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A maize non- infectious clone from *Sugarcane mosaic virus* can move in detached tobacco leaves

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Abstract

Background: Infectious clones are copies of a virus genome produced *in vitro* or inside a vector and can infect inoculated healthy plants or cells. They are important tools to study the molecular biology of viruses.

Questions: Is it possible to construct a *Sugarcane mosaic virus* infectious clone using methods suggested by other authors?

Studied species: *Sugarcane mosaic virus* isolate Veracruz 1, *Zea maize* line B73, *Nicotiana rustica.*

Methods: The Mexican viral isolate *Sugarcane mosaic virus* Veracruz 1 was used to construct an infectious clone after segment amplifications of the virus genome, intron insertion, and their further fusion by yeast homologous recombination. The clone was under the regulation of the *Cauliflower mosaic virus* 35S promoter and was tagged with the green fluorescent protein. Clones were then used to inoculate maize and tobacco plants and detached tobacco leaves.

Results: The viral clone obtained did not produce symptoms in inoculated maize plants. However, the viral Coat Protein and Viral Protein genome-linked cistrons, and the green fluorescent protein signal were detected in both inoculated maize and in tobacco plants.

Conclusions: We hypothesize that the ability of the clone obtained to infect tobacco is due to the use of the 35S promoter. This is the first monocot-infecting viral clone, that we are aware of, being able to be expressed in a dicot plant species.

Keywords: Infectious clone, maize, SCMV, tobacco.

Resumen

Antecedentes: Las clonas infecciosas son copias del genoma de un virus producidas *in vitro* o dentro de un vector. Cuando son inoculadas, pueden infectar plantas o células sanas. Son herramientas importantes en el estudio de la biología molecular de los virus.

Preguntas: ¿Es posible construir una clona infecciosa del *Sugarcane mosaic virus* usando métodos sugeridos por otros autores?

Especies de estudio: *Sugarcane mosaic virus* aislamiento Veracruz 1, *Zea maize* línea B73, *Nicotiana rustica.*

Métodos: El aislamiento viral mexicano *Sugarcane mosaic virus* Veracruz 1 se usó para construir una clona infecciosa basada en la amplificación de fragmentos del genoma viral, la inserción de un intrón y su posterior fusión mediante recombinación homologa en levadura. La clona está regulada por el promotor 35S del *Cauliflower mosaic virus y* también, fue marcada con la proteína verde fluorescente. Después, fue usada para inocular planta de maíz y plantas y hojas escindidas de tabaco.

Resultados: La clona no produjo síntomas en plantas de maíz. Sin embargo, los cistrones de la proteína de la cápside y la proteína viral unida al genoma y la señal de la proteína verde fluorescente se detectaron en plantas inoculadas de maíz y de tabaco.

Conclusiones: Se hipotetiza que la habilidad de la clona para infectar tabaco se debe al promotor 35S. Esta es la primera clona viral infecciosa de monocotiledóneas, de la que tenemos conocimiento, que es capaz de expresarse en una planta dicotiledónea.

Palabras clave: Clona infecciosa, maíz, SCMV, tabaco.

The potyviruses, RNA viruses that belong to the Potyviridae family, comprise the largest group of pathogenic RNA plant viruses (Roossinck 2012). The members of this family account for approximately 30 % of the known plant viruses. They possess a +ssRNA genome encoding for a polyprotein which, after being processed, releases eleven mature proteins (Revers & García 2015). The interactions of these proteins between themselves and with the host proteins trigger the establishment of an infection in susceptible individuals (Dunoyer *et al.* 2004, Kim *et al.* 2008, Kannan *et al.* 2020). Developing strategies against viral infections require fully understanding the function of the viral proteins in different hosts. For such understanding the use of infectious clones as a tool is an appealing alternative.

An infectious clone is a copy of a virus genome produced *in vitro* or inside a vector that maintains its ability to infect inoculated healthy plants (or cells derived thereof) (Vos *et al.* 1988, López-Moya *et al.* 1999). Infectious clones also help to understand the response of the plant to viral infections (Ullah & Grumet 2002, Plisson *et al.* 2003, Tatineni *et al.* 2014). If the infectious clones are tagged with a tracking protein, such as the green fluorescent protein (GFP), they can render valuable information about virus tropism (Dietrich & Maiss 2003, Takahashi *et al.* 2007, Bedoya *et al.* 2012). Infectious clones can be engineered to express host proteins at high levels that could lead to the silencing of host genes. These engineered vectors are used in viral-induced gene silencing (VIGS) (Liu *et al.* 2002, Lu *et al.* 2003a, Chen *et al.* 2014) which is especially useful in crops that are hard to genetically engineer as maize, tomato and pepper.

A variety of methodologies to obtain infectious clones have been used. Among them, the Gibson assembly has been shown to be efficient to obtain and engineer infectious clones. This method involves assembly in an isothermal reaction (~50 °C) with a mix of three enzymes: an exonuclease acting over 5' ends, a DNA polymerase, and a *Taq* DNA ligase. These enzymes join covalently PCR-generated fragments with overlapping ends (Gibson *et al.* 2009). Some examples of viral infections clones obtained using this methodology are *Lettuce mosaic virus* (LMV) (Bordat *et al.* 2015), *Tomato blistering mosaic virus* (ToBMV) (Blawid & Nagata 2015), *Bean golden mosaic virus* (BGMV) (Ferro *et al.* 2019), and *Beet chlorosis virus* (BChV) (Wetzel *et al.* 2018). However, Gibson assembly efficiency decreases in relation with the GC content (Li *et al.* 2018). A high GC content can lead to mismatches, high annealing temperatures, self-dimerization, and/or secondary structure formation not only in primer design but also leading to hairpin formation which interferes with primer annealing or overlapping ends (Kumar & Kaur 2014). Across potyvirus genomes, high or low GC content regions are not conserved (Nigam *et al.* 2019), which could be one of the reasons why Gibson assembly (or other sequence-homology based methods) cannot be globally used for cloning potyvirus genomes.

