Journal of
Antivirals & Antiretrovirals
ISSN: 1948-5964

Gulati-Sakhuja and Liu J Antivir Antiretrovir 2011, 3:1
DOI: 10.4172/jaa.1000026

Research Article Open Access

Infectious Full-Length Clones of Calibrachoa Mottle Virus (CbMV)

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Abstract

Full-length cDNA clones derived from genomic RNA (gRNA) and subgenomic RNAs (sgRNAs) of Calibrachoa mottle virus (CbMV) were constructed under the control of the T7 RNA promoter and ligated into plasmid pUC-18. The capped and uncapped *in vitro* transcripts, synthesized from full length genomic cDNA clone pUC-CbMV-FL generated chlorotic local lesions on mechanical inoculated *Chenopodium quinoa* within 10 to 15 days post-inoculation. The progeny virions recovered from inoculated symptomatic leaves had the same morphological properties as the parental virions. These virions are also reacted positively with CbMV antiserum in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and the viral coat protein was detected by Western blotting. The presence of five open reading frames (ORFs) on the CbMV genome and synthesis of corresponding proteins by gRNA and sgRNAs were confirmed by Northern blotting and *in vitro* translation. The transcription start site of three sgRNAs of CbMV was also determined. Successful construction of full-length infectious cDNA clone of CbMV makes it possible to develop molecular tools that can be used to understand the gene functions of this virus.

Keywords: CbMV; Calibrachoa mottle virus; Carmovirus; Full-length infectious clones; Geomic and subgenomic RNAs; *In vitro* transcription and translation; Northern and Western blotting

Introduction

Calibrachoa mottle virus (CbMV) infects Calibrachoa, an important new horticultural plant in Europe and in the United States [1]. It belongs to the genus *Carmovirus* of family *Tombusviridae* and has a single stranded, positive-sense RNA genome of 3.919 kb (Genbank accession number GQ244431) [2]. Molecular analysis of the CbMV genomic sequence revealed the presence of at least five open reading frames (ORFs) flanked by a short (34 nt) 5' untranslated region (UTR) and 234 nt non polyadenylated 3' UTR. The first 5' proximal ORF (ORF1) encodes a protein of 28 kDa (p28) and terminates with an amber stop codon, whose readthrough would result in the synthesis of a protein of 87 kDa (ORF2; p87) that encloses the motifs of the viral RNA dependent-RNA polymerase (RdRp) [2]. A comparison of amino acid sequences of CbMV with other carmoviruses has shown that both these proteins are involved with expression and/or regulation of the RdRp domain in viruses [3-5]. *In vitro* translation studies of *Carnation mottle virus* (CarMV) [6,7] showed that these two ORFs were expressed from the viral genomic RNA. Two small centrally located ORFs encode overlapping proteins of approximately 8 kDa (p8) and 9 kDa (p9), respectively. These proteins have been shown in *Turnip crinkle virus* (TCV) to be involved with viral cell-to-cell movement (movement proteins or MPs) [3]. The 3' proximal ORF5 encodes viral coat protein (CP) of 37 kDa (p37). There is no evidence for the presence of cap at 5' end of carmovirus genomes.

Construction of infectious clones corresponding to the genomes of RNA viruses, obtained either as cDNA clones or as *in vitro*-transcribed RNA copies, has allowed the study of virus-host interactions such as cell to cell movement. Important information is obtained about the expression and replication of RNA viruses by using *in vitro* mutagenesis (deletions, insertions, substitutions) and complementation tests. In the study of natural or induced RNA recombination, mechanisms of defect interfering RNA and satellite RNA genesis can also be obtained through infectious clones [8-10]. Infectious clones, considered as the pools of viral genes for designing antiviral strategies, are an essential source of material for the preparation of new viral vectors [8,10].

Plus-stranded RNA viruses synthesize subgenomic mRNAs (sgRNAs) from the single genomic mRNA (gRNA) for the expression of their 3'-proximal genes. These sgRNAs, with same 3' ends as gRNA, encode late viral genes that synthesize products required for pathogenesis and particle formation. The sgRNA promoter, an internal sequence in the minus strand of gRNA, is recognized by the RdRP for sgRNA synthesis. In CbMV, three 3' coterminal sgRNAs of sizes approximately 3.92, 1.63, and 1.26 kb are reported [2]. Studies of *Saguaro cactus virus* (SCV) and TCV have shown that two movement proteins (p8 and p9) are synthesized from 1.7 kb sgRNA and viral coat protein is synthesized from a 1.4 kb sgRNA [3,11-13].

