Construction of an infectious full-length cDNA clone of Sunflower chlorotic mottle virus

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ABSTRACT

- Sunflower chlorotic mottle virus (SuCMoV) belongs to the genus *Potyvirus,* and is the most prevalent sunflower virus present in Argentina. Two biologically different strains of SuCMoV have been described in Argentina: the common strain (C) and the chlorotic ringspot strain (CRS). Construction of full-length cDNAs of an RNA virus genome, from which infectious RNA can be transcribed *in vivo* is a crucial step not only towards mapping the genetic determinants involved in symptoms induction, but also to study the virus-sunflower interactions. Therefore, the objective of this work was to construct a full-length cDNA clone of the SuCMoV-C genome and test its infectivity.

- Three overlapping fragments which covered the complete genomic RNA of SuCMoV were amplified and assembled into a plasmid which contains a *Cauliflower mosaic virus* (CaMV) 35S promoter and the *nopaline synthase* (NOS) terminator. The full-length cDNA was constructed so that no extra nucleotide was present at the transcription initiation site and 20 adenine residues were present at the 3´ end of the SuCMoV cDNA clone, which was named p35SuCMoV.

- Mechanical inoculation on *Nicotiana occidentalis* seedlings with 5μg of p35SuCMoV DNA at a concentration of 100 ng/μl in DEPC water resulted in 20% infection. The chlorotic mottle symptoms induced by p35SuCMoV in *N. occidentalis* were identical to those caused by the wild-type SuCMoV on this host. However, a week delay in the establishment of infection was observed in those *N. occidentalis* infected with p35SuCMoV. Potyvirus like particles were observed in crude sap preparation of p35SuCMoV infected plants under electron microscopy. To further confirm that the symptoms observed in the p35SuCMoV-infected plants were induced by p35SuCMoV, we also carried out DAS-ELISA and RT-PCR analysis. All inoculated plants were analyzed for the presence of coat protein by DAS-ELISA using SuCMoV-C policlonal antisera. DAS-ELISA gave positive results only in those *N. occidentalis* showing chlorotic mosaic symptoms. Furthermore, the 5´ and 3´ end of the SuCMoV genome was detected using RT-PCR with sequence-specific primers. The 1495 nt and 1070 nt fragments, respectively, were amplified from p35SuCMoV-infected plants but not from a healthy plant. Finally, when the sap from p35SuCMoV-*N. occidentalis* infected plants was mechanically inoculated to sunflower seedlings, 100% plants developed chlorotic mottle symptoms, which were indistinguishable from that induced by the wild-type SuCMoV. No delay in the establishment of infection was observed. This indicates that the progeny virus from p35SuCMoV DNA inoculated plants is not phenotypically different from the original virus.

- These results demonstrate that the p35SuCMoV cDNA clone is infectious. Thus, this is the first report about the construction of a biologically active, full-length cDNA copy of the SuCMoV RNA genome.

- The infectious intact cDNA copy of the SuCMoV-C genome will be used to map the viral sequences involved in the virus symptoms induction by construction of hybrid viruses between SuCMoV-C and -CRS strains.

Key words: SuCMoV- infectious clone- mechanical inoculation - infectivity- progeny

INTRODUCTION

Sunflower chlorotic mottle virus (SuCMoV) belongs to the *Potato virus Y* (PVY) subgroup in genus *Potyvirus,* and is the most prevalent sunflower (*Helianthus annuus* L.) virus present in Argentina (Dujovny et al, 1998). Two biologically different strains of SuCMoV have been described in Argentina: the chlorotic ringspot strain (CRS), which causes chlorotic ringspots (Giolitti et al, 2010) and the common strain (C), which induces chlorotic mottling (Dujovny et al, 2000) and is the most widely distributed SuCMoV strain in Argentina. Their complete genome has been sequenced (Bejerman et al, 2010). A sunflower line tolerant to SuCMoV was detected and named L33 (Lenardon et al, 2005). Tolerance in L33 is controlled by a single dominant gene designated Rcmo-1 (Lenardon et al, 2005).

