Biological and Molecular Characterization of a Novel Carmovirus Isolated from Angelonia


ABSTRACT

A new carmovirus was isolated from Angelonia plants (Angelonia angustifolia), with flower break and mild foliar symptoms, grown in the United States and Israel. The virus, for which the name Angelonia flower break virus (AnFBV) is proposed, has isometric particles, ~30 nm in diameter. The experimental host range was limited to Nicotiana species, Schizanthus pinnatus, Myosotis sylvatica, Phlox drummondii, and Digitalis purpurea. Virions were isolated from systemically infected N. benthamiana leaves, and directly from naturally infected Angelonia leaves, using typical carmovirus protocols. Koch’s postulates were completed by mechanical inoculation of uninfected Angelonia seedlings with purified virions. Isometric particles were observed in leaf dips and virion preparations from both Angelonia and N. benthamiana, and in thin sections of Angelonia flower tissue by electron microscopy. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dissociated purified virus preparations, a major protein component with a molecular mass of 38 kDa was observed. Virion preparations were used to produce virus-specific polyclonal antisera in both Israel and the United States. The antisera did not react with Pelargonium flower break virus (PFBV), Carnation mottle virus (CarMV), or Sagaro cactus virus (SgCV) by either enzyme-linked immunosorbent assay or immunoblotting. In reciprocal tests, antisera against PFBV, CarMV, and SgCV reacted only with the homologous viruses. The complete nucleotide sequence of a Florida isolate of AnFBV and the coat protein (CP) gene sequences of Israeli and Maryland isolates were determined. The genomic RNA is 3,964 nucleotides and contains four open reading frames arranged in a manner typical of carmoviruses. The AnFBV CP is most closely related to PFBV, whereas the AnFBV replicate is most closely related to PFBV, CarMV, and SgCV. Particle morphology, serological properties, genome organization, and phylogenetic analysis are all consistent with assignment of AnFBV to the genus Carmovirus.

Additional keywords: host range, purification, serology.

MATERIALS AND METHODS

This research was completed in three laboratories: the U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS) in (i) Fort Pierce, FL, and (ii) Beltsville, MD, and (iii) the Department of Virology, Agricultural Research Organization, The Volcani Center, Israel. Similar methods were followed in all laboratories unless specified otherwise in the text.

Virus source, host range, and transmission. Leaf samples of symptomatic Angelonia grown in commercial greenhouses and/or outdoors in the United States and Israel were examined for virus infection using electron microscopy and commercially available virus group specific (degenerate) primers (Agdia, Elkhart, IN) followed by the manufacturer’s recommendations for reverse transcription-polymerase chain reaction (RT-PCR). Samples containing isometric particles and/or testing positive by RT-PCR with virus group specific primers were homogenized in 1% (wt/vol) K₂HPO₄, or in 20 mM sodium phosphate (pH 7.0), and the sap was used to inoculate leaves of Ageratum houstonianum (ageratum), Antirrhinum majus (snapdragon) cvs. Sonnet and Fordhook, Aquilegia vulgaris (columbine), Capsicum annuum, Centaurea cyanus (bachelor’s button), Chenopodium quinoa, C. amaranticolor, C. murale, Consolida ambigua (larkspur), Cucumis sativus, Cucurbita pepo, Datura stramonium, Digitalis purpurea (foxglove), Gomphrena globosa, Helenium hybrid (sneezeweed), Linum grandiflorum (flax), Lobelia erinus (lobelia), Lunaria