Up to date, full-length infectious cDNAs have been obtained in many carmoviruses e.g. *Melon necrotic spot virus* (MNSV) [14,15], TCV [16], SCV [13], *Hibiscus chorotic ringspot virus* (HCRSV) [17] and *Pelargonium flower break virus* (PFBV) [18]. The sgRNA promoters, with sizes ranging from 20 nucleotides to over 100 nucleotides, have been mapped and characterized in several carmoviruses [19,20,13]. In all these cases, the promoter sequence is present upstream of the transcription starting site.

We report here the construction of full length infectious cDNA clones of CbMV. In addition, CbMV gene expression strategy, and sequences of sgRNA promoter are presented.

Materials and Methods

Virus isolation and extraction of dsRNA and total RNA

The CbMV was originally isolated from symptomatic leaves of *Calibrachoa* plants and maintained in *Nicotiana benthamiana* by mechanical inoculations [1]. Virions were partially purified from CbMV infected *N. benthamiana* plants as described by Liu et al. [1]. RNA from purified virions and total RNA from CbMV infected *C.*

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Accepted January 19, 2011; **Published** January 21, 2011

Citation: Gulati-Sakhuja A, Liu HY (2011) Infectious Full-Length Clones of Calibrachoa Mottle Virus (CbMV). J Antivir Antiretrovir 3: 001-007. doi:10.4172/jaa.1000026

quinoa plants were isolated/extracted using RNeasy Plant Mini kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. Viral-associated dsRNA was prepared from CbMV infected *Calibrachoa* and *C.quinoa* plants according to Valverde et al. [21].

DsRNA electrophoresis

The dsRNAs isolated from Calibrachoa and *C. quinoa* were separated on a non-denaturing 1% agarose gel supplemented with 0.5 µg/ml ethidium bromide. All four bands corresponding to the CbMV gRNA (~ 3.9 kb) and sgRNA's (~3.1 kb, ~1.6 kb and ~1.3 kb) were excised and cleaned using Qiagen MinElute kit (Valencia, CA).

Construction of a full-length cDNA clone of CbMV- gRNA

To prepare the full length clone, two overlapping cDNA fragments (A and B), covering the complete genomic CbMV RNA (Figure 1(1)), were synthesized separately. The purified dsRNA was used as template to synthesize first-strand of cDNA. The dsRNA was denatured with 20 mM methyl mercury hydroxide for 6 minutes at room temperature, followed immediately by heating at 65°C for 4 additional minutes and incubation on ice for 1 minute. Reverse transcription (RT) was performed using primer Cb-3L and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA), per manufacturer's instructions. The RT product was

