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BRIEF REPORT

Development of a full-length infectious clone of sunflower chlorotic mottle virus (SuCMoV)

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Abstract A full-length cDNA clone (p35SuCMoV) of the sunflower chlorotic mottle virus common strain (SuCMoV-C) genomic RNA was constructed. Three cDNA fragments covering the whole genome of SuCMoV-C were cloned between a cauliflower mosaic virus 35S promoter and a nopaline synthase terminator. Mechanical inoculation of sunflower and *Nicotiana occidentalis* seedlings with p35SuCMoV DNA led to systemic infection. Symptoms induced by p35SuCMoV were similar to those caused by the wild-type SuCMoV-C but appeared four days later. Infection was confirmed by a western blot test, electron microscopy, RT-PCR and inoculation of progeny virions to sunflower seedlings. This is the first report about the construction of a biologically active, full-length cDNA copy of the SuCMoV-C RNA genome.

Keyword Sunflower · Potyvirus · Infectious clone · Mechanical inoculation · SuCMoV

Sunflower chlorotic mottle virus (SuCMoV) is a new species [1] in the Potato virus Y (PVY) subgroup in the genus *Potyvirus*. SuCMoV is the most prevalent sunflower (*Helianthus annuus* L.) virus present in Argentina, with several systemically infected weeds acting as natural virus reservoirs (Lenardon, personal communication). Potyvirus particles are flexuous rods that encapsidate a positive-sense, single-stranded polyadenylated RNA genome of about 9.5 kb that is covalently linked at the 5′ end to the virus-encoded VPg protein. The potyviral RNA genome encodes an open reading frame that is translated into a polyprotein, which is processed into 10 mature proteins by three viral-encoded proteases [26]. In addition, a "pretty interesting *Potyviridae* ORF" (PIPO), which is embedded in the P3 coding region as a plus 2 frameshift sequence, was discovered recently [7].

Two biologically different strains of SuCMoV have been reported: the chlorotic ringspot strain (CRS) [12] and the common strain (C) [10], the latter being the most widely distributed; both have been fully sequenced [4]. Moreover, a sunflower line (L33) that is tolerant to SuCMoV has been obtained, and segregation data have indicated the presence of a single dominant gene, designated Rcmo-1 [16].

To design sustainable strategies aimed at preventing SuCMoV infections, it is necessary to elucidate the basis of SuCMoV-sunflower interactions. Identifying SuCMoV domains involved in the interaction with the Rcmo-1 gene and those related to symptom induction and other biological properties requires the availability of infectious clones. Biologically active *in vitro* transcript RNAs from full-length cDNA clones with bacterial phage promoters [6, 14, 15, 17, 22–24] and *in vivo* infectious transcripts driven by a cauliflower mosaic virus (CaMV) 35S promoter [6, 13, 17, 19, 20, 24, 25, 30] have been reported for several potyviruses. Here, we report the construction of a full-length, stable, cDNA clone of the SuCMoV genome from which

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infectious RNA can be transcribed *in vivo*. The infectivity and symptomatology of viral RNA derived from the clone was tested and compared with those of wild-type SuCMoV-C.

SuCMoV-C was propagated in sunflower according to Dujovny et al. [9]. Total RNA from infected leaves was extracted using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and used as template for cDNA synthesis with the oligo-dT/*Not*I primer [29] and M-MLV reverse transcriptase (Promega, Madison, USA). Three fragments were amplified separately to construct a full-length cDNA clone of SuCMoV-C using specific primers (Table S1 in Supplementary material) and an Expand Long Template PCR System (Roche, Mannheim, Germany).

Each of the three amplified fragments was subcloned in pGEM-T Easy Vector (Promega), purified from a gel using a Wizard SV Gel and PCR Clean-Up System (Promega) and then cloned between a cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase (NOS) terminator in pAGUS1 [27] in the *E. coli* strain JM109 (Promega). The strategy for the construction of a full-length cDNA clone of SuCMoV-C is outlined in Fig. 1. First, pAGUS1 was cut with *Bam*HI and treated with mung bean nuclease (Promega) to create blunt ends. Fragment A (5′-end—1082) was digested with *Swa*I and *Hind*III and then inserted at the 5′ blunt end and the *Hind*III site of the plasmid vector to create p35SH. Second, p35SH was digested with *Age*I and *Sac*I, and fragment C (5408 -poly A) digested with the same enzymes was inserted into the plasmid vector to create p35SHAS. Finally, fragment B (1070-5419) was digested with *Mlu*I and *Bsi*WI and inserted into p35SHAS, which was cut with the same enzymes to obtain the full-length SuCMoV cDNA clone. The resulting construct was named p35SuCMoV (Fig. 1) and was further sequenced at Macrogen Inc. (Korea Republic) to confirm the junctions and the inserted sequences, and to determine the sequence similarity between p35SuCMoV and the previously reported sequence of SuCMoV-C (accession no. GU181199). As shown in Supplemental Fig.1 and its annex, sequence alignment showed that p35SuCMoV differs by 15 nucleotides from the reported sequence of SuCMoV-C. Twelve differences resulted in silent mutations, while three resulted in amino acid substitutions: C for A (nt 2877), leading to a Phe-for-Leu change in the P3 cistron (aa position 914); and T for A (nt 5098), leading to an Ser-for-Thr change; and A for T (nt 5249), leading to a Tyr-for-Phe change, both in the CI cistron (aa positions 1655 and 1705, respectively).

