Purification, Serology, and In Vitro Translation of an Alyce-clover Isolate of Blackeye Cowpea Mosaic Virus

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ABSTRACT

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An isolate of blackeye cowpea mosaic virus (BlCMV) from alyce-clover (*Alysicarpus vaginalis*), designated as BlCMV-AC, and a Florida isolate of BlCMV (BlCMV-FL) were purified from systemically infected leaves of cowpea or white lupine. The $A_{260/280nm}$ ratios obtained for purified preparations of BlCMV-AC and BlCMV-FL averaged 1.25 and 1.20, respectively. Analysis of purified BlCMV-AC by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a major and a minor protein component with relative molecular weights of 34,500 and 31,000, respectively. Isolate BlCMV-AC is serologically closely related to BlCMV-FL and to an isolate of BlCMV from South Carolina but is more distantly related to cowpea aphidborne mosaic virus, peanut stripe virus, and nine other potyviruses. The profiles of the translation products of BlCMV-AC and BlCMV-FL in rabbit reticulocyte lysate were similar but not identical. The genomes of the two viruses appeared to differ at their 5′ ends.

A viruslike disease of alyce-clover (*Alysicarpus vaginalis* (L.) DC.) characterized by mosaic, plant stunting, and leaf distortion was observed in 1983 in a research field at Gainesville, FL. A virus was isolated from these plants and identified as a potyvirus based on the presence of cytoplasmic cylindrical inclusions (CIs) in infected plants and the morphology of the virus particles (21). The virus has been identified as an isolate of blackeye cowpea mosaic virus (BlCMV), designated as BlCMV-AC.

Isolate BlCMV-AC and two other isolates of BlCMV, one from Florida (BlCMV-FL) (11) and the other from South Carolina (BlCMV-NR) (14), have similar host ranges and induce identical CIs (22). We report here some serological and biochemical properties of the three isolates of BlCMV and the relationship of BlCMV-AC to BlCMV-FL and BlCMV-NR.

MATERIALS AND METHODS

Source of virus isolates. BlCMV-AC (21,22) was isolated from naturally infected alyce-clover plants and maintained in cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*) and white lupine (*Lupinus albus* L.) by mechanical transmission. BlCMV-FL (11), originally isolated from field-grown cowpea in Florida, was subsequently subcultured in New York (18,19) and maintained in cowpea.

Purification of viruses and CIs. Isolates BlCMV-AC and BlCMV-FL were propagated in cowpea or white lupine by mechanical inoculation. Systemically infected leaves were harvested for virus purification 2–3 wk after inoculation. Isolates BlCMV-AC and BlCMV-FL were purified as described by Lima et al (11) with modifications. The harvested leaves (200–300 g) were homogenized in a blender containing 2 ml of 500 mM potassium phosphate buffer (pH 7.5), 0.33 ml of chloroform, 0.33 ml of carbon tetrachloride, and 5 mg of sodium sulfite per gram of tissue. The homogenized mixture was centrifuged at 2,000 *g* for 5 min. The supernatant fraction was filtered through four layers of cheesecloth, and the pellet was discarded. The filtrate was centrifuged at 11,000 *g* for 15 min, and both the resulting supernatant fraction and the pellet were saved. The inclusions were in the pellet and were further purified as described by Lima et al (11).

Virions in the supernatant fraction were precipitated by adding polyethylene glycol 8,000 (PEG) to a final concentration of 6% (w/v) and Triton X-100 to 2% (v/v) and stirring at 4 C for 60 min. The precipitated virions were collected by centrifugation at 11,000 *g* for 10 min. The resulting pellet was resuspended in 20 mM Tris-Cl buffer, pH 8.2, containing 0.1% (v/v) of 2-mercaptoethanol (2-ME). Triton X-100 (2%, v/v) was added to the suspension, and the mixture was stirred at 4 C for 30 min. The suspension was centrifuged at 23,500 *g* for 10 min, and the pellet was resuspended in 20 mM Tris-Cl buffer containing 0.1% 2-ME (v/v), pH 8.2. The resulting suspension was subjected to equilibrium density gradient centrifugation (at 150,000 *g* for 16–18 hr in a Beckman SW 41 rotor) in 30% (w/w) cesium sulfate prepared in 20 mM Tris-Cl buffer (0.1% 2-ME, v/v), pH 8.2.

