Biological and Molecular Characterization of a New Carlavirus Isolated from an *Aconitum* sp.

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ABSTRACT

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A new virus was isolated from symptomless *Aconitum napellus* plants. The virus, for which the name *Aconitum latent virus* (AcLV) is proposed, has flexuous particles 640 nm in length. The experimental host range was limited to *Nicotiana clevelandii*. Electron microscopy studies of ultrathin sections of infected *A. napellus* tissues revealed the presence of elongated virus particles. No inclusion bodies characteristic of potyvirus infection were observed. AcLV was purified from naturally infected *A. napellus* by cesium chloride step gradient centrifugation. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dissociated purified virus prepara-

tions, a major protein component with a molecular mass of 35 kDa was observed. Diagnostic antibodies that could specifically bind to virus particles were produced. The 5' terminus (620 nucleotides) of the viral RNA was cloned and sequenced. It comprised 71 nucleotides from the untranslated 5' terminus and 549 nucleotides of an open reading frame encoding 183 amino acids. Comparison of the predicted amino acid sequence with those of other plant viruses revealed 40 to 60% identity with several carlaviruses. Based on particle morphology, absence of inclusion bodies in ultrathin sections, the relative molecular weight of the coat protein, the nucleotide sequence, and predicted amino acid homology, it is suggested that this virus belongs to the carlavirus group.

Additional keywords: purification, serology.

Aconitum napellus (family Ranunculaceae) is a new geophyte attracting great demand in Europe as a cut flower. The crop, which is vegetatively propagated, was introduced into Israel in 1985 by importing tubers from the Netherlands. The growing area exceeded 15 acres; but among the factors that limit the expansion of the growing area are the contamination of the tubers with fungal diseases and the low natural propagation index. To the best of our knowledge, no viral diseases of *Aconitum* spp. have been reported.

During surveys of viral diseases conducted in Israel, *Aconitum* spp. plants grown in commercial farms were observed to exhibit mild mosaic symptoms. The causative agent was identified as *Cucumber mosaic virus* (CMV) (5). During 1997, plants with unusual viral symptoms of mosaic and necrosis on leaves were observed. These plants were found to be doubly infected with CMV and an unidentified elongated filamentous virus. Here, we report on the purification and characterization of a new carlavirus referred to as *Aconitum latent virus* (AcLV) isolated from *A. napellus*. We also report the cloning and sequencing of the 5'-terminal region of the genome of this virus and its comparison to the published sequences of other carlaviruses.

MATERIALS AND METHODS

Virus source, transmission, and host range. Leaf samples of *A. napellus* grown in commercial screenhouses in Israel were examined for virus particles using electron microscopy (EM). Samples containing elongated particles were homogenized in 1% K₂HPO₄, and the sap was inoculated to *Capsicum annuum*, *Chenopodium amaranticolor*, *Chenopodium murale*, *Chenopodium quinoa*, *Cucumis sativus*, *Cucurbita pepo*, *Datura stramonium*, *Eustoma grandi-*

florum, *Gomphrena globosa*, *Hyoscyamus negro*, *Lycopersicon esculentum*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, *N. sylvestris*, *N. tabacum* cv. Samsun, *N. tabacum* cv. Samsun NN, *N. tabacum* cv. Xanthi nc, *N. tabacum* cv. White Burley, *Petunia × hybrida*, and *Physalis floridensis* predested with Carborundum. These plants were maintained in the greenhouse and observed for symptom development.

The green peach aphid (*Myzus persicae* (Sulzer)) was used for transmission tests. A clone of *M. persicae* that originated from a single female was reared on healthy Japanese radish (*Raphanus sativus*) plants. Nonviruliferous apterous aphids were allowed to feed for 5 min either on *A. napellus* or on mechanically inoculated *N. clevelandii*. Five aphids were transferred to each of 10 healthy *N. clevelandii* plants and allowed to remain on the test plant for 12 h. These plants were then sprayed with insecticide, maintained in the greenhouse, and observed for symptom development.

Virus purification. Leaves (100 g) of *A. napellus*, collected in the field, were homogenized in 200 ml of 0.2 M potassium phosphate, pH 7.6, containing 3% (vol/vol) urea and 200 µl of thioglycolic acid for 4 min in an Omni-mixer (Sorval Inc., Newtown, CT). The virus was purified according to the method described by Zavriev et al. (19). For further purification, virus preparations were layered on cesium chloride step gradients (10 to 40% in 0.1 M phosphate buffer, pH 7, containing 20% sucrose) (2). The gradient columns were fractionated into 0.5-ml fractions with the aid of a peristaltic pump, and the virus-containing fractions were checked by EM. Fractions containing the highest concentration of virus were pooled and centrifuged.

Virus concentration was estimated spectrophotometrically using an extinction coefficient of 2.1 (mg/ml)⁻¹ cm⁻¹ at 260 nm for carlaviruses (18).

Production of antibodies. Partially purified virus (ppV) suspensions of AcLV (about 0.1 mg/ml) were emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into both hip muscles of 6-week-old female New Zealand

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white rabbits as described previously (20). Alternatively, the coat protein (CP) from purified virus preparations was denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Stained protein bands (SDS-PB) were excised from the gel and used for injection as described previously (7). Two weeks after the last injection, a booster injection with ppV was given. Antisera were collected 12 days after the last injection. These antisera were designated ppV/ppV and SDS-PB/ppV, respectively. Antisera were tested by western blotting (16), enzyme-linked immunosorbent assay (ELISA) (4), and immunoelectron microscope (IEM) decoration tests as described by Milne and Luisoni (10).