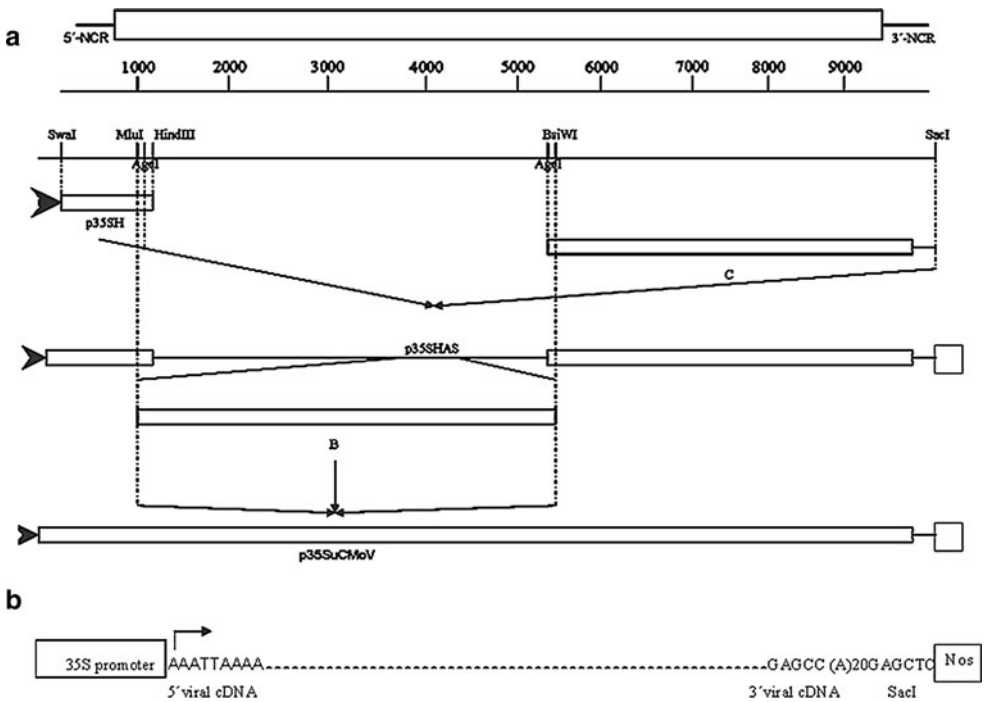

Fig. 1 Schematic diagram of the strategy for assembly of the SuCMoV-C cDNA clone. Panel A: Construction of the full-length cDNA clone, with the viral genome behind a CaMV 35S promoter. Rectangular boxes and the names below each one represent the positions of the inserts and the names of the cDNA clones (vector sequences are not represented). A scale (in base pairs) indicates the relative position of restriction enzyme sites used in the cloning protocol. Black triangles indicate the position of the CaMV 35S promoter, and the box indicates the Nos terminator. Panel B: nucleotide sequence and restriction site at the boundary of the full-length viral cDNA insert in p35SuCMoV. The CaMV35S promoter is shown as a box. The site of initiation of transcription is indicated by an arrow

The first nucleotide of the SuCMoV-C sequence was placed at the transcription initiation site to ensure that there would be no extra nucleotides at the 5′ end of the transcript generated *in vivo*. The 3′ end contained 20 adenine residues, followed by a NOS terminator. In general, the length of the poly (A) tail is considered to affect the infectivity of infectious clones [5, 21]. Furthermore, it has been reported that a cDNA clone lacking the NOS terminator was not infectious [11].