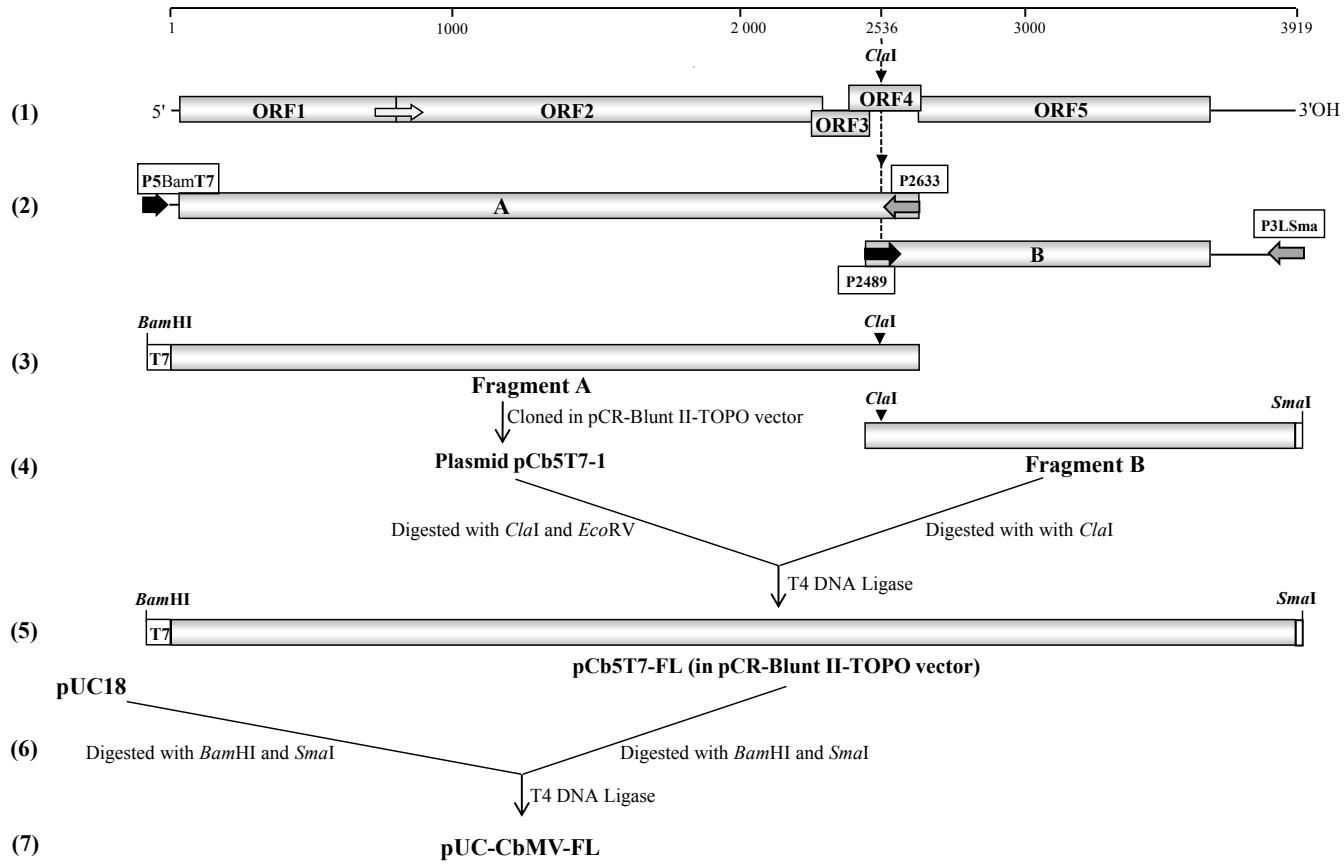

Figure 1:

Primer		Position[a]	Sequence[b] (5' to 3')
Cb-3L	antisense	3'-end (3919-3904)	CGGGCGGGGAGACTCC
P5BamT7-1	sense	5'-end (1-13)	GAGGATCCTAATACGACTCACTATAGCGATAAACTTAGC
P2489	sense	2489-2510	CCATTTTTGACTTCTTTCCCCC
P2633	antisense	2633-2609	TGGGTTTTACTGCTGTCGTGTGTAG
P3LSma	antisense	3'-end (3919-3904)	ATATCCCGGGCGGGCGGGGAGACTCC
PBamT7-3.1	sense	827-850	GAGGATCCTAATACGACTCACTATAGCACACCCTTACCTCAAAGAGAAAG
PBamT7-1.6	sense	2285-2308	GAGGATCCTAATACGACTCACTATAGAATCATGGAGCGCGAACATCCCAC
PBamT7-1.3	sense	2513-2538	GAGGATCCTAATACGACTCACTATAGTGAATATCCCTAACATAAACCAATCG
P3925	antisense	3502-3479	CCAAACTACCAGCCATTTACCCTC
P517	antisense	979-956	CGTAGAAAACCCGCTCCAGTAATC
P2527	antisense	2633-2609	TGGGTTTTACTGCTGTCGTGTGTAG
P2168	antisense	2992-2971	GGGGCTGTATTGACCTGGATTC
Oligo-dT	antisense	Poly(A)	GGCCACGCGTCGACTAGTAC$(T)_{17}$
Oligo-dG	antisense	Poly(C)	TACTACTCATATATTAATATA$(G)_{14}$

[a]Binding position on complete genome sequence of Calibrachoa mottle virus (CbMV, GQ244431)
[b]Shaded sequences are not from CbMV and were included to generate restriction sites *Bam*HI or SmaI (bold) and to incorporate T7 RNA polymerase promoter (underlined)

Table 1: Oligonucleotide sequences of primers used for reverse transcription and polymerase chain reaction.

