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

G. S. ZHAO, Graduate Research Assistant, D. D. BALTENSPERGER, Associate Professor, and E. HIEBERT, D. E. PURCIFULL, and J. R. EDWARDSON, Professors, Departments of Agronomy and Plant Pathology, University of Florida, Gainesville 32611

ABSTRACT

An isolate of blackeye cowpea mosaic virus (BiCMV) from alyce-clover (Alysicarpus vaginalis), designated as BICMV-AC, and a Florida isolate of BICMV (BiCMV-FL) were purified from systemically infected leaves of cowpea or white lupine. The $\Delta_{200nm}$ ratios obtained for purified preparations of BICMV-AC and BICMV-FL averaged 1.25 and 1.20, respectively. Analysis of purified BICMV-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 BICMV-AC is serologically closely related to BICMV-FL and to an isolate of BICMV from South Carolina but is more distantly related to cowpea aphiildrome mosaic virus, peanut stripe virus, and nine other potyviruses. The profiles of the translation products of BICMV-AC and BICMV-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 (CI) in infected plants and the morphology of the virus particles (21). The virus has been identified as an isolate of blackeye cowpea mosaic virus (BiCMV), designated as BICMV-AC.

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

MATERIALS AND METHODS
Source of virus isolates. BICMV-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. BICMV-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 CI's. Isolates BICMV-AC and BICMV-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 BICMV-AC and BICMV-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 ml 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 ml 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 ml 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 BICMV-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 BICMV-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: BICMV-FL (11) and BICMV-NR (13); papaya ringspot virus-W (PRV-W); clover yellow vein virus (CYVV) and pea mosaic virus (PMV) and their nuclear inclusions (NI's); 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 (ZYMV); 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 BICMV-AC and the following potyviruses: BICMV-FL, BICMV-NR, Scott isolate of bean yellow mosaic virus (BYMV-Scott), Moroccan...
isolette of cowpea aphid-borne mosaic virus (CAMV-MO), CYVV, PMV, PMoV, PSTVd, PYY, TEV, TuMV, WMV-2, ZYMV and ZYMV-R1. 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 antisera 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 y-globulins of antisera to BICMV-AC and BICMV-FL were conjugated with alkaline phosphatase. Microtiter plates were coated with 2 or 4 μg of y-globulins per milliliter. Each combination was replicated in four wells. The enzyme conjugate was diluted 1:200 for BICMV-AC and 1:250 for BICMV-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 BICMV-AC and BICMV-FL RNAs and translation in rabbit reticulocyte lysate. RNAs of BICMV-AC and BICMV-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 BICMV-AC and BICMV-FL. Purified preparations of BICMV-AC were obtained from infected leaf tissues of cowpea and white lupine. Yield of BICMV-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 BICMV-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 BICMV-FL, purified from infected leaves of cowpea, averaged 6-8 mg per 100 g of tissue.

The A_{260}/A_{280} ratios obtained for purified preparations of BICMV-AC and BICMV-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 BICMV-AC by SDS-PAGE (five gel runs on seven different preparations) revealed a major and a minor protein component with relative molecular weights (Mr) of 34,500 and 31,000, respectively.

Purification and properties of CI. CI's induced by BICMV-AC and BICMV-FL were purified from infected white lupine and cowpea, respectively. CI's containing only scrobes 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 Mr 68,000.

Seroxy. In SDS immunodiffusion tests, BICMV-AC was serologically closely related to BICMV-FL and BICMV-NR (Fig. 1A,B). BICMV-AC was also related to BYMV-Scott, CAMV-MO, CYVV, PMV, PMoV, PSTVd, PYY, TEV, TuMV, WMV-2, ZYMV-FL, and ZYMV-R1. Antiserum to BICMV-AC did not react with PRW or ZYFV. Spur formation was observed between BICMV-AC and CAMV-MO when tested against antiserum to BICMV-AC (Fig. 1A). Spur also formed with PSTV over BICMV-AC when tested against PSTV antiserum (Fig. 1A).

