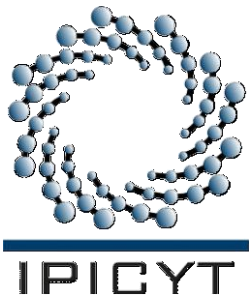


INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGÍA MOLECULAR

Development of VIGS vectors derived from broad-host range geminiviruses to induce post-transcriptional gene silencing in plants

Tesis que presenta

Marlene Taja Moreno

Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Gerardo Rafael Argüello Astorga

San Luis Potosí, S.L.P., 12 julio de 2011

IPICYT

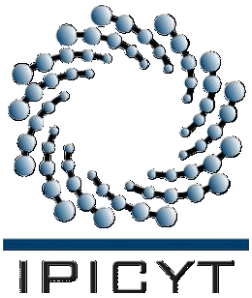
Constancia de aprobación de la tesis

La tesis "**Development of VIGS vectors derived from broad-host range geminiviruses to induce post-transcriptional gene silencing in plants**" presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por **Marlene Taja Moreno** y aprobada el **12 de julio de 2011** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Dr. Gerardo Rafael Argüello Astorga
(Director de la tesis)

Dr. Angel Gabriel Alpuche Solís
(Miembro del Comité Tutoral)

Dr. Juan Francisco Jiménez Bremont
(Miembro del Comité Tutoral)



Créditos Institucionales

Esta tesis fue elaborada en el Laboratorio de (Biología Molecular de Plantas) de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección del Dr. Gerardo Rafael Argüello Astorga.

Durante la realización del trabajo la autora recibió una beca académica del Consejo Nacional de Ciencia y Tecnología (No. de registro 230924) y del Instituto Potosino de Investigación Científica y Tecnológica, A. C.

El trabajo de investigación descrito en esta Tesis fue financiado con recursos otorgados al Dr. Gerardo Rafael Argüello Astorga por el CONACYT (PROYECTO: CB-2007-01-84004)

IPICYT

Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Acta de Examen de Grado

El Secretario Académico del Instituto Potosino de Investigación Científica y Tecnológica, A.C., certifica que en el Acta 083 del Libro Primero de Actas de Exámenes de Grado del Programa de Maestría en Ciencias en Biología Molecular está asentado lo siguiente:

En la ciudad de San Luis Potosí a los 12 días del mes de julio del año 2011, se reunió a las 12:00 horas en las instalaciones del Instituto Potosino de Investigación Científica y Tecnológica, A.C., el Jurado integrado por:

Dr. Ángel Gabriel Alpuche Solís	**Presidente**	**IPICYT**
Dr. Juan Francisco Jiménez Bremont	**Secretario**	**IPICYT**
Dr. Gerardo Rafael Argüello Astorga	**Sinodal**	**IPICYT**

a fin de efectuar el examen, que para obtener el Grado de:

MAESTRA EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustentó la C.

Marlene Taja Moreno

sobre la Tesis intitulada:

Development of VIGS vectors derived from broad-host range geminiviruses to induce post-transcriptional gene silencing in plants

que se desarrolló bajo la dirección de

Dr. Gerardo Rafael Argüello Astorga

El Jurado, después de deliberar, determinó

APROBARLA

Dándose por terminado el acto a las 13:15 horas, procediendo a la firma del Acta los integrantes del Jurado. Dando fe el Secretario Académico del Instituto.

A petición de la interesada y para los fines que a la misma convengan, se extiende el presente documento en la ciudad de San Luis Potosí, S.L.P., México, a los 12 días del mes de julio de 2011.

Dr. Marcial Bonilla Marín
Secretario Académico

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.
IPICYT
SECRETARIA ACADEMICA

Mtra. Ivonne Lizette Cuevas Vélez
Jefa del Departamento del Posgrado

Dedication

To my brother Edward, who passed away at age 10, for teaching me the importance of helping others and enjoying life's ups and downs.

To my mom for her love and for helping me to create a vision for my future, encouraging me to learn and supporting my education.

To all the women that struggle to have an education and equality.

Acknowledgements

I thank Dr. Gerardo R. Argüello-Astorga for all his helpful advice and guidance during the course of this work. I also thank Drs. Angel G. Alpuche-Solís, Juan Francisco Jiménez-Bremont, and Sergio Casas-Flores for their advice and critical review of this manuscript. Dr. Paul E. Riley of Peace Corps of the United States of America is gratefully acknowledged for his valuable review of the writing of this manuscript. I am grateful to Armando Mauricio-Castillo for kindly providing us with curtovirus isolates and to Bernardo Bañuelos-Hernández, who provided advice during the early part of this research. Finally, I want to make a special acknowledgement to Biol. Salvador Ambriz-Granados for his technical assistance. This research was supported by CONACYT (Project:CB-2007-01-84004)

Index

Table list

Figures list

Abbreviations

BMCTV	Beet mild curly top virus
bp	Base pairs
cDNA	Complementary DNA
DCL	Dicer -like ribonuclease
dpi	Days post infection
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
MCS	Multiple cloning site
nt	Nucleotides
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDS	Phytoene desaturase gene
PepCTV	Pepper curly top virus
PTGS	Post-transcriptional gene silencing
RCA	Rolling circle replication
RdRp	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RNAi	RNA interference
siRNAs	Small-interfering RNAs
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
SU	Tobacco sulfur gene
VIGS	Virus-induced gene silencing

Definitions

Agroinoculation. It is a procedure for systemic infection of plants with viruses, which consists in the usage of agrobacterium *(A.tumefasciens* or *A. rhizogenes*) as a delivery agent for viral genomes.

C1. Complementary sense gene required for viral replication.

C2. Complementary sense gene which specifies a protein that is important for pathogenesis and has silencing suppressor activity.

C3. Complementary sense gene which encodes a replication enhancer.

Curly top disease. A disease caused generally by curtoviruses. The curly top disease symptoms include stunted and distorted plant growth; leaf curling, crumpling, yellowing, vein swelling, distortion, necrosis, and hyperplasia of the phloem.

Dicotyledon. One of the two principal classes of flowering plants that is characterized by two cotyledons (primitive leaves) in the embryonic plant. Pepper, tomatoes, and beans are common dicots**.**

Geminiviruses. A specific group of viruses that have genomes composed of single-stranded DNA.

Hemi-dimer. A plasmid vector which has inserted a full viral genome plus a partial fragment of the same viral genome, also refered as multimeric or partial dimer clone.

Magnesium chelatase. A multi-subunit protein that catalyzes the insertion of magnesium into protoporphyrin IX required for chlorophyll production.

Meristem. The undifferentiated tissue at the tips of stems and roots in which new cell division is concentrated.

Tobacco sulfur gene. This gene was identified by transposon tagging and encodes the nucleotide binding subunit of magnesium chelatase. In tobacco, a mutated allele (Su) of one subunit of magnesium chelatase causes the phenotype known as “sulfur”, since tobacco plants homozygous for this allele are yellow.

V1. Virion sense gene that directs the synthesis of the capsid protein and is required for virus spread.

V2. Virion sense gene which encodes a protein required for the efficient accumulation of the viral ssDNA.

V3. Virion sense gene required for virus spread in the infected plant.

C4. Complementary sense gene which specifies a pathogenecity factor.

Resumen

Desarrollo de vectores VIGS derivados de geminivirus de amplio rango de hospederos para inducir silenciamiento post-transcripcional de genes en plantas

Los geminivirus pertenecientes al género *Curtovirus* son transmitidos por la chicharrita del betabel (*Circulifer tenellus*) e infectan a un amplio rango de plantas dicotiledóneas. Se presenta en este estudio la generación de dos clonas infectivas derivadas de una nueva cepa del curtovirus *Beet mild curly top virus,* (*BMCTV-[Mexico])* y de la nueva especie *Pepper curly top virus*, *(PepCTV-[Nuevo Mexico])*. Se demostró que el virus *BMCTV-[Mexico]* infecta a un amplio rango de hospederos al inocularlo, mediante bombardeo con micro-partículas y transmisión mecánica, en plántulas de *Nicotiana benthamiana, Aradidopsis thaliana (Col-0), Solanum habrochaites*, *Capsicum annuum* (cv. Serrano), *Cucurbita pepo* (cv. Grey Zucchini), y *Phaseolus vulgaris* (cv. Pinto Villa). Después de la infección con este curtovirus se observó remisión de síntomas solamente en plantas de *S. habrochaites* y *P. vulgaris*. El vector pPepCTV-[NM]1.4 se inoculó mediante transmisión mecánica en plantas de *Capsicum annuum* (cv. Serrano) e indujo síntomas de enrollamiento leve de las hojas y clorosis intervenal. Debido a que los sistemas de inducción de silenciamientos génico (VIGS) más confiables están basados en geminivirus, realizamos la construcción de los vectores de silenciamiento pBMCTV-SiV y pPepCTV-SiV. Se amplificaron fragmentos de los genes *SU*, *PDS*, y *PCNA* de *N. benthamiana* y del gen *PDS* de *C. pepo*, para insertarlos posteriormente en estos vectores y demostrar su funcionalidad. Considerando el amplio rango de hospederos de los curtovirus, y que los vectores pBMCTV-SiV y pPepCTV-SiV poseen un sitio múltiple de clonación para inserción de genes blanco, los vectores serán una herramienta molecular valiosa para estudios de genómica funcional y aplicada.

PALABRAS CLAVE: Vectores de silenciamiento; PTGS; curtovirus; *BMCTV*; *PepCTV*; *Arabidopsis thaliana*; Solanaceae; Cucurbitaceae; leguminosas; estructura secundaria del mRNA; transmisión mecánica.

Abstract

Development of VIGS vectors derived from broad-host range geminiviruses to induce post-transcriptional gene silencing in plants

Geminiviruses in the genus *Curtovirus* are vectored by the beet leafhopper (*Circulifer tenellus*) and infect a wide range of dicotyledonous plants. The generation of two multimeric infectious clones derived from a new strain of the curtovirus *Beet mild curly top virus,* (*BMCTV-[Mexico*]) and from the new curtovirus species *Pepper curly top virus, (PepCTV-[Nuevo Mexico]*, are presented. We demonstrated that *BMCTV-[Mexico]* infects a wide range of host plants, by either inoculating it by micro-particle bombardment or mechanical transmission into *Nicotiana benthamiana, Aradidopsis thaliana (Col-0), Solanum habrochaites*, *Capsicum annuum* (cv. Serrano), *Cucurbita pepo* (cv. Grey Zucchini), and *Phaseolus vulgaris* (cv. Pinto Villa) seedlings. Symptoms recovery was observed only in *S. habrochaites* and *P. vulgaris* plants, this is the second report of plants displaying recovery after infection with curtoviruses. The pPepCTV-[NM]1.4 was inoculated by mechanical transmission into *Capsicum annuum* (cv. Serrano) plants and induced mild leaf curling and interveinal chlorosis symptoms. Because geminiviruses have been used as reliable virus-induced gene silencing (VIGS) vectors, we carried out the construction of the pBMCTV-SiV and pPepCTV-SiV silencing vectors. We amplified fragments of the VIGS reporter genes *SU*, *PDS,* and *PCNA* from *N. benthamiana*, and the PDS from C. pepo, which may be inserted on the generated silencing vectors to further assess their functionality. Considering the curtoviruses broad host range, and that the vectors possess a multiple cloning site for insertion of target genes, these vectors may be valuable molecular tools for functional and applied genomics.

