PARTIAL MOLECULAR CHARACTERIZATION OF COWPEA STUNT ISOLATES OF CUCUMBER MOSAIC VIRUS AND BLACK EYE COWPEA MOSAIC VIRUS FROM ARKANSAS AND GEORGIA (USA)

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ABSTRACT

Partial molecular characterization of the coat protein of the cowpea stunt-causing isolates of Cucumber Mosaic Virus (CMV) from Arkansas and Georgia revealed that both isolates of CMV belong to CMV subgroup I and differ at eight nucleotides positions, resulting in two amino acids difference. There was only one amino acid difference for the Blackeye Cowpea Mosaic Virus (BlCMV) isolates from both locations. Differences in the coat protein genes of CMV and BlCMV isolates from Arkansas and Georgia could partially be responsible for the variation in the virus accumulation pattern. This is the first report on the taxonomic classification of the cowpea stunt disease-causing isolates of CMV and BlCMV in Arkansas and Georgia based on nucleotides and amino acid sequences analysis.

Keywords : cowpea, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*, USA, Côte d'Ivoire.

RESUME

CARACTERISATION MOLECULAIRE PARTIELLE D'ISOLATS DE CMV ET BICMV ASSOCIES A LA MALADIE DU NANISME DU NIEBE DANS L'ARKANSAS ET LA GEORGIE (USA)

La caractérisation partielle des gènes de la protéine de capside des isolats de CMV associés à la maladie du nanisme du niébé provenant de l'Arkansas et de la Georgie a révélé que les deux isolats de CMV appartiennent au sous-group I et qu'ils diffèrent au niveau de huit nucléotides, ce qui se traduit par une différence de deux acides aminés. Pour BlCMV, la différence entre les deux isolats porte seulement sur un acide aminé. Ces différences dans les gènes de la capside des isolats de CMV et de BlCMV des deux différentes localités pourraient être partiellement responsables de la différence entre le mode d'accumulation de ces virus. Ceci constitue le premier travail sur la classification taxonomique , basé sur l'analyse des séquences d'acides nucléiques et d'acides aminés des isolats de CMV et de BlCMV responsable du nanisme du niébé dans l'Arkansas et la Georgie.

***Mots clés :** Niébé,* Vigna unguiculata *(L.) Walp. sesp. unguiculata,* USA, Côte d'Ivoire.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp. subsp. *Unguiculata*) is an important crop worldwide with a global production covering approximately 12.5 million ha of which, 8 million ha are in west and central Africa (Singh *et al.*, 1997).

Cowpea stunt, a severe disease of cowpea, first reported in Georgia, Alabama and South Carolina (Pio-Ribeiro *et al.*, 1978 ; Pio-Ribeiro and Kuhn, 1980), is caused by a synergistic interaction between blackeye cowpea mosaic virus (BlCMV) and cucumber mosaic virus (CMV). Cowpea plants doubly infected with both viruses are severely stunted with small, blistered and malformed leaves.Stems and petioles of these infected plants become necrotic and show a significant reduction in the number of leaves and pods. Cowpea stunt caused important yield loss of 86.4 %

whereas only 2.5 and 14.2 % reduction occurred with single infections with CMV and BlCMV, respectively (Pio-Ribeiro *et al.*, 1978). Cowpea stunt was more recently found in Magnolia County, Arkansas (Anderson *et al.*, 1994).

Cucumber mosaic virus is the type virus of the cucumovirus group. It has a wide host range mostly composed of dicots. CMV can infect more than 800 plant species (Palukaitis *et al.*, 1992). The virus is characterized by small, icosahedral particles of about 30 nm in diameter. It genome is composed of three genomic, positive-sense RNA molecules (RNAs 1, 2 and 3) and a subgenomic RNA (RNA 4) coding for the 24.5 Kilodalton (Kd) coat protein (Matthews, 1991). The CMV coat protein (CP) is involved not only in symptom development, but also in the encapsidation of the RNAs as well as in aphid transmission (Mossop and Francki, 1977). Several strains of CMV have been isolated (Kapper and Waterworth, 1981; Rizos *et al.*, 1992). In order to differentiate and classify these strains, several methods have been used: serology (Devergne and Cardin, 1983), nucleic acid hybridation (Gould and Symons,1978; Piazzolla *et al.*, 1979 ; Owen and Palukaitis, 1988) and peptide mapping of the coat protein gene (Edwards and Gonsalves, 1983). However, only nucleotide sequence analysis of the CP gene and the 3' untranslated region was able to provide an accurate alternative differentiation between CMV strains and separate them into two subgroups, I and II (Quemada *et al.*, 1989; Rizos *et al.*, 1992).