Since development of the first infectious clone from a RNA plant virus [2], many other clones have been produced that function after transcription *in vitro* or *in vivo* [14, 15, 17, 19, 25]. The development of infectious clones is the first step in the design of virus-based expression vectors, which are useful in biotechnological applications [3]. Several potyvirus cDNA clones have been unstable when propagated in *E. coli*, as sequence alterations took place during culture [3]. However, p35SuCMoV was stable when propagated in *E. coli* and was recovered intact from three sequential subcultures of *E. coli* in LB medium incubated at 37°C. Restriction fragment length polymorphism (RFLP) analysis was performed with p35SuCMoV

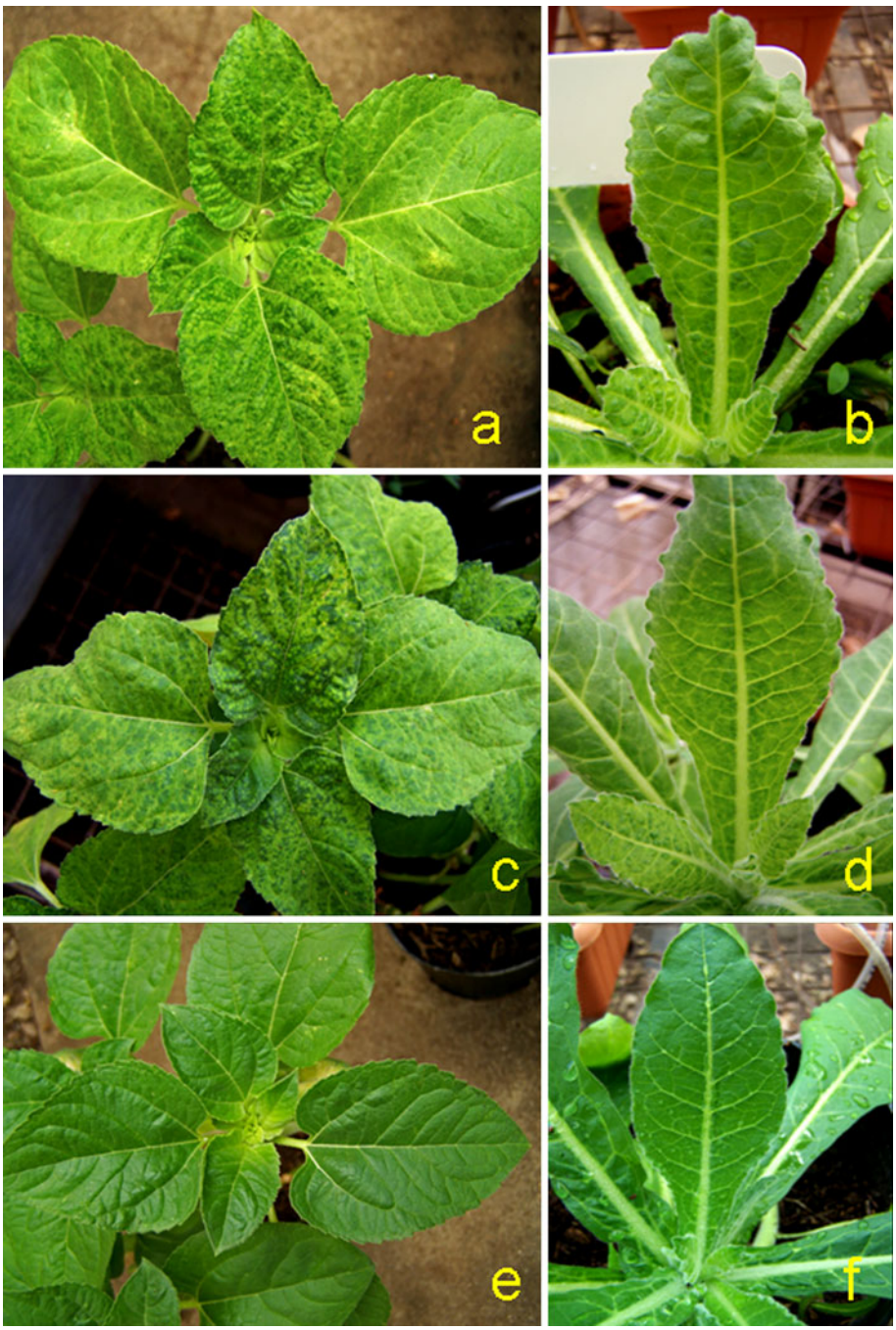

Fig. 2 Symptoms observed in sunflower and *N. occidentalis* inoculated with p35SuCMoV (a and b, respectively) or wild type SuCMoV-C (c and d, respectively). Mock-inoculated sunflower (e) and *N. occidentalis* (f). Photographs of upper leaves were taken at 21 days post-inoculation

using the restriction enzymes *Nco*I, *Mlu*I, *Bsi*WI, *Bam*HI and *Sac*I. The RFLP patterns of p35SuCMoV were identical to those expected from the complete nucleotide sequence of SuCMoV-C (data not shown).