Sugarcane mosaic virus (SCMV), a potyvirus, causes mosaics and dwarfing in sorghum, sugarcane, and maize plants. as with other potyviruses, it possess a single stranded positive sense RNA (+ssRNA) genome which encodes 11 viral proteins: P1, HC-Pro, P3, PIPO, 6K1, CI, 6K2, VPg, NIa, NIb, and CP (Revers & García 2015). Even though reports about the disease caused by SCMV date to 1900 (Teakle & Pritchard 1971) only a SCMV clone has been successfully produced in recent years (Mei *et al.* 2019b). The fact that in 40 years only one clone has been obtained for a long-known virus gives an insight into the difficulty of obtaining clones for this viral family. However, in 2012, Desbiez obtained an infectious clone of the *Turnip mosaic potyvirus* (TuMV) in less than two weeks. This methodology is based on the *in silico* division and amplification of segments of all the viral genome and their later joining by homologous recombination in yeast (Desbiez *et al.* 2012). This protocol also includes the insertion of introns in the potyviral P3 and CI cistrons, which have been reported to be toxic in *E. coli* cells, hindering further cloning steps (Johansen 1996). To obtain an infectious clone from a Mexican SCMV isolate (SCMV-Ver1), we followed the Desbiez methodology. Here, we report the development of two SCMV clones containing maize glutathione S-transferase (GST)-derived introns: the first, IC-SCMV and the second, IC-SCMV::GFP (GFP-tagged). IC-SCMV inoculated maize plants showed no mosaic symptoms even at 30 days post inoculation (dpi). As this clone failed to produce symptoms like the wild type (WT) virus it was deemed not functional (or non-infectious). However, when whole tobacco plants were used as bombardment controls, assuming they would not be infected, SCMV CP-derived cistrons and fluorescence were observed not only in the bombarded site but also in distal regions. These results suggest the ability of this IC-SCMV to replicate and move in tobacco tissue as opposed to the wild SCMV isolate.

Materials and methods

Virus, inoculation, and plant material. The *Sugarcane mosaic virus* Veracruz 1 isolated (SCMV-VER1; GenBank: EU091075.1) was used as the viral source to obtain an SCMV infectious clone. SCMV-symptomatic leaves were collected in maize fields in the state of Veracruz and stored at -70 °C. Approximately 500 mg of leaves were ground and mixed with1 ml of PBS buffer (0.15 mM K_2HPO_4, 15.5 mM NaCl, 0.27 mM Na_2HPO_4-$7H_2O$, pH 7.2), and 200 mg of carborundum (mesh 400; as abrasive agent). The mixture was used to inoculate maize plants (*Zea maize* ssp. *mays*) B73 line (CIMMYT seed bank, request numbers S2018_400684999 and S2020_623050816), or from a commercial Rogers cultivar. Plants were maintained in two safety greenhouses (devoted to plant-virus experiments) allowing to assess cross contamination. Leaves were collected when they showed the characteristic SCMV mosaic symptoms. To verify the SCMV presence in the sample, RT-PCR was conducted using a Taq polymerase recombinant (Thermo Scientific, Vilnius, Lithuania) and the SCMV-CP-Sn and SCMV-CP-An primers (Table 1) following the manufacturer instructions.

Production of clone fragments. To produce the SCMV infectious clone, the full-length genome sequence of the SCMV-Ver 1 isolate (9,583 bp) was divided *in silico* into four viral fragments with nucleotide positions in: fragment A (1 to 2,741), fragment C (2,752 to 4,802), fragment E (4,803 to 7,118), and fragment F (7,073 to 9,583). Then, primers were designed for the 5'and 3'ends of each fragment. To amplify the viral fragments, RNA was extracted from maize leaves showing SCMV mosaic symptoms. Leaves were ground in a mortar with a pestle with liquid nitrogen. Approximately 500 mg of virus-infected tissue was placed in a 1.5 ml Eppendorf tube and 1 ml of TRIzol (Thermo Fisher, Carlsbad, California, USA) reagent was added. The samples were processed following the manufacturer instructions and RNA was obtained. RNA integrity was evaluated by loading 2 µl of the sample in an agarose gel (2 %) and observed in a Gel Doc XR+ Imaging System (BioRad, Hercules, CA, USA). Also, RNA concentration was measured in a Nanodrop 2000 (Thermo Scientific, Washington, USA).

Then, the synthesis of the first cDNA strand was conducted using 2 µl (200 ng/µl) of total RNA and the Revert Aid H minus RT enzyme (Thermo fisher, Vilnius, Lithuania) following the manufacturer instructions. For the cloning of viral fragments C, E, and F a PCR reaction was performed using 2 µl of cDNA, the set of primers (Table 1), for each fragment (1 µl of each primer at 10 mM), and the recombinant Taq polymerase (5 u/µl, Thermo Scientific), following the manufacturer instructions and adjusting the $MgCl_2$ concentration at 2 mM. For the fragment A, the cloning was performed with the Phusion Green Hot Start II High-Fidelity polymerase (Thermo Scientific, Vilnius, Lithuania), the PCR mix contained 25 µl of the 2x Master Mix, 2.5 µl of FwA and RvA primers (10 mM), 0.5 µl of $MgCl_2$ (25 mM), 4 µl of cDNA, and 18 µl of sterile water. The PCR program was performed as recommended by the manufacturer with an annealing temperature of 60°C and an extension time of 2.5 min.