Corresponding author: S. Adkins; E-mail address: SAdkins@ushrl.ars.usda.gov

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annua (money plant, honest), Lycn尼斯 coronaria (dusty miller), Lycopersicon esculentum, Lyssmachia punctata (loosestrife), Myosotis sylvestica (forget-me-not), Nicotiana benthamiana, N. clevelandii, N. edwardsonii, N. glutinosa, N. megaloaphis, N. rustica, N. sylvestris, N. tabacum cv. NN, N. tabacum cv. Samsun, N. tabacum cv. Samsun NN, N. tabacum Xanthi nc, N. tabacum cv. White Burley, Petunia hybrida, Phlox drummondii, Physalis alkekengi, P. floridensis, Polemonium caeruleum (Jacob’s ladder), Raphanus sativus (Japanese radish), Salvia splendens (salvia), Saponaria ocymoides (trailing soapwort), Schizanthus pinnatus (butterfly flower), Verbascum phoenicicum (purple mullein), and Vigna sinensis (cowpea). These plants were maintained in a greenhouse and observed for symptom development. Inoculated as well as upper, noninoculated leaves were tested by enzyme-linked immunosorbent assay (ELISA), RT-PCR, or both 2 to 4 weeks after inoculation.

The green peach aphid (Myzus persicae (Sulz.)) was used for transmission tests. A clone of M. persicae that originated from a single female was reared on uninfected R. sativus plants. Non-viruliferous aperous aphids were allowed to feed for 5 min on naturally infected Angelonia. Five aphids were transferred to each of 20 uninfected Angelonia seedlings or 20 N. clevelandii plants and allowed to remain on the test plants for 12 h. These plants were then sprayed with insecticides, maintained in a greenhouse, observed for symptom development, and tested by ELISA 3 weeks postinoculation.

Pollen from uninfected or AnFBV-infected Angelonia plants was used to manually pollinate infected plants from which mature seeds were later collected. The presence of AnFBV in plants designated as seed sources was confirmed by ELISA prior to seed collection. Seeds were collected from uninfected plants to serve as controls. Seeds were washed in running tap water and dried at room temperature. Seeds were germinated and seedlings were subsequently transplanted to plastic pots in which they were grown for 5 to 8 weeks before being tested for AnFBV. Sixty-five seedlings were grown, observed weekly for AnFBV symptoms, and assayed by ELISA.

Virus purification. In Israel, virus was purified from naturally infected Angelonia, as described by Franck and Loebenstein (9), followed by sucrose gradient centrifugation (2). In Florida, virions were isolated from symptomless N. benthamiana leaves as described by Waterworth et al. (30). In Maryland, virions were isolated from N. benthamiana using a combination of methods based on polyethylene glycol concentration followed by high speed and CsCl gradient centrifugation (11,16). Virus concentration was estimated spectrophotometrically with an extinction coefficient of \( E_{260} \approx 0.18 \) mg/mL = 5 for carmoviruses (3).

Production of antibodies. Antibodies against AnFBV were synthesized by the replacement synthesis method using a Superscript Double-Stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA) at 37°C for 60 min using oligo(dT) primer and standard methods (22). First-strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) at 37°C for 60 min using oligo(dT) primer and standard methods (22). Second-strand cDNA was synthesized by the replacement synthesis method using a Superscript Double-Stranded cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. A second population of clones was prepared using primers derived from the initial population of clones, and RT-PCR by standard methods (22). Products from the initial cDNA synthesis reactions were ligated into the EcoRV site of pGEM-5Zf (Promega, Inc., Madison, WI), whereas those from the second population were ligated into pGEM-T (Promega). The 5′ terminus was amplified by random amplification of cDNA ends (RACE) using the specific AnFBV primer, 5′AnFBVRepvc (5′-GGCCACCACGACAAGGCGCCCTTG-3′), with the Generacer (Invitrogen) 5′ primer. The 3′ terminus was amplified by RACE using the specific AnFBV primer, 3′AnFBVendv (5′-CCGCGACAGGCGCCCTG-3′), with the Generacer (Invitrogen) 5′ primer. The genomic sequence of the Florida AnFBV isolate was used to design primers flanking the deduced CP gene for amplification of the corresponding genomic region from the Israeli and Maryland virus isolates by RT-PCR. The viral-sense primer, 5′AnFBVCP (5′-GCGGCAGGACGACGAGC-3′), was identical to nucleotides 2591 to 2610 just upstream of the CP initiation codon, and the viral complementary sense primer, 3′AnFBVCP (5′-GGGAAGA-
GTCGTTGACCGGAAGC-3'), was complementary to nucleotides 3740 to 3762 just downstream of the CP stop codon. The CP PCR products of the Israeli and Maryland isolates were cloned into pCR2.1 using the TA Cloning Kit (Invitrogen).