PCR amplified using PrimeSTAR HS DNA polymerase (Takara, Madison, WI) as per manufacturer's protocol. The sequences of all the oligonucleotides used are listed in Table 1. Fragment A (1- 2633 nt) was synthesized using primer pairs P5BamT7 and P2633. The oligonucleotide P5BamT7 contained restriction site *Bam*HI (Bold), the T7 RNA promoter sequence (underlined) plus 13 nucleotides of CbMV 5'- end sequence, and oligonucleotide P2633 located on the minus strand. Fragment B (2489-3919 nt) was synthesized using sense primer P2489 and antisense primer P3L*Sma* (which includes the last 16 nucleotides of 3'- end of CbMV sequence plus restriction site *Sma*I(bold)) (Figure 1(2)). Conditions for the PCR reactions were: initial denaturation at 98°C for 2 min followed by 35 cycles of 10 sec at 98°C, 10 sec at 53°C (fragment A) or 55°C (fragment B), and 105 sec (fragment B) to 3 min (fragment A) at 72°C. The final extension was 10 min at 72°C. The PCR fragment A ((Figure 1(3)) was cloned in vector pCR-Blunt II-TOPO using Zero Blunt TOPO PCR cloning kit (Invitrogen). The orientation and identity of clone pCb5T7-1 (containing fragment A) was checked by restriction digestion, and sequencing. To complete the full-length clone (fragment A+B), plasmid pCb5T7-1 was digested with restriction enzymes *Cla*I (a restriction site located at 2633 on fragment A) and *Eco*RV (a restriction site located at multiple cloning site of the vector pCR-Blunt II-TOPO), and fragment B was digested with restriction enzyme *Cla*I (a restriction site located at nt 48) allowing ligation with fragment A in pCb5T7-1 (Figure 1(4)). The identity of resulting clone pCb5T7-FL (Figure 1(5)) was confirmed by restriction digestion and sequencing. The complete full-length cDNA with T7 promoter at 5'- and *Sma*I at 3'-termini was isolated from pCb5T7-FL using restriction enzymes *Bam*HI and *Sma*I and cloned between *Bam*HI and *Sma*I sites of vector pUC18 (Figure 1(6)). The final clone containing the entire CbMV genome under the control of T7 promoter was named pUC-CbMV-FL (Figure 1(7)) and sequenced.

Construction of a cDNA clone of CbMV- sgRNAs

Full-length clones of different sgRNAs were prepared by using purified dsRNA as a template. The first strand of cDNA for all three sgRNAs was synthesized using the same method as discussed in section 2.3. To prepare full-length clone of 3.1 kb sgRNA, two overlapping fragments were generated, following the same procedure as for full-length cDNA clone of g-RNA. The primers used for RT and PCR were also the same, except primer PBamT7-3.1 was used in place of P5BamT7 (Table 1).

Full-length cDNA clones of 1.6 and 1.3 kb sgRNAs were synthesized as single fragments by using forward primers PBamT7-1.6 and PBamT7-1.3 for the 1.6 and 1.3 kb sgRNAs, respectively and Cb3LSma as the reverse primer. The oligonucleotides P5BamT7-3.1, PBamT7-1.6 and PBamT7-1.3 contained restriction site *Bam*HI (Bold), the T7 RNA promoter sequence (underlined) plus 24-26 nucleotides from internal regions of CbMV gRNA (Table 1). All the PCR products were cloned in pCR-Blunt II-TOPO vector prior to cloning in vector pUC18, as mentioned in section 2.3.

Sequencing of 5'-ends of sgRNAs

To confirm the sequence of the 5'-termini of the CbMV sgRNAs, poly(A)- or (poly)C-tails were added to the corresponding dsRNAs using rATP or rCTP (Promega), respectively and *E.coli.* poly(A) polymerase (Ambion, Foster City, CA) following the manufacturer's instructions. The tailed RNAs were denatured with 20 mM methyl mercury hydroxide and reverse transcribed with Superscript III-RT (Invitrogen) using the oligo-dT or oligo-dG reverse primers, respectively and the oligonucleotide forward primer P3925 as described in the method by Tzanetakis and Martin [22]. RT products of all three sgRNAs were PCR amplified with PrimeSTAR HS DNA polymerase (TAKARA) using the same oligo-dT or oligo-dG in combination with other primers derived from internal regions of CbMV gRNA: P517 (for 3.1 kb sgRNA), P2527 (for 1.6 kb sgRNA) and P2168 (for 1.3 kb sgRNA), homologous to nt 979-956, 2633-2608 and 2992-2971, respectively (Table 1). The products were sent for direct sequencing (TACGen, Richmond, CA) to confirm the complete 5'- termini of each sgRNA are present.

In vitro transcription and infectivity testing

Plasmid DNA, isolated from pUC-CbMV-FL clone, was linearized by digestion with *Sma*I, phenol/chloroform extracted, precipitated in ethanol and re-dissolved in nuclease free water. One microgram of linearized DNA was used as template for the synthesis of capped /or uncapped RNA transcripts *in vitro* using mMESSAGE mMACHINE T7 Ultra kit (Ambion) and T7 RiboMAX Express Large Scale RNA Production System (Promega), respectively, according to anufacturer's instructions. After the reaction, 1 µl of the product was run in a 1% agarose gel to check the quantity and integrity of the transcript.

