

CHARACTERIZATION OF ISOLATES OF THE CUCUMOVIRUS *CUCUMBER MOSAIC VIRUS* PRESENT IN BRAZIL

M. Eiras¹, A.J. Boari², A. Colariccio¹, A.L.R. Chaves¹, M.R.S. Briones³, A.R. Figueira² and R. Harakava¹

¹Instituto Biológico, Av. Conselheiro Rodrigues Alves, 1252, 04014-002, São Paulo, Brazil

²Universidade Federal de Lavras (UFLA), Minas Gerais, Brazil

³Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil

SUMMARY

Cucumber mosaic virus (CMV) is a naturally occurring virus that infects several crops in Brazil, although its economic and epidemiological impact has not been fully characterized. Samples of different host plant species from different regions of Brazil, including passion-fruit, sweet-pepper, black-pepper, *Peperomia*, melon, squash, tomato, pea, water-cress, zingiber, banana and *Commelina* sp., showing foliar symptoms of mosaic, deformations and chlorotic ringspots that resemble those induced by CMV, were collected for biological, serological and molecular analysis. DAS-ELISA, RT-PCR/RFLP, sequencing and phylogenetic analysis were used to characterize the isolates. *Nicotiana glutinosa* plants, that showed foliar mosaic after inoculation with extracts from the samples collected, were submitted to DAS-ELISA and total RNA extraction. RT-PCR, with specific primers for the 3' end of RNA 3 and part of the coat protein gene, yielded 486-499 base pair DNA fragments that were characterized by RFLP. ELISA and RT-PCR/RFLP results showed the isolates belong to CMV subgroup I. These results were confirmed by the sequences of the RT-PCR products, which were 92 to 99% identical to those of subgroup I CMV isolates. The multiple sequence alignment of the nucleotides and the translated amino acid sequences of these and other CMV strains, and phylogenetic analyses revealed three distinct clusters. Most of the Brazilian CMV isolates were closely related among themselves and clustered with other CMV subgroup IA isolates. One CMV isolate clustered together with CMV subgroup IB isolates. These results indicate the prevalence of the CMV subgroup I in Brazil.

Key words: CMV, RT-PCR, RFLP, sequencing, phylogenetic inference.

INTRODUCTION

Cucumber mosaic virus (CMV), the type species of the genus *Cucumovirus*, family *Bromoviridae*, is one of the most common plant viruses of substantial agricultural importance, infecting more than 1,000 plant species (Van Regenmortel *et al.*, 2000). CMV is a single-stranded positive-sense tripartite genome RNA virus. The genomic RNAs are designated as RNAs 1, 2 and 3. RNA 1 codes for the 1a protein, a viral component of the replicase complex that contains putative helicase and methyltransferase domains and is involved in virus movement. RNA 2 codes for the 2a protein, which is the viral RNA-dependent RNA polymerase subunit of the CMV replicase, and for the 2b protein, which is translated from a subgenomic RNA and is involved in systemic spread and in the virulence of the virus, possibly by suppressing gene silencing (Brigneti *et al.*, 1998). RNA 3 codes for two proteins involved in virus movement (3a protein) and encapsidation (coat protein). The latter is translated from subgenomic RNA 4. All RNAs have a cap structure at the 5' terminus and a highly conserved portion at the 3' terminus that can form a tRNA-like structure. Some isolates can encapsidate a small satellite RNA (satRNA) that can cause modifications to the symptoms induced by infection (Palukaitis *et al.*, 1992).

CMV is distributed worldwide. It has an extremely broad host range and has numerous strains. CMV isolates have been divided into two subgroups (named I and II) based on serological data, peptide mapping of the coat protein, nucleic acid hybridization, RT-PCR combined with RFLP and nucleotide sequence similarities (Edwards and Gonsalves, 1983; Palukaitis *et al.*, 1992; Rizos *et al.*, 1992; Wylie *et al.*, 1993; Singh *et al.*, 1995; Anonymous, 1998). Recently, Roossinck *et al.* (1999) suggested further division of subgroup I strains into IA and IB, on the basis of the results of phylogenetic analyses. Previously, Daniels and Campbell (1992) had proposed that CMV subgroup I be subdivided into Ia and Ib on the basis of host reaction and dsRNA patterns.

In Brazil, CMV isolates have been described in several hosts (Silberschmidt and Nóbrega, 1941; Maciel-Zambolim *et al.*, 1990, 1994; Dusi *et al.*, 1992; Duarte *et al.*, 1994; Boari *et al.*, 2000; Eiras *et al.*, 2000, 2001; Colariccio

et al., 1987, 1996, 2002). However, there is relatively little information about their geographical distribution in different regions of the country. Recently, Boari *et al.* (2000) characterized twenty-nine isolates from three States of the south-eastern Brazil by RT-PCR-RFLP and found that all of them were from subgroup I. Other authors have obtained similar results (Espinha and Gaspar, 1998; Eiras *et al.*, 2000, 2001, 2002).

In order to confirm the subgrouping and the genetic diversity of Brazilian CMV isolates, biological, serological (DAS-ELISA) and molecular tests (RT-PCR-RFLP and sequencing) were performed. Phylogenetic analyses were also done using the nucleotide sequences of part of the coat protein gene and the 3' untranslated region (UTRs) of RNA 3 of twenty-three Brazilian CMV isolates from different hosts and geographical regions. The most important commercial plant species affected by CMV, in Brazil, were included in this survey.