**Sequence analysis.** Selected clones were sequenced on an automated sequencer (ABI3730XL) at the U.S. Horticultural Research Laboratory (USHRL) DNA Sequencing Support Laboratory. Sequences were edited in VectorNTI (InforMax, North Bethesda, MD) and subjected to BLAST searches (1). Overlapping clones were aligned manually and with ClustalX version 1.8 (25) to assemble the complete AnFBV genome. Putative open reading frames (ORFs) were identified by comparison to related genomes identified using BLASTX and with ORF Finder (National Center for Biotechnology Information).

Selected recombinant plasmids containing the CP genes of the Israeli and Maryland isolates were sequenced using the BigDye 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and run on an Applied Biosystems 310 Genetic Analyzer (Hy Laboratories, Rehovot, Israel and Floral and Nursery Plant Research Unit, respectively). Multiple alignments of the CP genes and deduced proteins of the three AnFBV isolates and recognized carmovirus species were prepared using ClustalW version 1.82 (26). Percent nucleotide and amino acid identities were calculated using CLUSTALDIST. Phylogenetic analyses were performed using Phylip 3.6.4 (8). Bootstrap analyses were carried out with 1,000 replicates, and resulting trees were displayed with TreeView 1.6.6 (20).

**RESULTS**

**Virus transmission and host range.** Initially, AnFBV was detected by electron microscopy and/or RT-PCR in Angelonia plants that exhibited mild mottle symptoms on leaves (Fig. 1A and B) and overall stunting (Fig. 1A), accompanied by flower break (Fig. 1C). Only spherical particles were observed by electron microscopy, and RT-PCR products were only amplified with degenerate carmovirus primers. In further tests, the virus was also detected in symptomless Angelonia plants. All Angelonia plants with blue or dark blue flowers, representing the commercial stock grown in Israel and tested by electron microscopy and ELISA, were found to be infected. Similar symptoms were observed in the United States. Occasionally, mild green mottling in leaves developed when plants were exposed to high temperatures during the growing season.

Transmission of virus from Angelonia plants to the herbaceous host plants listed above resulted in obvious visible symptoms only in *N. benthamiana*. Systemic mild mottle and/or mild mosaic developed about 12 to 14 days after inoculation. These symptoms were often transient, with new growth frequently showing no obvious symptoms. Some infected plants, including *N. clevelandii*, developed a slight general chlorosis. No other viruses were detected by bioassay, supporting results from electron microscopy and virus group-specific PCR.

Testing of inoculated plants by electron microscopy, ELISA, and/or RT-PCR identified symptomless hosts; in some instances,
results were confirmed by bioassay on *N. benthamiana* and then ELISA. Only a local infection was found in *N. edwardsonii, N. rustica, N. sylvestris, M. sylvatica,* and *Digitalis purpurea.* Local and systemic infection was confirmed in *N. benthamiana,* *N. clevelandii,* *N. megalosiphon,* *Schizanthus pinnatus,* and *Phlox drummondii.*

Uninfected Angelonia seedlings were mechanically inoculated with AnFBV virions isolated from infected Angelonia. The symptoms that developed on these mechanically inoculated plants were identical to those observed on the original, naturally infected Angelonia plants. AnFBV infection was confirmed by ELISA thus completing Koch’s postulates.

No transmission of AnFBV by aphids occurred from naturally infected to uninfected Angelonia (0/20) or to *N. clevelandii* (0/20). All leaf samples tested by ELISA were negative.

No visible symptoms were observed in Angelonia seedlings grown from seeds harvested from naturally infected AnFBV plants that were pollinated with pollen from either uninfected or infected plants. None of the seedlings were found to be infected with AnFBV as determined by ELISA (0/65), up to 8 weeks after germination. These results indicate that the virus is not efficiently seed transmitted in Angelonia.

