Short communication

Characterization of cassava common mosaic virus and a defective RNA species

Lee A. Calvert, Maritza I. Cuervo, Manuel D. Ospina, Claude M. Fauquet and Bertha-Cecilia Ramirez

Centro Internacional de Agricultura Tropical (CIAT), A. A. 6713, Cali, Colombia, The Scripps Research Institute, 10666 North Torrey Pines, La Jolla, CA 92037, USA and Institut Jacques Monod, 2 Place Jussieu-Tour 43, 75251 Paris Cedex 05, France

The genome of cassava common mosaic potexvirus (CsCMV) has been sequenced from cDNA clones and consists of 6376 nucleotides (nt). A 76 nt untranslated region (UTR) at the 5' terminus was followed by ORF1 which potentially encodes a protein of 1449 amino acids (aa). ORFs 2, 3, and 4 were predicted to encode proteins of 231, 112 and 97 aa, respectively. ORF5 potentially encodes a 229 aa protein of 25 kDa that is similar to the coat proteins of other potexviruses. The 3'-terminal UTR of 114 nt was followed by a poly(A) tail. The genomic organization of the CsCMV genome is similar to that of other potexviruses. A cDNA clone that was apparently obtained from a defective RNA species contained both the 5' and 3' UTRs and an ORF that potentially encodes the first 263 aa of ORF1 and the last 33 aa of the coat protein. Defective RNA species were found both in purified preparations of the virus and in total nucleic acid isolated from CsCMV-infected plants.

Cassava (Manihot esculenta, Crantz) infected with cassava common mosaic virus (CsCMV) presents mosaic symptoms and chlorotic areas that are often limited by the veins. The disease can cause losses in yield of more than 30% and has been found in Brazil, Peru, Colombia (Costa & Kitajima, 1972) and Paraguay. The disease is most prevalent in southern Brazil and in Paraguay. Cassava is a vegetatively propagated crop and CsCMV is efficiently transmitted in the stem cuttings used for reproduction. The only other known method for transmission is mechanical inoculation of the virus.

CsCMV is classified as a potexvirus on the basis of particle morphology, serology and inclusion bodies. The virus particle is a 15 nm × 495 nm semiflexuous rod (Kitajima et al., 1965). The genome consists of one species of ssRNA of 2 × 10^6 Da, and the coat protein is a single species of molecular mass 21 kDa (Nolt et al., 1991). Cassava isolates of CsCMV cross-react weakly with potato virus X (PVX). Cytoplasmic inclusions typical of the potexvirus group are found in CsCMV-infected plants (Kitajima et al., 1965).

In contrast to the situation with CsCMV, detailed molecular information is available for many members of the potexvirus group. The complete nucleotide sequence has been established for PVX (Huisman et al., 1988), white clover mosaic virus (WCIMV) (Forster et al., 1988), narcissus mosaic virus (NMV) (Zuidema et al., 1989), papaya mosaic virus (PMV) (Sit et al., 1989), clover yellow mosaic virus (CYMV) (Sit et al., 1990), foxtail mosaic virus (FMV) (Bancroft et al., 1991) and strawberry mild yellow edge virus (SMYE) (Jelkmann et al., 1992). These viruses share common genomic organization and expression strategies.

The CsCMV isolates were propagated in Nicotiana benthamiana in a growth room. The CsCMV isolates were also maintained in cassava grown in greenhouses. Three CsCMV isolates originating from Colombia, Paraguay and southern Brazil were maintained.

Systemically infected leaves were collected 16–20 days after inoculation with CsCMV and were homogenized in 0.1 M-potassium phosphate (all potassium phosphate buffers were pH 7.5) containing 1% 2-mercaptoethanol (4 ml buffer/1 g leaf tissue) for 1 min in a blender (throughout the virus purification all steps were at 4 °C). The preparation was clarified by the addition of 50% chloroform, homogenized for 1 min and centrifuged at

* Author for correspondence. Fax +57 2 445 0273.
e-mail L.CALVERT@CGNET.COM

The nucleotide sequence data presented in this paper have been submitted to the GenBank database and assigned the following accession numbers: CsCMV RNA genome sequence, U23414; CsCMV defective RNA sequence, U23415.
Fig. 1. (a). For legend see facing page.
Characterization of CsCMV