KEY WORDS: Virus-induced gene silencing; PTGS; curtoviruses; *BMCTV*; *PepCTV*; *Arabidopsis thaliana*; Solanaceae; cucurbits; legumes; mRNA secondary structure; mechanical transmission.

Introduction

Geminiviruses are single-stranded circular DNA (ssDNA) viruses that cause economically significant diseases in a wide range of crop plants. Their genome replication is accomplished in the host cell nucleus through double-stranded DNA (dsDNA) intermediates (Gutierrez, 2000; Hanley-Bowdoin et al., 2000). Like plant RNA viruses, geminiviruses are both inducers and targets of post-transcriptional gene silencing (PTGS) or RNA interference (RNAi), a natural plant antiviral defense system (Napoli et al., 1990). The pathway for gene silencing adopted by virus-infected plants is cytoplasmic short interfering silencing, which is triggered by double-stranded RNA (dsRNA) (Baulcome, 2004). In the case of RNA viruses, the dsRNA could be a replication intermediate. While in DNA viruses, the dsRNA may be originated from “aberrant” RNA or single stranded RNA (ssRNA) , which are converted into dsRNA by the host RNA-dependent RNA polymerase (RdRp) (Baulcome, 2004). DICER-like ribonucleases (DCL) cleave the dsRNA, and generate 21-24 nucleotide (nt) small interfering RNA (siRNA) fragments. These fragments are further processed and incorporated into the RNA induced silencing complex (RISC) that targets complementary mRNAs for degradation (Chen, 2009; Rana 2007; Waterhouse and Helliwell, 2003). Moreover, a mobile silencing signal is produced in plants for specific degradation far from the inoculation point, thus making RNA silencing an effective natural defense mechanism (Muangsan et al., 2004).

It is not clear how geminiviruses trigger the RNA silencing mechanism since they do not generate dsRNA during their replication cycle. There are some hypotheses, such as (i) the overlapping of transcripts produced during bidirectional transcription, (ii) the high abundance of mRNAs from viral genes such as the replication gene C1, and (iii) secondary folding of viral messengers that could trigger the silencing machinery (Vanitharani et al., 2005). Whichever the case may be, different size classes of geminivirus-specific siRNAs (21, 22, and 24nt) have been reported in *A. thaliana* which express four DCL enzymes (Blevins et al., 2006).

Because it has been shown that viruses can carry and induce RNAi of exogenous gene sequences, this ability has been exploited in a technology known as "virus-induced gene silencing" (VIGS) (Waterhouse and Helliwell, 2003). VIGS has emerged as a powerful tool to study gene function in plants. Some of the main advantages of this tool over traditional functional genomic approaches are: it can identify a loss-of-function phenotype within a single generation, it allows the characterization of phenotypes that might be lethal in stable lines, and it has the potential to silence either an individual gene or multiple genes of the same family (Burch-Smith et al., 2004). In addition, it can be carried out with plant species which are difficult or impossible to transform (Burch-Smith et al., 2004; Tripp-Carrillo, 2006).

At the beginning, VIGS vectors were based only on RNA viruses like *Tobacco mosaic virus* (TMV) (Kumagai et al., 1995) and *Potato virus X* (PVX) (Ruiz et al., 1998). However, these vectors induced disease symptoms in inoculated plants that masked the silencing phenotype (Ratcliff et al., 2001). In order to overcome some of these constraints, several VIGS systems, based either on RNA or DNA viruses, have been developed. On the other hand, the possibility to replace the capsid protein gene (V1) of geminiviruses by a heterologous sequence has permitted their use as reliable VIGS vectors (Robertson, 2004). The gene V1 is also responsible for viral infectivity symptoms, so its replacement offers the advantage of generating VIGS vectors that may induce attenuated symptoms or no symptoms at all. In comparison with RNA viruses, geminiviruses cause an efficient and durable silencing of endogenous genes because they are not directly targeted by the RNA silencing system (Golenberg et al., 2009). Although usually both DNA and RNA viruses are excluded from plant meristems, geminiviruses such as *Tomato golden mosaic virus* (TGMV) (Peele et al., 2001) and *Beet curly top virus* (BCTV) (Golenberg et al., 2009), were successfully used to silence genes in meristematic tissues. Interestingly, it has been possible to silence simultaneously two essential genes in *N. benthamiana* with TGMV as well as in *A. thaliana* with *Cabbage leaf curl virus* (CbLCuV) (Peele et al., 2001; Turnage et al., 2002). Furthermore, VIGS systems based on *African cassava mosaic virus* (ACMV)

(Fofana et al., 2004) and on *Pepper huasteco yellow vein virus* (PHYVV) (Abraham-Juárez et al., 2008) have been very useful in transiently transforming recalcitrant crops (such as cassava and pepper), and in studying genes involved in certain biochemical pathways (Tripp-Carrillo, 2006). Despite these achievements, there is still more work to do since most of the reliable VIGS vectors are based on geminiviruses with limited host range, are functional only in some plant species, and may in fact suppress silencing. Hence, increasing the availability of infectious clones of geminiviruses with a wider host range to develop VIGS vectors may help circumvent some of these limitations.

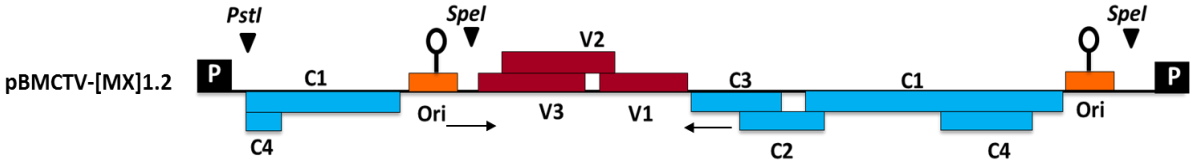
Herein, we report the generation and characterization of infectious clones derived from a new strain of the *Beet mild curly top virus* (*BMCTV-[Mexico*]) and the new species *Pepper curly top virus-Nuevo Mexico* (*PepCTV-[Nuevo Mexico]),* geminiviruses that belong to the genus *Curtovirus*, which have a monopartite genome (~2.9kb) and are vectored by the beet leafhopper (*Circulifer tenellus*) (Soto and Gilbertson, 2003). Considering that curtoviruses can infect over 300 dicotyledonous plant species from at least 44 families (Bennett, 1971), and also that Golenberg et al. (2009) have recently demonstrated the reliability of a VIGS vector based on a curtovirus, these viruses may be useful as vectors for gene silencing. Thus, we present in this study the construction of the gene-silencing vectors pBMCTV-SiV and pPepCTV-SiV, which are based upon these curtoviruses.

Results

Generation of a 1.2-mer infectious *BMCTV-[Mexico]* clone

In order to define the biological properties of *BMCTV-[Mexico],* a 1.2-mer clone (pBMCTV-[MX]1.2) was generated for infectivity studies. This infectious clone has two replication origins which allow the Rep-mediated release of a full-length virus genome upon inoculation of the plant. A 600 bp fragment containing the *BMCTV-[Mexico]* viral replication origin was amplified by PCR and cloned in the *PstI/SpeI* restriction sites of a modified pBlueScript plasmid. Then, the complete viral genome was obtained by rolling circle amplification (RCA) and further inserted into the previous construct in the *SpeI* restriction site. The resulting 6555 bp construct, designated as pBMCTV-[MX]1.2 (Fig. 1A), was tested by restriction analysis using *PstI* and *SpeI* (Fig. 1B), and positive clones were verified by sequencing.

(A)



(B)

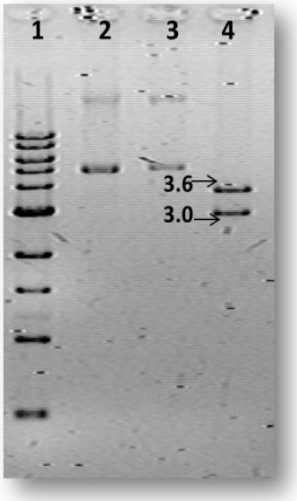
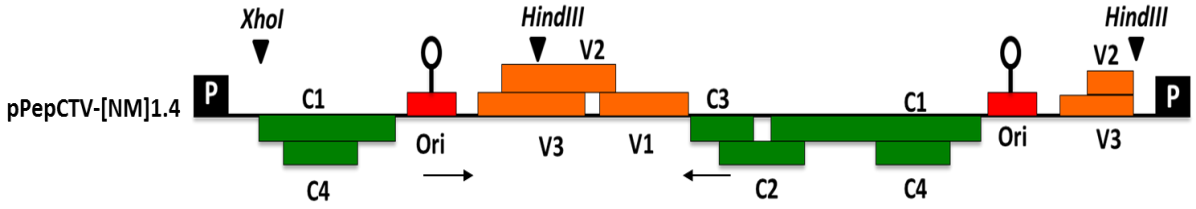


Fig.1. Map of the *BMCTV-[Mexico]* multimeric construct and its restriction pattern assay. (A) The restriction sites *PstI* and *SpeI*, where the complete and partial genomes were inserted into the pBlueScript plasmid (pBS), are indicated: solid arrows indicate the location and polarity of open reading frames encoded by the virion (V1, V2, and V3) or complementary (C1, C2, C3, and C4) sense strand. (B) Restriction profile of a positive multimeric clone: lane 1, 1kb DNA ladder (NEB); lane 2, undigested pBMCTV-[MX]1.2 vector; lane 3, infectious clone digested with *HindIII* (verification of restriction site removal from pBS); lane 4, vector digested with *PstI* showing the correct orientation of the hemidimer in the construction.

Generation of a 1.4-mer infectious *PepCTV-[New Mexico*] clone

For the generation of a *PepCTV-[New Mexico*] multimeric construct, a similar approach as applied to the *BMCTV-[Mexico]* was used. A 1.3kb fragment containing the viral origin of replication from the *PepCTV-[New Mexico]* was amplified by PCR. Consequently, the construct pPepCTV-[NM].4 was obtained by cloning this fragment into the *HindIII* and *XhoI* restriction sites from a modified pBlueScript vector. The viral full length genome was amplified by rolling circle replication and cloned into the previous construct, generating the 7079 bp multimeric clone pPepCTV-[NM]1.4 (Fig. 2A). Restriction pattern assay to verify the proper orientation of the hemi-dimer was performed using *HindIII* and *XhoI* endonucleases (Fig. 2B). Positive clones were then verified by sequencing.

(A)



(B)

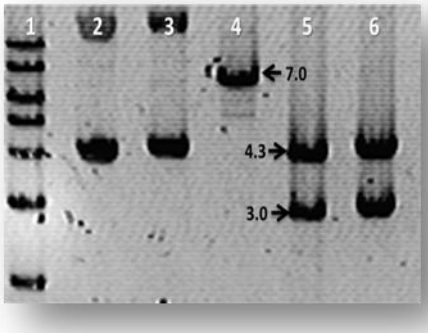


Fig.2. Map of the *PepCTV-[Nuevo Mexico]* multimeric construct and its restriction pattern assay**.** (A) The restriction sites *XhoI* and *HindIII* where the complete and partial genome, were inserted into the modified pBlueScript plasmid (PBS) are indicated: solid arrows indicate the location and polarity of open reading frames encoded by the virion (V1, V2, and V3) or complementary (C1, C2, C3, and C4) sense strand. (B) Restriction profile of a positive multimeric clone: lane 1,1kb DNA ladder (NEB); lane 2, shows undigested pPepCTV[NM]1.4 vector; lane 3, shows infectious clone digested with *EcoRV* for verification of restriction site removal from pBS; lane 4, shows vector digested with *EcoRI* (this enzyme cuts the construction once and linearizes it); lanes 5 and 6, show vector digested with *XhoI and HindIII*, respectively, with the proper orientation of the hemi-dimer in the construction.