Infectivity testing

Chenopodium quinoa plants of 12-14 days old were inoculated mechanically by rubbing leaves with the capped or uncapped transcription products (approximately 4-5 µg RNA per leaf) in the presence of inoculation buffer (1.6 µg/µl Bentonite, 0.05 M Phosphate buffer, pH 7.2) and carborundum. Inoculated plants were kept in a greenhouse under natural lighting with a temperature range of 24 to 30°C. After 21 days post inoculation, viral total RNA and dsRNA were isolated from symptomatic leaves for further analysis.

In vitro translation

Plasmid DNAs, isolated from clones pUC-CbMV-FL, pUC-CbMV-3.1, pUC-CbMV-1.6, and pUC-CbMV-1.2, were linearized by digestion with *Sma*I, purified with phenol/chloroform and precipitated with ethanol. Uncapped RNA transcripts from each clone were synthesized using Ribomax T7 kit (Promega) and purified by ethanol precipitation. Purified viral RNA and synthetic RNA transcripts of each construct were translated *in vitro* using Rabbit Reticulocyte Lysate System (Promega). Approximately 2 µg of synthetic transcript or 1 µg of viral RNA was translated in 25 µl of rabbit reticulocyte lysate using 2 µl Transcend t-RNA. After incubation at 25°C for 2 hrs, 10 µg RNaseA was added followed by incubation at 30°C for 5 min. Ten microliters of biotin-labeled *in vitro* translation products were mixed with equal volume of 2X Tricine sample buffer, boiled for 15 min at 70°C and resolved by Tricine SDS-PAGE as mentioned by Gulati-Sakhuja et al. [23]. The product was detected using Transcend™ Colorimetric Translation Detection System (Promega) as per supplier's recommendations.

Northern and Western blot analyses

For Northern blot analysis, NorthernMax kit (Ambion) was used. Seven µg of total RNA and 1-2 µg of viral RNA preparations were denatured with Formaldehyde Load Dye, electrophoresed in 1% denaturing agarose gel, and blotted onto nylon membrane (Roche Applied Science). Pre hybridization and hybridization was done at 68°C in preheated ULTRAhyb buffer in roller bottle hybridization oven as per manufacturer's recommendations. Three Digoxigenin (DIG) - labeled RNA probes encompassing nucleotides 608 to 851 (5' end of CbMV), nucleotides 2428 – 2634 (central portion of genome), and nucleotides 2807 to 2993 (3' end of CbMV) were prepared using DIG RNA -labelling kit (Roche). Three probes were used separately for hybridization (overnight). DIG wash and block buffer set (Roche) was used for washing, blocking and detection with CSPD (Roche).

For Western blots, leaves from healthy and infected *C. quinoa* plants and lyophilized leaf tissue of infected Calibrachoa were ground with 1XPBS. Crude leaf extracts and CbMV virions were mixed with equal volume of 2X Tricine sample buffer and boiled for 5 min. The denatured samples were separated by 10% Tricine SDS-PAGE. After transfer onto a nitrocellulose membrane (BioRad), CbMV coat protein was detected using anti-CbMV serum and AP-goat-anti rabbit second antibody following procedures as described previously [23].

ELISA

DAS-ELISA was performed as described [23]. Crude leaf extracts from healthy and inoculated *C. quinoa* plants and lyophilized leaf tissue of infected Calibrachoa were added to microtitre plates coated with CbMV-IgG. AP conjugated-CbMV IgG was used as the second antibody and color was developed by adding PNP (*p*-nitrophenyl phosphate) substrate. The absorbance at 405nm was recorded after 2h incubation.

Results

Construction and testing of a biologically active full-length cDNA clone

Four clones, each containing the full-length cDNA of CbMV viral RNA in the pCRII-TOPO blunt vector were constructed by joining two overlapping cDNA fragments as shown in Figure1. Sequencing of two clones confirmed that each clone had the expected T7 promoter sequence at the 5' end, the *Sma*I restriction sequence at the 3' end, and were cloned in the correct orientation (data not shown). The DNA fragment was isolated by digesting with restriction enzymes *BamH*I and *Eco*RV and ligated into pUC-18 vector between *BamH*I and *Sma*I restriction sites. Two clones, sequenced at both the ends, showed the expected sequence with no extra or deleted nucleotides. One clone was selected for infectivity testing. Both capped and uncapped *in vitro* RNA transcripts were synthesized under the control of the T7 promoter using *Sma*I linearized full-length cDNA template of pUC-CbMV-FL clone. After inoculation of *C. quinoa* with the RNA transcripts, plants developed typical symptoms of chlorotic local lesions within 10 to 15 days post-inoculation. These lesions turned to mottling at the later stage of infection (Figure 2) Uncapped CbMV transcripts were as infectious as the capped transcripts and the time interval for symptom development was the same in both events (data not shown).