To test the infectivity of p35SuCMoV plasmid DNA was purified according to Del Sal et al. [8] and 5 µg of DNA per plant, at a concentration of 100 ng/µl in diethylpyrocarbonate (DEPC)-treated water, was mechanically inoculated to sunflower at the two-true-leaf stage, and to *Nicotiana occidentalis* at the six-leaf stage. Control seedlings were inoculated with wild-type SuCMoV, whereas other seedlings were mock-inoculated. All inoculated plants were analyzed by DAS-ELISA using SuCMoV-C polyclonal antisera according to Dujovny et al. [9]. In addition, infection was confirmed by western blot test, electron microscopy, RT-PCR and inoculation of progeny virus to healthy sunflower seedlings.

Typical chlorotic mottling symptoms appeared on an average of 15% (7 out of 44, the total number of two independent experiments) and 20% (6 out of 30, the total number of two independent experiments) of the sunflower and *N. occidentalis* seedlings, respectively, inoculated with p35SuCMoV DNA. The efficiency of infectivity of other potyvirus infectious cDNA clones ranged from 5% to 100% [6, 11, 17, 22, 24, 25, 28, 30]. The variability in the efficiency of infection among infectious cDNA clones have been reported as to be dependent on the amount of DNA used to inoculate each leaf, as well as on the host and inoculation method [24]. Mechanical inoculation is highly variable, probably due to differences in the damage caused to the plant in each experiment [18]. Biolistic technology based on particle bombardment greatly increased infection rates with cloned viral material as compared to mechanical inoculation [11, 18]. Therefore, infectivity percentages of p35SuCMoV may be improved by optimizing inoculation procedures.

Symptoms on sunflower and *N. occidentalis* plants inoculated with p35SuCMoV appeared 12 days post-inoculation (dpi), whereas symptoms on plants inoculated with wild-type SuCMoV appeared 8 dpi. Sunflower and *N. occidentalis* plants inoculated with p35SuCMoV showed chlorotic mottling symptoms on inoculated and upper leaves (Fig. 2a and b, respectively) that were similar to those induced by the native virus (Fig. 2c and d, respectively). No symptoms were observed in mock-inoculated plants (Fig. 2e and f, respectively). Therefore, the biological properties of p35SuCMoV tested so far were identical to those of SuCMoV-C. However, symptoms