**Electron microscopy.** Spherical isometric particles, ≈30 nm in diameter, were consistently observed in negatively stained samples from leaves of naturally infected Angelonia or systemically infected *N. benthamiana* (data not shown). Electron microscopy studies using ultrathin sections of infected Angelonia tissues revealed the presence of clusters of isometric particles, resembling virus particles, in the cytoplasm of leaf epidermal, mesophyll, and vascular parenchyma cells (data not shown) and flower petals (Fig. 2C); no viral inclusions were observed.

**Virus purification, physicochemical and serological properties.** Using the isolation procedures outlined above, highly purified virions were obtained both from leaves of naturally infected Angelonia and from mechanically inoculated *N. benthamiana* plants. When the virus was centrifuged in an isopycnic cesium chloride gradient, a single opalescent band was formed, from which highly purified virus was recovered following dialysis to remove cesium chloride. Yields of purified virus ranged from 50 to 60 mg/kg to about 300 mg/kg for infected Angelonia and *N. benthamiana* tissue, respectively. Electron microscopy revealed large numbers of virus particles with relatively little contamination (Fig. 2A and B). The virus particles gave a distinct decoration with the specific antisera produced against AnFBV (Fig. 2D). The \( A_{260/280} \) was found to be 1.46, uncorrected for light scattering. The reported \( A_{260/280} \) ratio for carmoviruses is 1.50 to 1.60 (3).

Denaturing SDS-PAGE of SDS-disrupted purified virus preparations revealed one major polypeptide band with an estimated Mr of 38 kDa (Fig. 3A, lane 2). Immunoblots with AnFBV antisera gave a clear and strong reaction with the 38-kDa band and therefore it was identified as the AnFBV CP (Fig. 3B, lane 2).

![Fig. 2](image-url). *Angelonia flower break virus (AnFBV) virion morphology is typical of recognized carmoviruses.* **A and B,** Virion preparations resulting from typical carmovirus isolation protocols stained with 2% (wt/vol) uranyl acetate and analyzed by electron microscopy. Mean particle diameter of 100 measured virions was 30 nm. Scale bar represents 100 nm. Similar results were obtained with *A,* Florida, *B,* Maryland, and *D,* Israeli isolates. **C,** Ultrathin section of Angelonia flower petal tissue showing AnFBV particles in the cytoplasm (particles are indicated by arrow). Scale bar represents 500 nm. **D,** Immunodecoration of AnFBV virions with rabbit polyclonal antiserum prepared to AnFBV. Scale bar represents 50 nm.
In comparing the mobility of the AnFBV CP in SDS-PAGE with those of other carmoviruses, the estimated size of PFBV, CarMV, and SgCV was found to be 35, 38, and 39 kDa, respectively (data not shown). In reciprocal tests with PFBV, CarMV, and SgCV antisera in immunoblots, only homologous reactions were obtained (data not shown).

In ELISA tests, the reactions with AnFBV antiserum were highly specific and efficient in detecting the virus even when infected plant material was used at a dilution of 1:10,000. Average DAS-ELISA values (OD405) of samples from naturally infected Angelonia, and mechanically inoculated N. benthamiana, N. clevelanditii, and N. edwardsonii diluted 1:100, were 1.75 ± 0.09, 1.30 ± 0.15, 0.87 ± 0.05, and 0.65 ± 0.03, respectively. The antiserum did not cross-react with extracts from plants infected with the carmoviruses PFBV, CarMV, or SgCV.