The aqeous phase was collected and Triton X-100 was added to a final concentration of 1%. After 15 min of incubation, polyethylene glycol (PEG 8000) and NaCl were added to final concentrations of 8% (w/v) and 0.25 M, respectively. After low speed centrifugation, the pellet was resuspended in 0.05 M-potassium phosphate, and the preparation was centrifuged at 40000 r.p.m. in a SW-65 rotor, a band consisting primarily of viral particles was collected and dialysed against 0.05 M-potassium phosphate. The RNA was extracted from virions by degradation of the coat protein using 2% SDS and 0.2 mg/ml proteinase K at 60 °C in the presence of vanadyl ribonuclease complex. The RNA was purified by centrifugation using caesium chloride (Chirgwin et al., 1977) and the pellet was recovered by resuspension in sterile water.

The Brazilian isolate of CsCMV was used for all the cDNA cloning experiments. Cloning of the 3' portion of the genome was performed using oligo(dT) as a primer. Cloning of the 5' half of the genome was performed using the oligonucleotide primer 5' TTGCAGGTTCAGGCT-TGT 3' (complementary to nucleotides 3086-3068). Complementary DNA synthesis was performed as described by Gubler & Hoffman (1983). Subclones were prepared by restriction enzyme digestion and auto-ligation. Using clone pCsCMV28 a series of deletions was produced using an exonuclease III digestion method (Henikoff, 1984). The cDNA clones were sequenced using the dideoxynucleotide chain-termination method (Sanger et al., 1977). The sequence data was analysed and the predicted polyadenylation signal and a six base conserved motif are underlined. (A > 60) represents a poly(A) tail. (b) Predicted genomic organization of CsCMV based on the nucleotide sequence of cloned viral RNA. The ORFs are represented by boxes. The predicted size of the proteins are written either above or below the boxes. NTP-binding motifs are indicated by the letter N and the GDD RNA-dependent RNA polymerase motif is indicated by the letter G. Excluding the poly(A) tail, the length of the genome is 6376 nt (GenBank accession number U23414). The independent dashed boxes represent the portions of the 5' and 3' termini that are found in the defective RNA species. (c) A defective RNA species that has an ORF with the potential to encode a fusion protein with the amino terminus of ORF1 and the carboxy terminus of ORF5. Excluding the poly(A) tail, this defective RNA is 1093 nt (GenBank accession number U23415).
using Sequaid II (D. D. Rhoads & D. J. Roufa, 1989, Kansas State University, Manhattan, Kansas, USA) and DNASIS (Pharmacia).

Comparisons of regions of the proteins that contain the conserved motifs for the RNA-dependent RNA polymerase were made for CsCMV and seven other potexviruses. The amino acid sequences were aligned and the phylogenetic analyses were carried out by the maximum parsimony method (PAUP program: Phylogenetic Analysis Using Parsimony, version 3.0, developed and released in 1990 by D. L. Swofford, Illinois Natural History Survey, Champaign, Ill., USA). The most parsimonious trees were determined using the heuristic search, branch swapping and majority-rule options of the program. The results were confirmed by a 100 cycle bootstrap analysis (Felsenstein, 1985). The regions of ORF1 that were compared were CsCMV amino acids (aa) 918–1383, PVX aa 921–1386 (Huisman et al., 1988), NMV aa 1056–1522 (Zuidema et al., 1989), WCIMV aa 756–1212 (Forster et al., 1988), PMV aa 1006–1474 (Sit et al., 1989), FMV aa 787–1252 (Bancroft et al., 1991), CYMV aa 1139–1605 (Sit et al., 1990) and SMYE aa 791–1254 (Jelkmann et al., 1992). A similar analysis was made using the core section of the coat proteins of these potexviruses. The core section was determined by starting at the amino terminus and ending at the carboxy terminus of WCIMV coat protein.