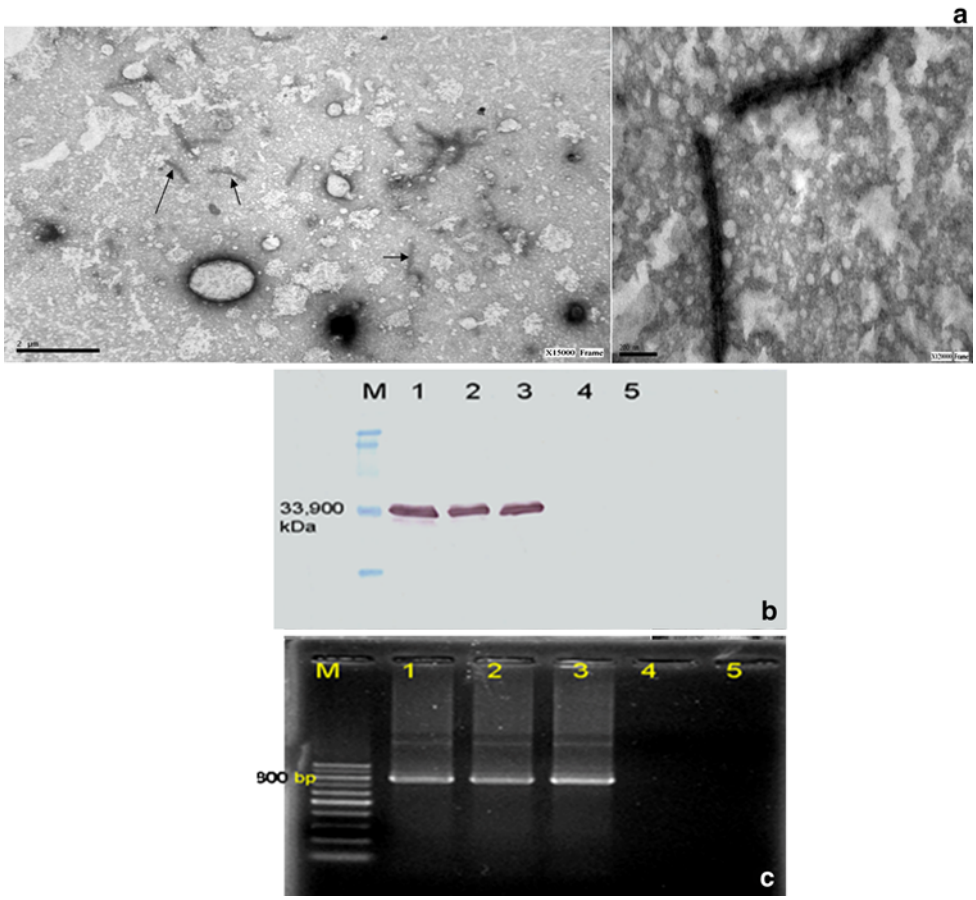

Fig. 3 Electron microscopy (a), western blot (b) and RT-PCR (c) analysis of sunflower plants infected with p35SuCMoV. **(a)** Decorated particles of SuCMoV, with SuCMoV-C polyclonal antisera, observed in the systematically infected leaves of sunflower plants inoculated with p35SuCMoV. The scale bar represents 500 nm. **(b)** Detection of SuCMoV coat protein by western blot and **(c)** amplification of SuCMoV coat protein by RT-PCR. *Lane 1*, inoculated leaf of a plant infected with p35SuCMoV; *lane 2*, systemic upper leaf of a plant infected with p35SuCMoV; *lane 3*, systemic upper leaf of a plant infected with wild-type SuCMoV; *lane 4*, systemic upper leaf of an uninfected plant after inoculation with p35SuCMoV; *lane 5*, mock-inoculated plant as a negative control; *lane M*, protein size marker (b); 100-bp DNA ladder (c)

appeared four days later in plants infected with p35SuCMoV than in those inoculated with wild-type SuCMoV-C. Delayed symptom appearance when potyvirus full-length cDNA clones were inoculated has been reported [6, 13, 15, 19, 25, 30]. One of the possible reasons for delayed appearance of symptoms is the inefficiency of delivery of the full-length cDNA clone to the plant nucleus, where the 35S promoter is active [19]. Gal-On et al. [11] reported a 10-day delay in symptom appearance in plants that were mechanically inoculated compared with plants that were subjected to particle bombardment. Therefore, introducing p35SuCMoV DNA by particle bombardment might avoid a delay in the appearance of symptoms.

Using the immunosorbent electron microscopy with decoration technique (ISEM-D) [31] with SuCMoV-C polyclonal antiserum, numerous filamentous virus particles of about 770 nm in length were found in samples from p35SuCMoV-infected plants (Fig. 3a). In addition, when all inoculated plants were tested by DAS-ELISA using SuCMoV-C polyclonal antiserum, positive results were detected only in plants showing chlorotic mottle symptoms. A 33-kDa protein of SuCMoV was detected in plants infected with p35SuCMoV DNA by western blot analysis, which was performed according to Dujovny et al. [9]. The 33-kDa protein was identical in size to the coat protein (CP) detected in plants infected with SuCMoV wild-type (Fig. 3b). Moreover, RT-PCR analysis performed using primers CP1 (5′-GGTGACAACATAGATGCAGG-3′) and CP2 (5′-ACATGTTACGAACCCCAAGC-3′) showed that a fragment corresponding to the SuCMoV-C CP coding region (807 nt) was only amplified from p35SuCMoV-infected and wild-type-infected plants (Fig. 3c).

When sap from p35SuCMoV- infected sunflower and *N. occidentalis* plants was mechanically inoculated to sunflower seedlings, all plants developed chlorotic mottle symptoms at 8 dpi that were similar to those at symptom onset in plants infected with wild-type SuCMoV (20 plants per inoculum tested, two independent experiments). These results indicate that progeny viruses derived from p35SuCMoV are not phenotypically different from the original virus.

Our results demonstrate the development of a full-length infectious SuCMoV clone that can be transmitted to sunflower and *N. occidentalis* by mechanical inoculation. The full-length cDNA clone of SuCMoV-C and its progeny are infectious, with biological and biochemical properties similar to those of wild type-virus. Development of a full-length infectious SuCMoV cDNA clone will enable functional analysis and virus-tagging experiments. Therefore, p35SuCMoV may be a powerful tool to study not only the genomic determinants involved in the induction of symptoms, but also the viral components that interact with the Rcmo-1 gene.

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