating in their arthropod vectors/hosts. Most viruses in the order *Bunyavirale* have 3 genomic RNAs, except tenuiviruses and emaraviruses that have 4 to 8 genomic RNAs [13]. The extra RNAs of some emaraviruses and of MeCSV, showing no similarity with known sequences, may have been acquired to facilitate virus-host or virus-vector interactions [13]. Recent studies on virus diversity and evolution in the *Bunyavirale* suggest that arthropods are the original host of viruses in this order, and some species have evolved to dual host infection [5, 8, 10].

To our knowledge, MeCSV is the first tenuivirus described as naturally infecting a dicotyledonous host. It is also the first tenuivirus observed in France and the first octopartite virus in the *Phenuiviridae*. Within Europe, the only tenuivirus described so far was the tentative European wheat striate mosaic virus (EWSMV), first described in 1958, infecting monocotyledonous hosts and transmitted by the planthoppers *Javesella pellucida* and *J. dubia* [1]. EWSMV has not yet been characterized molecularly and was not available for comparison with MeCSV. However the major differences in host ranges strongly support that they are distinct entities. Further studies are required to characterize the vector of MeCSV and to estimate its prevalence and potential agronomic impact on melon or other vegetable crops.

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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 caused by MeCSV: a : Chlorotic spots observed on the original melon sample ; b : general yellowing observed on *Petunia* x *hybrida*; c: systemic necrotic spots observed on tomato; d: chlorotic local lesions observed following mechanical inoculation of *Vigna unguiculata*

Fig 2 Maximum-likelihood tree based on the amino-acid sequence of the nucleocapsids of viruses in the family *Phenuiviridae*. Bootstrap values (500 bootstraps) above 60% are indicated for each node. The scale bar represents a genetic distance of 0.5.

Table 1 Genetic organization of E11-018 and protein similarity with other viruses