Total RNA was extracted from N. benthamiana (CsCMV-infected and control plants) using a modified LiCl precipitation method (Thompson et al., 1983). The total RNA preparations were electrophoresed in formaldehyde gels and transferred to membranes. The cDNA clone of the 5' terminus was used to prepare RNA probes. Labelled RNA transcripts were produced using T7 RNA polymerase, and [7-32P]ATP. To prepare RNA probes complementary to nucleotides 5761–6376 of the 3' terminus of CsCMV RNA, plasmid pCsCMV28 was linearized and synthesis performed with T7 RNA polymerase. A subclone of pCsCMV28 (complementary to nucleotides 5761–6080) was used to distinguish between subgenomic RNA and defective RNA species. Hybridization conditions were 50% formamide, 6 x SSPE (3 M-NaCl, 0.23 M-NaH2PO4 and 0.02 mM-EDTA pH 7.4), 7 x Denhardt’s solution, 0.2% SDS and herring sperm DNA at 42 °C. The filters were washed at 37 °C for 30 min in SSC (0.15 M-NaCl and 0.015 M-sodium citrate) containing 0.2% SDS, and three times at 65 °C for 30 min in 0.2 x SSC containing 0.1% SDS.

A library of cDNA clones was produced: clones pCsCMV28, pCsCMVX7, pCsCMVSP2 and pCsCMV64 represent the complete cDNA of CsCMV. Excluding the 3'-terminal poly(A) tail, the sequence of CsCMV was 6379 nucleotides (nt) (Fig. 1). The 5'-terminal residues were not directly determined, but by analogy with the established 5' termini of other potexviruses, clone pCsCMV64 starts at the first nucleotide. The 76 nt UTR at the 5' terminus was followed by ORF1 that potentially encodes a protein of 1449 aa (166 kDa). This putative protein contained consensus sequences for NTPase helicases and RNA-dependent RNA polymerases. ORFs 2, 3 and 4 were predicted to encode proteins of 231, 112, and 97 aa of 25 kDa (25K protein), 14 kDa (14K protein) and 9 kDa (9K protein), respectively. The 25K and 14K proteins share amino acid similarities with the corresponding proteins encoded by other potexviruses. ORF2 extended into ORF3 which in turn overlapped ORF4 (Fig. 1). ORF5 potentially encodes a 229 aa protein of 25 kDa. The deduced protein presented amino acid similarity with the coat proteins of other potexviruses. Within ORF5 but in another reading frame, lies ORF6 with the potential to encode a protein of 89 aa of 10 kDa (Fig. 1). The 3' UTR region of 114 bases was followed by a poly(A) tail. The clones that were synthesized using oligo(dT) as a primer included a poly(A) tail that contained from 20 to more than 60 nt. Starting at nucleotide 6272 (Fig. 1), a motif typical of the polyadenylation signal (Guilford et al., 1991) was found. Also present was a six base consensus sequence (Fig. 1, nucleotides 6338–6343) that is found 30–60 nt from the end of the 3' UTR of other potexviruses (Bancroft et al., 1991).

The genomic arrangement of CsCMV is similar to that of other potexviruses including ORF 2, 3 and 4 that overlap in a manner consistent with the triple gene block proteins involved in cell-to-cell movement (Beck et al., 1991). The Mw of 25000 of the coat protein calculated from the sequence data is intermediate between the 21 kDa reported by Nolt et al. (1991) and our results of 27 kDa (data not shown). A small ORF6 within the coat protein of CsCMV has the potential to encode a unique protein. Similar ORFs within the coat protein gene are reported for NMV (Zuidema et al., 1989), WCIMV (Forster et al., 1988), SMYE (Jelkmann et al., 1992) and lily virus X (Memelink et al., 1990).

From the analyses of a region of ORF1 (of 463 to 468 aa depending on the virus) that included the GDD consensus motif, the predicted relationship between eight potexviruses was determined and a phylogenetic tree obtained (Fig. 2). CsCMV, FMV, CYMV and PMV were grouped together on one branch of the tree whereas WCIMV, NMV and SMYE were in another group. Based on the calculated branch distances, PVX was most closely related to SMYE. This region of ORF1 of CsCMV was found to have the most similarity with the polymerase protein of FMV. From the analysis of the core regions of the coat proteins, these eight potexviruses were grouped in a similar pattern. From the combination of analyses, FMV, PMV, CYMV and CsCMV constitute
Characterization of CsCMV

Fig. 2. Illustration of the relationship between eight potexviruses (CsCMV, PVX, WCIMV, FMV, PMV, NMV, CYMV and SMYE) based on a region of the RNA-dependent RNA polymerase including the GDD-containing consensus motif. The mean assigned branch lengths represent relatedness (the more closely related the lower the value) and are written above each branch.