dsRNA and northern blot analyses. dsRNA isolated from AnFBV-infected N. benthamiana plants and fractionated on an agarose gel showed four major bands (Fig. 4A, lane 2). The largest band, of about 4.0 kbp, co-migrated with the equivalent dsRNA of CarMV (Fig. 4A, lanes 1 and 3), and is considered the putative dsRNA form of the AnFBV genomic RNA. Three additional dsRNA species of about 1.75, 1.60, and 1.31 kbp were observed on the gel. A comparable northern blot of total RNA from AnFBV-infected N. benthamiana and Angelonia revealed the presence of a 3.96-kb RNA when probed with an AnFBV CP-specific DIG-labeled probe (Fig. 4B, lanes 3 and 4). A similar sized RNA was observed by northern blot analysis of AnFBV viral RNA (Fig. 4B, lane 5). Smaller RNAs ranging from 0.93 to 2.59 kb were also detected in AnFBV-infected N. benthamiana and Angelonia plants.

Sequence of the AnFBV genome. The AnFBV genomic RNA is 3,964 nucleotides (GenBank accession no. DQ219415), a size consistent with other carmoviruses (15,21). The RNA contains four ORFs, and 5' and 3' untranslated regions (UTRs) of 24 and 257 nucleotides, respectively (Fig. 5). The 5' proximal ORF1 (nucleotides 25 to 759), encodes a 26.8-kDa protein (p27) and terminates with an amber codon which may be read through to generate an 86-kDa protein (p86; nucleotides 25 to 2307). The p86 protein also terminates with an amber codon that may potentially be read through to yield an 87.8-kDa protein (p88; nucleotides 25 to 2349). Two small ORFs, ORF2 and ORF3 (nucleotides 2298 to 2483 and nucleotides 2389 to 2655, respectively), located in the central part of the viral genome, may encode proteins of 6.7-kDa (p7) and 9.7-kDa (p10), respectively. The 3' proximal ORF4 (nucleotides 2652 to 3707) encodes the 38-kDa CP (or p38), in agreement with SDS-PAGE estimates from purified virions.

The nucleotide and deduced amino acid sequences of the AnFBV CP and replicase were compared with those of various carmovirus species. The AnFBV CP nucleotide and deduced amino acid sequence exhibited 34 to 48% and 19 to 37% identities, respectively, with the sequences of other carmoviruses (Table 1). On the other hand, the AnFBV replicase nucleotide and deduced amino acid sequences had identity percentages ranging from 44 to 57% and 36 to 55%, respectively (Table 2). The highest overall CP and replicase nucleotide and amino acid identities were obtained with PFBV. The Florida AnFBV CP gene sequence was more similar to the Israeli sequence (98.2% nucleotide identity; GenBank accession no. DQ223771) than to the Maryland sequence (92.0% nucleotide identity; GenBank accession no. DQ221212). However, the deduced CP sequences of all three isolates shared a high level of amino acid identity (96.6 to 98.9%).

Multiple alignments with the sequences of the different carmovirus CP and replicases were generated and used for phylogenetic analyses. In all cases, AnFBV clustered together with carmoviruses (Fig. 6). The only sequences producing significant alignments from BLASTX queries were those of PFBV and CarMV. However, during review of this manuscript, the CP sequence of a similar carmovirus isolated from Angelonia in Germany and tentatively named Angelonia flower mottle virus (AnFMV) was released (31).
DISCUSSION

AnFBV was found to be similar to other carmoviruses in particle size and morphology, the apparent molecular mass of the CP, genome organization, and the nucleotide and predicted amino acid sequences of the different genes. Like many carmoviruses, AnFBV caused flower break and mild foliar symptoms in its natural host (Fig. 1) and has a relatively narrow range of experimental hosts. Attempts to find alternative symptomatic hosts for AnFBV have so far failed. The experimental host range was limited to Nicotiana species, Schizanthus pinnatus, Myosotis sylvatica, Phlox drummondii, and Digitalis purpurea. No consistent symptoms were observed despite the high virus yields obtained from N. benthamiana. Attempts to passage the virus by aphids to Angelonia or N. clevelandii were unsuccessful. No visible symptoms were observed in seedlings grown from seed harvested from AnFBV-infected Angelonia plants up to 2 months after germination, and none of the seedlings were infected with AnFBV as confirmed by ELISA. These results indicate that AnFBV is not seedborne in Angelonia with high efficiency.