The virus zone, located 24–25 mm from the bottom of the tube, was collected drop by drop through a hole punched in the bottom of the tubes. The collected material was diluted with an equal volume of the Tris-Cl buffer (0.1% 2-ME, v/v) and centrifuged at 6,000 *g* for 8 min. The virions in the supernatant fraction were precipitated by adding 30% (w/v) PEG solution (until the preparation became cloudy) and were collected by centrifugation at 12,100 *g* for 10 min.

Purification of coat and CI proteins. The coat protein and CI protein of BlCMV-AC were purified by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (9). The running buffer and gradient were prepared as described by Laemmli (10). Samples were electrophoresed at constant 55 V for 15–16 hr. Molecular weights of proteins were determined by analysis in SDS-PAGE (10).

Serology. Antisera to the coat and CI proteins of BlCMV-AC were prepared in New Zealand white rabbits (9). The rabbits were bled at weekly intervals after the final injection.

Antisera to the following antigens were used in this study: BlCMV-FL (11) and BlCMV-NR (13); papaya ringspot virus-W (PRV-W); clover yellow vein virus (CYVV) and pea mosaic virus (PMV) and their nuclear inclusions (NIs); peanut mottle virus (PMoV); peanut stripe virus (PStV); potato virus Y (PVY); tobacco etch virus (TEV) and its NI; turnip mosaic virus (TuMV); watermelon mosaic virus-2 (WMV-2); zucchini yellow fleck virus (ZYFV); isolates of zucchini yellow mosaic virus from Florida (ZYMV-FL) (obtained from D. E. Purcifull and E. Hiebert) and from Réunion Island (ZYMV-RI) (provided by H. Lecoq); and helper component protein (HCP) of tobacco vein mottling virus (TVMV) (obtained from T. P. Pirone).

SDS immunodiffusion tests (16) were conducted with BlCMV-AC and the following potyviruses: BlCMV-FL, BlCMV-NR, Scott isolate of bean yellow mosaic virus (BYMV-Scott), Moroccan

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isolate of cowpea aphidborne mosaic virus (CAMV-MO), CYVV, PMV, PMoV, PRV-W, PStV, PVY, TEV, TuMV, WMV-2, ZYFV, ZYMV-FL, and ZYMV-RI. The antigens were tested in freshly prepared extracts or resuspended freeze-dried extracts from infected plants.

In intragel cross-absorption tests (20), center wells were charged with SDS-treated extracts from infected cowpea leaves and emptied 15 hr later. After the removal of cross-absorbing antigen, appropriate antiserum was added to the center wells, and test antigens were added to peripheral wells.

In direct double-antibody sandwich enzyme-linked immunosorbent assays (ELISAs) (4), the γ-globulins of antisera to BlCMV-AC and BlCMV-FL were conjugated with alkaline phosphatase. Microtiter plates were coated with 2 or 4 μg of γ-globulin per milliliter. Each combination was replicated in four wells. The enzyme conjugate was diluted 1:200 for BlCMV-AC and 1:250 for BlCMV-FL. The absorbance values at 405 nm were measured with a Biotek automated microplate reader (model EL 309) 1 hr after the substrate (*p*-nitrophenyl phosphate) was added.

Isolation of BlCMV-AC and BlCMV-FL RNAs and translation in rabbit reticulocyte lysate. RNAs of BlCMV-AC and BlCMV-FL were isolated from purified virus preparations as described by Brakke and Van Pelt (2,3). Translation conditions in RNA-dependent rabbit reticulocyte lysate (RRL) were those used by Dougherty and Hiebert (7). The translation products were immunoprecipitated with a variety of antisera to virus-specific proteins (7,8). The immunoprecipitated products were analyzed by SDS-PAGE (10) and detected on dried gels by fluorography.

RESULTS

Purification and properties of BlCMV-AC and BlCMV-FL. Purified preparations of BlCMV-AC were obtained from infected leaf tissues of cowpea and white lupine. Yield of BlCMV-AC from cowpea ranged from 3 to 5 mg per 100 g of tissue when plants were harvested 13–14 days after inoculation. The yield of purified BlCMV-AC from white lupine ranged from 13 to 20 mg per 100 g of tissue when plants were harvested 3 wk after inoculation. Yield of BlCMV-FL, purified from infected leaves of cowpea, averaged 6–8 mg per 100 g of tissue.