Blackeye cowpea mosaic virus belongs to the most economically important group of plant viruses, the potyvirus group. These viruses particles are long and flexuous rod-shape, measuring approximately 12 x 900 nm. Potyviruses have their genome composed of a linear, single-stranded, positive-sense RNA, approximately 9.500 nucleotides (nt) in length. A genome-linked protein (Vpg) is located at the 5' terminus and at the 3'-end, there is a 200 nt untranslated region (3'-UTR), followed by a poly-A tail (Matthews, 1991). The virus genome consists of a large open reading frame. The coat protein gene is located at the 3'-end. BlCMV has been reported as the cowpea strain of bean yellow mosaic virus (BYMV) based on microagglutination test (Gay and Winstead, 1970 ; Harrison and Gudauskas, 1968a ; Harrison and Gudauskas, 1968b ; Kuhn, 1964 ; Kuhn *et al.*, 1965). However, based on comparative studies of cytological inclusions and host range, these viruses have been shown to be different (Edwardson *et al.*, 1972 ; Zettler and Evans, 1972). BlCMV and cowpea aphid-born mosaic virus (CaBMV), sometimes considered closely related or similar (Bock and Conti, 1974), have been differentiated based on their reactions on resistant cowpea cultivars (Taiwo *et al.*, 1982). For a better differenciation and classification of potyviruses, methods based on coat protein and/ or 3'-UTR region sequences seem to give good results (Frenkel *et al.*, 1989, 1991; Khan *et al.*, 1990, 1993 ; Lana *et al.*, 1988 ; Shukla and Ward, 1988 ; Shukla and Ward, 1989 ; Van der Vlugt *et al.*, 1993).

Since cowpea stunt was first discovered in Georgia and later in Arkansas, it was important to compare the disease-causing isolates of CMV and BlCMV from both geographical locations. In previous studies where the stunt-causing isolates of CMV and BlCMV from Arkansas and Georgia were compared biologically, it was found that although all four types of mixed infections produced similar symptoms on cowpea plants, the viruses behaved differently based on their accumulation patterns in the leaves and stems (Diallo, 1998). The objective of this study was to molecularly characterize the Arkansas and Georgia cowpea stunt isolates of CMV and BlCMV.

MATERIALS AND METHODS

VIRUSES AND PLANTS

The Arkansas cowpea isolates of BlCMV ($BlCMV_{AR}$) and CMV (CMV_{AR}) were originally obtained from field samples taken in Columbia County in 1994. The Georgia isolates of cowpea stunt viruses ($BlCMV_{GA}$ and CMV_{GA}) were provided by Dr. A. G. Gillaspie, Jr., USDA-ARS Genetic Resources Unit, University of Georgia, Griffin, GA. All viruses were maintained as dried infected tissues at 4 °C or in the cowpea cultivar 'Coronet' (Brantley, 1976) under greenhouse conditions with temperatures ranging from 20-30 °C. 'Coronet' cowpea plants were grown individually in 3-inch pots containing Redi-Earth 3CP potting mixture (Grace Sierra, Milpitas, CA).

CHARACTERIZATION OF CMV AND BlCMV COAT PROTEIN GENES

Total nucleic acids from plants infected with CMV_{AR}, CMV_{GA}, $BlCMV_{AR}$, $BlCMV_{GA}$ and healthy

cowpea plants were extracted according to the procedure described by Pappu *et al.* (1993). Each plant sample was frozen in liquid nitrogen and ground in 300µl of extraction buffer (2 % SDS, 0.1 M Tris, 0.002M EDTA, pH 8.0). The viral RNAs were extracted in phenol/ chloroform-isoamyl alcohol (1:1). Crude RNA extracts were purified on a sephadex G 50 column equilibrated with TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0). The nucleic acid eluants were collected and used for reverse transcriptase polymerase chain reaction (RT-PCR) amplification and cloning of the coat protein (CP) genes of all four isolates of CMV and BlCMV according to the procedure described by Pappu *et al.* (1993). For CMV, two oligonucleotide primers were designed based on the published sequence of CMV strain Q and FNY (Quemada *et al.*, 1989). The entire CP gene including the 3' flanking sequence was amplified using the following upstream primer EA 39 (5'-TTC TCC GCG AGA TTG C-3') corresponding to positions 1167-1182 of the published sequence of FNY-CMV (CMV FNY). The downstream primer, CMV3'CP (5'-CGT AAG CTG GAT GGA C-3') corresponding to positions 2021-2036 of FNY-CMV was used. Using these two primers the predicted length of the CMV fragments is about 860 base pairs (bp).