MATERIALS AND METHODS

Virus samples and natural hosts. CMV isolates were obtained from various hosts in 7 States of Brazil (Table 1, Fig. 1). They derived from samples showing different symptoms that were collected between 1996 and 2002. The isolates MelCEBR and ErviMSBR were kindly provided by Dr. J.A. Lima (University of Ceará, Brazil) and Dr. A.N. Dusi (Embrapa-Hortaliças, Brazil), respectively. Samples were considered infected after mechanically inoculation experimental and host plants were confirmed as infected by DAS-ELISA and RT-PCR/RFLP tests.

Biological tests. Extracts of naturally diseased leaves made in cold (~4°C) 0.5% sodium sulphite were rubbed on carborundum-dusted leaves of the healthy indicator host plants: *Capsicum annuum* L., *Chenopodium amaranticolor* Coste & Reyn., *Cucurbita pepo* L., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Nicotiana benthamiana* L., *N. glutinosa* L., *N. tabacum* L. ('Samsun NN', 'Turkish' and 'White Burley'), *Petunia hybrida* Vilm., *Vigna unguiculata* L. After inoculations, the plants were maintained in greenhouse conditions for symptoms to develop. The differentiation by host reactions into CMV subgroup I and II was done according to Daniels and Campbell (1992).

Serology. The identification of the CMV subgroup was done by DAS-ELISA (Eiras *et al.*, 2001), using monoclonal antibodies (Agdia, Elkhari, IN, USA). Absorbance (A_{405nm}) evaluations were done after substrate (p-nitrophenylphosphate) addition, using a microplate reader 3550-UV (Bio-Rad Laboratories, Hercules, CA, USA). The results were assessed as the relation between the readings (mean of 3 readings) of the infected samples and the readings of the healthy ones (I/H). Extracts

of dehydrated leaves of plants infected with CMV subgroups I or II were employed as positive controls (Agdia, Elkhari, IN, USA) and healthy leaves of *N. glutinosa* were used as negative controls.

Total RNA extraction and RT-PCR. Extraction of total RNAs was performed according to Eiras *et al.* (2001) from 1 g of systemically infected leaves from mechanically inoculated *N. glutinosa*. The complementary DNA (cDNA) strands were synthesized by mixing 1 µg of total RNAs with the "Pre-amplification System First Strand cDNA Synthesis" (Gibco BRL, Rockville, MD, USA) according to the manufacturer's instructions and using the primer CMV1 (5' GCCGTAAGCTGGATGGACAA 3'). PCR was done using 10 µl of the cDNA, 10 ng µl⁻¹ of the CMV1 and CMV2 (5' TATGATAAGAAGCTTGTTCGCG 3') primers (Wylie *et al.*, 1993), 1 unit of *Taq* DNA polymerase (Gibco BRL, Rockville, MD, USA), 1 ml of deoxynucleotide mixture (0.03M) and 5 µl of enzyme buffer (Gibco BRL, Rockville, MD, USA), according to Wylie *et al.* (1993). Samples were then placed in a PTC-100 MJ-Research (Waltham, MA, USA) thermocycler and after an initial heating at 92°C for 3 min, the amplification was by 35 cycles of 95°C for 1 min followed by 60°C for 1 min and 72°C for 1.5 min and by a final extension at 72°C for 7 min. The amplified DNA fragments were subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide (0.01%) and visualized by using a UV transilluminator (Sambrook and Russel, 2001). The size of the PCR product expected was 486 to 488 for CMV subgroup I and 499 to 502 for CMV subgroup II isolates (Wylie *et al.*, 1993).

RT-PCR/RFLP products. PCR-amplified products were digested by restriction endonucleases *Hind*III, *Eco*RI and *Msp*I (Gibco BRL, Rockville, MD, USA) using 10 µl amplified DNA, 2 µl of each indicated buffer (10X) and 1 µl (1 unit) of enzyme (Singh *et al.*, 1995). The digestion results were analyzed by electrophoresis as described earlier. The criteria used to differentiate the CMV subgroups were according to Singh *et al.* (1995).

Cloning and sequencing. Amplified RT-PCR products were purified from the agarose gel by the "Concert Rapid Gel Extraction System kit" (Life Technologies, Rockville, MD, USA), cloned into the pGEM-T *Easy vector* (Promega Corp, Madison, WI, USA) and used for transforming competent *E. coli* cells (DH5- α). Procedures were according to Sambrook and Russel (2001) or to suppliers recommendations. Amplified products were sequenced by using the dideoxy chain termination reaction (Sanger *et al.*, 1977), using the automatic ABI 377 sequencer and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit - Ampli *Taq* DNA polymerase, FS (Applied Biosystem, Foster City, USA) according to the manufacturer's instructions.



Fig. 1. Locations of seven states of Brazil where *Cucurbit mosaic virus* isolates were collected. MS, Mato Grosso do Sul; SP, São Paulo; RJ, Rio de Janeiro; ES, Espírito Santo, MG, Minas Gerais; CE, Ceará, PE, Pernambuco. The municipalities are described in Table 1. The number of isolates collected from each state is indicated in brackets.