Determination of host range and symptomatology of the *BMCTV-[Mexico]*

A host range experiment was next performed by inoculating with micro-particle bombardment the construct pBMCTV-[MX]1.2 in the following plant species: *Nicotiana benthamiana*, Arabidopsis *[Arabidopsis thaliana* (Col-0)], wild tomato [*Solanum habrochaites*] and pepper [*Capsicum annuum* (cv. Serrano)] with 4-6 true leaves from each of these species. Since squash [*Cucurbita pepo* (cv. Grey Zucchini)] and bean [*Phaseolus vulgaris* (cv. Pinto Villa)] seedlings with 2-3 true leaves did not fit on the bombardment chamber and to use a simpler inoculation method, they were inoculated by mechanical transmission (carborundum) with the same construct. Symptoms appearance was observed in all infected plants approximately 25 days post infection (dpi). Symptoms were observed in *N. benthamiana* plants, including severe stunted growth and leaf curling, crumpling, and yellowing (Fig. 3A-a). On the other hand, Serrano pepper and squash plants showed similar but less severe symptoms than in *N. benthamiana* plants (Fig. 3A-d,e). Arabidopsis plants developed smaller and distorted siliques and bolt curling, but not leaf yellowing (Fig. 3A-b). Chlorosis and vein purpling were observed in wild tomato (Fig. 3A-c); while leaf crumpling, vein swelling, and enations were identified in beans (Fig. 3A-f). Interestingly, symptoms remissions were observed in both wild tomato and bean plants approximately 2-3 weeks after symptoms appearance. Infectivity of the pBMCTV-[MX]1.2 construct in selected plant species after inoculation by either particle bombardment or mechanical transmission was between 50% and 75% (Table 1). The presence of curtovirus DNA in newly emerged leaves from symptomatic plants was confirmed by PCR with the universal curtovirus primer pair (Cur Rep 2GQ Fw and Cur CP 450 Rv) showing a 1.6 kb product, but not observed in asymptomatic or mock inoculated plants (Fig. 3B). Together, these results showed that *BMCTV-[Mexico]* can infect a broad range of hosts and thus can be used to develop a versatile gene silencing vector. Finally, our results extend the BMCTV host range to Arabidopsis and wild tomato.

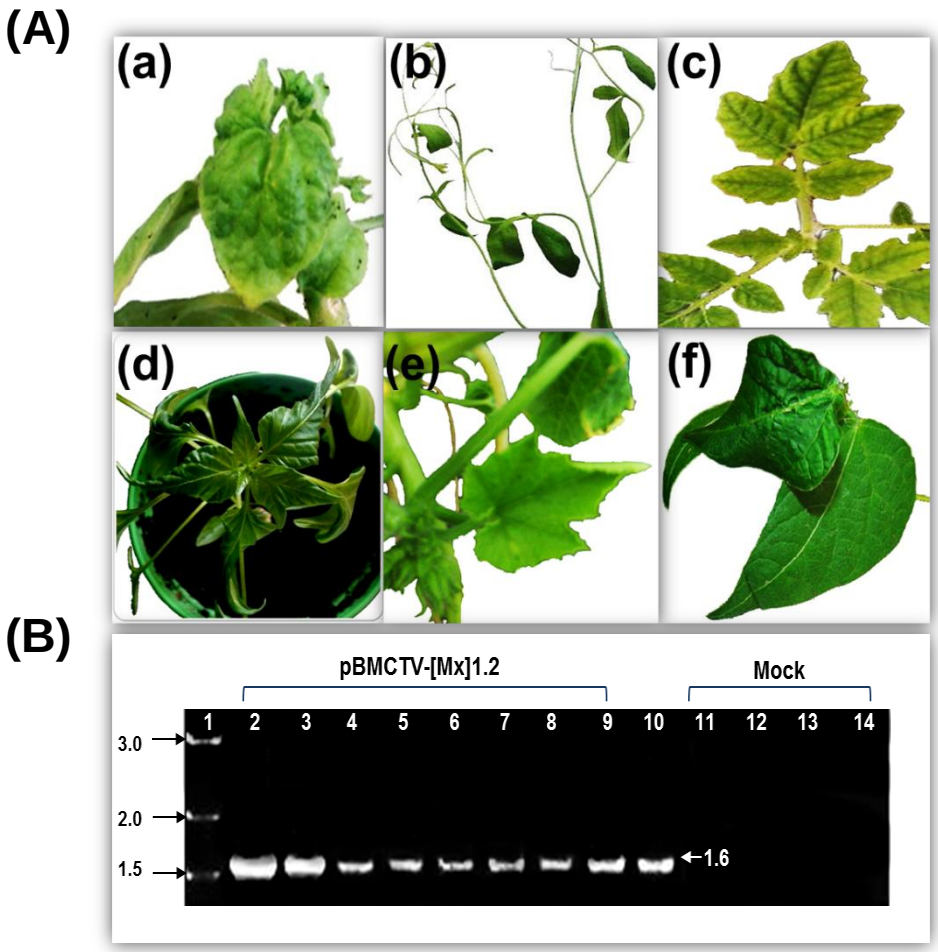


Fig. 3. Disease symptoms induced in selected plant species infected with the multimeric construct *pBMCTV[MX]1.2*, 25 days post infection, and PCR detection of the curtovirus with universal primers. (A) Symptoms observed in: (a) *Nicotiana benthamiana*, (b) Arabidopsis [*Arabidopsis thaliana* (Col-0)], (c) wild tomato [*Solanum habrochaites*], (d) pepper [*Capsicum annum* (cv. Serrano)], (e) squash [*Cucurbita pepo* (cv. Grey Zucchini)], and (f) bean [*Phaseolus vulgaris* (cv. Pinto Villa)]. (B) Ethidium-bromide-stained gel showing DNA fragments amplified by PCR with curtovirus-universal primers Cur Rep 2GQ Fw and Cur CP 450 Rv, from DNA extracts of pBMCTV[MX]1.2 infected plants showing disease symptoms as well as mock inoculated plants with pBlueScript. Lanes are as follows: 1, 1-kb ladder (NEB); 2-3, *N. benthamiana* ; 4-5, Arabidopsis; 6-7, wild tomato; 8, pepper serrano; 9-10, squash and bean, 11-14 mock inoculated plants.

Table 1. Infectivity of cloned *BMCTV-[Mexico]* DNA in selected plant species after inoculation by particle bombardment or mechanical transmission, respectively.

Plant species	**Infectivity[a]**	
	Particle bombardment	**Mechanical transmission**
Nicotiana benthamiana	3/4	0/5
Arabidopsis thaliana Col-01	2/4	NT[b]
Solanum habrochaites	2/4	NT[b]
Capsicum annuum (cv. Serrano)	3/4	NT[b]
Cucurbita pepo cv.	NT[b]	3/5
Phaseolus vulgaris (cv.PintoVilla)	NT[b]	3/5
Negative control[c]	0/12	0/12

[a]Recombinant plasmid pBMCTV-[MX]1.2, containing a multimeric BMCTV-[Mexico] clone, was used as inoculum. Infectivity indicates the number of plants with disease symptoms [(systemically infected)/(total number of plants inoculated)]. Symptom-less infections were not detected.

[b]NT= not tested.

[c]Negative controls consisted of 2 plants from each species which were mock inoculated with the plasmid pBlueScript either by bombardment with tungsten particles or by mechanical transmission.

Infectivity assay of the pPepCTV[NM]1.4 construct in pepper plants

To determine the infectivity of the *PepCTV-[Nuevo Mexico]*, the pPepCTV[NM]1.4 multimeric clone was inoculated by mechanical transmission in pepper [*Capsicum annuum* (cv. Serrano)] seedlings at 4 leaf stage. Symptoms appearance was observed in 3 of 4 inoculated pepper plants after 25 dpi. Pepper seedlings developed leaf mild curling and interveinal chlorosis (Fig. 4A). Since we worked previously with the *BMCTV-[Mexico],* and we wanted to avoid any risk of contamination, we selected a specific primer pair instead of the curtovirus-universal primer pair to detect the presence of the *PepCTV-[Nuevo Mexico]* in these plants. The specific primer pair used was: Cur NM-*XhoI* Fw1 and CurNM-*HindIII*-Rev1. The *PepCTV-[Nuevo Mexico]* presence was detected by PCR in all the symptomatic plants (Fig. 4B). PCR products of 1.3 kb were obtained from symptomatic infected plants. While, no curtovirus presence was detected by PCR in nor mock inoculated plants with pBlueScript (pBS) neither in the negative control. Whereas, it was detected in all infected pepper plants that showed disease symptoms but not in an asymptomatic inoculated plant (Fig. 4B). Together, these results indicate that pPepCTV[NM]1.4 construct is infectious in Serrano pepper plants. Nevertheless, to further characterize this curtovirus we are currently developing new infectivity assays for this curtovirus in different pepper cultivars and *N. benthamiana* seedlings. Moreover, we may extend the infectivity assay to other plants species to study the *PepCTV-[Nuevo Mexico]* host range and we may test it as well as a silencing vector.

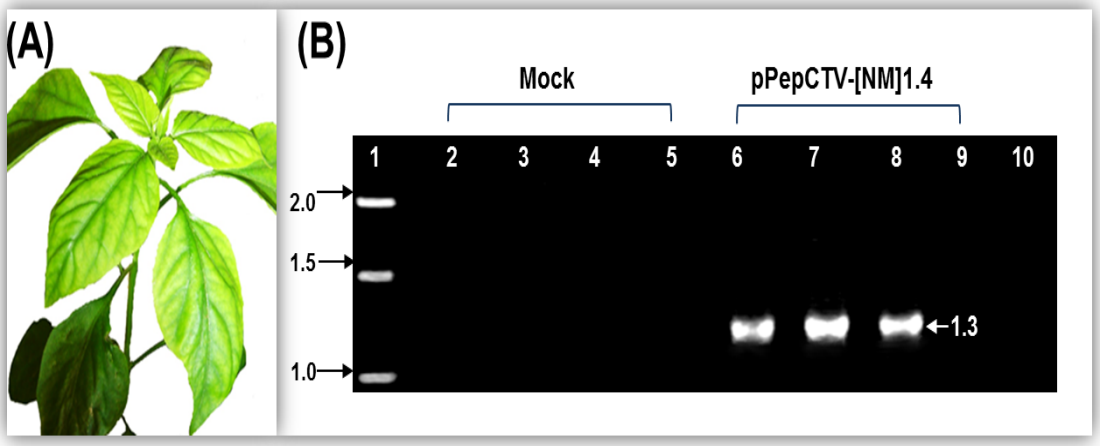


Fig. 4. Disease symptoms induced in pepper plants inoculated with the multimeric construct pPepCTV[NM]1.4, 25 days post infection, and PCR detection of the curtovirus with specific primer pair. (A) Symptoms observed in pepper [*Capsicum annuum* (cv. Serrano)]. (B) Ethidium-bromide-stained gel showing DNA fragments amplified by PCR with primers Cur NM-*XhoI* Fw1 and CurNM-*HindIII*-Rev1 from DNA extracts of pepper mock inoculated plants with pBlueScript, and pepper plants showing disease symptoms after inoculation with pPepCTV[NM]1.4. Lane 1, 1-kb ladder (NEB); lanes 2-5, mock inoculated plants; lanes 6-8, symptomatic pepper plants inoculated with pPepCTV[NM]1.4; lane 9, asymptomatic pepper plant inoculated with pPepCTV[NM]1.4; lane 10, negative control.