Total nucleic acid extracts were heat denatured at 70 °C for 3 min and placed on ice. Each extract (20 µl) was added to 73 µl of the PCR mix [1X Taq DNA polymerase buffer (Promega, Madison, WI), 0.01M dithiothreitol (DTT), 0.04M dNTP mix, 2.5mM $MgCl_2$, 16 units of RNAsin (Promega), 20 units of AMV-reverse transcriptase (Promega), 2.5 units of Taq DNA polymerase (Promega), and 100 pmol of each oligonucleotide primer]. Samples were incubated at 41 °C for 1 h for the synthesis of the first strand complementary DNA (cDNA) and placed in a thermal cycler (Barnstead Thermolyne). Initial denaturation was done at 96 °C for 2 min. Thirty-five cycles were performed with periods of 1 min for annealing at 44 °C, 1 min for synthesis at 72 °C and 1 min for melting at 94.5 °C. The reverse transcriptase (RT-PCR) products were analysed by a 1 % agarose gel electro-phoresis and visualized by ethydium bromide staining.

The entire CP gene and the 3'-untranslated regions (3'-UTR) of BlCMV isolates from Arkansas and Georgia were amplified using a degenerate primer EA 13 (5'-AAG ATT GAA GAG TTA GC-3') designed based on the published sequence of BlCMV strain W (Khan *et al.*, 1993) corresponding to positions (1-17) on the viral polymerase gene portion of the published sequence and an oligo-dT primer. The predicted length of the RT-PCR fragment was approximately 1200 bp. Total nucleic acids were denatured at 70 °C for 3 min, and 55.3 µl added to tubes containing 36.9 µl of PCR mix (same as before) and 100 pmol of each primer. The reactions were incubated at 42 °C for 1 h for the synthesis of the first strand cDNA. Thirty cycles were run in a thermal cycler with periods of 45 s at 93.5 °C for denaturation, 45 s at 36 °C for annealing and 1 min at 72 °C for extension. The RT-PCR fragments were analysed by a 1 % agarose gel electrophoresis.

CLONING AND SEQENCING OF CMV AND BLCMV COAT PROTEIN GENES

The RT-PCR fragments of CMV and BlCMV isolates from Arkansas and Georgia were blunt-ended with DNA polymerase I (Klenow fragment), extracted from 0.8 % low melting point agarose gel in 0.05 M Tris-borate/EDTA electrophoresis buffer (TBE), and phosphorylated. CMV and BlCMV fragments were ligated into the *Sma* I site of pUC118 and pUC119 plasmids respectively and transformed into competent *Escherichia coli* DH5a cells. Transformed bacteria were cultured on solid 2XYT medium containing 75 µg/ml ampicillin, 40 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside, United States Biochemical) and 8 µg/ml IPTG (isopropylthio-b-D-galactoside, United States Biochemical) and incubated at 37 °C for 16 h. Recombinants were screened by blue and white colour reaction. White colonies were cultured in 2XYT liquid medium containing ampicillin. Plasmids were digested with restricted enzymes *EcoR*I and *Pst*I according to the supplier's recommendations. Inserts were detected by agarose gel electrophoresis. Plasmids with inserts from selected clones were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the Sequenase v.2.0 kit (USB, Cleveland, OH) and the dideoxynucleotide sequencing method. At least 2 independent clones from each virus were sequenced in both directions. Sequence data were analysed and compared using PC/Gene v.6.85 (Intelligenetics, Campbell, CA).

RESULTS

PARTIAL MOLECULAR CHARACTERIZATION OF THE ARKANSAS AND GEORGIA ISOLATES OF CMV

PCR-amplified viral products of CMV$_{AR}$ and CMV$_{GA}$ were analysed by agarose gel electrophoresis. Both viral coat protein genes displayed identical migration patterns and sizes which corresponded to the expected size of 860 nucleotides (Diallo,1998).The nucleotide sequence comparison of the coat protein genes and portion of the 3' flanking regions of the CMV$_{AR}$ and CMV$_{GA}$ isolates showed that CMV$_{AR}$ differed from CMV$_{GA}$ isolate at only eight positions (figure 1), with seven of the differences located in the coding region. There were 14 nucleotide differences between CMV$_{AR}$ and CMV$_{FNY}$ and 12 between CMV$_{GA}$ and CMV$_{FNY}$. The CP fragment nucleotide sequences homology of the two CMV isolates was 99 %, 90 % with subgroup I CMV and only 77-80 % with subgroup II CMV. The CMV CP genes encoded the predicted 218 amino acid residues for CMV$_{AR}$, CMV$_{GA}$ and CMV$_{FNY}$. The deduced amino acid sequences of CMV$_{AR}$ and CMV$_{GA}$ revealed only two amino acid differences located at positions 61 and 124 (figure 2). CMV$_{FNY}$ on the other hand differed from CMV$_{AR}$ and CMV$_{GA}$ at 14 and 12 amino acid residues, respectively, with the differences not restricted to any specific area of the coat protein gene.