The $A_{260/280nm}$ ratios obtained for purified preparations of BlCMV-AC and BlCMV-FL (subjected twice to equilibrium density gradient centrifugation in cesium sulfate) averaged 1.25 and 1.20, respectively, after correction for light scattering. Analysis of purified preparations of BlCMV-AC by SDS-PAGE (five gel runs on seven different preparations) revealed a major and a minor protein component with relative molecular weights (M_rs) of 34,500 and 31,000, respectively.

Purification and properties of CIs. CIs induced by BlCMV-AC and BlCMV-FL were purified from infected white lupine and cowpea, respectively. CIs containing only scrolls were observed in the purified preparations by electron microscopy. In SDS-PAGE, SDS-dissociated CI proteins of the two viruses were found to consist of a single subunit with M_r 68,000.

Serology. In SDS immunodiffusion tests, BlCMV-AC was serologically closely related to BlCMV-FL and BlCMV-NR (Fig. 1A,B). BlCMV-AC was also related to BYMV-Scott, CAMV-MO, CYVV, PMV, PMoV, PStV, PVY, TEV, TuMV, WMV-2, ZYMV-FL, and ZYMV-RI. Antiserum to BlCMV-AC did not react with PRV-W or ZYFV. Spur formation was observed between BlCMV-AC and CAMV-MO when tested against antiserum to BlCMV-AC (Fig. 1A). Spurs also formed with PStV over BlCMV-AC when tested against PStV antiserum (Fig. 1A).

Isolate BlCMV-AC was serologically related to but different from BlCMV-FL and BlCMV-NR, as determined by intragel cross absorption (Fig. 1B) and direct ELISA (Table 1). After absorption of the antiserum to BlCMV-FL with sap from BlCMV-AC-infected plants, no reactions were observed with BlCMV-AC, but reactions occurred with BlCMV-FL. Similarly, no reaction was observed with BlCMV-FL but a reaction occurred with BlCMV-AC after the absorption of antiserum to BlCMV-AC with BlCMV-FL. In direct ELISA, BlCMV-AC reacted strongly with its homologous antiserum but less strongly with antiserum to BlCMV-FL (Table 1). Isolates BlCMV-FL and BlCMV-NR reacted strongly with BlCMV-FL antiserum and less strongly with BlCMV-AC antiserum (Table 1).

RNA isolation and translation in RRL system. After 4 hr of centrifugation of the SDS-dissociated purified preparations of BlCMV-AC and BlCMV-FL on sucrose gradients, three peaks—designated as top, middle, and bottom—were resolved. The top peak contained dissociated viral coat protein, which was confirmed by SDS immunodiffusion tests (16). The middle peak was digested in the presence of DNase, indicating that it contained host DNAs (*data not shown*). The bottom peak containing viral RNA was collected and used in the translation systems.