Glutathione-S-transferase intron cloning. Following previous suggestions of intron addition to obtain infectious clones (Johansen 1996, Desbiez *et al.* 2012), we chose the maize-GST intron sequence. To clone and add overlapping ends in the GST intron, 2 µl (200 ng/µl) of total DNA extracted with the Murray protocol (Murray & Thompson 1980) from B73 maize plants were used as a template in a PCR reaction with the recombinant Taq polymerase (Thermo Scientific, Vilnius, Lithuania) and the FwB/RvB or the FwD/RvD primers following the manufacturer instructions.

Fragment fusion. To fuse either fragments B with fragment C or fragment D with E, a fusion PCR was conducted. The PCR reaction was performed using the Fw B and Rv C primers and a mix of 1 µl of each B (100 ng/µl) and C (100 ng/µl) purified fragments as templates. The same strategy was used for the fusion of fragments D and E.

Even though both fragments B and D have the sequence of the GST intron, they differ in their overlapping ends. Fragment B possesses overlapping sequences at its 5' and 3' ends with fragments A and C, respectively. Fragment D has overlapping sequences at its 5'and 3'ends with fragments C and E, respectively. After the PCR fusion, fragments B+C and D+E were obtained. The fused fragments were purified and stored at -20 °C until use.

Table 1. List of Primers used in this work.

Name	Sequence	Viral Fragments Overlaped by the primers	Amplicons
35S Sn	GATAAGCTTGATATCGAATTCCTGCAGCCCAA-CATGGTGGAGCACGACAC	PRS424	CaMV promoter
35S An	TGTTGTGTTGTGTTGAGTTTTGTTGTTTTT-GTCCAAATGAAATGAACTTCCTTA	Frag. A	
FwA	AAAAACAACAAAACTCAACACAAC	-	Viral frag. A
RvA	ACAGTATGATAGTTCTGCCCACG	-	(P1/HC-Pro/P3)
FwB	AACCAATCGTGGGCAGAACTATCATACTGT-GTCGACCAGTTGATTGTCAA	Frag. A	GST intron
RvB	GAACACACGCCATATTGCTGAAAATTTTCCCT-GCAGAGAGAGATAGATAACC	Frag. C	
FwC	CAGCAATATGGCGTGTGTT	-	Viral Frag. C (P3/6K1/CI)
RvC	AATCTGTGGGTGCATAGAGC	-	
FwD	AAATTTGACGGCTCTATGCACCCACAGATT-GTCGACCAGTTGATTGTCAA	Frag. C	GST intron
RvD	TCTCAATTTGTACTTGGTTAACGCTTCATGCT-GCAGAGAGATAGATAACC	Frag. E	
FwE	CATGAAGCGTTAACCAAGTAC	-	Viral Frag. E (CI/6K2/NIa/NIb)
RvE	TGCCTTTTCAAAGATATCGTAG	Frag. F	
FwF	GCCGATTTACGTAGGTGAAA	Frag. E	Viral Frag. F (NIb/CP)
RvF	GGTCTCTTACCAAGACACTCG	-	
GFP-Sn	[TCAGGTACA]ATGGTGAGCAAGGGCGAG	-	GFP +
GFP-An	[TTGAAAGTAGAGGTTCTCTGTAGTTT]ACTT-GTACAGCTCGTC	-	[proteolytic motif]
Tnos-Sn	GTAAGAGAC-CAAAAAAAAAAAAAAAAAAAACAGCTC-GAATTTCCCCGA	Frag. F (CP region)	NOS terminator
Tnos-An	GGCGGCCGCTCTAGAACTAGTGGATCCCCC-GAGCTCCACCGCGGTGGCGG	PRS424	
NIb-GFP	CTCCTCGCCCTTGCTCACCATTGTACCTGATT-GATGGAAAACATCTTC	Frag. F-one	5' GFP
GFP-CP	AAGTAAACTACAGAGAACCTC-TACTTTCAATCCGGTTCTGTGGATGCA	3' GFP	3'GFP
1090	CTCCTCGCCCTTGCTCACCATTGTACCTGATT-GATGGAAAACATCTTC	Frag. F-three	5' NI-b
1095	AAGTAAACTACAGAGAACCTC-TACTTTCAATCCGGTTCTGTGGATGCA	Frag. F-three	5'CP
SCMV-CP Sn	AACCAGTGGCTCAGGAAC	-	SCMV cistron
SCMV-CP An	TTGTTCATCTCCGTCCATCA	-	SCMV cistron
SCMV-VPg Sn	CACCAAGGGAAGAACAAGCG	-	VPg cistron
SCMV-VPg An	GGTGCTGTCACGCTGCTCTCTG	-	VPg cistron

The 35S promoter fragment was cloned from the pTRV2 plasmid (Liu *et al.* 2002). For the terminator, the nopaline synthase terminator (Tnos) sequence was cloned from the Gateway plasmid pJCV51 (VIB-UGent Center for Plant Systems Biology; Mansour Karimi and Julian Verdonk). Each fragment was cloned by PCR with the Taq polymerase recombinant following the manufacturer instructions and using the respective set of primers (Table 1). Fusion PCR was also used to add overlapping sequences to the 5'and 3' ends of the 35S fragment with the 3' end of the PRS424 shuttle vector and the 5' end of the fragment A, respectively. In the same way, we performed fusion PCR to add overlapping sequences to the Tnos fragment 5' and 3' ends with the 3' end of fragment F and the 5' end of PRS424, respectively.