Construction and description of *BMCTV-[Mexico]* VIGS vector

A VIGS plasmid vector was constructed by cloning into pBMCTV[MX]1.2 construct a fragment of the *BMCTV-[Mexico]*, which lacked most of the V1 gene, but instead included a MCS to allow insertion of targeting sequences. The absence of the V1 function, leads to a competent fully replicated but truncated *BMCTV-[Mexico]* genome when it is released from the plasmid vector. The replacement fragment was generated by PCR splicing of two fragments designed to have a MCS overlapping region (Fig. 5A-a, b). The first overlapping fragment isolated by PCR, included a portion of the IR, the viral genes V2 and V3, and the 5' portion of the V1 gene. The V1 gene directs synthesis of the capsid protein, and, as well as V3, is required for the spread of the virus during infection (Stanley et al., 1992). Gene V2 encodes a protein required for efficient accumulation of viral ssDNA (Hormuzdi and Bisaro, 1993). In order to generate an additional insertion site, this fragment included a point mutation in the 5' portion of V1 that is close to the MCS region.

The second PCR fragment included the 3' end of the V1 gene to retain mRNA processing signals. Since the MCS was introduced before the V1 stop codon, the targeting sequences should be transcribed as part of the virion-sense transcription unit that includes the V1. Additionally, this fragment contained the genes C2 and C3, and the 3' end of the C1 gene. The C2 protein is important for pathogenesis and has silencing suppressor activity. The C3 gene encodes a replication enhancer (REn) and the C1 gene encodes a replication protein (Rep) (Hormuzdi and Bisaro, 1995). The completed plasmid was designated pBMCTV-SiV. A map of the vector is shown in Fig. 5B and the sequences in the vicinity of the MCS for insertion of targeting sequences are given in Fig. 5C. This construct will be further tested as a VIGS vector in our laboratory.

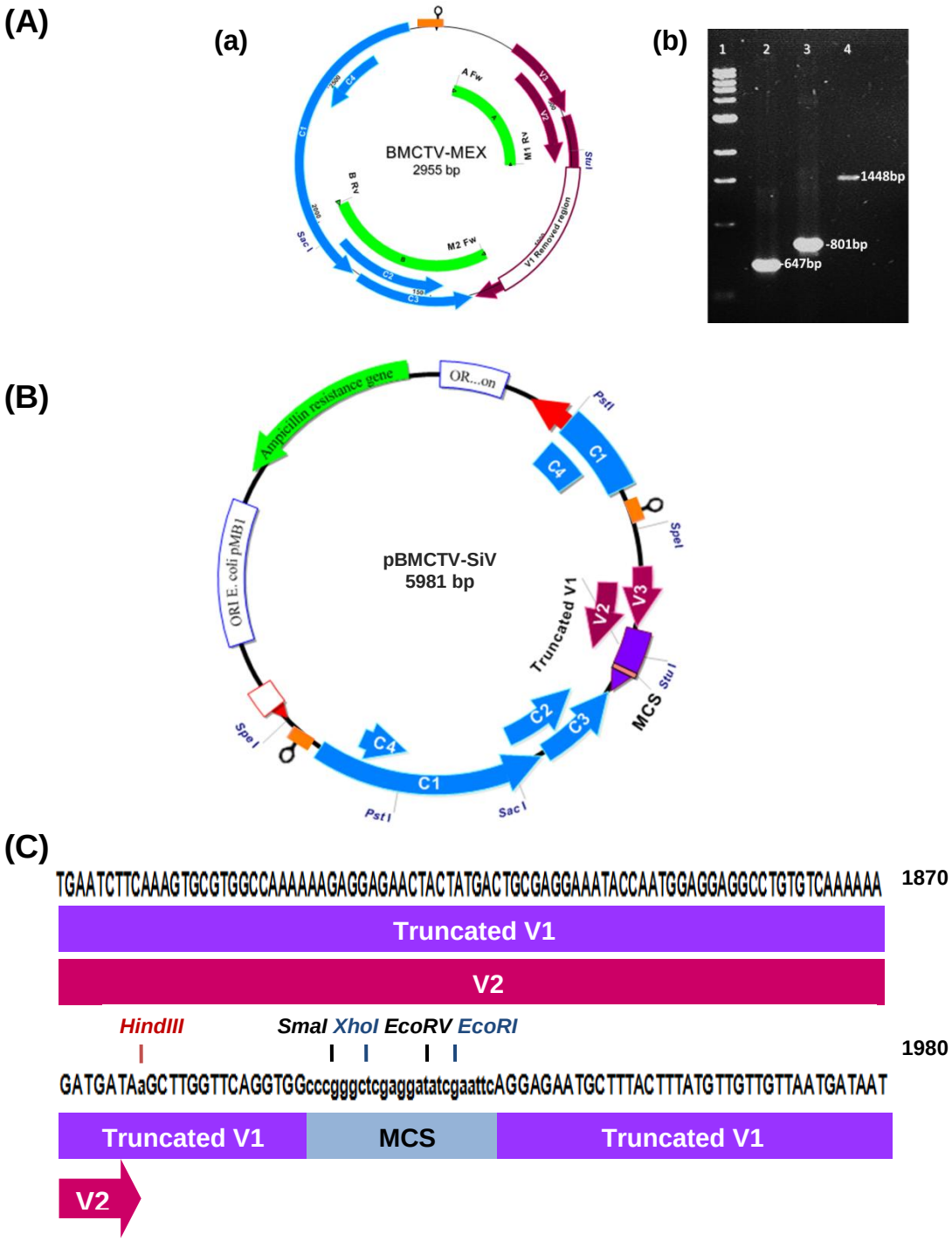


Fig.5. Map of pBMCTV-SiV and its construction. (A) PCR splicing to generate the *BMCTV-[Mexico]* replacement fragment: (a), Monomer map and specific primers used to amplify two specific regions of the genome (shown with green boxes), and (b), Ethidium-stained-gel showing DNA fragments amplified by PCR with specific primer pairs first to generate a 647 bp product (lane 2) and an 801 bp product (lane

3); and then to generate by PCR splicing a third fragment of 1448 bp (lane 4), that has a MCS and that lacks 489 bp from V1 gene. (B), Multimeric construct map shows *StuI* and *SacI* replacement sites with the 1448 bp product. (C), Replacement of the V1 gene coding region with a MCS. Restriction sites (*SmaI, XhoI, EcoRI* and *EcoRV*) of the MCS are shown, as well as an additional insertion site (*HindIII*) generated by-introducing a single point mutation (A to T) in the *BMCTV-[Mexico]* genome at position 744.

To further determine the *BMCTV-[Mexico]* vector utility as an effective inducer for the silencing of endogenous genes, we first chose to target the tobacco sulfur (SU) gene that encodes a magnesium chelatase subunit required for chlorophyll production. A 324 bp fragment of this gene was amplified from cDNA of *N. benthamiana* (Fig. 6A). Then we targeted a ~ 400bp fragment of the phytoene desaturase (PDS) gene from cDNAs of *N.benthamiana* and squash (Fig. 6A-B). Each fragment will be inserted separately into the *XhoI* and *EcoRI* sites of the MCS within the truncated V1 gene of the pBMCTV-SiV vector. The resulting vectors will be further inoculated into *N. benthamiana* plants (6-8 true leaf stage) and into squash cotyledons.

Finally, to determine whether this silencing vector can be used to silence genes expressed in meristematic tissues, we isolated from cDNA of *N. benthamiana* a fragment of 379 bp of the proliferating cell nuclear antigen (*PCNA*) gene (Fig. 6A), since this gene is expressed in meristematic tissue. This fragment will be inserted into the pBMCTV-SiV vector in the *XhoI* and *EcoRI* sites and inoculated into *N. benthamiana* plants.

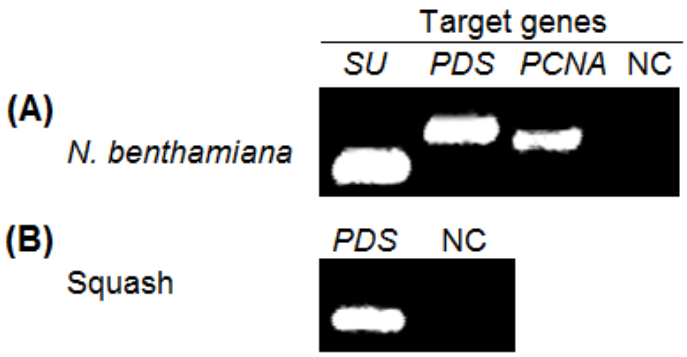
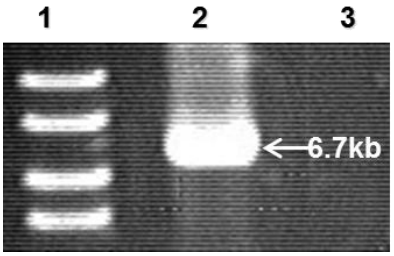


Fig.6. Isolation by RT-PCR of targeting sequences of *SU*, *PDS* and *PCNA* cDNAs in leaves of (A) *N. benthamiana* and (B) squash for insertion into pBMCTV-SiV vector. Lane NC represents the negative controls.