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CMVFny   TTCTCCGCGAGATTGCGTTATTGTCTACTGACTATATAGA    40
CMVAR    ...................-..........G----..TT.    35
CMVGA    ...................-..........G----..TT.    35

CMVFny   GAGTGTTTGTGCTGTGTTT-TCTCTTTTGTGTCGTAGAAT    79
CMVAR    ...A.......T.......C.......CT...T.....TA    75
CMVGA    ...A.......T.......C.......C....T.....TA    75

CMVFny   T--GAGTCGAGTCATGGACAAATCTGAATCAACCAGTGCT   117
CMVAR    .TC........................C...G....C..C   115
CMVGA    .TC........................C...G....C..C   115

CMVFny   GGTCGTAACCGTCGACGTCGTCCGCGTCGTGGTTCCCGCT   157
CMVAR    ........................................   145
CMVGA    ........................................   145

CMVFny   CCGCCCCCTCCTCCGCGGATGCTAACTTTAGAGTCTTGTC   197
CMVAR    ....TT...............T.............C....   195
CMVGA    ....TT...............T.............C....   195

CMVFny   GCAGCAGCTTTCGCGACTTAATAAGACGTTAGCAGCTGGT   237
CMVAR    ......A........G........................   235
CMVGA    ......A........G........................   235

CMVFny   CGTCCAACTATTAACCACCCAACCTTTGTAGGGAGTGAAC   277
CMVAR    .....T..C.........................A...G.   275
CMVGA    .....T................................G.   275

CMVFny   GCTGTAGACCTGGGTACACGTTCACATCTATTACCCTAAA   317
CMVAR    .T....A......A...........G..............   315
CMVGA    .T....A......A...........G..............   315

CMVFny   GCCACCAAAAATAGACCGTGGGTCTTATTACGGTAAAAGG   357
CMVAR    ..................C...........T.........   355
CMVGA    ..................C...........T.........   355

CMVFny   TTGTTACTACCTGATTCAGTCACGGAATATGATAAGAAGC   397
CMVAR    ....CTT.......G.............TC........A.   395
CMVGA    ....CTT.......G.............TC........A.   395

CMVFny   TTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAA   437
CMVAR    ........................................   435
CMVGA    ........................................   435

CMVFny   ATTTGATTCTACCGTGTGGGTGACAGTCCGTAAAGTTCCT   477
CMVAR    ...............T.......T................   475
CMVGA    ........................................   475

CMVFny   GCCTCCTCGGACTTATCCGTTGCCGCCATCTCTGCTATGT   517
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CMVAR    .....T........G..............T...A......    515
CMVGA    .....T........G.....C........T...A......    515

CMVFny   TCGCGGACGGAGCCTCACCGGTACTGGTTTATCAGTATGC    557
CMVAR    .T......................................    555
CMVGA    .T......................................    555

CMVFny   CGCATCTGGAGTCCAAGCCAACAACAAACTGTTGTATGAT    597
CMVAR    T.....C.................T...T...........    595
CMVGA    T.....C.................T...T.......C...    595

CMVFny   CTTTCGGCGATGCGCGCTGATATAGGTGACATGAGAAAGT    637
CMVAR    ......C................T..C......C.T....    635
CMVGA    .....AC................T..C......C.T....    635

CMVFny   ACGCCGTCCTCGTGTATTCAAAAGACGATGCGCTCGAGAC    677
CMVAR    .......A.....................A.A..T...T.    675
CMVGA    .......A.....................A.A..T...T.    675

CMVFny   GGACGAGCTAGTACTTCATGTTGACATCGAGCACCAACGC    717
CMVAR    ...T........G........C.....T...........T    715
CMVGA    ...T........G........C.....T...........T    715

CMVFny   ATTCCCACATCTGGAGTGCTCCCAGTCTGATTCCGTGTT-    756
CMVAR    ..........................T...A........T    755
CMVGA    ..........................T...A........T    755

CMVFny   CCCAGAATCCTCCCTCCGATCTCTGTGGCGGGACGTGAGT    796
CMVAR    .....G.C.........AT.T....A.......GC.....    795
CMVGA    .....G.C.........AT.T....A.......GC.....    795

CMVFny   TGGCAGTTCTGCTATAAACTGTCTGAAGTCACTAAA-CGT    835
CMVAR    .......ATC...........C..............G.--    833
CMVGA    .......ATC...........C..............G.--    833

CMVFny   TTTTTACGGTGAACGGGTTGTCCATCCAGCTTACG         870
CMVAR    --...G.............................         866
CMVGA    --...G.............................         866
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Figure 1 : Comparison of the nucleotide sequences of the coat protein and untranslated regions of the published data for CMVFNY (Quemada *et al.,* 1989), the Arkansas isolate of CMV (CMVAR) and the Georgia isolate of CMV (CMVGA).