The sap from leaves inoculated with capped and uncapped transcripts from the pUC-CbMV- FL clone was tested by ELISA using CbMV antiserum, and showed positive reaction. Electron microscopy also showed spherical particles (30-35 nm in diameter), which are the same size as CbMV virions. SDS-PAGE and Western blot analysis using CbMV antiserum showed a single protein band of 37 kDa, the same molecular weight as the CbMV coat protein (Figure 3). These results indicate that the full-length cDNA clone of CbMV (pUC-CbMV-FL) was infectious.

Analysis of viral RNA

Total RNAs were extracted from *C. quinoa* leaves inoculated with *in vitro* RNA transcripts of the pUC-CbMV-FL clone and analyzed by Northern blot hybridization with three different DIG-labeled probes corresponding to 5'-end, central and 3'-end regions of the CbMV genome. In addition to the 3.9 kb gRNA, CbMV infected tissue showed three sgRNA species of approximately 3.1, 1.6 and 1.4 kb as estimated by regression analysis of the electrophoretic mobility in comparison to known RNA markers. The 3.9 kb gRNA hybridized with all three probes and its size was similar to RNA extracted from purified virions and original CbMV infected Calibrachoa leaves (Figure 4 A, B, and

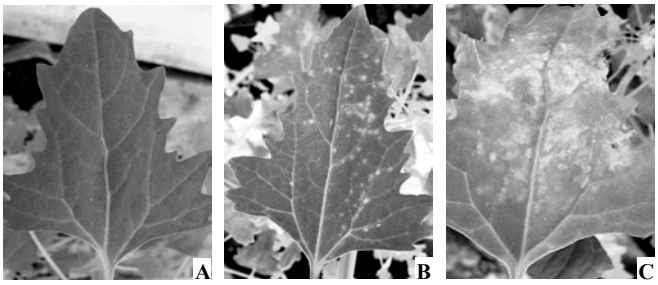

Figure 2:

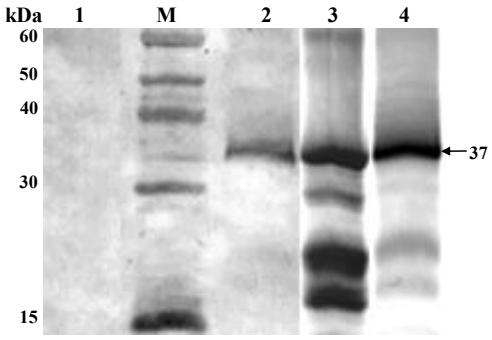

Figure 3:

C). The other three sgRNAs showed hybridization only with central and 3' end probes. Similar results were observed with total RNA of Calibrachoa indicating that CbMV replication synthesizes three 3'-coterminal sgRNAs in addition to a gRNA. The intensity of the hybridization signal of sgRNAs was low as compared to gRNA. Analysis of dsRNA isolated from infected leaves of *C. quinoa* and Calibrachoa also showed the presence of four dsRNA species whose estimated sizes were consistent with those of gRNA and sgRNAs (data not shown) as detected by Northern hybridization.

In vitro Translation of CbMV RNAs

To investigate whether the predicted ORFs of CbMV were translatable, *in vitro* RNA 11 transcripts of pUC-CbMV-FL, pUC-CbMV-3.1, pUC-CbMV-1.6, and pUC-CbMV-1.2 containing cDNA sequences corresponding to gRNA, 3.1 kb, 1.6 kb, and 1.3 kb sgRNAs , respectively, were translated *in vitro* in the rabbit reticulocyte lysate system. RNAs from pUC-CbMV-FL and pUC-CbMV-3.1 produced a major band of 38 kDa that corresponds to CbMV coat protein. Two other relevant bands of 28 kDa and 87 kDa representing ORF1 and ORF2 of CbMV genome were also produced from pUC-CbMV-FL RNA. In addition, a protein with a molecular mass of 40 kDa was synthesized from the translation of pUC-CbMV-3.1 RNA. Two small protein bands of 8 kDa and 9 kDa could not be resolved on this gel (Figure 5A). RNAs from pUC-CbMV-1.6, and pUC-CbMV-1.2 produced 38 kDa and 8-9 kDa bands (Figure 5B). Unexpectedly, two other protein species with apparent masses of 32 kDa and 25kDa were also observed from translation of pUC-CbMV-FL RNA. It is possible that these proteins are a premature termination of p38 and p28. Translation of CbMV viral RNA in rabbit reticulocyte lysate yielded patterns identical to those of cDNA clones (data not shown).