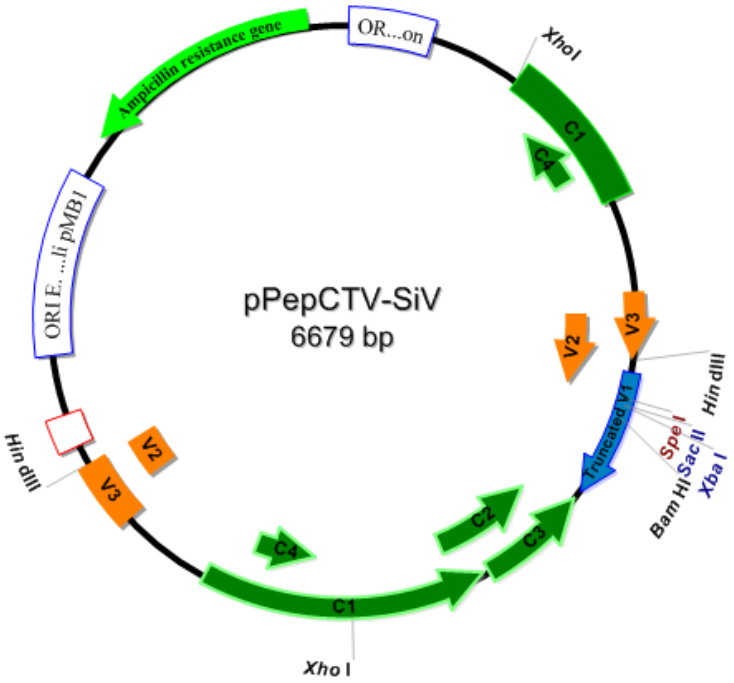
Construction and description of *PepCTV-[Nuevo Mexico]* VIGS vector

A *PepCTV-[Nuevo Mexico]* silencing vector was generated by using PCR site-directed mutagenesis to modify the pPepCTV-[NM]1.4 vector. The modifications included: (i) the removal from most of V1 gene, which conserved the 3' end to retain the mRNA processing signals; (ii) a single point mutation at the 5' start of V1 gene to generate the *SpeI* insertion site; (iii) and the generation of a liker containing the *SacII* and *XbaI* cloning sites (Fig. 7B). PCR site-directed mutagenesis was used instead of PCR splicing, because it is an easier and faster procedure for the generation of mutants and has more than 80% efficiency. First, the mutant strand synthesis was performed by amplifying with the primer pair MU1 (Fw) and MU1 (RV), that contained the desired modifications, the parental pPepCTV-[NM]1.4 plasmid DNA strand using a high-fidelity polymerase. The resultant PCR product was 6.7 kb in size since it excluded 305 bp from the V1 gene, while the original plasmid was 7 kb (Fig. 7A). In order to remove the parental DNA template from the PCR product, it was digested with *DpnI* enzyme. The *DpnI* endonuclease is specific for methylated and hemi-methylated DNA and is used to digest the parental DNA template and to select for mutations containing synthesized DNA (Nelson and McClelland, 1992). Since the pPepCTV-[NM]1.4 DNA was isolated from an *E. coli* strain that methylates DNA (dam methylation), therefore it was susceptible to *DpnI* digestion. After digestion with *DpnI*, the PCR product was ligated and transformed into *E.coli* competent cells. The restriction pattern assay with *SpeI*, *SacII*, *XbaI* and *BamHI* restriction sites, confirmed the generation of the pPeCTV-SiV silencing vector (Fig. 7C). The resultant vector will be further tested as a VIGS vector.

(A)



(B)



(C)

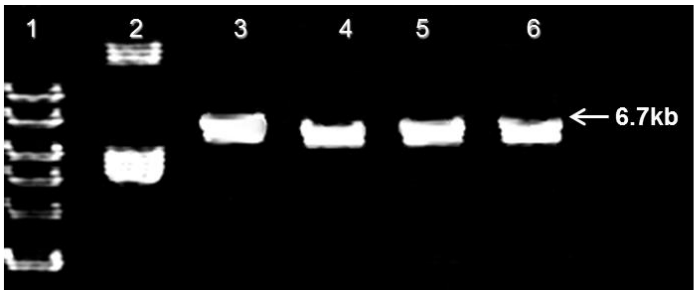


Fig.7. Map of pPepCTV-SiV and its restriction pattern assay. (A) Ethidium-stained-gel showing product amplification of pPepCTV[NM]1.4 vector with primer pair MU1 (FW) and MU2 (RV) by PCR directed-mutagenesis: lane 1, 1-kb ladder (NEB;

lane 2, pPepCTV-SiV 6.7kb product; lane 3, negative control. (B), Multimeric construct map from the generated pPepCTV-SiV silencing vector shows : truncated V1 gene region (blue arrow), *SpeI* restriction site generated by a single point mutation, *SacII* and *XbaI* linker cloning sites, and, *BamHI* unique restriction site near the linker cloning sites. (C), Restriction profile of a positive pPepCTV-SiV multimeric clone: lane 1,1kb DNA ladder (NEB); lane 2, undigested pPepCTV-SiV; lanes 3 to 6, vector digested with the unique restriction sites *SpeI*, *SacII, XbaI and BamHI*, respectively, shows the right restriction pattern from the vector.

To further determine the *PepCTV-[Mexico]* vector utility as an effective inducer for the silencing of endogenous genes, we first chose to target the tobacco sulfur (*SU*) gene. A 323 bp fragment of this gene was amplified from cDNA of *N. benthamiana* leaves (Fig. 8A). Then we targeted a 257bp fragment of the phytoene desaturase *(PDS*) gene from cDNA of *N.benthamiana* (Fig. 8A). The *SU* gene fragment will be inserted into the *SpeI* and *XbaI* linker sites of pPepCTV-SiV vector. The *PDS* fragment will be inserted in to the *SpeI* and *BamHI* unique sites of the silencing vector. The resulting vectors will be further inoculated into *N. benthamiana* plants (6-8 true leaf stage).

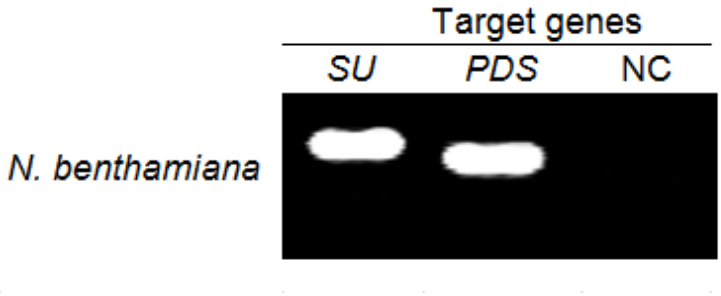


Fig.8. Isolation by RT-PCR of targeting sequences of *SU* and *PDS* cDNAs in leaves of *N. benthamiana* for insertion into pPepCTV-SiV vector. Lane NC represents the negative controls.

Analysis of predicted *N. benthamiana SU* RNA secondary structure

Careful selection of the nucleotide sequence of a target gene is an important factor for determining VIGS efficacy (Burch-Smith et al, 2004). By analyzing the RNA secondary structure of a gene and considering the results from previous studies, it may be possible to predict which regions or positions within a target gene may be better exposed to form dsRNA and induce silencing. Here, we chose for this analysis a prediction of the secondary RNA structure of the fragment of the *N. benthamiana SU* gene, which we isolated by PCR; however, this analysis must be performed for all target genes as well. Several predicted RNA folded structures for this gene using the Mfold software (http://mfold.rit.albany.edu/?q=mfold/RNA-Folding-Form) were obtained. The structure used, was the one with the highest free energy for analysis, because it reflects the overall stability of the target sequence and has higher RNAi efficiency (Vig et al., 2009). Figure 9 shows the *N. benthamiana SU* secondary structure, which has a free energy of -65.58 kcal/mol and, as shown, contains various stem loops, internal bulges, and hairpin structures.

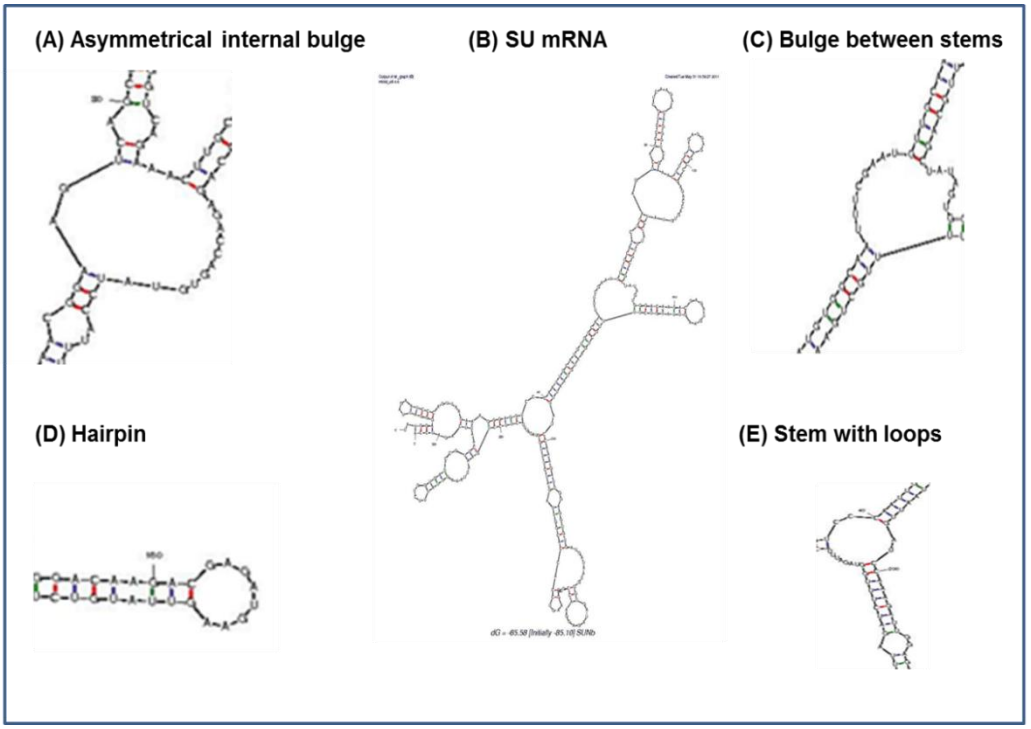


Fig.9. Predicted secondary structure of *N. benthamiana* SU mRNA (B). The structure was predicted with Mfold software. Different local structures from the predicted structure are shown: (A) Asymmetrical internal bulge between two stem loops, (C) a large asymmetric bulge between two adjacent stems, (D) Hairpin structure, and (E) stem region with two loops.

Discussion

Most of the known curtoviruses have been isolated and characterized in the United States. There exist only two reports of a curtovirus isolate characterized at the molecular level in Mexico (Chen et al., 2011; Mauricio-Castillo, 2011). Increasing reports of curly top disease distribution in economically important crops in the United States and Northern Mexico have raised the interest in the isolation and biological characterization of curtoviruses. Previously, Mauricio-Castillo et al, isolated in 2007 a new curtovirus strain named *BMCTV-[Mexico]* (GenBank accession No.: EU193175) and a new curtovirus species *PepCTV-[Nuevo Mexico]* (GenBank accession No.: EF501977). The successfully developed multimeric infectious clones derived from the*se* two new curtoviruses are significant contribution, since higher inoculation efficiencies were achieved with respect to monomeric clones. The generated pBMCTV-[MX]1.2 and pPepCTV-[NM]1.4 infectious clones have an additional origin of replication, which may reflect an inhibition of degradation of the monomeric genome upon delivery to host cells.

With the employment of the 1.2-mer infectious *BMCTV-[Mexico]* clone, we have been able to extend the biological characterization of this new strain into other plants species. The host range and symptomatology of *BMCTV-[Mexico]* are similar to those previously described for BMCTV isolates, including severe stunted growth, leaf curling, and crumpling in *N. benthamiana*, pepper and squash (Chen et al., 2010; Mauricio-Castillo, 2011; Soto et al., 2005; Stenger et al., 1990). These findings are consistent with reports that *BMCTV-[Mexico]* may be responsible for causing the curly top disease outbreak in commercial pepper fields of San Luis Potosi, Mexico (Mauricio-Castillo et al, 2007). However, the symptom phenotype in bean was different from that induced by a closely related isolate (*BMCTV-[MX-P24]),* which induces stunted growth, downward curling, and yellowing symptoms, but neither vein swelling nor general symptoms recovery (Chen et al., 2011). Nevertheless, vein swelling is included among the symptoms already reported for curly top disease (Bennett, 1971).