Yeast homologous recombination. The six fragments synthesized by PCR (A, B+C, D+E, F, Tnos, and 35S), and the linearized PRS424 vector (each at 10 ng/µl) were mixed. The mix was used to transform the W303a yeast line, following the Gietz protocol (Gietz *et al.* 1995). The transformed yeast cells were spread using glass pearls in a Petri dish containing minimum yeast medium lacking tryptophan (previously treated by immersion in 1 M HCl and oven-dried at 200 °C overnight). The Petri dishes were incubated for 2 days at 30 °C and the largest yeast colonies were chosen for DNA extraction. Yeast DNA was further used to transform electrocompetent DH5α *E. coli* cells to increase the copy number of the SCMV-clone. Transformed bacterial cells were cultivated in LB medium with carbenicillin (1 mg/ml). Antibiotic-resistant colonies were chosen, and plasmids were extracted by alkaline lysis (Ehrt & Schnappinger 2003).

GFP-tagged clone. Three new fragments were produced to insert the GFP sequence between the viral NI-b and CP cistrons. The fragment F-one, synthesized by PCR (Fw F/1090), binds to the viral NIb cistron and includes an overlapping region with the GFP fragment. The fragment F-two, also synthesized by PCR (1,095/Rv F), binds to the CP cistron and includes an overlapping region with the GFP fragment. And finally, fragment F-three with the GFP sequence cloned by PCR (GFP-Sn/GFP-An) from the pCAMBIA vector. Additionally, the fragment F-three includes the P1 (DNA sequence "TCAGGTACA" coding for SGT) and the NIa artificial proteolytic site (DNA sequence "TTGAAAGTAGAGGTTCTCTGTAGTTT" coding for TTENLYFQ/SGT). Those viral self-proteolytic domains guarantee the release of the mature protein according to Bedoya protocol to tag infectious clones (Bedoya *et al.* 2012). SGT and TTENLYFQ/SGT sites had overlapping sequences with the F-one and F-two fragments added by fusion PCR.

The SCMV clone fragments (A, B+C, and D+E) were mixed with the GFP fragments (F-one, F-two, and F-three) and used to transform yeast. The selection of colonies and the purification of the clone was conducted in the same way as the untagged clone.

SCMV clone integrity. To discard SCMV clones assembled with incomplete fragments, plasmids were used as templates in PCR reactions with each pair of primers for the fragments. Only the plasmids amplifying all segments were selected for further work. After the first step of selection, 2 µl of the plasmids were digested with the Eco57I enzyme (Thermo Scientific, Vilnius, Lithuania). Only colonies with digestion patterns matching the *in-silico* predictions made in the SnapGene software were chosen.

Maize inoculation with SCMV clone. Two inoculation methods were evaluated. In the first, maize plants from the susceptible Rogers and B73 lines were mechanically inoculated at the third true leaf with a mix of 200 mg of carborundum, the plasmid containing the SCMV clone (1 ng/µl), and 1.5 ml of 1X PBS buffer. After inoculation, plants were maintained in isolated chambers inside the safety greenhouse and were daily monitored for the appearance of the viral mosaic symptoms.

In the second method maize and tobacco plants (*Nicotiana rustica*) were bombarded with tungsten particles. Particles were prepared following the protocol mentioned in Méndez-Lozano *et al.* (2003). Maize plants were bombarded in the third true leaf, around the ligule zone. The tobacco plants were bombarded approximately 1 mm from

the central vein. After bombardment, plants were placed in a growing chamber (16/8 h day/night period at 30 °C) and daily observed for the presence of mosaics.

Detection of viral CP and VPg cistrons. For the cloning of the CP and VPg viral cistrons, a PCR reaction was performed using 2 µl of cDNA, and the set of primers of SCMV-CP Sn and SCMV-CP An, and SCMV-VPg Sn and SCMV-VPg An, at 10 mM each (Table 1). The recombinant Taq polymerase (5 u/µl, Thermo Scientific) was used following the manufacturer instructions and adjusting the $MgCl_2$ concentration to 2 mM. The PCR program was performed with an initial denaturation at 95 °C for 3 min, followed by a step of denaturation at 95 °C for 30s, annealing at 60 °C for 30s; finally, an extension step at 72 °C for 1 min, in 35 cycles. The PCR products were visualized in an agarose gel (2 %) with a Gel Doc XR+ Imaging System (BioRad, Hercules, CA, USA).

Inoculation of tobacco detached leaves. To inoculate tobacco leaves, fully mature leaves of *Nicotiana rustica* were visually divided into four sections (L1-L4). Section L1 was mechanically inoculated with a mix of 200 mg of carborundum, the plasmid solution containing the SCMV clone (1 ng/µl), and 1.5 ml of 1X PBS buffer. After inoculation, leaves were cut in the petiole at the most proximal section to the stem. Then cut leaves were incubated in a wet chamber at 28 °C for 6 days.

GFP detection. After 6 dpi in the growing chamber, discs from the inoculated leaves were cut and placed in a glass slide adding 50 µl of water to maintain tissue turgor. Different regions within each disc were observed using an Olympus BX60 microscope (Shibuya-ku, Tokyo, Japan) coupled with BX-FLA reflected light fluorescence attachment (high-pressure mercury burner and halogen lamp), with a UM41014 cube, a BP460-490 exciter filter, and a BA510IF barrier filter. Images of the observed region were captured, and the scale bar was added using the Image-Pro Plus software v. 4.0.

Results

Production of an SCMV infectious clone. We generated an SCMV clone following the methodology suggested by Desbiez (Desbiez *et al.* 2012). The whole viral genome (9.6 kb) was divided *in silico* into four fragments of comparable size, named A (2.7 kb), C (2kb), E (2.3kb), and F (2.5kb) (Figure 1A, B). The A, C, E, and F fragments were cloned from cDNA synthesized from total RNA extracted from SCMV-infected maize leaves. The C, E, and F fragments were obtained using a recombinant Taq polymerase, but the Phusion Green Hot Start II High-Fidelity polymerase PCR master mix, was used for the A fragment. Following the same methodology, we cloned and purified the sequence of the maize GST intron which was inserted between the CI and P3 viral sequences (Desbiez *et al.* 2012). After cloning and purification, all fragments were joined by homologous recombination in W303 alpha yeast strain which yielded twenty-seven candidate colonies.