AnFBV can be purified directly from Angelonia leaves (Fig. 2D) and from mechanically inoculated N. benthamiana leaves (Fig. 2A and B). Purified virus preparations were infectious to both Angelonia and N. clevelandii by mechanical inoculation. The Mr of 38 kDa observed in SDS-PAGE for the CP (Fig. 3A) was in agreement with those published for other carmoviruses, including PFBV (33 to 37 kDa), CarMV (37.8 kDa), Hibiscus chlorotic ringspot virus (44.1 kDa), and SgCV (38.9 kDa) (4,9,21), and with estimates from our sequence data (Fig. 5).

Serological reactions in western blot and decoration tests with AnFBV antisera were very specific and gave clear and strong reactions with the polypeptide corresponding to the CP (Figs. 2D and 3B). The antiserum did not react with the carmoviruses PFBV, SgCV, and CarMV in either ELISA or western blot assays. Furthermore, antisera against PFBV, SgCV, and CarMV reacted with the homologous virus but not with AnFBV, indicating that these viruses are serologically distinct.

Sequence analysis of AnFBV isolates from Florida, Maryland, and Israel showed a high degree of similarity in the CP coding region. The Florida AnFBV CP gene sequence is more similar to the Israeli sequence (98.2% nucleotide identity) than to the Maryland sequence (92.0% nucleotide identity) suggesting a common origin (geographic and/or cultivar) for the Florida and Israeli isolates and a distinct origin for the Maryland isolate. However, the deduced CP sequences of all three isolates share a high level of amino acid identity (96.6 to 98.9%). The deduced CP sequence of amino acid identity (96.6 to 98.9%). The deduced CP sequence of the three AnFBV isolates reported here, of amino acid identity (96.6 to 98.9%). The deduced CP sequence of the three AnFBV isolates reported here, suggesting that these are the same virus.

TABLE 1. Amino acid and nucleotide sequence homology (percent identity) between the coat protein gene of Angelonia flower break virus (AnFBV) strains and related carmoviruses

| Virus          | AnFBV-FL | AnFBV-MD | AnFBV-IS | PFBV | CarMV | ELV | SgCV | TCV | CCMV | HCRSV | JINRV | GaMV | CPMoV | PSNV | MNSV | PVM |
|----------------|----------|----------|----------|------|-------|-----|------|-----|------|-------|-------|------|------|------|------|------|-----|
| AnFBV-FL       | 98.9     | 96.6     | 98.9     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| AnFBV-MD       | 97.4     | 95.9     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.0 |
| AnFBV-IS       | 96.6     | 96.6     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| PFBV           | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| CarMV          | 98.9     | 98.9     | 98.9     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| ELV            | 87.6     | 87.6     | 87.6     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| SgCV           | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| TCV            | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| CCMV           | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| HCRSV          | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| JINRV          | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| GaMV           | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| CPMoV          | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| PSNV           | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |
| MNSV           | 97.4     | 97.4     | 97.4     | 36.5 | 33.4  | 29.4| 30.6 | 25.6| 25.5 | 27.8  | 28.7  | 23.9| 18.8 | 22.6| 20.9| 9.3 |