Phylogenetic inference. Nucleotide sequences were aligned using the Sequencer 3.1 program (Gene Codes Corporation, Ann Arbor, MI, USA). Comparisons with other sequences from GenBank were obtained using the Basic Local Alignment Search Tool, BLASTn program, of the National Center for Biotechnology Information (NCBI). Multiple alignments were done for the deduced amino acid sequences using Clustal X 1.8 (Thompson *et al.*, 1997) and the similarities were determined using the GeneDoc program (Nicholas *et al.*, 1997). Nucleotide sequences of part of the coat protein gene and 3' UTR of RNA 3 from 60 CMV isolates (Tables 1 and 2) were used for phylogenetic inference. Trees were constructed by maximum likelihood using PAUP 4.0b10 and modelfit tests using the hierarchical likelihood ratio test implemented in MODELTEST 3.04 (Posada and Crandall, 1998). For the CP data set the selected model assumed

equal base frequencies (0.25 each), transition/transversion ratio of 3.2057, proportion of invariable sites=0 and gamma distribution shape parameter alpha=0.3452. For the 3'UTR data set the model parameters used were base frequencies $f(A)=0.2121$, $f(C)=0.2306$, $f(G)=0.2205$ and $f(T)=0.3368$, transition/transversion ratio of 2.2644, proportion of invariable sites=0 and gamma distribution shape parameter alpha=0.7589. Searches were done using a combination of TBR and neighbor-joining. The standard errors of branch lengths were estimated using PAUP 4.0.

RESULTS

Biological tests. The symptoms in indicator plants were similar to those induced by isolates of CMV subgroup I. All CMV isolates induced mosaic symptoms in

Table 1. CMV isolates from Brazil: hosts, origin, restriction sites, subgroup and the GenBank accession numbers of the nucleotide sequences.

CMV-isolate	Original host	<i>MspI/EcoRI</i> sites	Subgroup	Origin	GenBank
BanMSPBR	Banana (<i>Musa</i> sp.)	1/0	IA	Miracatu, São Paulo (SP)	AY048748
BanRSPBR	Banana (<i>Musa</i> sp.)	1/0	IA	Registro, São Paulo (SP)	AF547219
BanMGBR	Banana (<i>Musa</i> sp.)	1/0	IA	Pirapora, Minas Gerais (MG)	AY153425
BanPEBR	Banana (<i>Musa</i> sp.)	1/0	IA	Recife, Pernambuco (PE)	AF547218
CommSPBR	<i>Commelina</i> sp.	1/0	IA	Registro, São Paulo (SP)	AF418580
MarMSPBR	Passion-fruit (<i>Passiflora edulis</i>)	1/0	IA	Marília, São Paulo (SP)	AF418576
MarVCSPBR	Passion-fruit (<i>P. edulis</i>)	1/0	IA	Vera Cruz, São Paulo (SP)	AF418574
MarSPSPBR	Passion-fruit (<i>P. edulis</i>)	1/0	IA	São Paulo, São Paulo (SP)	AF418577
MarASPBR	Passion-fruit (<i>P. edulis</i>)	1/0	IA	Amparo, São Paulo (SP)	AF418575
AbobMGBR	Squash (<i>Cucurbita pepo</i>)	1/0	IA	Viçosa, Minas Gerais (MG)	AY153426
MelCEBR	Melon (<i>Cucumis melo</i>)	1/0	IA	Fortaleza, Ceará (CE)	AF547220
PCMGBR	<i>Peperomia caperata</i>	1/0	IA	Lavras, Minas Gerais (MG)	AF172843
PimSPBR	Sweet-pepper (<i>Capsicum annuum</i>)	1/0	IA	São Paulo (SP)	AY153419
CA5MGBR	Sweet-pepper (<i>C. annuum</i>)	1/1	IA	Viçosa, Minas Gerais (MG)	AF172842
P15MGBR	Sweet-pepper (<i>C. annuum</i>)	1/0	IA	Viçosa, Minas Gerais (MG)	AY153422
Bass4ESBR	Black-pepper (<i>Piper nigrum</i>)	1/0	IA	Soretama, Espírito Santo (ES)	AY153423
Bass22ESBR	Black-pepper (<i>P. nigrum</i>)	1/0	IA	Soretama, Espírito Santo (ES)	AY153424
GengSPBR	<i>Zingiber officinali</i>	1/0	IA	São Paulo (SP)	AF521104
TomRJBR	Tomato (<i>Lycopersicon esculentum</i>)	1/0	IA	Itaperuma, Rio de Janeiro (RJ)	AY153421
TomESBR	Tomato (<i>L. esculentum</i>)	1/0	IA	V.N. Emi, Espírito Santo (ES)	AY153420
ErvMSBR	Pea (<i>Pisum sativum</i>)	1/0	IA	Mato Grosso do Sul (MS)	AF547217
AgrMGBR	Water Cress (<i>Nasturtium officinale</i>)	1/0	IA	Lavras, Minas Gerais (MG)	-
CA21MGBR	Sweet-pepper (<i>C. annuum</i>)	3/1	IB	Pará Minas, Minas Gerais (MG)	-

Nicotiana glutinosa and *Cucurbita pepo* plants. However, *Vigna unguiculata* plants reacted showing some differences. Thus, all passion fruit isolates, the banana isolate from Miracatu (BanMSPBR), and the ginger (GengSPBR) and sweet pepper (PimSPBR) isolates from São Paulo, induced mosaic, while the other isolates induced necrotic local lesions. However, host responses depended on temperature and the time of year; in particular symptoms in *V. unguiculata* varied in summer. Thus we were unable to classify effectively all the isolates according to Daniels and Campbell (1992) into CMV-Ia and CMV-Ib, on the basis of systemic versus local infection on *V. unguiculata*.