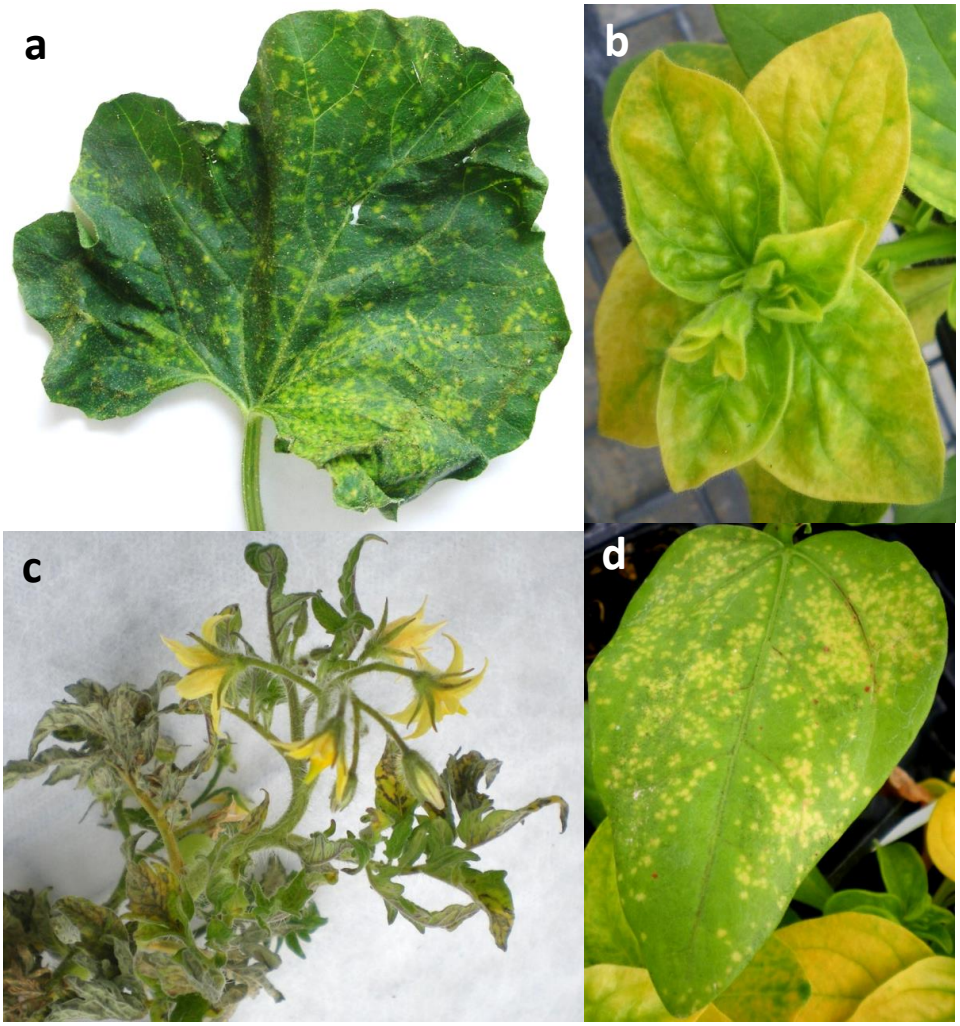

Fig. 1

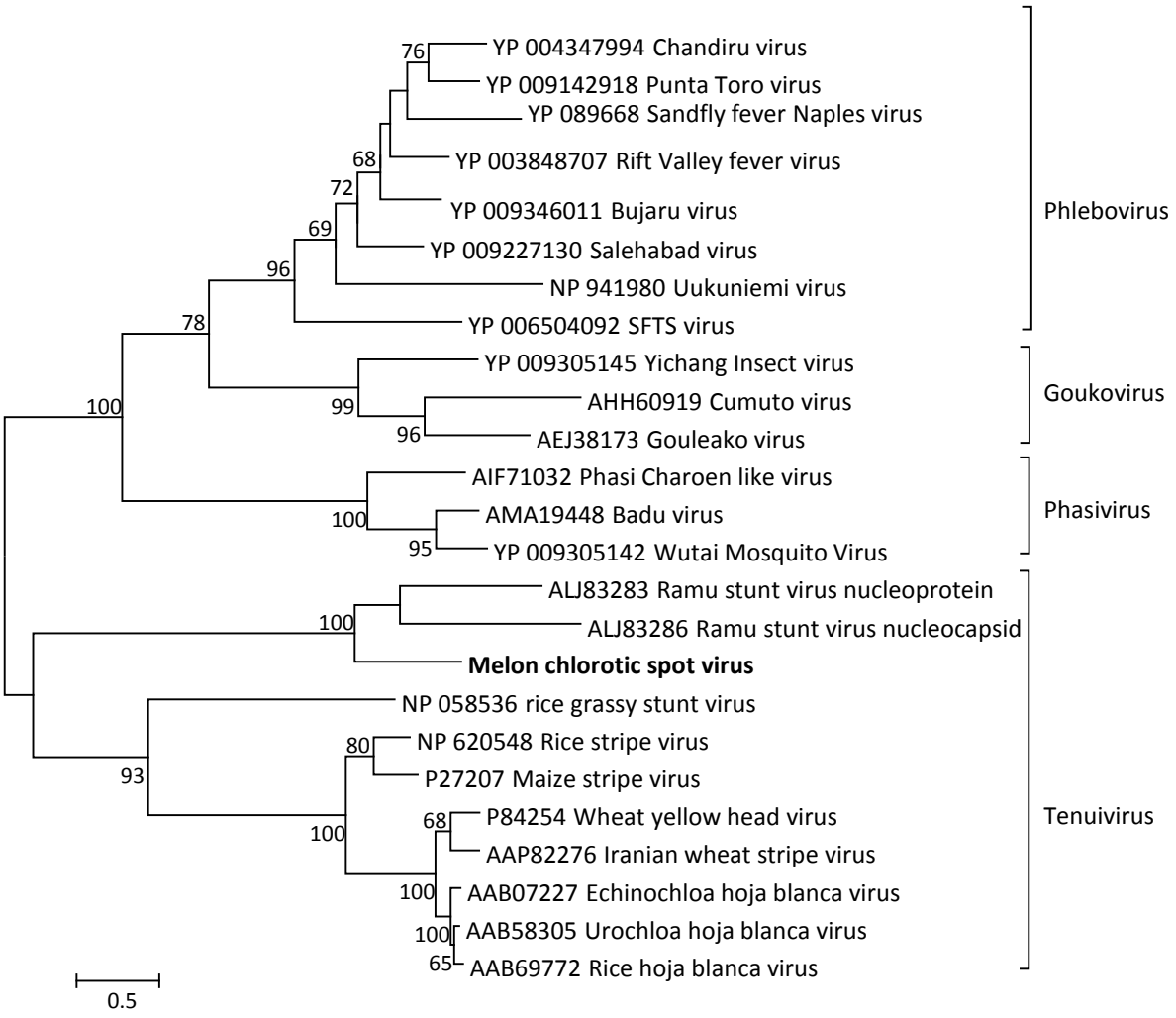
YP 004347994 Chandiru virus
YP 009142918 Punta Toro virus
YP 089668 Sandfly fever Naples virus
YP 003848707 Rift Valley fever virus
YP 009346011 Bujaru virus
YP 009227130 Salehabad virus
NP 941980 Uukuniemi virus
YP 006504092 SFTS virus
YP 009305145 Yichang Insect virus
AHH60919 Cumuto virus
AEJ38173 Gouleako virus
AIF71032 Phasi Charoen like virus
AMA19448 Badu virus
YP 009305142 Wutai Mosquito Virus
ALJ83283 Ramu stunt virus nucleoprotein
ALJ83286 Ramu stunt virus nucleocapsid
Melon chlorotic spot virus
NP 058536 rice grassy stunt virus
NP 620548 Rice stripe virus
P27207 Maize stripe virus
P84254 Wheat yellow head virus
AAP82276 Iranian wheat stripe virus
AAB07227 Echinochloa hoja blanca virus
AAB58305 Urochloa hoja blanca virus
AAB69772 Rice hoja blanca virus
Phlebovirus
Goukovirus
Phasivirus
Tenuivirus
0.5