Dots (...) indicate nucleotides identical to CMVFNY sequences and dashes (---) represent missing residues. The ATG and AGT codons corres pond to the initiation and termination sites for the putative CMV coat protein open reading frame. Nucleotide differences between CMVAR and CMVGA are in bold.

Comparaison des séquences de nucléotides des régions de la protéine de capside et non-codante du CMVFNY publié (Quemada et al., 1989), de l'isolat de CMV de l'Arkansas (CMVAR) et de l'isolat de CMV de la Georgie (CMVGA).

Les (…) indiquent les nucléotides identiques aux séquences de CMVFNY et les tirets (---) représentent les résidus manquants. Les codons ATG et AGT correspondent aux sites d'initiation et de terminaison du cadre de lecture. Les différen ces en nucléotides entre CMVAR et CMVGA sont en gras.

CMV$_{Fny}$	MDKSESTSAGRNRRRRPRRGSRSAPSSADANFRVLSQQLS	40
CMV$_{AR}$	....D.A.................S....V..........	40
CMV$_{GA}$	....D.A.................S....V..........	40
CMV$_{Fny}$	RLNKTLAAGRPTINHPTFVGSERCRPGYTFTSITLKPPKI	80
CMV$_{AR}$	....................**N**...K...............	80
CMV$_{GA}$	........................K...............	80
CMV$_{Fny}$	DRGSYYGKRLLLPDSVTEYDKKLVSRIQIRVNPLPKFDST	120
CMV$_{AR}$	..........S..E....F.....................	120
CMV$_{GA}$	..........S..E....F.....................	120
CMV$_{Fny}$	VWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGV	160
CMV$_{AR}$	...**I**................T...................	160
CMV$_{GA}$	....................T...................	160
CMV$_{Fny}$	QANNKLLYDLSAMRADIGDMRKYAVLVYSKDDALETDELV	200
CMV$_{AR}$	...........P....................T..S....	200
CMV$_{GA}$	...........P....................T..S....	200
CMV$_{Fny}$	LHVDIEHQRIPTSGVLPV	218
CMV$_{AR}$	....	218
CMV$_{GA}$	....	218

Figure 2 : Comparison of the deduced amino acid sequences of the coat protein genes of the published data for CMVFNY (Quemada *et al.*, 1989), the Arkansas isolate of CMV (CMVAR), and the Georgia isolate of CMV (CMVGA).

Dots (...) indicate amino acids identical to the CMVFNY sequence. Amino acid differences between CMVAR and CMVGA are in bold.

Comparaison des séquences d'acides aminés des gènes de la protéine de capside de CMVFNY publié (Quemada et al., 1989), de l'isolat de CMV de l'Arkansas (CMVAR), et de l'isolat de CMV de la Georgie (CMVGA).

Les points (...) indiquent les acides aminés identiques à la séquences de CMVFNY. Les différences d'acides aminés entre CMVAR et CMVGA sont en gras.

PARTIAL MOLECULAR CHARACTERIZATION OF THE ARKANSAS AND GEORGIA ISOLATES OF BLCMV

PCR-amplified products of BlCMV$_{AR}$ and BlCMV$_{GA}$ were analysed by agarose gel electrophoresis. Similar migration patterns and sizes (approximately 1200 bases) corresponding to the expected sizes were observed for both BlCMV isolates (Diallo, 1998). Nucleotide sequence comparisons between the Arkansas and Georgia isolates of BlCMV showed only one nucleotide difference located at position 281 (99 % homology). Alignment with BlCMV$_{W}$ (Khan *et al.*, 1993) indicated only one nucleotide difference with BlCMV$_{GA}$ at that position (figure 3). The amino acid sequences of both isolates of BlCMV (from Arkansas and Georgia) were deduced from the nucleotide sequences. The predicted amino acid sequence data for the coat protein genes of BlCMV$_{AR}$, BlCMV$_{GA}$ and BlCMV$_{W}$ were aligned (figure 4). Both predicted BlCMV$_{AR}$ and BlCMV$_{GA}$ coat proteins were identical in size (239 amino acid residues) while the deduced amino acid sequence of BlCMV$_{W}$ was a few amino acids longer (244 residues). The Arkansas and Georgia isolates of BlCMV differed by only one amino acid located at position 19 where the glutamine on BlCMV$_{AR}$ is replaced by a proline on BlCMV$_{GA}$. At that same position BlCMV$_{W}$ had also a Q residue similar to BlCMV$_{AR}$. The Arkansas and Georgia isolates differed from BlCMV$_{W}$ at 10 and 11 amino acid positions, respectively. The 3' end of all three isolates of BlCMV displayed some important differences. There seemed to be a five amino acids deletion or insertion depending on the virus isolate (figure 4). The overall similarity of the amino acid sequence of BlCMV$_{AR}$, BlCMV$_{GA}$ and BlCMV$_{W}$ was 93 %.