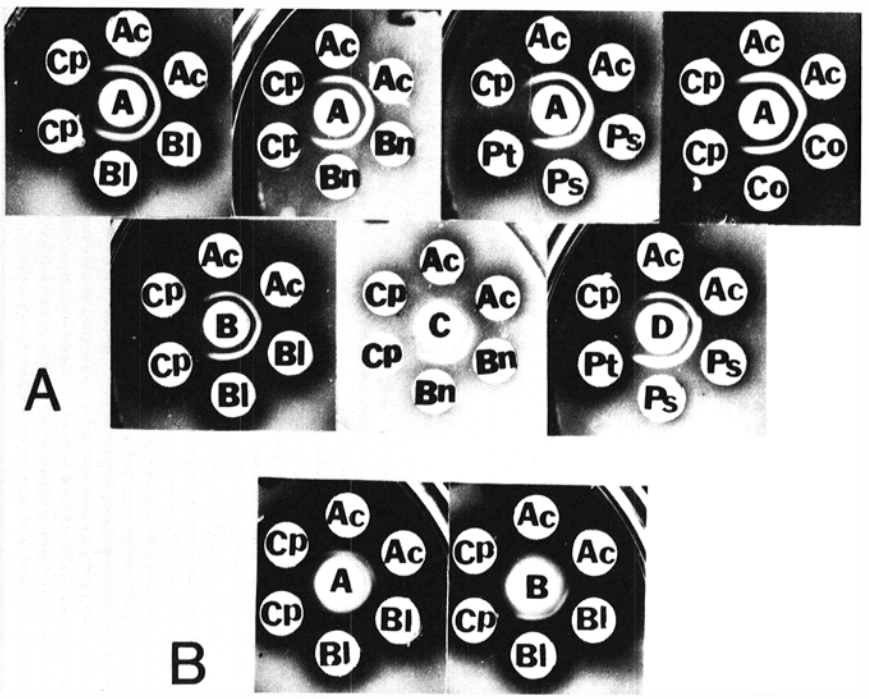

Fig. 1. Serological relationships of blackeye cowpea mosaic virus (BlCMV) isolate from alyceclover (BlCMV-AC) to two other isolates of BlCMV (BlCMV-FL and BlCMV-NR), a Moroccan isolate of cowpea aphidborne mosaic virus (CAMV-MO), and peanut stripe virus (PStV) in sodium dodecyl sulfate (SDS) immunodiffusion tests. In **A,** the center wells contained antiserum to BlCMV-AC (A), BlCMV-FL (B), BlCMV-NR (C), and PStV (D). The peripheral wells contained SDS-treated extracts from cowpea infected with BlCMV-AC (Ac), BlCMV-FL (Bl), BlCMV-NR (Bn), and CAMV-MO (Co); healthy cowpea (Cp); peanut infected with PStV (Ps); and healthy peanut (Pt). **B,** Intragel cross-absorption test with BlCMV-AC and BlCMV-FL. The center wells were charged with BlCMV-FL-infected cowpea and 15 hr later, antiserum to BlCMV-AC (A) and with BlCMV-AC-infected cowpea and 15 hr later, antiserum to BlCMV-FL (B). The peripheral wells contained SDS-treated extracts from cowpea infected with BlCMV-AC (Ac) and BlCMV-FL (Bl) and healthy cowpea (Cp).

Translation of BlCMV-AC and BlCMV-FL RNAs under identical conditions in RRL resulted in at least 20 products (Fig. 2). Isolated RNAs of BlCMV-AC and BlCMV-FL stimulated incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable products at levels 25- and 12-fold, respectively, above endogenous levels. The major translation products of BlCMV-AC RNA in RRL had M_rs of 104,000 and 49,000, whereas the major products of BlCMV-FL RNA translation had M_rs of 101,000 and 49,000.

Figure 2 shows the results of analysis of products of BlCMV-AC RNA translation in RRL by immunoprecipitation. Antiserum to the coat protein of BlCMV-AC selectively precipitated five major products with M_r 91,000, 84,000, 77,000, 49,000, and 26,000, as well as seven minor products. A product with M_r 34,500 was presumed to be the viral coat protein. Antiserum to the M_r 54,000 NI protein (NIP) of TEV immunoprecipitated four major products with M_r 91,000, 84,000, 77,000, and 49,000 and three minor products. The three minor products were also immunoprecipitated by antiserum to the coat protein of BlCMV-AC and were considered to be the polyproteins of the large NI gene and the viral coat protein gene. Antiserum to TEV M_r 49,000 NIP immunoprecipitated two major products of M_r 49,000 and 46,000 and four minor products. Antiserum to BlCMV-AC CI protein immunoprecipitated one major product of M_r 118,000 and several minor products. The minor products with M_r larger than 118,000 were also immunoprecipitated by antiserum to TEV M_r 49,000 NIP and were believed to be polyproteins of the small NI gene and CI gene. Antiserum to TVMV HCP immunoprecipitated a major product of M_r 104,000 and a minor product of M_r 48,000. The minor M_r 48,000 product was presumed to be HCP, and the major M_r 104,000 product was believed to be a polyprotein encoded by the helper component (HC) gene and an adjacent gene toward the 5′ portion of the viral genome.