Serological tests. The twenty-three Brazilian CMV isolates reacted positively with specific CMV-I monoclonal antibodies in DAS-ELISA. The absorbance values ranged from 1 to 1.6 (data not shown). No reactions were observed with CMV-II monoclonal antibodies for all isolates analyzed, except for CMV subgroup II positive control.

RT-PCR/RFLP. DNA products were successfully amplified by RT-PCR for all CMV isolates tested. The DNA fragments were the size expected for subgroup I (486 base pairs), except for the tomato isolate from Espírito Santo (TomESBR), which was 499 base pairs in

length. After enzyme digestion, the majority of the isolates showed restriction patterns typical of subgroup I isolates with a unique *MspI* site and no *EcoRI*, *BamHI* or *HindIII* sites. However, the sweet-pepper isolates from Minas Gerais (CA21MGBR and CA5MGBR) showed restriction patterns that differed from those of isolates from subgroup I (FNyUSA) and II (WLUSA). The CA21MGBR RT-PCR products were not cut by *EcoRI*, *BamHI* or *HindIII*, but *MspI* digestion resulted in just one fragment of approximately 150 base pairs. In contrast, CA5MGBR DNA was cut by *MspI*, to give a pattern typical of subgroup I, but it was also cut by *EcoRI*, to give fragments of approximately 390 and 90 base pairs. Sequencing results, confirmed the results of RT-PCR/RFLP tests. CA21MGBR DNA contained of three *MspI* sites, but the fragments of 165, 152, 138 base-pairs co-electrophoresed and that of 28 base pairs was invisible (data not shown).

Nucleotide and deduced amino acid sequences analysis. Nucleotide sequences of the 23 Brazilian CMV isolates were compared to 37 CMV sequences stored in the GenBank using the BLASTn search tool. Similarity values above 92% were obtained for all Brazilian isolates in comparison with CMV subgroup I and below 75% when compared with CMV isolates from subgroup II. The CMV sequences of the 23 Brazilian isolates were stored in the GenBank and the accession codes are indicated in Table 1.

The multiple alignment of the deduced amino acid sequences of the C-terminal regions of the coat proteins of the Brazilian CMV isolates revealed few differences in sequence and allowed them to be separated into three different groups that presented the same amino acid sequence: Group 1: CommSPBR, BanRSPBR and BanMSPBR; Group 2: BanMGBR and AbobMGBR; Group 3: Bass4ESBR, Bass22ESBR, ErviMSBR, TomR-JBR, P15MGBR, MarM/VC/SPSPBR, GengSPBR, PCMGBR and the FnyUSA; QAUST is a subgroup II CMV isolate from Australia. In addition, eight different isolates were found, including the CA21MGBR sweet-pepper isolate that belongs to subgroup IB (Fig. 4).

Phylogenetic analysis. In comparisons with 37 CMV isolate sequences stored in the GenBank, all Brazilian CMV isolates clustered with other CMV isolates of subgroup IA, except the Brazilian sweet-pepper isolate from Minas Gerais State (CA21MGBR) that clustered with Asian CMV isolates of the subgroup IB (NT9Taiwan, IXFilip, C72JP, PhysIndia) (Fig. 2, 3). Correlation between geographical region and nucleotide sequences was evident for two northeastern isolates (melon from Ceará – MelCEBR and banana from Pernambuco – BanPEBR), three southeastern isolates (sweet-pepper - P15MGBR and CA5MGBR and squash - AbobMGBR) from Minas Gerais and two southeastern black-pepper

isolates (Bass4ESBR and Bass22ESBR) from Espírito Santo. They shared specific clusters in the coat protein phylogenetic tree (Fig. 2). In addition, CMV banana isolates (BanRSPBR and BanMSPBR) and *Commelina* sp. CMV isolate (CommSPBR), collected from the most important region producing banana in Brazil, were closely related, grouping in the same cluster in both phylogenetic trees (Fig. 2, 3).

DISCUSSION

Based on DAS-ELISA with monoclonal antibodies, RT-PCR/RFLP patterns and phylogenetic analyses of partial coat protein and 3' UTR of RNA 3 nucleotide sequences, all Brazilian CMV isolates analyzed here belong to subgroup I. The results of host range and symptomatology did not permit the classification of all the isolates according to Daniels and Campbell (1992) into CMV-Ia and CMV-Ib, on the basis of systemic *versus* local infection on *V. unguiculata*. Despite the simplicity of this sub-