Isolate	Sequence	Position
BlCMV_{W}	AAGATTGAAGAGTTAGCCAAGTATCTTGAAGTGTTTGACT	40
BlCMV_{AR}	.................A..A..C..G......C.C..T.	40
BlCMV_{GA}	.................A..A..C..G......C.C..T.	40
BlCMV_{W}	TTGACTATGATGTAGGATGCGGAGAATCTGTGCACCTACA	80
BlCMV_{AR}	..........C.............................	80
BlCMV_{GA}	..........C.............................	80
BlCMV_{W}	ATCTGGAACTGGACAGCCGCAACCACCAATAGTGGATGCT	120
BlCMV_{AR}	...A.................G.....G.....T......	120
BlCMV_{GA}	...A.................G.....G.....T......	120
BlCMV_{W}	GGTGTGGATGCTGGAAAGGACAAGAGAGAGAGAAGCAATA	160
BlCMV_{AR}	..............G......................GC.	160
BlCMV_{GA}	..............G......................GC.	160
BlCMV_{W}	GAGGAAAAGACCCTGAAAGCAGGGAGGGGTCAGTAAACAA	200
BlCMV_{AR}	.........................A..T....GG.....	200
BlCMV_{GA}	.........................A..T....GG.....	200
BlCMV_{W}	CAACCGTGGTGCAGGGGATTCAACAATGAGAGACAAGGAT	240
BlCMV_{AR}	...............A........................	240
BlCMV_{GA}	...............A........................	240
BlCMV_{W}	GTGAACGCAGGCTCCAGGGGAAAAGTTGTCCCGCGGCTTC	280
BlCMV_{AR}	........G.......AA..G...........A.......	280
BlCMV_{GA}	........G.......AA..G...........A.......	280
BlCMV_{W}	AAAAGATCACAAAAAGGATGAACTTGCCCATGGTGAAAGG	320
BlCMV_{AR}	....	320
BlCMV_{GA}	**C**.......................................	320
BlCMV_{W}	GAATGTTATTTTAAATCTAGATCATCTGTTGGATTACAAG	360
BlCMV_{AR}	................T....C.....A............	360
BlCMV_{GA}	................T....C.....A............	360
BlCMV_{W}	CCAGTTCAAACTGACCTTTTTAACACAAGAGCAACAAGGG	400
BlCMV_{AR}	....AA........T.....C................A.A	400
BlCMV_{GA}	....AA........T.....C................A.A	400
BlCMV_{W}	ACCAGTTTGAAATGTGGTACAATGCTGTGAAGGGCGAGTA	440
BlCMV_{AR}	TG.............................A........	440
BlCMV_{GA}	TG.............................A........	440
BlCMV_{W}	TGAAATAGATGATGATCAGATGTCAATTGTAATGAACGGA	480
BlCMV_{AR}	...G..........CA..A.................T..C	480
BlCMV_{GA}	...G..........CA..A.................T..C	480
BlCMV_{W}	TTCATGGTGTGGTGTATTGACAATGGCACTTCACCGGATG	520
BlCMV_{AR}	....	520
BlCMV_{GA}	....	520
BlCMV_{W}	TGAATGGTACATGGGTGATGATGGATGGAGATGAGCAAGT	560
BlCMV_{AR}	.......C.......................C........	560
BlCMV_{GA}	.......C.......................C........	560
BlCMV_{W}	TGAATACCCACTCAAACCAATGGTTGAAAATGCAAAGCCA	600
BlCMV_{AR}	...G....................................	600
BlCMV_{GA}	...G....................................	600
BlCMV_{W}	ACACTCCGTCAAATCATGCACCATTTCTCAGATGCAGCTG	640
BlCMV_{AR}	....	640
BlCMV_{GA}	....	640
BlCMV_{W}	AAGCATACATTGAGATGAGAAATTCCGAAAGGCCGTACAT	680
BlCMV_{AR}	............................G.AA........	680
BlCMV_{GA}	............................G.AA........	680