Although plant recovery has been reported for other geminiviruses, for curtoviruses there has been only one report for *N. benthamiana* plants inoculated with two mutants (L2 and L3) of the *BCTV-Logan* strain, which demonstrated recovery after infection (Hormuzdi and Bisaro, 1995). Thus, the recovery phenotype displayed by bean and wild tomato plants, which initially exhibited severe symptoms of infection with *BMCTV-[Mexico]*, appears to be an isolated finding. There are several factors that could explain this recovery phenomenon. For example, Chellapan et al. (2004) suggested that PTGS is the main mechanism associated with the recovery of a plant from geminivirus infection, since they found that viral accumulation and associated symptoms were inversely correlated with the presence of specific siRNAs. Carrillo-Tripp et al. (2007) proposed that a viral threshold concentration must be attained to generate or induce persistent symptoms of infection. Moreover, they reported that PepGMV-infected pepper plants showed lower percentage of recovery after inoculation with biolistic high pressure methods compared to low-pressure-hand-held procedures. On the other hand, even the inoculation efficacy of the two methods which we employed in this work was not high; in fact it does not explain the recovery phenotype that we observed, since it was not shown by all of the selected plant species. Clearly, additional factors may be involved, such as host-virus interactions that may block viral movement and replication and perhaps the efficiency of viral silencing suppressors as well.

The fact that neither micro-particle bombardment nor mechanical transmission of cloned multimeric *BMCTV-[Mexico]* DNA provided high rates of infectivity in the experimental hosts, may reflect the phloem-limited nature of curtovirus infections (Soto et al., 2005). Since it is likely that the primary cell targets of these methods are mesophyll cells, the virus may not have been able to access the phloem of inoculated leaves. In order to achieve higher infectivity rates, we may try to establish an agro-inoculation system. In general, the most efficient inoculation method for curtoviruses is leafhoppers transmission, but special facilities are required to maintain these insect populations (Soto and Gilbertson, 2003).

Previously, Mauricio-Castillo (2011) reported the characterization of the *PepCTV-[Nuevo Mexico]* in Anaheim pepper and chard. However, no clear symptoms of infection were reported in Pasilla pepper and beet, probably due to the use of a monomeric infectious clone. In order to overcome this difficulty, we developed a 1.4-mer infectious clone of the *PepCTV-[Nuevo Mexico]*, since multimeric clones have higher infectivity efficiency. Our results confirmed the infectivity of this multimeric construct in Serrano pepper plants. In contrast with the symptoms previously described for this curtovirus in Anaheim pepper, which included leaf mild crumpling and deformation (Mauricio-Castillo, 2011), the symptoms we observed in Serrano pepper included mild curling and interveinal chlorosis. Thus, indicating a difference of symptoms induced by *PepCTV-[Nuevo Mexico]* between pepper cultivars. Furthermore, we may extend this assay to different pepper cultivars, *N. benthamiana* and other plant species to fully characterize this curtovirus species. Therefore, if we confirm that pPepCTV-[NM]1.4 is infectious in a wide range of host plants, that finding may be very important, since this is the seventh new species from curtovirus isolated so far.

On the other hand, it seems that the use of the *BMCTV-[Mexico]* as a VIGS vector may offer several advantages over other VIGS vectors reported to date. First, it can facilitate efficient specific gene silencing among a broad range of plants, including model plants such as *A. thaliana* and *N. benthamiana* species, economically important crops such as tomato, cucurbits, and recalcitrant species such as pepper and bean. Second, the pBMCTV-SiV is an adaptable silencing vector, which contains several insertion sites for targeting sequences. Third, since this silencing vector has a truncated V1 gene, the virus will not be able to produce symptoms after infection that could mask the silencing phenotype. However, it may have a limited spread and perhaps a secondary helper plasmid expressing V1 should be generated. Nevertheless, Golenberg et al 2009 developed a VIGS vector based on a V1 and V2 truncated curtovirus and concluded that viral DNA replication, but not virus spread, was required for effective silencing. Fourth, if it is confirmed that this silencing vector can successfully silence a meristem gene like the *PCNA* in *N. benthamiana*, then it may be effective in assessing the function of

genes involved in shoot, leaf, flower, and fruit development (Burch-Smith et al., 2004).

In comparison with pBMCTV-SiV silencing vector, the pPepCTV-SiV has several similitudes. First, it can be used to study gene functions in some recalcitrant crops since it has been demonstrated that it can successfully infect pepper; however, it is still required to determine how wide is the *PepCTV-[Nuevo Mexico]* host range in order to use it as a competent VIGS vector. Second, even the pPepCTV-SiV only has a linker containing two insertion sites; it also has 2 additional unique insertion sites near to the linker cloning region. This fact makes it a fexible silencing vector as well. Third, no symptoms of infection that may mask the silencing phenotype will be produced since the pPepCTV-SiV also has a truncated V1 gene. As in the case of the *BMCTV-[Mexico]* silencing vector, it may have a limited spread and if necessary a second helper plasmid may be generated. In contrast, the pPepCTV-SiV vector was generated using PCR site-directed mutagenesis instead of PCR splicing. Although this is a faster and simpler procedure, perhaps some random mutation may occur in the sequence of the generated plasmid. Nevertheless, the small amount of starting DNA template required to perform the method, the high fidelity of the Phusion H-F polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency, decreases potential for random mutations during reaction.

One of the limitations of the pBMCTV-SiV and pPepCTV-SiV silencing vectors is that they may not produce a highly uniform knockout phenotype due to the expression of a silencing suppressor. The curtovirus C2 protein has been identified as a silencing suppressor of both PTGS and transcriptional gene silencing by a mechanism that involves adenosine kinase (ADK) inactivation (Bisaro, 2006; Buchman et al., 2004; Wang et al., 2005; Zhang et al., 2011). In order to overcome this limitation, we suspect that it may be useful to develop a version of these VIGS vectors which includes silent point mutations that may disrupt the C2 silencing suppressor function, without altering the function of genes C1 and C3.

To further test the *BMCTV-[Mexico]* and *PepCTV-[Nuevo Mexico]* silencing vectors, we may insert the amplified fragments of the *SU* and *PDS* genes into it. These genes have been used as reporter genes in previous VIGS studies, because their silencing produces an unambiguous phenotype (Kumagai et al., 1995; Ratcliff et al. 2001). Thus, proper silencing these genes with our vector may allow easily assess to silencing in selected plant species. The *SU* gene encodes a magnesium chelatase subunit required for chlorophyll production, while the *PDS* gene is essential for the production of carotenoid pigments. The length of the fragments isolated from *SU* and *PDS* genes are in the range usually used in VIGS systems, which fluctuates between 300-800 bp (Waterhouse and Helliwell, 2002). However, our vectors may not be functional if fragments longer than 300-498 bp are inserted, which corresponds to the length of the replaced region of the gene V1. In fact, insert length has been shown to influence stability of VIGS vectors (Bruun-Rasmussein et al., 2007). Moreover, geminiviruses may have constrained movement if they carry long inserts. In contrast, shorter sequences can be effective to induce silencing, but this depends upon their nucleotide composition (Igarashi et al., 2009). Thus, to further investigate the influence of insert length and nucleotide composition on efficiency and stability of VIGS, we may generate *BMCTV-[Mexico]* and *PepCTV-[Nuevo Mexico]* vectors carrying different lengths of a *SU* gene in *N. benthamiana*.

Based on the results of the prediction of the mRNA secondary structure from the *SU* gene in *N. benthamina*, we can design smaller target regions within this gene sequence. In addition, we may select regions of the predicted secondary RNA structure that may have a high potency of gene silencing. We may avoid selecting regions of the *N. benthamiana SU* gene with a high presence of hairpins or stem regions with many loops. Schubert et al. (2005) reported that the presence of hairpin structures within the target region reduced the strength of gene silencing. On the other hand, Vig et al. (2009) found that a stem region with two loops make silencing less efficient; however, they observed substantial gene silencing when regions with loops and asymmetrical bulges were targeted. To further investigate if there is an association between secondary mRNA predicted structure and VIGS

silencing efficiency, we may generate vectors carrying different fragments of similar length of the *SU* gene with different nucleotide compositions. Nevertheless, there is still a need for the development of software to define target mRNA secondary structure with a higher level of confidence.

Conclusion

This study provides evidence that the generation of multimeric infectious clones may be helpful in the investigation of biological properties of the curtoviruses. Moreover, our results demonstrate that *BMCTV-[Mexico]* clone derived from a new strain curtovirus, has a broad range of plant species and that the VIGS vector developed may be valuable to determine the resultant functions of various genes inserted into plants. In particular, the silencing vector reported here may be very helpful for functional genomics of cucurbits and legumes in-which systems to generate transgenic plants are not fully established. The successful assessment of the *PepCTV-[Nuevo Mexico]* host range in additional plant species may unveil its potential use as a silencing vector. Finally, a better understanding of the effect of insert length and mRNA secondary structure on VIGS stability and efficiency may provide the basis for further improvement and development of *BMCTV-[Mexico]* and *PepCTV-[Nuevo Mexico]* gene silencing vectors.

Materials and methods

Generation of multimeric infectious clones

To generate a multimeric infectious clone, a fragment from the *BMCTV-[Mexico]* genome (GenBank accession no.EU193175) from position 2454 to 110 was isolated by PCR. The fragment consisted of the viral replication origin and was ~600 bp in length. The primers used for amplification were Cur1-*PstI*-Fw 5' GCA TTT AGT GCC TCT GCA GCA TC 3' and Cur1-*SpeI*-Rv 5' TCG GCC ACA ACT TTT GAC TAG TC 3'. This fragment was cloned in the *PstI/SpeI* restriction sites of a modified pBlueScript plasmid (pBS) to generate the pBMCTV-[MX].2 construct. The full length BMCTV-[Mexico] genome was excised from a plasmid vector by digesting with *XmnI* and *XbaI* restriction enzymes. The digested fragments were size-separated on a 1.5% agarose gel. The 2.9 kb fragment corresponding to the full genome, was cut from the gel and extracted using the Wizard SV Gel Clean Up System (Promega, Madison, WI). This fragment was ligated overnight using T4 DNA ligase at 4°C. In order to increase the quantity of ligated molecules, it was subjected to rolling circle amplification (RCA) using the TempliPhi kit (GE Healthcare, USA) according to the manufacturer's instructions. Subsequently, the RCA amplified genome was digested with *SpeI* and cloned into pBMCTV-[MX].2. The resulting construct, designated pBMCTV-[MX]1.2, was tested by restriction analysis using *PstI* and *SpeI* to verify the correct orientation of the monomer, and then verified by sequencing.

For the generation of a *PepCTV-[New Mexico*] multimeric construct, a similar approach as applied to the *BMCTV-[Mexico]* clone was used. A fragment from the *PepCTV-[New Mexico*] genome (EF501977.1) from position 2283-504 was amplified by PCR. This fragment contained a viral origin of replication and was ~1.3kb in length. The primer pair used for amplification was: CurNM-*XhoI*-Fw1 5' ATT TCT GGA ACT CGA GTG AAA G 3' and CurNM-*HindIII*-Rev1 5' CAG AAG CTT CTT CAC GTC CTT 3'. Subsequently, the construct pPepCTV-[NM].4 was obtained by cloning the PCR fragment into the *HindIII* and *XhoI* restriction sites from a modified pBS. The viral full length genome cloned into pGEM-T-Easy vector was liberated by digesting with *EcoRI/ScaI*. The fragment corresponding to the

virus genome was extracted from gel, purified, ligated, and subjected to RCA. Then, the RCA product was cloned into the previous construction generating the construct pPepCTV-[NM]1.4. Restriction pattern assay to verify the proper orientation of the hemi-dimer was performed using *HindIII* and *XhoI* endonucleases. The positive clones were verified by sequencing.