* Percent identity calculated using CLUSTALDIST based on ClustalW alignments. Sources of sequences and GenBank accession numbers are as follows: AnFBV-FL, -MD, and -IS (DQ219415, DQ221212, and DQ223771); Pelargonium flower break virus (PFBV; AJ514833); Carnation mottle virus (CarMV; AF19277); Elderberry latent virus (ELV; AY038066); Saguaro cactus virus (SgCV; U72322); Turnip crinkle virus (TCV; M29671); Cardamine chlorotic fleck virus (CCIFV; L16015); Hibiscus chlorotic ringspot virus (HCRSV; YX60448); Japanese iris necrotic ringspot virus (JINRV; D86123); Galinsoga mosaic virus (GaMV; Y13463); Cowpea mottle virus (CPMoV; U20976); Pea stem necrosis virus (PSNV; AB086951); Melon necrotic spot virus (MNSV; M29671); and Potato virus M (PVM; D14449).
Several features of the analysis indicate that AnFBV is most closely related to, but distinct from, PFBV. Guidelines for demarcation of virus species cannot be exactly defined and have to be established for each virus genus or family (29). In the plant virus families Geminiviridae and Potyviridae, isolates with less than 85 to 90% nucleotide identity over the entire genome would normally be regarded as distinct virus species (29); these criteria are exceeded substantially for the carmoviruses most closely related to AnFBV (i.e., PFBV and CarMV; ≈57% in the replicase; 45 to 48% in the CP), indicating that the differences are of an order that separates distinct carmoviruses (24). These sequence identities also exceed the levels reported by Castaño and Hernández (5) for viruses in different genera of the family Tombusviridae, supporting assignment of AnFBV to the genus Carmovirus.

In conclusion, based on particle morphology, the Mr of the CP and the genome sequence and organization, it is suggested that AnFBV belongs to the genus Carmovirus. Moreover, the differences in host range, serological reactions, genome sequence, and predicted protein sequence provide sufficient justification to consider AnFBV a novel carmovirus. In pondering a suitable designation for the Angelonia carmovirus, we propose that it be called Angelonia flower break virus (AnFBV) to designate the natural host of the virus and the most prominent symptom it incites on this host.

TABLE 2. Amino acid and nucleotide sequence homology (percent identity) between the replicase gene of Angelonia flower break virus (AnFBV) and related carmoviruses

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a Amino acid identities (above the diagonal) and nucleotide identities (below the diagonal) were calculated using CLUSTAL DIST based on ClustalW alignments. Sources of sequences and GenBank accession numbers are as follows: AnFBV-FL (DQ219415), Pelargonium flower break virus (PFBV; AJ514833), Carnation mottle virus (CarMV; AF192772), Saguaro cactus virus (SgCV; U72332), Hibiscus chlorotic ringspot virus (HCRSV; X86448), Turnip crinkle virus (TCV; M22445), Pea stem necrosis virus (PSNV; AB086951), Melon necrotic spot virus (MNSV; M29671), Cardamine chlorotic fleck virus (CCIFV; L16015), Japanese iris necrotic ringspot virus (JINRV; D86123), Cowpea mottle virus (CPMoV; U20976), Gallinsoga mosaic virus (GaMV; Y13463), and Potato virus M (PVM; D14449).

Fig. 6. Phylogenetic analysis of the amino acid sequences of the A, replicase and B, coat protein of Angelonia flower break virus (AnFBV) and other carmoviruses. Sequences were aligned using CLUSTALW with gap opening and extension penalties of 10.0 of 0.2, respectively, and Gonnet series protein weight matrix. Phylogenetic trees were inferred using the PMB model and neighbor-joining method using Phylip version 3.6 with Potato virus M (PVM, D14449) as an outgroup. Bootstrap analysis was performed with 1,000 replicates. Trees were displayed using Treeview. Viruses included (listed with abbreviation and GenBank accession number for nucleotide sequence) are as follows: Florida (FL), Maryland (MD), and Israeli (IS) isolates of AnFBV (DQ219415, DQ221212, DQ223771), Pelargonium flower break virus (PFBV; AJ514833), Carnation mottle virus (CarMV; AF192772), Elderberry latent virus (ELV; AY038066), Saguaro cactus virus (SgCV; U72332), Turnip crinkle virus (TCV; M22445), Cardamine chlorotic fleck virus (CCIFV; L16015), Hibiscus chlorotic ringspot virus (HCRSV; X86448), Japanese iris necrotic ringspot virus (JINRV; D86123), Cowpea mottle virus (CPMoV; U20976), Gallinsoga mosaic virus (GaMV; Y13463), and Potato virus M (PVM; D14449).
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LITERATURE CITED