Figure 2 shows the translation profile of BlCMV-FL RNA in RRL, which is similar to that of BlCMV-AC. Antiserum to BlCMV-FL coat protein immunoprecipitated only three major products of M_r 91,000, 76,000, and 28,500. In BlCMV-AC translation, a major product of M_r 26,000, which was immunoprecipitated by antiserum to the coat protein of BlCMV-AC, was missing in BlCMV-FL translation. A major product of M_r 46,000 in BlCMV-AC translation was immunoprecipitated by antiserum to TEV M_r 49,000 NIP, but it was barely detectable in BlCMV-FL translation. Antiserum to TVMV HCP immunoprecipitated a major product of M_r 101,000 and two minor products of M_r 84,000 and 50,000. The M_r 50,000 product was believed to be HCP.

Table 1. Serological comparison of isolates of blackeye cowpea mosaic virus (BlCMV) from alyce-clover (BlCMV-AC), Florida (BlCMV-FL), and South Carolina (BlCMV-NR) in direct enzyme-linked immunosorbent assay[a]

Virus concentration (μg/ml)	BlCMV-AC antiserum[b]			BlCMV-FL antiserum[b]		
	FL	AC	NR	FL	AC	NR
0.1	0.27	0.27	0.09	1.72	0.68	0.62
0.3	0.45	0.51	0.21	2.28	0.92	1.06
1.0	0.90	1.44	0.78	2.94	1.72	2.68
3.0	1.41	2.31	1.26	3.50	2.10	3.32

[a] Plates were coated with 4 and 2 μg of immunoglobulin G purified from antisera to BlCMV-AC and BlCMV-FL, respectively, per milliliter. Enzyme conjugates were diluted 1:200 for BlCMV-AC and 1:250 for BlCMV-FL.

[b] Values are the mean absorbance (405 nm) of four replicates. FL = BlCMV-FL, AC = BlCMV-AC, and NR = BlCMV-NR.