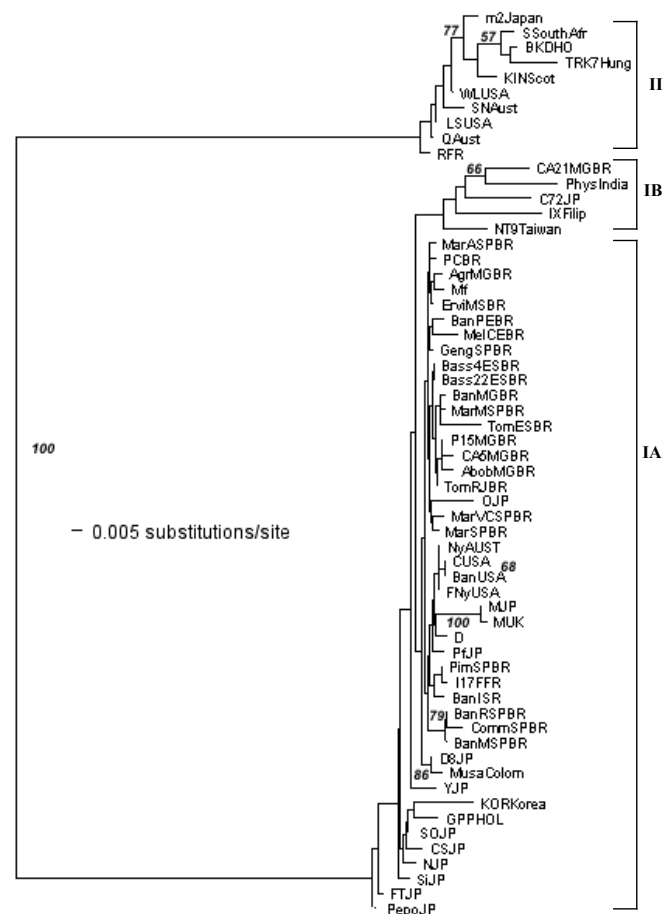


Fig. 2. Unrooted phylogenetic tree based on the partial coat protein ORF sequences of different *Cucurbit mosaic virus* isolates. Bootstrap percentage values are shown in the branches. The GenBank accession numbers of the isolates are described in Table 1 and 2. The isolates from Brazil have ending "BR".

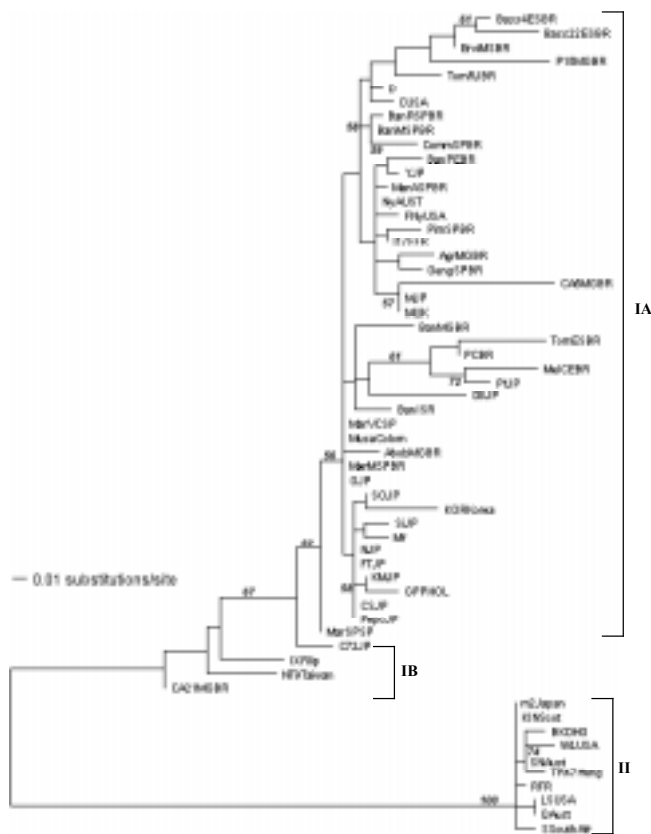


Fig. 3. Unrooted phylogenetic tree based on the 3' end sequences of different *Cucumbers mosaic virus* isolates. Bootstrap percentage values are shown in the branches. The GenBank accession numbers of the isolates are described in Table 1 and 2. The isolates from Brazil have ending "BR".

grouping classification method, there was much variation in host response, mainly depending on the temperature and the time of year. Therefore, the use of host range and symptomatology to define strains is not practical. The symptom expression can vary according to temperature, age of the indicator plants and source of inoculum. CMV concentrations can vary between plants and between leaves of the same plant (Hu *et al.*, 1995) and a single amino acid change in the coat protein can also alter the development and diversity of symptoms (Carrère *et al.*, 1999; Takeshita *et al.*, 2001). Our results did not show correlation between symptom expression and coat protein and 3'UTR RNA 3 phylogenetic analysis.

DAS-ELISA using specific antisera against CMV subgroup I and II seems to be the most appropriate method to detect and subgroup CMV isolates, mainly for routine tests of large number of samples (Anonymous, 1998). RT-PCR followed by RFLP is the most sensitive method and might be used to confirm negative results of ELISA. However, Varveri and Boutsika (1999) commented that using only restriction analysis of PCR products to characterize viruses such as CMV could lead to inconclusive results. CMV isolates may exhibit important genomic variation (Rodriguez-Alvarado *et al.*, 1995).

In our approach, correlation between geographical region and nucleotide sequences was evident for some isolates. Brazil is a continental country with five different geographical regions that have equatorial, tropical and subtropical climates. This variety of climate is very important and could allow CMV isolates to occupy specific niches with different natural hosts and vectors. Our survey, carried out in seven States from three different Regions of Brazil and twelve cultivated species, indicated high genetic conservation among CMV isolates on the coat protein ORF and 3'UTR of RNA 3 nucleotide sequences. Only CMV subgroup IA isolates were found, except for one sweet-pepper isolate from Minas Gerais that belongs to subgroup IB (Figs. 2-3). Few changes of nucleotide sequence of the coat protein ORF resulted in amino acid substitution. Amino acid changes in coat protein ORFs among the Brazilian CMV isolates are indicated in Fig. 4, although there is a high degree of similarity among them.