Figure 2

Table 1 : genetic organization of E11-018 and protein similarity with other viruses

	RNA size	ORF position	Number of aa	Size (kDa)	blastp, tblastx or tblastn results
RNA1	9096	74-8905	2943	341	45% identity with RmSV pC1 (RdRp); 32-33% identity with RSV, RHBV, RGSV, 30% with MYSV (partial sequence).
RNA2	1847	48-1169	373	43.2	No blast; putative valyl-tRNA synthetase domain ($p=10^{-3}$)
		1810-1220	196	21.2	No blast
RNA3	1598	92-679	195	23.2	No blast
		1544-837	235	27.7	No blast
RNA4	1592	48-965	305	33.3	35% identity with RmSV pC2 (nucleoprotein) ; 33% with RmSV nucleocapsid
ARN5	1545	62-694	210	23.1	47% identity with MYSV pC4 (partial); 32% identity with RmSV (77% cover)
		1495-1141	114	13	38% identity with protein from RNA4 of MYSV (76-471)
RNA6	1509	47-1249	400	45.8	31% identity with MYSV pC5 ; 25% identity with RmSV pC4
RNA7	1486	1411-982	143	16	54% identity with protein from RNA3 of MYSV; 25% identity with protein from RNA6 of RmSV
		47-301	84	9.7	No blast
RNA8	1406	65-346	93	11.2	No blast
		1317-970	115	12.9	No blast

RmSV : Ramu stunt virus ; MYSV : maize yellow stripe virus ; RSV : rice stripe virus ; RHBV : rice hoja blanca virus ; RGSV : rice grassy stunt virus