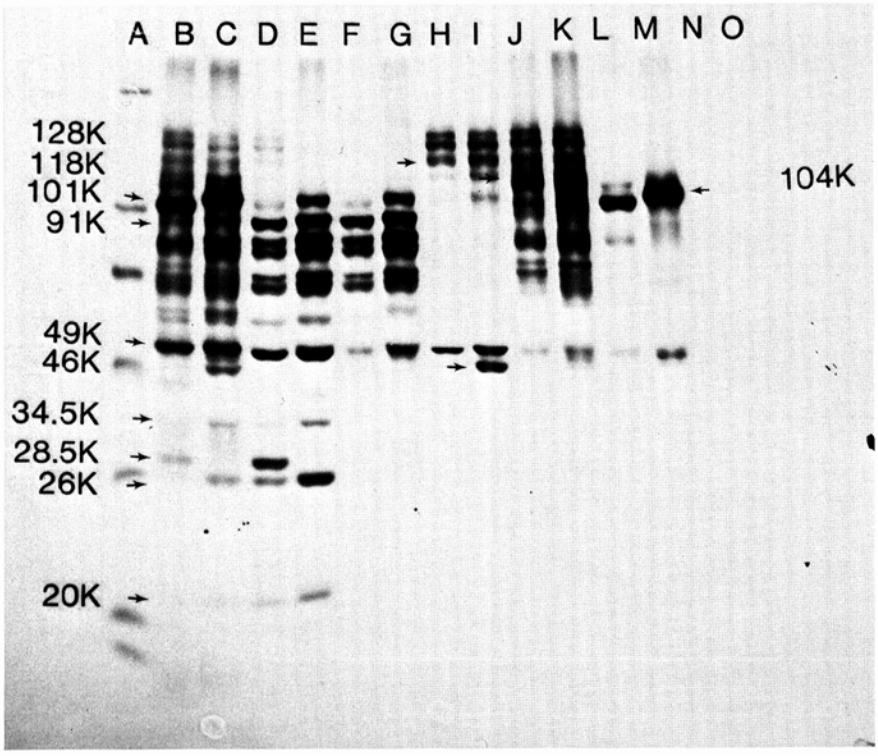

Fig. 2. The in vitro translation of RNAs from isolates of blackeye cowpea mosaic virus (BlCMV) from alyce-clover (BlCMV-AC) and from Florida (BlCMV-FL) in rabbit reticulocyte lysate. The [^{35}S]methionine-labeled products were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and detected by fluorography. Lane A shows the following ^{14}C-labeled protein markers (from the top): myosin (200,000), phosphorylase b (93,000), serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), and lysozyme (14,300). Lane B was BlCMV-FL total products, and lane C was BlCMV-AC total products. Lanes D, F, H, J, and L were translation products of BlCMV-FL immunoprecipitated by antiserum to BlCMV-FL coat protein (lane D), tobacco etch virus (TEV) M_r 54,000 nuclear inclusion protein (NIP) (lane F), TEV M_r 49,000 NIP (lane H), BlCMV-AC cylindrical inclusion protein (CIP) (lane J), and tobacco vein mottling virus (TVMV) helper component protein (HCP) (lane L). Lane N was translation products of BlCMV-FL immunoprecipitated by preimmune serum. Lanes E, G, I, K, and M were translation products of BlCMV-AC immunoprecipitated by antiserum to BlCMV-AC coat protein (lane E), TEV M_r 54,000 NIP (lane G), TEV M_r 49,000 NIP (lane I) BlCMV-AC CIP (lane K), and TVMV HCP (lane M). Lane O was translation products of BlCMV-AC immunoprecipitated by preimmune serum. Relative molecular weights of the major translation products are indicated on the sides of the figure.

DISCUSSION

At least four potyviruses—CYVV, PMoV, PStV, and WMV-2—have been reported to infect alyce-clover (1,13,22). Antiserum to BlCMV-AC reacted with CYVV, PMoV, and WMV-2. In reciprocal tests, antisera to these viruses did not react with BlCMV-AC, indicating that they are very distantly related. PStV (5) was serologically related to but different from BlCMV-AC by spur formation.

Another potyvirus, CAMV (12), was also serologically related to BlCMV-AC, but the relationship between CAMV and BlCMV remains confusing. Lima et al

(11) compared BlCMV-FL with CAMV-MO and concluded that they were two distinct potyviruses. Later, Taiwo and co-workers (18,19) assigned several potyvirus isolates to BlCMV or CAMV, based on serology and the reactions of different cultivars of cowpea. More recently, Dijkstra et al (6) in The Netherlands concluded that all isolates of BlCMV and CAMV studied should be considered BlCMV. Our results show that BlCMV-AC was serologically related to but different from CAMV-MO because of spur formation when tested against antiserum to BlCMV-AC.

Isolates BlCMV-FL and BlCMV-NR were serologically related to BlCMV-AC. Based on the results of the intragel absorption and ELISA, BlCMV-AC was serologically closely related but not identical to BlCMV-FL. Because of the lack of sufficient antiserum to BlCMV-NR, it was not feasible to concentrate it in order to compare BlCMV-AC with BlCMV-NR in reciprocal tests. However, antiserum to BlCMV-NR, which was diluted eightfold in glycerol (14), reacted very weakly with BlCMV-AC, although it reacted very strongly with its homologous antigen (Fig. 1A). BlCMV-AC was also distinguishable from BlCMV-NR in ELISA. This study, along with our previous one (22), clearly indicates that BlCMV-AC, BlCMV-FL, BlCMV-NR, CAMV-MO, and PStV have both common and unique antigenic determinants, and they are biologically more closely related to one another than to many other potyviruses. Our study also indicates that BlCMV-AC is more closely related serologically and biologically to BlCMV-FL and BlCMV-NR than to CAMV-MO and PStV.

In vitro translation profiles of BlCMV-AC and BlCMV-FL RNAs in RRL systems were similar but not identical, indicating that they differed from each other at the genomic level. Such differences have been reported in other potyvirus isolates (15,17). Based on the results of the analysis of in vitro translation products of BlCMV-AC and BlCMV-FL RNAs in RRL, we propose the following genetic maps for BlCMV-AC and BlCMV-FL from the 5′-terminus to the 3′-terminus: 5′–56,000 unknown protein–48,000 HC–50,000 unknown protein–68,000 CI–49,000 NI–56,000 NI–34,500 coat protein–3′ (for BlCMV-AC) and 5′–51,000 unknown protein–50,000 HC–50,000 unknown protein–68,000 CI–49,000 NI–54,000 NI–34,500 coat protein–3′ (for BlCMV-FL). Clearly, BlCMV-AC and BlCMV-FL differed from each other primarily on the 5′ portions of their genomes.

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