DNA was extracted from the candidates and used as a template in PCR for all the SCMV clone fragments. Only two colonies (10 and 14) showed amplification for all the SCMV clone components and the DNA of each was used to transform electrocompetent DH5α *E. coli* cells and sixty-one colonies were recovered. Their plasmids were extracted, purified, and digested with the Eco57I restriction enzyme. A total of thirty-five colonies that did not match the predicted restriction pattern for the full clone were discarded. The presence of the promoter, terminator, and the other components was also evaluated in the selected colonies. Only two colonies (numbered as 15 and 19), amplified all the viral components. Both colonies came from the same yeast transformation event from colony 10 (mentioned above). The two plasmids containing all the clone's fragments were named IC-SCMV-15 and IC-SCMV-19 and used in further experiments.

IC-SCMV Inoculated maize plants do not show mosaic symptoms. IC-SCMV-15 and IC-SCMV-19 were mechanically and ballistically inoculated in leaves of B73 and Rogers susceptible maize plants. Also, maize leaves were inoculated with wild SCMV-WT as an inoculation control. The SCMV-WT inoculated plants showed the presence of

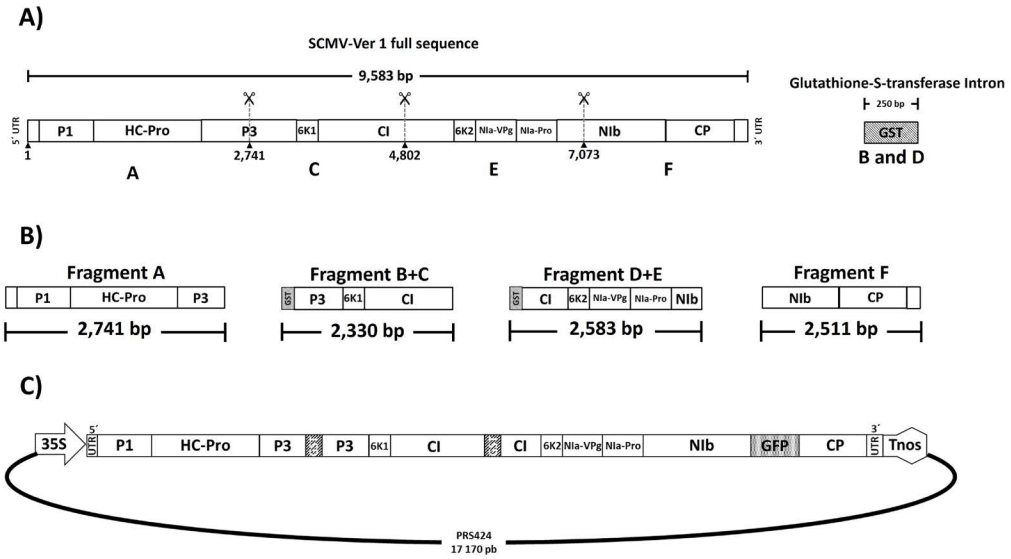

Figure 1. Schematic representation of the SCMV-VER1 genome division for fragments generation resulting in a genomic clone after homologous recombination. A) The genome of the SCMV-Ver1 isolate (9,583 bp) was divided *in silico* into four segments at specified nucleotide positions: A (1 to 2,741), C (2,752 to 4,802), E (4,803 to 7,118), and F (7,073 to 9,583). B) Four fragments of similar length were generated, and the intron from the Glutathione-S-transferase gene (250 bp) was cloned from maize B73 plants and fused to fragments C and E creating the B+C and D+E fragments. C) After homologous recombination, a cDNA clone derived from SCMV-Ver1 (10,130 bp) with introns between P3 and CI viral proteins, the 35S promoter of the CaMV virus, and the nopaline synthase terminator (Tnos) inside the PRS424 vector was generated. P1 = Protein 1; HC-Pro = Helper component protein; P3 = Protein 3; 6K1 = 6 kDa protein 1; CI = Cytoplasmic inclusions protein; 6K2 = 6 kDa protein 2; NIa-VPg = Nuclear inclusion protein a, genome linked viral protein domain; NIa-Pro = Nuclear inclusion protein a, proteolytic domain; Nib = Nuclear inclusion protein b; CP=coat protein.

mosaic symptoms 9 days post-inoculation (dpi). In contrast, no mosaic symptoms were observed with IC-SCMV-15 or IC-SCMV-19 inoculation, either mechanically or ballistically (Figure 2A). In biolistic inoculation experiments, only leaves inoculated with IC-SCMV-19 showed discoloration in the vicinity of the inoculated area after 15 dpi (Figure 2A). Distinctively, the mechanically inoculated plants showed a pale mosaic discoloration pattern along the leaf blade (Figure 2A). As these discoloration patterns have not been reported as SCMV symptoms the plants were considered non infected.

Due to the absence of a clear mosaic pattern to visually ascertain the viral infection in maize plants, RNA extraction, and RT-PCR were conducted using primers for the proteins of the CP and VPg viral cistrons. Both cistrons were detected in susceptible infected B73 maize plants symptomatic for SCMV. Infected B73 was further used as a positive control (C+). Both IC-SCMV clones inoculated in plants of the two maize lines (B73 and Rogers) showed amplification of the two viral cistrons (Figure 2B, C). To discard cross contamination between wild type SCMV (WT) and IC-SCMV-15, RNA extraction and RT-PCR were performed. Inoculated IC-SCMV-15 and non-inoculated B73 plants grown in separate greenhouses were used as samples. The CP and VPg viral cistrons from SCMV were only detected in the IC-SCMV inoculated plants (Figure 2C).