Clone pCsCMV64 was 1093 bases [excluding the poly(A) tail] and contained portions of both the 5' (nucleotides 2–877) and 3' (nucleotides 6161–6376) termini (Fig. 1c). It contained both the 5' and 3' UTRs and an ORF that potentially encodes the first 263 aa of ORF1 and the last 33 aa of the coat protein. To determine if pCsCMV64 was an artifact of cDNA cloning, RNA probes homologous to the 5' and 3' regions were prepared. The 5' terminus probe detected both genomic and defective CsCMV RNA in total RNA extractions and purified preparations of the virus of the Brazilian and Paraguay isolates (Fig. 3a, lanes 1, 3, 4 and 6). In the Brazilian isolate, the genomic CsCMV RNA was at a low concentration (as determined by all the probes tested) and longer exposure times were needed to detect it (longer exposures not shown). The 5' terminus probe detected the genomic RNA and a species (weak reaction) that could be a defective CCMV RNA species in the Colombian isolate (Fig. 3a, lanes 2 and 5). The 3' terminus probe detected genomic CsCMV RNA, defective CsCMV RNA and a subgenomic CsCMV RNA in total RNA extracts and purified preparations of the virus of the Brazilian and Paraguay isolates (Fig. 3b, lanes 1, 3, 4 and 6) but only the genomic and subgenomic species in the Colombian isolate (Fig. 3b, lane 2). It was possible that a defective CsCMV RNA species were present in the total RNA extraction in the Colombian isolate (Fig. 3b, lane 5). A 3'-end deletion probe was prepared that would not hybridize with the predicted defective RNA. This probe hybridized with the genomic and subgenomic species in all three isolates. The largest RNA species was the same size as genomic length RNA and the smallest species was a subgenomic species of approximate 1000 nt that is probably the subgenomic RNA encoding the coat protein (the size estimate data are not shown). The intermediate-length RNA species

![Image]

Fig. 3. Autoradiograms of total RNA extracted from mock-infected or CsCMV-infected N. benthamiana leaves and RNA extracted from purified preparations of CsCMV particles. The genomic length RNA and the defective RNA species were detected using RNA probes prepared from both the 5' (a), 3' (b) termini and 3' deletion clone within the coat protein (C) of CsCMV. Lanes 1, 2, and 3: total RNA extracted from the CsCMV-Brazilian isolate; CsCMV-Colombian isolate and CsCMV-Paraguay isolate respectively; lanes 4, 5 and 6: RNA extracted from purified virus preparations of the CsCMV-Brazilian, CsCMV-Colombian isolate and CsCMV-Paraguay isolate, respectively.
that was present in the Brazilian and Paraguay isolates was estimated to be 1200 nt and contained portions from both the 5' and 3' termini.

The cDNA clone pCsCMV64 appeared to correspond to a complete defective RNA containing an ORF that potentially encodes a fusion protein of the amino terminus of the RNA-dependent RNA polymerase protein and the carboxy terminus of the coat protein (Fig. 1c). A six nucleotide (UUGUUU) and a four nucleotide (GAGG) sequence were repeated on both sides of the proposed junction site of the defective RNA. It was not determined whether these repeats are important in the formation of defective RNA species. The defective CsCMV RNA is similar to the defective RNAs that were found in plants infected with CYMV (White et al., 1991). In both cases, the lengths of the RNA species are similar, they encode for polymerase/coat protein fusion proteins and the RNA species are found in purified virus preparations, implying that they are encapsidated. There was no apparent attenuation of symptoms in either the cassava or N. benthamiana plants that were infected with the CsCMV isolates, but the presence of the defective RNA did seem on occasion to lower the amount of virus in the plant (Fig. 3). Since defective RNA species were detected (it is probable but not certain that the defective species was not present in the Colombian isolate) in only two of the three CsCMV isolates, it is likely that the defective RNA species are not essential for viral functions.

We conclude that CsCMV is a typical member of the potexvirus group. This is the second report of a defective RNA associated with a potexvirus, but it remains to be determined whether the defective RNAs have any role in pathogenicity.

The authors are indebted to Catalina Masuda for maintenance and purification of CsCMV. We would also like to thank Guillermo Guzman in helping in the preparation of the photographs.

References


(Received 17 July 1995; Accepted 31 October 1995)