Determination of 5' termini sequence of CbMV sgRNAs

To determine the 5'-terminal region of sgRNAs, poly(A) and poly(C) tails were added to the dsRNAs and reverse transcription followed by PCR amplification of resulting cDNAs complementary to

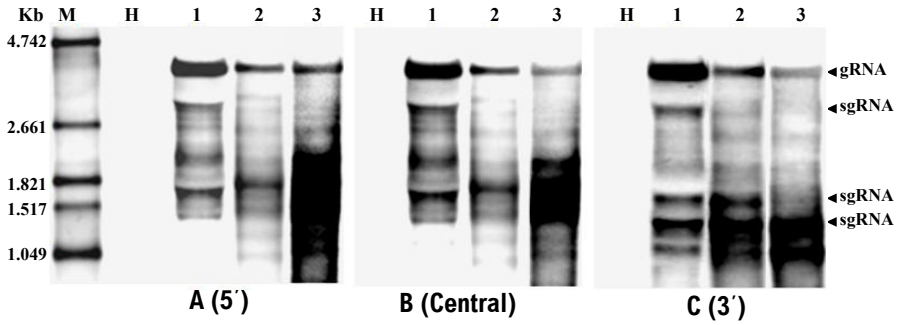

Figure 4:

negative viral RNA strands was performed. Eight to ten clones derived from 3.1, 1.6 and 1.3 kb sgRNAs cDNAs (with A-tail or C-tail) obtained from two different cloning events, were sequenced. Variations in length were found in the clones derived from all three sgRNAs. These differences in length could be due to either mismatching of PCR primers or degradation of template RNA. The most consistently obtained type of clone was considered to contain the right 5' end sequence.

Sequence analysis showed that the transcription site of 1.6 kb sgRNA is located at position 2161 of the gRNA which is 128 nucleotides upstream of start codon of p8 gene. The 1.3 kb sgRNA maps 215 nucleotides (at position 2442 of gRNA) upstream of start codon of the CP gene. Therefore the correct size of 1.6 kb and 1.3 kb sgRNAs is 1758 and 1477 nucleotides, respectively and these sizes have also been confirmed by Northern hybridization. The 5' terminal of 3.1 kb was mapped to position 825 of gRNA, therefore 3.1 kb sgRNA is composed of 3094 nucleotides. The presence of this additional sgRNA is rare but not uncommon for carmoviruses and its molecular analysis has not been reported. However, there is a possibility that the sgRNA of 3.1 kb is involved in the synthesis of a polypeptide of 40 kDa by translating a part of readthrough portion of RdRp corresponding to C-terminus of p87 that might be required for infection in CbMV.