Biolistic bombardment of plants

The construct pBMCTV[MX]1.2i (at a concentration of 0.7 µg/µl) was precipitated onto tungsten particles by combining 50 µl of a tungsten/water slurry with 17 µl of viral DNA, and then sonicated. For mock-inoculation, tungsten particles were coated with plasmid pBlueScript. Then, 2.5M $CaCl_2$ and 20 µl of 0.1M spermidine were added. The mixture was centrifuged at 10,000 rpm for 10 seconds and the liquid was removed. The slurry was then washed with 400 µl of ethanol and centrifuged. The remaining slurry was sonicated and re-suspended in 60 µl of ethanol and 10µl of this suspension were applied to the screen of the luer lock adaptor of the particle gun. Six seedlings, with 4-6 true leaves, from each of the following species; *Nicotiana benthamiana*, *Arabidopsis thaliana* (ecotype Col-0), *Solanum habrochaites* and pepper (*Capsicum annuum* cv. Serrano) were inoculated with micro-particle bombardment. Two seedlings from each of the selected plant species were used as mocks. The target plants were placed in the vacuum chamber approximately 5 cm from the adapter. A vacuum was drawn and the particles (coated with either 2 µg of viral DNA or pBlueScript) were expelled from the particle gun under a helium pressure of 650 psi onto each plant. The plants were placed in a greenhouse and covered with plastic wrap for 2 days to prevent desiccation during recovery.

Mechanical transmission

Five seedlings with 2-3 true leaves from squash (*Cucurbita pepo* cv. Grey Zucchini) and bean (*Phaseolus vulgaris* cv. Pinto Villa), which were not able to fit into our bombardment chamber due to their height and because it is a cheaper and

easier method, were inoculated with mechanical transmission with the construct pBMCTV[MX]1.2i. Carborundum powder was evenly distributed onto 2-3 young leaves ~2 cm in length. Subsequently, a solution of the construct pBMCTV[MX]1.2i (5 µg of DNA in 20 µl of water) was placed at the leaf base from selected leaves and spread by rubbing with soft but firm strokes from the base to the tip of each leaf, in accordance to Ascencio-Ibañez and Settlage (2007) method. At the end of these treatments, the leaves were gently washed with a stream of distilled water to remove excess carborundum. The plants were covered with plastic for 2 days and placed in a greenhouse. Additionally, four pepper (*Capsicum annuum* cv. Serrano) seedlings (4 leaf stage) were mock inoculated with pBlueScript plasmid. Four pepper seedlings from the same leaf stage were inoculated by mechanical transmission with the construct pPepCTV[MX]1.4 (5 µg of DNA in 20 µl of water). A similar inoculation procedure was followed as used for squash and bean seedlings previously.

PCR assay of viral infection

The plants, inoculated by either of the two methods mentioned above, were evaluated for symptoms of disease. First, the infection status of the inoculated plants was assessed by visual inspection for symptoms. Total genomic DNA was extracted from newly emerged leaves from symptomatic plants using the Dellaporta method (Dellaporta et al., 1983). Then, the DNA extracts of squash and bean were used in a PCR with the universal curtovirus primer pair (Cur Rep 2GQ Fw and Cur CP 450 Rv), which can direct the amplification of a ~1.6 kb fragment from a conserved region of the curtovirus genome. DNA extracts of pepper were used in a PCR with the primer pair (Cur NM-*XhoI* Fw1 and CurNM-*HindIII*-Rev1) which can direct the amplification of a ~1.3 kb fragment from the *PepCTV-[Nuevo Mexico]* genome.

Construction of *BMCTV-[Mexico]* VIGS vector

Construction of the pBMCTV-SiV vector was carried out in several steps using PCR splicing. First, a fragment of the *BMCTV-[Mexico]* genome from positions 107 to 754 (647 bp) was isolated by PCR. This fragment contains part of the intergenic region (IR), the complete V3 and V2 genes, the beginning of the V1 gene, which starts at position 578, and a 24 nt multiple cloning site (MCS) tail. The primers used in this reaction were AFw 5' TAG TCA AAA GTT GTG GCC GAC 3'and M1Rv 5' GAA TTC GAT ATC CTC GAG CCC GGG TGA ACC AAG CTT ATC 3'. M1Rv had a 24 nt tail with four unique restriction sites (*SmaI, XhoI, EcoRV* and *EcoRI*) to generate a MCS. This primer was designed to deviate from the *BMCTV-[Mexico]* sequence at position 744 (A to T) to create an additional restriction site (*HindIII*) near the MCS. Then, a second fragment from the same genome was isolated by PCR, which included position 2046 to 1245 (801bp). This fragment included the 3'end of the V1 gene, which ends at position 1342, the complete C2 and C3 genes, and the end of C1 gene from position 2046 to 1784. The fragment was also designed to have a 24 nt overlapping MCS with the first fragment. The primers used to amplify the reaction were M2Fw 5' CCC GGG CTC GAG GAT ATC GAA TTC AGG AGA ATG CTT TAC 3' and BRv 5' GCA TTG GAA ACA CCT CAT AGG 3'. M2Fw also had a 24 nt MCS tail. Subsequently, the two fragments that were generated (647 bp and 801 bp, respectively) were purified and mixed in a third PCR reaction using primer pair AFw and BRv. The resultant fragment was 1448 bp in length. This fragment included a *HindIII* site, the 24 nt MCS but excluded the gene V1 from position 755 to 1244 (489 bp). Then, this third fragment and the multimeric construct pBMCTV[MX]1.2 were digested with *StuI* and *SacI* restriction enzymes. The digested fragments were ligated overnight using T4 DNA ligase at 16°C. Finally, the resultant pBMCTV-SiV vector was generated and further tested by restriction digest.

Construction of *PepCTV-[Mexico]* VIGS vector

The pPepCTV-[NM]1.4 vector was used as a template to generate a *PepCTV-[Mexico]* silencing vector by site-directed mutagenesis. The site –directed mutagenesis PCR reaction was performed using 0.4 µl of Phusion (Finnzymes, Filand) High-fidelity DNA polymerase, 8 µl of 5XPhusionHf buffer, 10 mM of dNTPs, 0.8 µl of each primer (forward and reverse primer pair), 1 µl of pPepCTV-[NM]1.4 DNA and distilled water to a volume of 40 µl. The PCR amplification was performed for 20 cycles, using the following temperatures: 98°C, 58°C, and 72°C, for denaturation, annealing and extension, respectively. The primers used in this reaction were MU1 (Rv) 5' TCT AGA CCG CGG CTC CAC TGA TAC TTA CTA G 3' and MU1 (FW) 5' CCG CGG TCT AGA GAT ATT CCA GAT AAT GGT CAG G 3'. These primers were complementary to the opposite strands of the vector, and excluded 306 bp from the V1 coding region in the pPepCTV-[NM]1.4 plasmid. Both primers were also designed to have a liker tail containing the *SacII* and *XhoI* restriction sites. MU1 (Rv) was designed to deviate from the plasmid sequence at position 1968 (A-T) to generate an insertion site *SpeI*. Following amplification, the PCR product was treated with the enzyme *DpnI*. Then it was ligated at 4°C overnight using T4 DNA ligase. The resultant nicked vector DNA incorporating the desired mutation and linker was subsequently transformed iinto *E. coli* competent cells. Finally, the pPepCTV-SiV silencing vector was obtained by selecting positive clones tested by restriction digest with *SpeI*, *XbaI* and *SacII*.

Isolation of targeting sequences

The sequences of the *SU*, *PDS*, and *PCNA* genes were amplified from cDNA samples from *N. benthamiana* leaves using primer pairs for insertion into pBMCTV-SiV vector; (1) NbSU27*XhoI* (Fw) (5' CCG CTC GAG TAT GGA GGG ATT AGA ATC 3' corresponding to nt positions 27-44) and NbSU351*EcoRI* (Rv) (5' CCG AAT TCC TGG ATC TGA ATT GAA CGG 3' complementary to nt positions 333-350) for *SU* gene (Genebank accession no. AJ571699), (2) NbPDS938*XhoI*

(Fw) (5’ CCG CTC GAG TTT GCT ATT GGA CTC TTG 3’ to nt positions 938-955) and NbPDS1345EcoRI (Rv) (5’ CGG GAA TTC AGC ATC TCC TTT AAT TGT ACT GC 3’ complementary to nt positions 1323-1345) for *PDS* gene (EU165355), and (3) NbPCNA113*Xho*I (Fw) (5’ CCC TCG AGT TGG AAT TAC GGC TTG TT 3’ corresponding to nt positions 113-130) and NbPCNA491*EcoRI* (Rv) (5’ CCG AAT TCT CCC AAG ATG TTC ACT GTC 3’ complementary to nt positions 473-491) for *PCNA* gene (AF305075), respectively. The amplified DNAs were cloned into pGEM-T-Easy vectors.

For the insertion into the pPepCTV-SiV vector the sequences of the SU, and PDS, were amplified from cDNA samples from *N. benthamiana* leaves using primer pairs; (1) NbSU27*SpeI* (Fw) (5‘ CCG ACT AGT TAT GGA GGG ATT AGA ATC 3’ corresponding to nt positions 27-44) and NbSU350*XbaI*(Rv) (5’ CCT CTA GAC TGG ATC TGA ATT GAA CGG 3’ complementary to nt positions 333-350) for SU gene (Genebank accession no. AJ571699), (2) NbPDS*SpeI*(Fw) (5’ CCG ACT AGT TTC ATA AAC CCT GAC GAG CTT TCG 3’ to nt positions 1088-1115) and NbPDS*BamHI* (Rv) (5’ CGG GGA TCC AGC ATC TCC TTT AAT TGT ACT GC 3’ complementary to nt 1323-1345 positions) for PDS gene (EU165355).

The sequence of squash *PDS* gene for insertion into pBMCTV-SiV vector, was amplified a from cDNA sample of squash leaves using primer pair: cuPDS7*Xho*I (Fw) (5’ CCC TCG AGT ATG AGA CAG GTC TGC AC 3’ corresponding to nt positions 7-24) and cuPDS330*EcoRI* (Rv) (5’ CCG GAA TTC TCT CAT CCA CTC TTG CAC 3’ complementary to nt positions 313-330) for a *PDS* gene (EF159942). The amplified DNAs were cloned into pGEM-T Easy vectors for sequencing.

RNA extraction and RT-PCR

N. benthamiana and squash leaves samples (0.1 g) were homogenized with 1 ml of Trizol Reagent (Invitrogen, Carlsband, CA, USA) and incubated for 5 minutes at 4°C. Then, 0.2 ml of chloroform was added to each sample. The samples were centrifuged and RNA was precipitated with 0.5 ml of isopropyl

alcohol. The pellets were collected by centrifugation, washed with 1 mL of 75% ethanol, and dissolved in 50 µl of DEPC water (Diethylpyrocarbonate RNAse free water). The nucleic acid samples were then treated with DNAseI (Invitrogen, Carlsband, CA, USA) and the RNA samples were finally dissolved in DEPC water at a concentration of 1µg/µl. Concentrations of RNA samples were estimated by spectrophotometry. First strand cDNA was synthesized using 2 µl of RNA, oligo (dt), and SuperScript II RT Reverse transcriptase (Invitrogen, Carlsband, CA, USA), following the manufacturer's protocol.