Breeding programs for disease resistance, using either the traditional genetic methods or engineering approaches based on pathogen-derived sequences, would be greatly assisted by a lack of genetic variability (Boari *et al.*, 2000). Fraile *et al.* (1997) characterized 217 CMV isolates from Spain by RNase protection assay and found no correlation between the place or year of sampling and the frequency composition of the different haplotypes analyzed. The authors also pointed to pathogen-derived resistance by transgenic plants as an

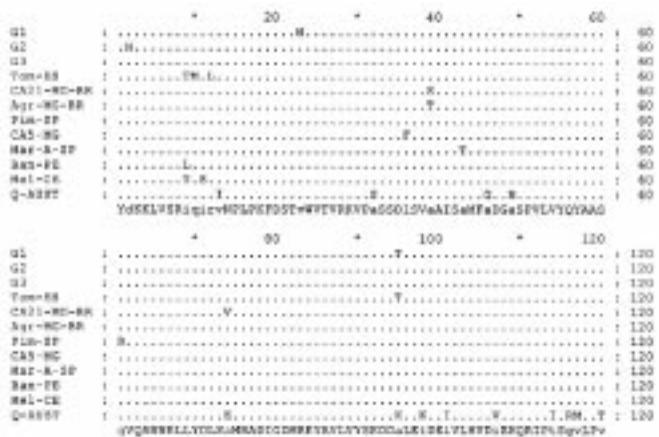


Fig. 4. Multiple alignment of the deduced amino acid sequences of the C-terminal region of the coat protein of CMV isolates from different hosts and regions of Brazil. The groups 1 (G1), 2 (G2) and 3 (G3) correspond to isolates that present the same amino acid sequence: Group 1: CommSPBR, BanR-SPBR and BanMSPBR; Group 2: BanMGBR and AbobMG-BR; Group 3: Bass4ESBR, Bass22ESBR, ErviMSBR, TomR-JBR, P15MGBR, MarM/VC/SPSPBR, GengSPBR, PCMGBR and the FnyUSA; QAUST is a subgroup II CMV isolate from Australia. The consensus amino acids sequence is shown at the bottom and amino acids that differ from the consensus sequence in each isolate are indicated. The GenBank accession numbers are indicated in Table 1.

Table 2. Origin and GenBank accession numbers of the nucleotide sequences of CMV isolates used in phylogenetic analysis and comparisons.

CMV isolates	Accession Number	Subgrup	Origin
Fny	D10538	IA	Usa
Ny	U22821	IA	Australia
I17F	Y18137	IA	France
Mf	AJ276481	IA	Korea
D	AJ131624	IA	-
C	D00462	IA	Usa
Ban	U43888	IA	Israel
O	D00385	IA	Japan
Y	M22710	IA	Japan
Si	AB063333	IA	Japan
SO	AF103992	IA	Japan
CS	D28489	IA	Japan
D8	AB004781	IA	Japan
N	D28486	IA	Japan
FT	D28487	IA	Japan
Pf	AJ237850	IA	Japan
PEPO	D28488	IA	Japan
KM	AB004780	IA	Japan
C7-2	D42079	IB	Japan
M	D10539	IA	Australia
GPP	AJ131623	IA	Netherlands
BANANA	U31219	IA	Usa
KOR	L36251	IA	Koréa
NT9	D28780	IB	Taiwan
MUSA	U32859	IA	Colombia
IX	U20219	IB	Philippines
Phys	X89652	IB	India
WL	D00463	II	Usa
M2	AB006813	II	Japan
KIN	Z12818	II	Scotland
LS	AF127976	II	Usa
R	Y18138	II	France
BKD	AJ131621	II	Netherlands
SN	U22822	II	Australia
Q	M21464	II	Australia
S	AJ131620	II	South Africa
TRK7	L15336	II	Hungary

efficient method for CMV disease control. Recently, Chen *et al.* (2001) showed high coat protein sequence conservation among CMV isolates from ornamentals found in different geographical regions, suggesting probable host specificity. According to them, in general, nucleotide sequence homology among CMV isolates of the same area is much higher than among isolates from different areas. Correlation between geographical region and nucleotide sequences was evident in our approaches for some isolates. The bootstrap values (79

and 69) for the cluster of the three isolates from São Paulo (BanRSPBR, CommSPBR and BanMSPBR), in the CP and 3' UTR trees, respectively, support the suggestion of a relationship among them, which has epidemiological importance. These data may indicate the importance of weeds as reservoirs of CMV in banana crops. Recently, we have also observed *Commelina* sp. plants growing in passion fruit crops (Eiras *et al.*, 2000, 2002). For the other CMV isolates analyzed in this work, it was not possible to establish relationship among virus isolate, host specificity and geographic region. The coat protein seems to interact with movement protein but this interaction is still not completely clear (Nagano *et al.*, 2001). Lin *et al.* (2003) did not find correlation between CP gene variation and geographical origin for 81 CMV isolates from California.