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

RNA isolation and translation in RRL system. After 4 hr of centrifugation of the SDS-dissociated purified preparations of BICMV-AC and BICMV-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. Seroxy relationships of blackeye cowpea mosaic virus (BICMV) isolate from white clover (BICMV-AC) to two other isolates of BICMV (BICMV-FL and BICMV-NR), a Moroccan isolate of cowpea aphid-borne mosaic virus (CAMV-MO), and peanut stripe virus (PSTV) in sodium dodecyl sulfate (SDS) immunodiffusion tests. A, the center wells contained antiserum to BICMV-AC (A), BICMV-FL (B), BICMV-NR (C), and PSTV (D). The peripheral wells contained SDS-treated extracts from cowpea infected with BICMV-AC (Ac), BICMV-FL (B), BICMV-NR (Bn), and CAMV-MO (Co); healthy cowpea (C); peanut infected with PSTV (Ps); and healthy peanut (P). B, Intrage gel-cross-absorption test with BICMV-AC and BICMV-FL. The center wells were charged with BICMV-FL-infected cowpea and 15 hr later, antiserum to BICMV-AC (A) and with BICMV-AC-infected cowpea and 15 hr later, antiserum to BICMV-FL (B). The peripheral wells contained SDS-treated extracts from cowpea infected with BICMV-AC (Ac) and BICMV-FL (B) and healthy cowpea (C)].
Translation of BiCMV-AC and BiCMV-FL RNAs under identical conditions in RRL resulted in at least 20 products (Fig. 2). Isolated RNAs of BiCMV-AC and BiCMV-FL stimulated incorporation of $[^35]$S methionine into tri-chloroacetic acid-precipitable products at levels of 25- and 12-fold, respectively, above endogenous levels. The major translation products of BiCMV-AC RNA in RRL had $M_s$ of 104,000 and 49,000, whereas the major products of BiCMV-FL RNA translation had $M_s$ of 101,000 and 49,000.

Figure 2 shows the results of analysis of products of BiCMV-AC RNA translation in RRL by immunoprecipitation. Antiserum to the coat protein of BiCMV-AC selectively precipitated five major products with $M_s$ 91,000, 84,000, 77,000, 49,000, and 26,000, as well as seven minor products. A product with $M_s$ 34,500 was presumed to be the viral coat protein. Antiserum to the $M_s$ 54,000 NI protein (NIP) of TEV immunoprecipitated four major products with $M_s$ 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 BiCMV-AC and were considered to be the polyproteins of the large NI gene and the viral coat protein gene. Antiserum to TEV $M_s$ 49,000 NIP immunoprecipitated two major products of $M_s$ 49,000 and 46,000 and four minor products. Antiserum to BiCMV-AC CI protein immunoprecipitated one major product of $M_s$ 118,000 and several minor products. The minor products with $M_s$ larger than 118,000 were also immunoprecipitated by antiserum to TEV $M_s$ 49,000 NIP and were believed to be polyproteins of the small NI gene and CI gene. Antiserum to TVM HCP immunoprecipitated a major product of $M_s$ 104,000 and a minor product of $M_s$ 48,000. The minor $M_s$ 48,000 product was presumed to be HCP, and the major $M_s$ 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 BiCMV-FL RNA in RRL, which is similar to that of BiCMV-AC. Antiserum to BiCMV-FL coat protein immunoprecipitated only three major products of $M_s$ 91,000, 76,000, and 28,500. In BiCMV-AC translation, a major product of $M_s$ 26,000, which was immunoprecipitated by antiserum to the coat protein of BiCMV-AC, was missing in BiCMV-FL translation. A major product of $M_s$ 46,000 in BiCMV-AC translation was immunoprecipitated by antiserum to TEV $M_s$ 49,000 NIP, but it was barely detectable in BiCMV-FL translation. Antiserum to TVM HCP immunoprecipitated a major product of $M_s$ 101,000 and two minor products of $M_s$ 84,000 and 50,000. The $M_s$ 50,000 product was believed to be HCP.

**DISCUSSION**

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

Another potyvirus, CAMV (12), was also serologically related to BiCMV-AC, but the relationship between CAMV and BiCMV remains confusing. Lima et al.
(11) compared BICMV-FL with CAMV-MO and concluded that they were two distinct potyviruses. Later, Taiwo and co-workers (18, 19) assigned several potyvirus isolates to BICMV 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 BICMV and CAMV studied should be considered BICMV. Our results show that BICMV-AC was serologically related to but different from CAMV-MO because of spur formation when tested against antiserum to BICMV-AC.

Isolates BICMV-FL and BICMV-NR were serologically related to BICMV-AC. Based on the results of the intragel absorption and ELISA, BICMV-AC was serologically closely related but not identical to BICMV-FL. Because of the lack of sufficient antiserum to BICMV-NR, it was not feasible to concentrate it in order to compare BICMV-AC with BICMV-NR in reciprocal tests. However, antiserum to BICMV-NR, which was diluted eightfold in glyc erol (14), reacted very weakly with BICMV-AC, although it reacted very strongly with its homologous antigen (Fig. 1A). BICMV-AC was also distinguishable from BICMV-NR in ELISA. This study, along with our previous one (22), clearly indicates that BICMV-AC, BICMV-FL, BICMV-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 BICMV-AC is more closely related serologically and biologically to BICMV-FL and BICMV-NR than to CAMV-MO and PSTV.

In vitro translation profiles of BICMV-AC and BICMV-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 BICMV-AC and BICMV-FL RNAs in RRL, we propose the following genetic maps for BICMV-AC and BICMV-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 BICMV-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 BICMV-FL). Clearly, BICMV-AC and BICMV-FL differed from each other primarily on the 5' portions of their genomes.

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LITERATURE CITED