Further inoculations showed SCMV CP or VPg cistron presence in more of the IC-SCMV-19 inoculated plants than in IC-SCMV-15. Thus, IC-SCMV-15 was not used in further experiments. As the IC-SCMV-19 clone did not produce SCMV-WT like symptoms, it was considered non-infectious.

IC-SCMV was tagged with a fluorescent (GFP) protein. SCMV clone can be used as a vector for the transient expression of genes in maize. Even with the absence of symptoms, viral cistrons were detected in clone-inoculated plants. To evaluate if the IC-SCMV-19 clone can be used as an expression vector, the insertion of GFP was done. First, we obtained new fragments containing the GFP sequence (F-two), and two viral self-proteolytic domains, one cor-

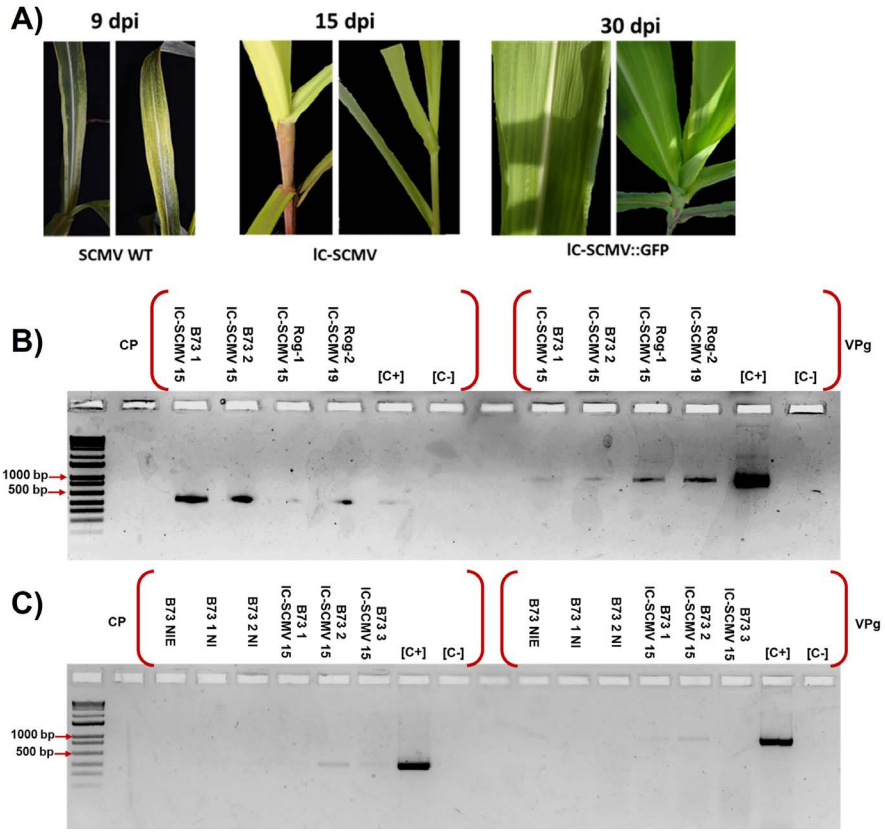

Figure 2. Presence and absence of symptoms in mechanically wild type-SCMV and ballistically IC-SCMV/IC-SCMV::GFP-inoculated plants and detection of the CP and VPg viral cistrons in maize leaves. A) Symptoms were observed in SCMV WT inoculated plants at 9 dpi (left). In contrast, plants inoculated with IC-SCMV (center) or IC-SCMV::GFP (right) do not show mosaic symptoms at 30 dpi. B) SCMV-CP and SCMV-VPg cistrons were detected in IC-SCMV bombarded maize plants in susceptible B73 and Roger (Rog) lines. A B73 SCMV infected plants was used as positive control (C+) and a PCR reaction without DNA was used as negative control (C-). C) Absence or presence of CP and VPg in three non-inoculated (NI) and three IC-SCMV 15 inoculated B73 plants. Letter E refers to plants from a separate greenhouse. SCMV-CP and SCMV-VPg cistrons were detected in IC-SCMV15 in two inoculated B73 plants.

responding to P1 (SGT; F-one) and other the NIa (TTENLYFQ/SGT; F-three) (Bedoya *et al.* 2012) in the 5' and 3' ends. To assemble the clone, a mixture containing fragments A, B + C, D + E, and the newly synthetized fragments (F-one, F-two, and F-three) were used to transform yeast. A clone containing GFP inserted between the potyviral NI-b and CP was generated and named IC-SCMV::GFP.

GFP fluorescence was detected in maize and tobacco leaves IC-SCMV::GFP inoculated. Initially, the IC-SCMV::GFP clone was ballistically inoculated in two B73 maize plants. Tobacco plants were also inoculated as a technical control. After 4 days post-bombardment (dpb), tissue sections from both plant species were cut off from the immediate vicinity and the regions around the bombarded lesion. The sections were mounted on a slide and observed under a fluorescence microscope. Fluorescence was detected in both maize and tobacco tissue samples (Figure 3) as early as 4 dpb. Surprisingly, tobacco leaves showed fluorescence, not only in the vicinity of the lesion (Figure 3E) but also in distal regions (Figure 3F) of the inoculation site.