Recently, Roossinck *et al.* (1999) suggested a further division of subgroup I strains into IA and IB, according to 5'UTR RNA 3 and coat protein ORF phylogenetic analyses. This classification was partially supported in our results, mainly in the coat protein phylogenetic tree (Fig. 2), probably because only part of the coat protein was employed in our analysis, although a great number of isolates were analyzed, which might be reflected in the relatively low bootstrapping values obtained. Roossinck (2002), studying the entire genomes of 15 CMV isolates by phylogenetic analyses of the different ORFs and 3' non-coding regions, suggested that each RNA appears to have a different evolutionary history. The author discussed the importance of reassortment for the high genetic diversity, broad host range and worldwide distribution. Roossinck (2002) also showed that coat protein and 2a ORFs present have evolved by more rapid evolution than the 1a and 3a ORFs; the evolution rate of 3'UTR was higher than coat protein ORF. We obtained similar results as judged by comparing the length of the branches in the respective phylogenetic unrooted trees.

CMV is widespread and infects several commercial and wild plant species in Brazil (Boari *et al.*, 2000; Eiras *et al.*, 2002). The high sequence conservation among Brazilian CMV isolates might allow specific control strategies to avoid their dispersion and increase in new areas. A single distinct isolate, the sweet-pepper isolate from Minas Gerais (CA21MGBR), was closely related to subgroup IB Asian isolates. Probably, it was originated from Asia by dispersion of infected plants or by the introduction of an exotic CMV-infected plant, contaminated seeds or bulbs into Brazil. This is the first report of the occurrence of a CMV isolate from subgroup IB, in Brazil. Recently, a CMV subgroup IB isolate was found for the first time by Lin *et al.* (2003) among different CMV isolates from California. In spite of the occurrence of CMV subgroup II isolates in tropical countries (Hord *et al.*, 2001), our survey found no CMV subgroup II isolates in Brazil, indicating the prevalence of the CMV subgroup IA.

ACKNOWLEDGMENTS

The authors are indebted to Dr. A.N. Dusi (Embrapa-hortaliças, Brazil) and Dr. J. Albérico A. Lima (Universidade Federal do Ceará, Brazil) for kindly provided the CMV isolates from Mato Grosso do Sul (pea - ErviMSBR) and Ceará (melon - MelCEBR), respectively, and to Dr. M.C. Gonçalves (Instituto Biológico de São Paulo, Brazil) for critical review of the manuscript; M.R.S. Briones received grants from FAPESP and CNPq, Brazil and the International Research Scholar grant from the Howard Hughes Medical Institute, USA.

REFERENCES

- Anonymous 1998. Detection and biodiversity of Cucumber mosaic cucumovirus. Conclusions from a ringtest of European Union Cost 823 (New technologies to improve phyto-diagnosis). *Journal of Plant Pathology* **80**: 133-149.
- Boari A.J., Maciel-Zambolim E., Carvalho M.G., Zerbini F.M., 2000. Caracterização biológica e molecular de isolados do *Cucumber mosaic virus* provenientes de oito espécies vegetais. *Fitopatologia Brasileira* **25**: 49-58.
- Brigneti G., Voinnet O., Li W.X., Ji L.H., Ding S.W., Baulcombe D.C., 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO Journal* **17**: 6739-6746.
- Carrère I., Tepfer M., Jacquemond M., 1999. Recombinants of cucumber mosaic virus (CMV): determinants of host range and symptomatology. *Archives of Virology* **144**: 365-379.
- Chen Y.K., Derks A.F.L.M., Langeveld S., Goldbach R., Prins M., 2001. High sequence conservation among cucumber mosaic virus isolates from lily. *Archives of Virology* **146**: 1631-1636.
- Colariccio A., Chagas C.M., Mizuki M., Vega J., Cereda E., 1987. Infecção natural do maracujá amarelo pelo vírus do mosaico do pepino no Estado de São Paulo. *Fitopatologia Brasileira* **12**: 254-257.
- Colariccio A., Eiras M., Vicente M., Chagas C.M., Harakava R., 1996. Caracterização parcial de um isolado do vírus do mosaico do pepino de *Musa* sp. "nanição". *Fitopatologia Brasileira* **21**: 268-274.
- Colariccio A., Chagas C.M., Ferrari J.T., Eiras M., Chaves A.L.R., 2002. Molecular Characterization and Phylogenetic analysis of *Cucumber mosaic virus* in *Zingiber officinale* in Brazil. In: *Proc. XII International Congress of Virology. The World of Microbes, Paris, France*, 443-444.
- Daniels J., Campbell R.N., 1992. Characterization of *Cucumber mosaic virus* isolates from California. *Plant Disease* **76**: 1245-1250.
- Duarte L.M.L., Rivas E.B., Alexandre M.A.V., Ferrari J.T., 1994. Detection of CMV isolates from *Commelinaceae* species. *Fitopatologia Brasileira* **19**: 248-253.
- Dusi A.N., Maciel-Zambolim E., Gama M.I.C., Giordano L.B., Santos J.R.M., 1992. Ocorrência e caracterização do vírus do mosaico do pepino (CMV) em ervilha. *Fitopatologia Brasileira* **17**: 286-291.
- Edwards M.C., Gonsalves D., 1983. Grouping of seven biologically defined isolates of cucumber mosaic virus by peptide mapping. *Phytopathology* **73**: 1117-1120.
- Eiras M., Araujo J., Colariccio A., Chaves A.L.R., Harakava R., Chagas C.M., 2002. Caracterização molecular de isolados do *Cucumber mosaic virus* de maracujazeiros no Estado de São Paulo. *Summa Phytopathologica* **28**: 346-349.
- Eiras M., Colariccio A., Chaves, A.L.R. Araujo, J. Moreira, S.R. 2000. Partial characterization of *Cucumber mosaic virus Commelina* sp. isolate from banana crops. *Virus: Reviews and Research* **5**: 192.
- Eiras M., Colariccio A., Chaves A.L.R., 2001. Isolado do vírus do mosaico do pepino obtido de bananeira no Estado de São Paulo pertence ao subgrupo Ia. *Fitopatologia Brasileira* **26**: 53-59.
- Espinha L.M., Gaspar, J.O., 1998. Caracterização molecular de dois isolados do vírus do mosaico do pepino por IC/RT-PCR/RFLP e produção de sonda para os RNAs 3 e 4. *Fitopatologia Brasileira* **23**: 316.
- Fraile A., Alonso-Prados J.L., Aranda M.A., Bernal J.J., Malpica J.M., García-Arenal F., 1997. Genetic Exchange by recombination or reassortment is infrequent in natural populations of a tripartite RNA plant virus. *Journal of Virology* **71**: 934-940.
- Hord M.J., García A., Villalobos H., Rivera C., Macaya G., Roossinck M.J., 2001. Field survey of *Cucumber mosaic virus* subgroups I and II in crop plants in Costa Rica. *Plant Disease* **85**: 952-954.
- Hu J.S., Li H.P., Barry K., Wang M., Jordan R., 1995. Comparison of dot blot, ELISA and RT-PCR assays for detection of two cucumber mosaic isolates infecting banana in Hawaii. *Plant Disease* **79**: 902-906.
- Lin H.X., Rubio L., Smythe A., Jimenez M., Falk, B.W., 2003. Genetic diversity and biological variation among California isolates of *Cucumber mosaic virus*. *Journal of General Virology* **84**: 249-258.
- Maciel-Zambolim E., Carvalho M.G., Matsuoka K., 1990. Caracterização parcial do vírus do mosaico do pepino isolado de pimenta-do-reino. *Fitopatologia Brasileira* **15**: 220-225.
- Maciel-Zambolim E., Assis M.I.T., Zambolim L., Ventura J.A., Carvalho M.G., 1994. Infecção natural da bananeira cultivar "prata" (AAB) pelo vírus do mosaico do pepino no Estado de Minas Gerais. *Fitopatologia Brasileira* **19**: 483-484.
- Nagano H., Kazuyuki M., Furusawa I., Okuno T., 2001. Conversion in the requirement of coat protein in cell-to-cell movement mediated by the cucumber mosaic virus movement protein. *Journal of Virology* **75**: 8045-8053.
- Nicholas K.B., Nicholas H.B., Deerfield D.W., 1997. GeneDoc: analysis and visualization of genetics variation. *EMBL-NEW News* **4**: 14.
- Palukaitis P., Roossinck M.J., Dietzgen R.G., Francki R.I.B., 1992. Cucumber mosaic virus. *Advances in Virus Research* **41**: 281-341.
- Posada D., Crandall K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