$BlCMV_{W}$	GCCTAGGTACGGACTACTTCGGAATTTGAGGGATAAAAAT	720
$BlCMV_{AR}$	..T............G........................	720
$BlCMV_{GA}$	..T............G........................	720
$BlCMV_{W}$	CTAGCTCGCTACGCTTTTGATTTCTATGAGGTGACATCAA	760
$BlCMV_{AR}$	......................................C.	760
$BlCMV_{GA}$	......................................C.	760
$BlCMV_{W}$	AAACATCGGATCGAGCCAGAGAAGCAGTAGCACAGATGAA	800
$BlCMV_{AR}$	....	800
$BlCMV_{GA}$	....	800
$BlCMV_{W}$	GGCAGCAGCCCTCAGCAACGTTAGCAGCAAGTTGTTTGGA	840
$BlCMV_{AR}$	....	840
$BlCMV_{GA}$	....	840
$BlCMV_{W}$	CTTGACGGTAATGTTGCAACAACCAGCGAGAATACTGAAA	880
$BlCMV_{AR}$	.....T.......................A..........	880
$BlCMV_{GA}$	.....T.......................A..........	880
$BlCMV_{W}$	GGCACACTGCAAGGGACGTTAACCAAAACATGCACACACT	920
$BlCMV_{AR}$	................T..C..T.................	920
$BlCMV_{GA}$	................T..C..T.................	920
$BlCMV_{W}$	TCTTGGCATGGTTCTCC-GCAGTAAAGGTTGGGTAAACTG	960
$BlCMV_{AR}$	...........GC.C...................G...C.	960
$BlCMV_{GA}$	...........GC.C...................G...C.	960
$BlCMV_{W}$	ACCACAGTTAGCATCTCGCGTCGCTGAATAATTTCATATA	1000
$BlCMV_{AR}$	..............T.........................	1000
$BlCMV_{GA}$	..............T.........................	1000
$BlCMV_{W}$	GTAATCTTTTATGTTCTCTTTAGTTTCTGTGTGGTTTTAC	1040
$BlCMV_{AR}$	...............T...........A........AC..	1040
$BlCMV_{GA}$	...............T...........A........AC..	1040
$BlCMV_{W}$	CACCTTTGTGTTACTATTGTGATAGCGTGGTTAGTCCACC	1080
$BlCMV_{AR}$	.........................T..............	1080
$BlCMV_{GA}$	.........................T..............	1080
$BlCMV_{W}$	AACATATTGTGAGTACTTTATGTTTATGAGTAAGCCGGAA	1120
$BlCMV_{AR}$	................................G.......	1120
$BlCMV_{GA}$	................................G.......	1120
$BlCMV_{W}$	GAACCATTGCAATGGTGAGGGCATGCAGAGTGATTTGATC	1160
$BlCMV_{AR}$	......G.................................	1160
$BlCMV_{GA}$	......G.................................	1160
$BlCMV_{W}$	ATGTGTCATGAAGTAGCTACGGCAATGTTTGTTGTT--	1198
$BlCMV_{AR}$	.C.C.C.....G.....C....................CC	1198
$BlCMV_{GA}$	.C.C.C.....G.....C....................CC	1198

Figure 3 : Comparison of the nucleotide sequences of the coat protein genes, 3' untranslated regions and portion of the polymerase of the published data for BlCMVW (Khan *et al.*, 1993), the Arkansas solate of BlCMV (BlCMVAR), and the Georgia isolate of BlCMV (BlCMVGA).

Dots (...) indicate nucleotide identical to the BlCMVW sequence, and dashes (---) represent missing residues. The ATG and TAG codons correspond to the initiation and termination sites for the putative BICMV coat protein open reading frame. Nucleotide differences between the Arkansas and Georgia isolates of BICMV are in bold.

Comparaison des séquences de nucléotides des gènes de la protéine de capside et d'une partie de la polymérase du BlCMVW publié (Khan et al., 1993), de l'isolat de BlCMV de l'Arkansas (BlCMVAR) et de l'isolat de BlCMV de la Georgie (BlCMVGA).

Les (...) indiquent les nucléotides identiques aux séquences de CMVFNY et les tirets (--) représentent les résidus manquants. Les codons ATG et AGT correspondent aux sites d'initiation et terminaison du cadre de lecture. Les différence de nucléotides entre BlCMVAR et BlCMVGA sont en gras.

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BlCMVW   MRDKDVNAGSRGKVVPRLQKITKRMNLPMVKGNVILNLDH   40
BlCMVAR  ..........K.............................   40
BlCMVGA  ..........K.......P.....................   40

BlCMVW   LLDYKPVQTDLFNTRATRDQFEMWYNAVKGEYEIDDDQMS   80
BlCMVAR  ......E..........KM.................A...   80
BlCMVGA  ......E..........KM.................A...   80

BlCMVW   IVMNGFMVWCIDNGTSPDVNGTWVMMDGDEQVEYPLKPMV  120
BlCMVAR  ........................................  120
BlCMVGA  ........................................  120

BlCMVW   ENAKPTLRQIMHHFSDAAEAYIEMRNSERPYMPRYGLLRN  160
BlCMVAR  ............................K...L.......  160
BlCMVGA  ............................K...L.......  160

BlCMVW   LRDKNLARYAFDFYEVTSKTSDRAREAVAQMKAAALSNVS  200
BlCMVAR  ........................................  200
BlCMVGA  ........................................  200

BlCMVW   SKLFGLDGNVATTSENTERHTARDVNQNMHTLLGMVLRSK  240
BlCMVAR  ...................................-----  235
BlCMVGA  ...................................-----  235

BlCMVW   GWVN     244
BlCMVAR  .PPQ     239
BlCMVGA  .PPQ     239
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Figure 4 : Comparison of the amino acid sequences of the coat protein genes of the published data for BlCMVW (Khan *et al.*, 1993), the Arkansas isolate of BlCMV (BlCMVAR), and the Georgia isolate of BlCMV (BlCMVGA).