To achieve a better control of SuCMoV the basis of the SuCMoV-sunflower interactions needs to be elucidated. The identification of SuCMoV domain(s) involved in the interaction with the Rcmo-1 gene and those responsible for symptoms induction and other biological properties requires the availability of infectious clones. Biologically active *in vitro* transcripts RNAs from full-length cDNA clones with bacterial phage promoters (Johansen, 1996; Nicolás et al, 1996; Puurand et al, 1996; Chiang and Yeh, 1997; Sanchez et al, 1998; Lin et al, 2002; Lee et al, 2011) and *in vivo* infectious transcripts, which are driven by a *Cauliflower mosaic virus* (CaMV) 35S promoter that can be transcribed by an endogenous host RNA polymerase (Maiss et al, 1992; Chiang and Yeh, 1997, Jakab et al, 1997; Sanchez et al, 1998; Yang et al, 1998; Masuta et al, 2000; Lin et al, 2002; Seo et al, 2009) , have been reported for several potyviruses. These tools have contributed to deepen our knowledge of potyvirus genomic organization and to clarify the involvement of each viral genomic protein in potyvirus basic biology (Schaad and Carrington, 1996; Kasschau et al, 1997). In the present study we report the construction of full-length, stable, cDNA clone of SuCMoV genome, from which infectious RNA can be transcribed *in vivo*. The infectivity and symptomatology of the clones were analyzed and compared to those of wild-type SuCMoV-C.

MATERIAL AND METHODS

SuCMoV-C was propagated in sunflower according to Dujovny et al, (1998). Total RNA from sunflower leaves infected with SuCMoV-C was extracted using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and used as template for cDNA synthesis using oligo-dT/*Not*I primer (Tsuneyoshi et al, 1998) 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 1) and Expand Long Template PCR System (Roche, Mannheim, Germany).

Table 1. Primers used to construct the SuCMoV-C cDNA clon and to detect SuCMoV-C.

Primer	Sequence	Purpose	Position	Sense
SwS	CAC<u>ATTTAAAT</u>TAAAACAAAACATCAC AAC	Amplification of fragment A	5´-end	5´-3´
HAMS	CAAG<u>AAGCTT</u>ACCGGTACGCGTGCACA AGTTTTGTGAC		1061-1082	3´-5´
MlS	CTTGTGC<u>ACGCGT</u>TGCTGTTC	Amplification of fragment B	1070-1090	5´-3´
BsS	CATACT<u>CGTACG</u>CTGACATC		5400-5419	3´-5´
AgBS	GAGT<u>ACCGGT</u>CGTACGAGTATGAAAG AATTGGTG	Amplification of fragment C	5408-5431	5´-3´
SaS	CAGC<u>GAGCTC</u>T$_{(20)}$GGCTCCTGATTGAG ATCTGC		3´-end	3´-5´
P1F	CAAAACATCACAACACAAC	Detection of SuCMoV	10-28	5´-3´
P1R	TGCTGTGAGTTACTAATGC		1486-1504	3´-5´

Restriction sites are underlined

Each of the three amplified fragments, named A to C in order from 5´ to 3´, was subcloned in pGEM-T Easy Vector (Promega), purified from gel using the 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 (Skuzeski et al, 1990) 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 leave 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 then