Discussion

Advances in molecular biology techniques have made it possible to analyze and modify genomes at the molecular level and therefore, gaining deeper insight into their organization and expression. Infectious transcripts are biologically amplifiable synthetic molecules, and are subjected to RNA recombination events and/or genetic modifications resulting from the viral replication process (e.g. point mutations and template-switching activity from the error prone viral replicase), like their wild-type viral RNA counterpart. To date, infectious *in vitro* transcripts have been synthesized from many plus-stranded RNA viruses. Based on transcription place, infectious cloned RNA viruses can be divided into two categories: infectious RNA (*in vitro* transcripts) and infectious cDNA (based on the transcription *in vivo*). Several RNA polymerase promoters have been used e.g. *E. coli* Pm promoter derived from bacteriophage λ and promoters of bacteriophages SP6, T3 and T7. Bacteriophage promoters are mainly used as they give high yield of transcripts [24, 25]. However, T7 promoter is preferred over the SP6 promoter probably due to more thoroughly studied genetics of bacteriophage T7. To get high infectivity, the 5' end of the transcript should be identical or have high similarity with wild type sequence of virus. According to Boyer and Haenni [8], presence of non-viral nucleotides at the 5' end of synthetic viral transcripts substantially decreases or even abolishes infectivity whereas 3' extensions are more easily tolerated. Sometimes, the presence of one or two nucleotides (usually G) at the 5' end of the construct might significantly lower or eliminate the infectivity [10]. However, there might be many other mechanisms that inhibit the expression, but no details are known [26,27]. In the present report, RNA transcripts derived from full-length cDNA clone of CbMV under the control of T7 promoter were infectious. T7 promoter was fused with 5'end sequence of the virus without the addition of any extra non-viral sequence. Similar results of preparation of infectious clones under the control of T7 promoter have been reported in other carmoviruses, SCV [13], MNSV [14,15], TCV [16], and HCRSV [17]. The transcripts of CbMV produced by *in vitro* transcription driven by the T7 promoter were proven infectious when mechanically inoculated to *C. quinoa* where local lesions were produced. In addition, ELISA, western blotting, and electron microscopy confirmed that the artificial viruses created from *in vitro* transcripts were indistinguishable to the native CbMV. Similar results of infectivity assay of *in vitro* infectious transcripts has been obtained in other carmoviruses [14,15,16,17,13] In polycistronic RNA viruses with genome of positive polarity, only ORFs located near the 5' are translated efficiently, whereas, 3'-proximal ORFs are translationally silent. The sgRNAs, which are 3'-coterminal with the genomic RNA, are therefore synthesized during infection [28]. The transcription of viral sgRNA is carried out by their RdRps. Three possible mechanisms have been proposed for the synthesis of sgRNAs: (i) initiation where replicase initiates transcription of positive strand of sgRNA internally on the minus-strand of gRNA (29-33); (ii) discontinuous transcription (leader priming and recombination during minus strand synthesis) [34-36]; and (iii) premature termination during minus strand synthesis from gRNA [37-40]. The sgRNA promoters are localized in the middle part of viral genome and play an important role in sgRNA synthesis. A different (higher) strength of subgenomic promoter may serve as a regulatory mechanism affecting the quantity of expressed proteins [41]. In CbMV, three 3' coterminal sgRNAs of sizes approximately 3.1, 1.63, and 1.26 kb are reported [2]. Northern blot analysis of CbMV RNA from leaves with lesions also revealed that the viral RNAs included gRNA

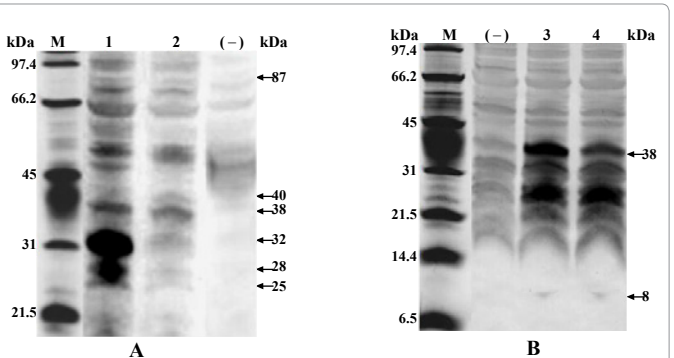

Figure 5:

in abundance and the three sgRNAs in low abundance. As reported earlier, CbMV cluster phylogenetically with AnFBV, SCV, *Pelargonium flower break virus* (PFBV) and Carnation mottle virus (CarMV) [2]. Northern hybridization of SCV RNA revealed two sgRNAs (1.614 and 1.396 kb) and RNAs of PFBVand AnFBV showed four sgRNAs (3.2, 2.9, 1.7 and 1.4 kb in PFBV and 2.59, 1.30, 1.10 and 0.93 in AnFBV). There is possibility that CbMV is a link between SCV, AnFBV and PFBV.

In vitro translation of 3.1 kb sgRNA in CbMV had shown an extra protein of 40 kDa. Upon analysis of the sequence of CbMV gRNA, we found an AUG codon located at nucleotide 1282-1284 within ORF2 (RdRp) that might be used as translation initiation signal for the synthesis of 40 kDa protein by a leaky translation mechanism. Similar results of presence of extra ORFs have been reported in other carmoviruses [15,17]. However, the biological significance of 3.1 kb sgRNA in CbMV is unknown.

Our results indicate that an infectious full-length clone of CbMV had been successfully constructed. The preparation of biologically functional RNA transcripts from these full length cDNA clones provides a powerful tool to conduct detailed analysis of the gene functions and replication in CbMV by mutation or recombination.

Acknowledgements

The authors thank John Sears for doing mechanical inoculations, maintaining plants in the green house and extracting total RNAs and Laura L. Hladky for her critical review of the manuscript.

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