SCMV clone infects tobacco leaves while wild type SCMV cannot. Because the tobacco plants used as controls showed fluorescence, we wanted to discard the bombardment effect and tried the mechanical inoculation method for the SCMV clone delivery. Tobacco plants were mechanically inoculated with the IC-SCMV :: GFP clone (in a mix

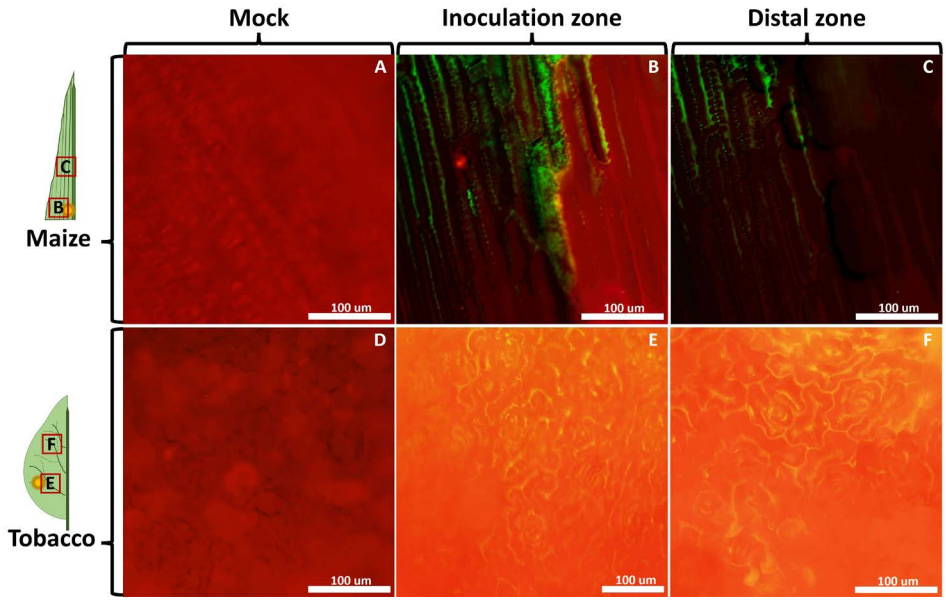

Figure 3. GFP fluorescence in maize and tobacco plants after ballistic inoculation with the SCMV-GFP clone. The IC-SCMV::GFP clone was ballistically inoculated in B73 *Zea mays* and *N. tabacum* (initial Bombardment control). GFP fluorescence was detected in the vicinity (B and E) and in a distal (C and F) region to the inoculation area (yellow spot) at 4 dpb (days post bombardment) in maize and tobacco plants. Mock inoculated controls (A and D) do not show fluorescence.

with 1X PBS buffer and carborundum) and samples were taken at 6 dpi. Fluorescence was detected in all mechanically inoculated leaves showing that the fluorescence was not due to the inoculation method.

To evaluate the ability of the IC-SCMV :: GFP clone to move, a new assay was conducted. Tobacco leaves were visually divided into L1 to L4 sections (Figure 4A). L1 section was mechanically inoculated with the IC-SCMV :: GFP clone, then the whole leaf was detached from the tobacco plant and placed in a wet chamber at 28 °C for six days. A leaf tissue disc was cut from each section (L1 to L4), mounted on a slide, and observed under a fluorescence microscope. Fluorescence was detected in all four sections (Figure 4A). After fluorescence detection, RT-PCR was conducted using RNA extracted from each section and the presence of the SCMV-CP and SCMV-VPg cistrons was searched. SCMV-CP was detected in all the leaf sections confirming the presence of the clone (Figure 4B). In contrast, RT-PCR assays using RNA derived from tobacco plants mechanically inoculated with wild type SCMV did not show SCMV-CP cistron amplification even at the L1 inoculation site (data do not show).

Also, to evaluate if the nature of the inoculum interfered with the establishment of an SCMV infection, viral like particles (VLPs) were extracted from maize infected leaves to inoculate tobacco. However, no CP cistron was detected after using SCMV-VLPs as viral inoculum.

Discussion

The development of infectious clones represents an advantageous tool in the understanding of the complex interactions between viruses and plant hosts. Especially when such interactions lead to the establishment (or not) of an infection. By using infectious clones, the study of the members of the *Potyviridae* family is showing to be especially enlightening. The viral cycle of potyviruses relies heavily in the interactions between viral and host proteins (Revers & García 2015). Dissecting how, where and when this interaction occurs could derive in the development of resistant lines to viral infection.

Furthermore, infectious clones can become vectors to the expression of host proteins or reporter genes. Viral clones expressing host protein triggers the silencing mechanism of the host (Waterhouse *et al*. 2001) through viral