fragment C (5408 -Poly A) digested with the same enzymes was inserted in the plasmid vector to create p35SHAS. The 3′ terminus of the cDNA was modified to contain a long poly(A) tract and a *Sac*I site to proviede a 5′overhang to aid in transcription. In the final step, fragment B (1070-5419) was digested with *Mlu*I and *Bsi*WI and inserted in p35SHAS, which was cut with the same enzymes to obtain the full-length SuCMoV cDNA clone under the control of CaMV 35S promoter and NOS terminator. The resulting vector was denominated p35SuCMoV (Fig. 1), which was further sequenced by genome walking at Macrogen Inc.
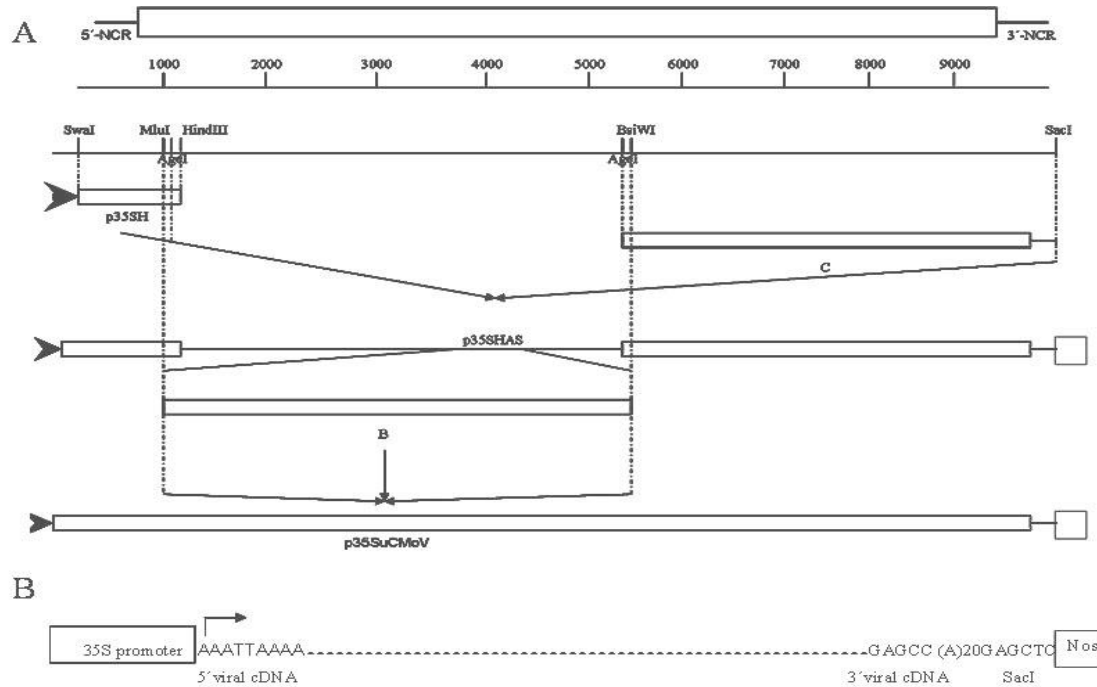
Fig.1. Schematic diagram of the strategy for assembly of SuCMoV-C cDNA clon. Panel A: Construction of the full length cDNA cone behind CaMV 35S promoter. Rectangular boxes and the name below represent the position of the insert and the name of its cDNA clone (vector sequences not represented). A scale (in base pairs) indicates the relative position of restriction enzymes 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 border of the full-length viral cDNA in p35SuCMoV. CaMV35S promoter is shown as a box. The initiation of transcription is indicated by an arrow.

To test p35SuCMoV infectivity, its DNA was purified according to Del Sal et al, (1989) and 5 μg per plant, at a concentration of 100 ng/μl in Diethyl pyrocarbonate (DEPC) treated water, were mechanically inoculated into *Nicotiana occidentalis* at the six-leaf stage. Control seedlings were inoculated with SuCMoV wild-type and other plants were mock-inoculated. Following inoculation, the course of virus infection was followed by the observation of symptoms appearance. All inoculated plants were analyzed by DAS-ELISA using SuCMoV-C policlonal antisera according to Dujovny et al, (1998). RT-PCR analysis of viral RNA was performed following extraction of total RNA from systematically infected leaves using RNeasy Plant Mini Kit (QIAGEN). Total RNA was used as template for one-step RT-PCR, which was performed using the Access RT-PCR System (Promega) with primers P1F and P1R (Table 1) and CP1 (Dujovny et al, 2000) and SaS (Table 1). Moreover, a leaf dip of one of the infected plants was observed under transmission electron microscopy according to Dujovny et al, (1998).