- Rizos H., Gunn L.V., Pares R.D., Gillings M.R., 1992. Differentiation of cucumber mosaic virus isolates using polymerase chain reaction. *Journal of General Virology* **73**: 2099-2103.
- Rodriguez-Alvarado G., Kurath G., Dodds J.A., 1995. Heterogeneity in pepper isolates of cucumber mosaic virus. *Plant Disease* **79**: 450-455.
- Roossinck M.J., 2002. Evolutionary history of *Cucumber mosaic virus* deduced by phylogenetic analyses. *Journal of Virology* **76**: 3382-3387.
- Roossinck M.J., Zhang L., Hellwald K.H., 1999. Rearrangements in the 5' nontranslated region and phylogenetic analyses of *Cucumber mosaic virus* RNA 3 indicate radial evolution of three subgroups. *Journal of Virology* **73**: 6752-6758.
- Sambrook J., Russel D.W., 2001. Molecular cloning: a laboratory manual. Third Edition, Cold Spring Harbor, New York, USA.
- Sanger F., Nicklen S., Coulson A.R., 1977. DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences* **74**: 5463-5467.
- Silberschmidt K.M., Nóbrega N.R., 1941. Sobre uma doença de vírus da bananeira. *O Biológico* **7**: 216-219.
- Singh Z., Jones R.A.C., Jones M.G.K., 1995. Identification of Cucumber mosaic virus subgroup I isolates from banana plants affected by infectious chlorosis disease using RT-PCR. *Plant Disease* **79**: 713-716.
- Swofford D.L., 1999. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0. Sinauer Associates, Sunderland, Massachusetts, USA.
- Takeshita M., Suzuki M., Takanami Y., 2001. Combination of amino acids in the 3a protein and the coat protein of cucumber mosaic virus determines symptom expression and viral spread in bottle gourd. *Archives of Virology* **146**: 697-711.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmogin F., Higgins D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality tools. *Nucleic Acids Research* **24**: 4876-4882.
- Van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., Wickner R.B., 2000. Virus Taxonomy Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, California, USA.
- Varveri C., Boutsika K., 1999. Characterization of cucumber mosaic cucumovirus isolates in Greece. *Plant Pathology* **48**: 95-100.
- Wylie S., Wilson C.R., Jones R.A.C., Jones M.G.K., 1993. A polymerase chain reaction assay for cucumber mosaic virus in lupin seeds. *Australian Journal Agricultural Research* **44**: 41-51.

Received 9 September 2003

Accepted 6 February 2004