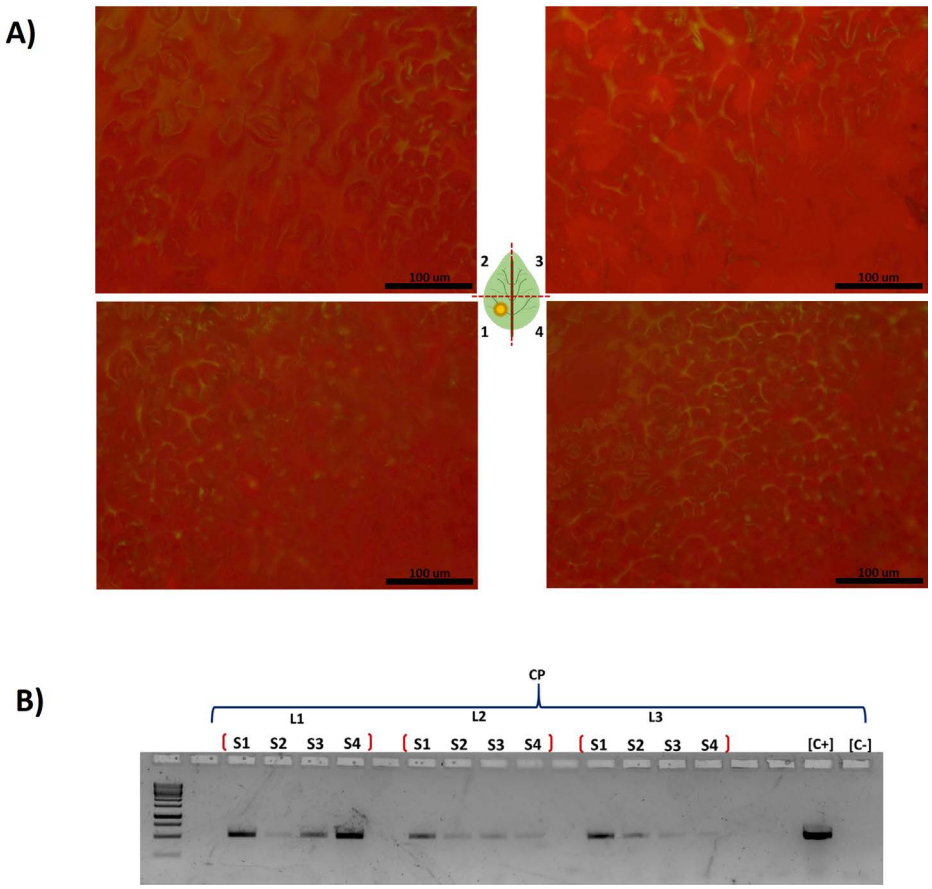

Figure 4. GFP detection of IC-SCMV::GFP away from the inoculated foci. Tobacco leaves were mechanically inoculated in the L1 section (S) with the IC-SCMV::GFP (yellow mark). Fluorescence (A) and the presence of the SCMV-CP (B) cistron were detected in all the sections (S1-S4) of three different leaves (L1-L3).

gene silencing (VIGS) (Robertson 2004). This method allows the evaluation of the gene function by reverse genetics. VIGS has been used to discover the function of a myriad of defense related genes in plants (Lu *et al.* 2003b). The development of a VIGS vector for crops that are hard to genetically modify (like pepper, bean, and maize) is especially appealing, particularly for maize, a monocot with a big and complex genome (Haberer *et al.* 2016), and one of the mayor sources of calorie uptake by humans. In spite of to their importance, only four clones from viruses affecting this species clade were not available until recently: Wheat streak mosaic virus (WSMV), Johnson grass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV) (Choi *et al*. 1999, 2002, Kim *et al*. 2003, Stewart *et al*. 2012), and Foxtail mosaic virus (FoMV) (Mei *et al.* 2019a). Thus, obtaining a clone of SCMV is of special interest.

Recently a SCMV infectious clone was obtained and reported (Mei *et al.* 2019b). Plants inoculated with this clone showed mosaic symptoms after 6 dpi. Subsequently, the clone was tagged with GFP, to evaluate its potential as a maize expression vector, and its high efficacy was later reported (Chung *et al.* 2021). We developed an SCMV clone from a Mexican isolate (SCMV-Ver1) that was also tagged with GFP. We used the methodology proposed by Desbiez, for which the viral genome was divided into four fragments, a GST-intron was added, and the fragments were fused by homologous recombination in yeast. The process is quick and more cost-friendly than the other reported for SCMV. Also, the Desbiez methodology can be used for other viral species.

Trying to emulate the tagging of the TEV clone in the Desbiez work, we decided to tag our SCMV clone with GFP. However, contrary to the TEV clone, our clone was deemed not functional because it is unable to cause mosaic symptoms in maize plants. Even if the clone was not biologically functional, the GFP presence was putatively detected in

maize cells. The absence of symptoms in maize plants and the faint amplification of the bands for the CP and VPg viral cistrons in the inoculated plants with the non-infectious clone, could be related to the use of the 35S promoter in maize (Schledzewski & Mendel 1994). To understand how SCMV moves inside maize cells and the role of the promoter in the clone, more experimental evidence, and the use of a functional clone are necessary.

To our surprise, when the IC-SCMV::GFP was used as an inoculation bombardment control in whole tobacco plants, GFP signal and SCMV associated cistrons were found. Both signals and cistrons could be detected not only on the site of inoculation but also in the distal parts of the plants. Further experiments showed that both inoculation methods, particle bombardment and mechanical inoculation, with the IC-SCMV::GFP led to the observation of both cistrons and GFP signals. Tobacco leaf inoculation with maize tissue infected with SCMV-WT or isolated VLPs did not show any viral cistron presence, thus confirming tobacco is not a host for SCMV. However, our evidence is not enough to discard the possibility that an element present in maize crude extracts could affect the onset of infection in tobacco species. Also, using VLPs as inoculum source was ineffective to infect tobacco plants. The possibility that the technical procedure in VLP's purification could affect infection was not evaluated and more studies are needed in this regard.

We hypothesized that the apparent ability of our IC-SCMV::GFP, deemed as not functional, to spread in tobacco leaves is related to the use of the 35S promoter used in the clone construction. This promoter has shown its influence in the transcriptional activity (Amack & Antunes 2020) and has shown to act as an IRES-type structure (Martinez-Salas *et al.* 2018). Both characteristics could facilitate the transcription and translation in the studied system. Although more experimental evidence is needed, it is tantalizing to speculate that the 35S CaMV promoter might be the only cause of the IC-SCMV::GFP spread in the tobacco leaves, however, future work using a different promoter is necessary. Understanding if only the promoter affects the transcription of the SCMV will lead to improve the use of infectious clones as tools; for example, leading to the development of VIGS vector with reduced symptoms or the use of tobacco, a known and reliable system for the expression of different proteins in the biotech industry.

In conclusion, a SCMV-Ver1 clone was developed. Even when deemed non-infectious, as no viral symptoms were observed in its maize host, the clone was able to spread in tobacco detached leaves, as evidenced by the presence of SCMV-derived cistrons and fluorescent GFP confirming its transient protein expression and conceiving it as a possible viral expression vector in tobacco plants for biotechnical applications.

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