Sap from *N. occidentalis* showing symptoms of viral infection after inoculation with p35SuCMoV was used to mechanically inoculate sunflower plants. Control seedlings were inoculated with SuCMoV wild-type and other plants were mock-inoculated. All mechanical inoculations were performed according to Dujovny et al, (1998).

RESULTS and DISCUSSION

The full length SuCMoV-C cDNA was cloned under the control of CaMV 35S promoter and named p35SuCMoV. The first nucleotide of the SuCMoV-C sequence was placed at the transcription initiation site, and there would thus not be extra nucleotides at the 5´ end of the transcript generated *in vivo*. The 3´ end contained 20 adenine residues, followed by a NOS terminator. It is generally considered that the length of the poly (A) tail affects the infectivity of infectious clones (Boyer and Haenni, 1994). Gal-On et al (1995) showed a cDNA clone lacking the NOS terminator was not infectious. When *E. coli* transformed with p35SuCMoV was incubated at 37ºC, small colonies from which colonies containing the full-length cDNA clone were recovered and good plasmid yields were obtained.

Since the development of the first RNA plant virus biologically active cDNA clone (Ahlquist et al, 1984), many biologically actives clones have been produced which function after transcription *in vitro* or *in vivo*. Despite these many successes, generating biologically active, full-length cDNA clones is still quite a laborious procedure for viruses in the genus *Potyvirus* (Lee et al, 2011). Infectious clones of potyviruses are the starting point to design virus-based expression vectors useful in biotechnological applications (Bedoya and Daros, 2010). Nevertheless, several potyvirus cDNA clones resulted unstable when propagated in *E. coli* and sequence alterations are poduced during culture (Bedoya and Daros, 2010). Among the reasons proposed to explain this instability, the toxicity of the viral gene products expressed from promoters either present in the plasmid or cryptic in the viral cDNA but active in bacteria has been mentioned (Boyer and Haenni, 1994). However, p35SuCMoV was stable when propagated in *E. coli*. p35SuCMoV was recovered intact from three sequential subcultures of *E.coli* in LB medium incubated at 37ºC. Restriction fragment-length polymorphism (RFLP) analysis was performed with p35SuCMoV 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).

Nicotiana occidentalis seedlings mechaniclly inoculated with p35SuCMoV DNA developed symptoms in 20% of the tested plants, whereas 100% of the seedlings inoculated with wild-type SuCMoV developed symptoms. The infectivity efficiency of other potyvirus infectious cDNA clones ranged from 5% to 100% (Gal-On et al, 1995; Nicolás et al, 1996; Chiang and Yeh, 1997; Sanchez et al, 1998; Yang et al, 1998; Lin et al, 2002; Seo et al, 2009). Variability in infectivity efficiencies among infectious cDNA clones have been reported as dependant of the amount of DNA applied per inoculated leaf, host and method of inoculation (Sanchez et al, 1998). Lin et al, (2002) obtained a 100% infection after mechanical inoculation of *Chenopodium quinoa* with a full-length cDNA clone; whereas 0% of infection was obtained when they mechanically inoculated zucchini. Mechanical inoculation is highly variable, probably due to differences in the damage caused to the plant in each experiment (Lopez-Moya et al, 2000). Therefore, infectivity percentages of p35SuCMoV could be improved by optimization of the inoculation procedures and/or the quality of the plasmid preparation. Biolistic technology based on particle bombardment greatly increased infection rates with cloned viral material as compared to mechanical inoculation, because particle bombardment delivers foreign DNA directly to the host nucleus (Gal-On et al, 1995; Lopez-Moya et al, 2000). Gal-On et al, (1995) showed that particle bombardment increased the infectivity of a full-length cDNA clone from 19% by manual inoculation to 100%.