N. benthamiana SU RNA secondary structure prediction

The *N. benthamiana SU* RNA secondary structure was obtained using default settings on the mfold web server version 3.2 (http://mfold.rit.albany.edu/?q=mfold/RNA-Folding-Form). The partial mRNA sequence for gen *SU* from the *N. benthamiana* (AJ571699) from positions 27-350 was used for this analysis.

REFERENCES

Abraham-Juárez, M.R., Rocha-Granados, M.C., López, M.G., Rivera-Bustamante, R.F., and Ochoa-Alejo, N., 2008. Virus-induced silencing of Comt, pAmt and Kas genes results in a reduction of capsaicinoid accumulation in chilli pepper fruits. Planta 227,681-695.

Ascencio-Ibañez, J.T., Settlage, S.B., 2007. DNA abrasion onto plants is an effective method for geminivirus infection and virus induced gene silencing. J. Virol. Met. 142,198-203.

Baulcome, D., 2004. RNAi silencing in plants. Nature 409, 363-366.

Bennett, C.W., 1971. The curly top disease of sugar beet and other plants. The American Phytopathological Society, St. Paul, MN. Monogr. 7

Bisaro, D.M., 2006. Silencing suppression by geminivirus proteins. Virology 344,158-168.

Blevins, T., Rajeswaran, R., Shivaprasad, P. V., Beknazariants, D., Si-Ammour, A., Park, H.S., Vazquez, F., Robertson, D., Meins Jr, F., Hohn, T., Pooggin, M.M., 2006. Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Res. 34,21, 6233-6246.

Bruun-Rasmussen, M., Madsen, C.T., Jessing, S., Albrechtsen, M., 2007. Stability of Barley stripe mosaic virus-induced gene silencing in barley. Mol. Plant-Microbe Interact. 20, 1323–1331.

Buchmann, R.C., Asad, S., Wolf, J.N., Mohannath, G., and Bisaro, D.M., 2009. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. J. Virol. 83, 5005–5013.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B., Dinesh-Kumar, S.P., 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J. 39, 734–746.

Carrillo-Tripp, J., Lozoya-Gloria, E., Rivera-Bustamante, R. F., 2007. Symptom remission and specific resistance of pepper plants after infection by Pepper golden mosaic virus. Phytopathology 97, 51-59.

Chellappan, P., Vanitharani, R., Fauquet, C. M. 2004. Short interfering RNA accumulation correlates with host recovery in DNA virusinfected hosts, and gene silencing targets specific viral sequences. J. Virol. 78,7465-7477.

Chen, L.-F., Vivoda, E., Gilbertson, R.L., 2011. Genetic diversity in curtoviruses: a highly divergent strain of *Beet mild curly top virus* associated with an outbreak of curly top disease in Mexico. Arch. Virol. 156, 4, 546-555.

Chen, X., 2009. Small RNAs and their roles. Annu. Rev. Cell Dev. Biol. 35, 21–44.

Dellaporta, S.L.,Wood,J., Hicks, J.B., 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1, 19-21.

Fofana, I. B.F., Sangare, A., Collier, R., Taylor,C., Fauquet, C., 2004. A geminivirus-induced gene silencing system for gene function validation in cassava. Plant Mol. Biol. 56, 613–624.

Golenberg, E.M, Sather, D.N., Hancock, L.C., Buckley, K.J., Villafranco, N.M., Bisaro, D.M., 2009. Development of a gene silencing DNA vector derived from a broad host range geminivirus. Plant Methods 5:9.

Gutierrez, G., 2000. DNA replication and the plant cell cycle: learning from geminiviruses. EMBO J. 19, 792-799.

Hanley-Bowdoin, L., Settlage, S., Orozco, B., Nagar, S., Robertson, D., 2000. Geminiviruses: models for plant DNA replication, transcription, and cell-cycle regulation. Crit. Rev. Biochem. Mol. Biol. 35,105-140.

Hormuzdi, S.G., Bisaro, D.M., 1993**.** Genetic Analysis of Beet Curly Top Virus: Evidence of three virion sense genes involved in movement and regulation of single- and double-stranded DNA levels. Virology 193, 900-909.

Hormuzdi, S.G., Bisaro, D.M., 1995**.** Genetic Analysis of Beet Curly Top Virus: Examination of the Roles of L2 and L3 Genes in Viral Pathogenesis. Virology 206,1044-1054.

Igarashi, A., Yamagata, K., Sugai, T., Takashi, Y., Sugawara, E., Tamura, A., Yaegashi, H., Yamagishi, N., Takahashi, T., Isogai, M., Takashi, H., Yoshikawa, N., 2009. Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, Arabidopsis thaliana, cucurbits, and legumes.Virology 386, 407-416.

Kumagai, M.H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., Grill, L.K., 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc. NatI. Acad. Sci. USA 92,1679-1683.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.P, Dolja, V.V., Fernández-Calvino, L., López-Moya, J., Burgyán, J., 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. The EMBO J. 25, 2768-2780.

Mauricio-Castillo, 2011. Caracterización de nuevas especies y cepas de geminivirus por un método molecular que optimiza la detección de infecciones mixtas. Tesis de Doctorado, IPICYT, División de Biología Molecular.

Muangsan, N., Beclin, C., Vaucheret, H., Robertson, D., 2004. Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. Plant J 38, 1004-1014.

Napoli, C.A., Lemieux, C., Jorgensen, R.A., 1990. Introduction of chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2, 279-289.

Nelson, M., McClelland, M., 1992. Methods Enzymol. 216, 279-303.

Peele, C., Jordan, C.V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., Robertson, D., 2001. Silencing of a meristematic gene using geminivirus-derived vectors. Plant J. 27, 357-366.

Rana, T.M., 2007. Illuminating the silence: understanding the structure and function of small RNAs. Nat. Rev. Mol. Cell Biol. 8, 23-36.

Ratcliff, F., Martin-Hernandez, A.M., Baulcome, D.C., 2001.Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J. 25, 237-245.

Robertson, D., 2004. VIGS vectors for gene silencing: many targets, many tools. Annu. Rev. Plant. Biol. 55, 495-519.

Ruiz, M. T., Voinnet, O., Baulcombe, D.C., 1998. Initiation and maintenance of virus-induced gene silencing. Plant Cell 10, 937-946.

Schubert, S., Grunweller, A., Erdamann, V. A., Kurreck, J., 2005. Local RNA target structure influences siRNA efficacy: Systematic analysis of intentionally designed binding regions. J. Mol. Biol. 348, 883-893.

Soto, M.J., Gilbertson, R.L., 2003. Distribution and rate of movement of the curtovirus *Beet mild curly top virus* (Family *Geminiviridae*) in the beet leafhopper. Phytopathology 93, 478-484.

Soto, M.J., Chen, L.-F., Seo, Y.-S., Gilbertson, R.L., 2005. Identification of regions of the Beet mild curly top virus (Family Geminiviridae) capsid protein involved in systemic infection, virion formation and leafhopper transmission. Virology 341, 257-270.

Stanley, J., Latham, J.R., Pinner, M.S., Bedford, I., Markham, P.G., 1992. Mutational analysis of the monopartite gemnivirus beet curly top virus. Virology 191, 396-405.

Stenger, D.C., Carbonaro, D., Duffus, J.E., 1990. Genomic characterization of phenotypic variants of beet curly top virus. J. Gen. Virol.71, 2211 – 2215.

Tripp-Carrillo, J., Shimada-Beltrán, H., Rivera-Bustamante, R., 2006. Use of geminiviral vectors for functional genomics. Curr. Opin. Plant Biol., 9, 209-215.

Turnage, M. A., Muangsan, N., Peele, C. G., Robertson, D., 2002. Geminivirus-based vectors for gene silencing in *Arabidopsis*. The Plant Journal 30, 107–114.

Vanitharani, R., Chellappan, P., Fauquet, C.M., 2005. Geminiviruses and RNA silencing. Trends Plant Sci. 10, 1360-1385.

Vig, K., Lewis, N., Moore, E.G., Pillai, S., Dennis, V.A., Singh, S.R., 2009.Secondary RNA structure and its role in RNA interference to silence the respiratory syncytial virus fusion protein gene. Mol. Biotechnol. Published online

Wang, H., Buckley, K.J., Yang, X., Buchmann, R.C., Bisaro, D.M., 2005. Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. J. Virol. 79, 7410–7418.

Waterhouse, P.M., Helliwell, C.A., 2003. Exploring plant genomes by RNA-induced gene silencing. Nat. Rev. Genet. 4, 29-38.

Zhang, Z., Chen, H., Huang,X., Xia, R., Zhao,Q., Lai,J., Teng,K., Li,Y., Liang,L.,Du,Q., Zhou,X., Guo,H., Xiea,Q., 2011.BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in Arabidopsis. Plant Cell 23, 273–288.

APPENDIX

Presentation of the poster**: "Isolation and characterization of a pepper-infecting curtovirus from Mexico"** at the 6th International Geminivirus Symposium and 4th International ssDNA Comparative Virology Workshop

Virus Family: *Geminiviridae*

Category: Taxonomy/Emerging viruses

Title: Isolation and characterization of a pepper-infecting curtovirus from Mexico

Authors: **M. TAJA**, J.A. MAURICIO-CASTILLO, B. BAÑUELOS-HERNANDEZ AND G.R. ARGUELLO-ASTORGA

Address: Instituto Potosino de Investigación Científica y Tecnológica, A.C. Camino a la Presa San José 2055, Colonia Lomas 4ta. Sección, San Luis Potosí, S.L.P., México C.P. 78216

Abstract: Geminiviruses in the genus *Curtovirus* are vectored by the beet leafhopper (*Circulifer tenellus*) and infect a wide range of dicotyledonous plants. Most of the known curtoviruses have been isolated and characterized in the United States; however, none of the curtoviruses isolated in Mexico have been previously characterized at the molecular level. To search for curtoviruses in the dry lands of North-Central Mexico, where the viral vector is widely distributed, two sets of degenerated primers were designed that made feasible the amplification by PCR of the complete genomic DNA of these viruses. Pepper plants which displayed upwardly rolled and yellow leaves, as well as stunted growth were collected during the summer of 2006 in commercial fields of San Luis Potosi, Mexico. Several curtovirus-positive samples were identified, and PCR-RFLP analyses showed that a single type of virus was present in all of them. The complete genome from this curtovirus was sequenced (GenBank No. EU193175). Full-length genome comparisons revealed that the isolate was a new strain of *Beet mild curly top virus* (BMCTV) that shared 88.1% of nucleotide sequence identity with its closest relative

BMCTV-[Worland]. Moreover, to determine experimentally the host range of *BMCTV-[Mexico],* the multimeric construct pBMCTV-[MX]1.2 was generated. This infectious clone was inoculated by either micro-particle bombardment or mechanical transmission into *Nicotiana benthamiana*, Serrano pepper, and chard seedlings. Infected plants of all the species developed disease symptoms. The known courtoviruses are geographically restricted to the north hemisphere, and interestingly, the *BMCTV-[Mexico]* is the member of this viral group which has been isolated close to the earth tropical belt.