Dots (...) indicate amino acids identical to the BlCMVW sequence, and dashes (---) represent missing residues. Amino acids differences are in bold.

Comparaison des séquences d'acides aminés des gènes des protéines de capside de l'isolat de BlCMVW publié (Khan et al., 1993), de l'isolat de BlCMV de l'Arkansas (BlCMVAR), et de l'isolat de BlCMV de la Georgie (BlCMVGA).

Les points (…) indiquent les acides aminés identiques à la séquences de BlCMVW , et les tirets (- --) les résidus manquants. Les différences d'acides aminés entre BlCMVAR et BlCMVGA sont en gras.

DISCUSSION

Cowpea stunt disease, first observed in Georgia, was found later in Arkansas (Anderson *et al.*, 1994). It was therefore necessary to compare the cowpea stunt-causing isolates of CMV and BlCMV from both geographical locations. Based on a biological study conducted previously, it was found that although the four types of mixed infections resulted in similar cowpea stunt disease symptoms on inoculated cowpea plants, the different virus accumulation patterns differed (Diallo, 1998). It was concluded from that study that the CMV and BlCMV isolates associated with the cowpea stunt disease from Arkansas and Georgia, behave differently. In order to not only better understand that difference, but also accurately classify these virus isolates, it was important to characterize them molecularly since in all the previous reports on cowpea stunt viruses, they were classify only based on symptom observation and serology (Pio-Ribeiro *et al.*, 1978 ; Pio-Ribeiro and Kuhn, 1980; Anderson *et al.*, 1994).

Indeed there used to be a lot of confusion about the taxonomic classification of BlCMV. However, with the methods based on the analysis of the nucleotide and amino acid sequences of the coat protein gene and 3'-UTR, it was shown that accurate results could be obtained (Khan *et al.*, 1990, 1993; Frenkel *et al.*, 1989, 1991; Lana *et al.*, 1988; Van der Vlugt, 1993). These methods were therefore used in this study to characterized and compare the Arkansas and Georgia isolates of BlCMV, respectively, $BlCMV_{AR}$ and $BlCMV_{GA}$. Nucleotide sequence comparison of the coat protein genes and 3' untranslated regions of BlCMV isolates from Arkansas and Georgia revealed only one nucleotide difference which in turn resulted in one amino acid difference in the coat protein. The coat protein of both BlCMV isolates displayed more than 93 % homology with $BlCMV_{W}$. This result confirms that the potyvirus

isolates involved in the cowpea stunt disease in Arkansas and Georgia are indeed BICMV. However, only one nucleotide difference was found between the BICMV isolates, indicating that there was not much variation in the BICMV isolates from both locations and associated with the disease.

Similarly, since it was shown that the analysis of the nucleotide sequence of the coat protein gene and 3' flanking region of CMV provides a good differentiation between isolates (Quemada *et al.*, 1989; Rizos *et al.*, 1992), this method was also used in this study. Nucleotide sequence comparison of the coat protein genes and 3' flanking regions of both CMV isolates showed 8 nucleotides differences between the Arkansas and the Georgia isolates, resulting in 90 % homology with CMVs belonging to subgroup I and only 70-80 % homology with subgroup II CMVs. Therefore, It could be concluded that based on nucleotide sequence comparison, both CMV isolates belong to subgroup I of CMV. Amino acids comparison revealed only two nucleotides differences between the cowpea stunt disease-causing isolates of CMV from Arkansas and Georgia.

CONCLUSION

Cucumber mosaic virus (CMV) isolates from Arkansas and Georgia belong to CMV subgroup I and have seven nucleotides differences, resulting in two amino acids differences in their coat protein genes and 3' flanking regions. For the BICMV isolates from both locations, there was only one nucleotide difference, leading to one amino acid difference in the coat protein genes and 3'-untranslated region. This result shows that even with differences in amino acids, the different virus isolates can still cause the disease. The epidemiological implication of the results could be even more important if it is shown that all mixed infections involving any strain or isolate of CMV and BICMV could cause cowpea stunt disease.

This work is the first report of the taxonomic classification of cowpea stunt-causing viruses from Arkansas and Georgia based on nucleotide and amino acid sequences analysis.

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