Symptoms on *N. occidentalis* inoculated with p35SuCMoV consisted in a chlorotic mottle, which were detected 17 days post-inoculation (dpi) (Fig. 2a). Such symptoms were identical to those induced by the wild-type SuCMoV on this host (Fig 2b), which appeared at 7 dpi. No symptoms were observed in mock-inoculated plants (Fig. 2c). Therefore, the biological properties of p35SuCMoV tested so far were identical to those of SuCMoV-C. However, in *N. occidentalis* infected with p35SuCMoV, symptoms appeared 7 days later than in plants inoculated with wild-type SuCMoV. Maiss et al, (1992); Chiang and Yeh, (1997); Jakab et al, (1997); Yang et al, (1998); Seo et al, (2009) and Lee et al, (2011) also reported a delay in symptom establishment when they inoculated potyvirus full-length cDNA clones. Possible reasons for symptoms appearance include the inefficiency of delivery of the full-length cDNA clone to the plant nucleus where the 35S promoter is active, or the possible introduction of sequence errors in the cDNA copy (Maiss et al, 1992). Such errors may have introduced mutations that altered the performance of p35SuCMoV. Therefore the p35SuCMoV complete sequence was determined. 11 nucleotide differences were found between SuCMoV-C and p35SuCMoV. 9 differences in the coding region resulted in silent mutations while 2 resulted in amino acid substitutions, which were located in CI coding region.
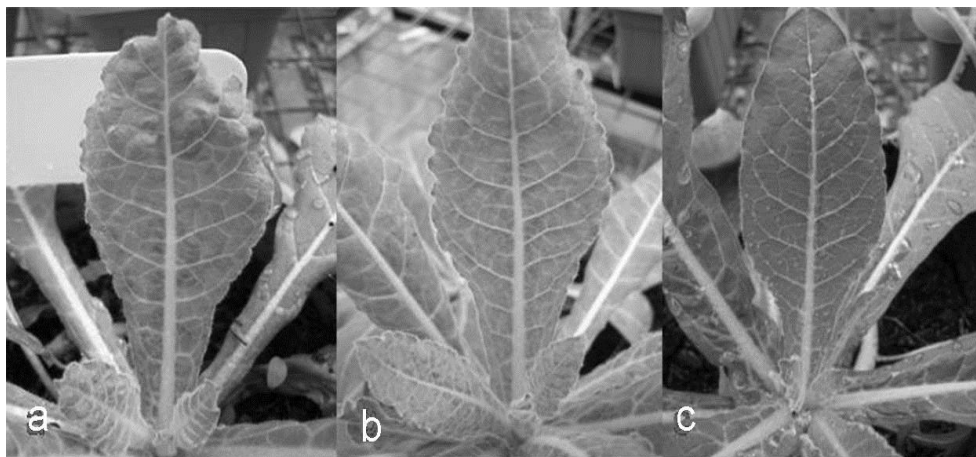
Fig. 2. Symptoms in *N. occidentalis* inoculated with p35SuCMoV (a) and wild type SuCMoV-C (b). *N. occidentalis* mock inoculated (c). Photographs were taken 24 days post-inoculation.

Characteristic flexuous filamentous potyvirus-like particles were observed in crude sap preparation of p35SuCMoV infected plants (data not shown). Plants were analyzed for the presence of coat protein by DAS-ELISA. Positive results were observed only in plants showing chlorotic mottle symptoms. The 5´ and 3´ ends of the SuCMoV-C genome were detected using RT-PCR with sequence specific primers. The 1495 nt and 1070 nt fragments, respectively, were amplified from p35SuCMoV-infected plants but not from a healthy plant (data not shown).

When the sap from p35SuCMoV-*N. occidentalis* infected plants was mechanically inoculated to sunflower seedlings, 100% of the inoculated plants, developed chlorotic mottle symptoms 10 days after inoculation, which were indistinguishable from those induced by the wild-type SuCMoV. A delay in the establishment of infection was not observed. This indicates that the progeny derived from p35SuCMoV is not phenotypically different from the original virus.

These results demonstrate that the p35SuCMoV cDNA clone is infectious. Therefore p35SuCMoV will be a powerful tool to study not only the genomic determinants involved in the virus symptoms induction, but also the domains that interact with Rcmo-1 gene. To our knowledge, this is the first report of the construction of a biologically active, full-length cDNA copy of the SuCMoV RNA genome.

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