Physicochemical properties. The relative molecular weight (M_r) of AcLV CP was determined by SDS-PAGE of purified virus preparation; 12% polyacrylamide gels (75 × 5 mm) were prepared as described previously (7). The CPs of *Potato virus M* (PVM) and *Potato virus S* (PVS) were also analyzed. The PBs were either stained with 0.1% Coomassie brilliant blue or electroblotted to nitrocellulose (16) and probed with the polyclonal antiserum. Antibody binding was detected with anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (BioMakor, Rehovot, Israel).

Crude plant extracts in 0.1 M phosphate buffer or AcLV preparations were negatively stained with 20 g of uranyl acetate per liter and examined for virus in a JEOL 100CX II electron microscope (JEOL Ltd., Tokyo). Particle length was measured on ×50,000 enlarged photographs, and the normal length was calculated from the measurements of 100 particles. For calibration of magnification, a cross-line grating replica with spacing of 2,160 lines per millimeter was used.

For ultrathin sections, segments (2 × 1 mm) were excised from young leaves of *A. napellus* and fixed in 2.5% glutaraldehyde buffered with 75 mM potassium phosphate, pH 7, for 2 h. The samples were then rinsed in the same buffer and postfixed in 1% OsO₄ for 3 h. After a buffer rinse, the samples were dehydrated in a graded acetone series, embedded in an Epon-Araldite resin mixture, and polymerized as described by Orion and Franck (12). Sectioned material was stained with uranyl acetate followed by lead nitrate.

IEM decoration tests were conducted as described by Milne and Luisoni (10). For identification of the serological reactions of the virus with other carlaviruses, antisera against PVM and PVS, diluted 1:10 (provided by G. Loebenstein, ARO, Volcani Center, Bet Dagan, Israel) were tested. Homologous and heterologous decoration reactions were determined using antigen from crude extracts of infected plants.

Fig. 1. Severe mosaic symptoms accompanied with necrosis on leaves of *Aconitum napellus* naturally doubly infected with *Aconitum latent virus* and *Cucumber mosaic virus*.

cDNA synthesis and molecular cloning. RNA was extracted from purified AcLV particles by phenol/chloroform extraction as described previously (13).

The universal Riboclone cDNA Synthesis System Kit (Promega Corp., Madison, WI) with both random and oligo deoxyribosylthymine (dT) primers were used. cDNA synthesis was carried out according to the manufacturer's instructions. Double-stranded cDNA was blunted with T4 DNA polymerase and ligated into the *Sma*I site of a Bluescript plasmid vector using standard procedures (15). *Escherichia coli* DH5 α cells were transformed with the recombinant plasmid and plated on Luria-Bertani agar containing ampicillin, isopropyl- β -D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. White colonies were selected for plasmid analysis. Minipreps of plasmid were cleaved with *Eco*RI and *Xba*I to screen for cDNA inserts by gel electrophoresis. Plasmid DNA of clones containing the insert was prepared by using the plasmid Midi-Kit (Qiagen Inc., Valencia, CA).

Sequence analysis. Selected recombinant plasmids were sequenced at the Weizmann Institute of Science, Rehovot, Israel, by the dideoxynucleotide chain termination method, using either forward or reverse M13 primers. Nucleotide and amino acid sequences were performed with the DNAMAN package, version 4 (Lynnon Biosoft, Quebec, Canada). AcLV-related sequences were initially identified using the BLASTP search program of GenBank (1). The sequence of the N-terminus 183 amino acids was compared with the following carlaviruses: *Apple stem pitting virus*, BAA04853 (8); *Blueberry scorch virus*, AAA68984 (3); *Garlic latent virus*, CAA92815 (C. Jin Nam, S. Sang Ik, S. Jong Tae, L. Jong Seob, and C. Yang Do, unpublished data); *Grapevine rupestris stem pitting associated virus*, AAC62910 (21); PVM, PN0093 (19); PVM Russian isolate, P17965 (19); *Rupestris stem pitting associated virus*, AAC35498 (9); and *Sour cherry green ring mottle virus*, AAC35433 (22).

Phylogenetic analysis was performed to determine the relationship of AcLV with other carlaviruses using the neighbor-joining algorithm in DNAMAN according to Saito and Nei (14). The data set was subjected to 1,000 bootstrap replicates. All nodes supported by >45% confidence values are shown.

RESULTS

Virus transmission and host range. Initially, AcLV was detected in *A. napellus* plants that exhibited unusual viral symptoms of mosaic on leaves accompanied by necrosis (Fig. 1). These plants were found to be doubly infected with CMV and AcLV. In further tests, AcLV was detected in symptomless *A. napellus* plants. All *A. napellus* plants, representing the commercial stock grown in several different locations in Israel and tested by EM and ELISA, were found to be infected. Occasionally, mild green mottling in leaves developed when plants were exposed to high temperatures during the growing season.

Fig. 2. Electron micrograph of purified *Aconitum latent virus* particles **A**, stained with uranyl acetate and **B**, decorated with specific antiserum. Bar = 250 nm.

AcLV was not mechanically transmitted to any of the 23 herba-
ceous plant species listed above, as determined by symptomology,
for 1 month following mechanical inoculation. Tests by EM and
ELISA confirmed occurrence of infection only in *N. clelandii*.
Attempts to transmit the virus mechanically from inoculated to
healthy *N. clelandii* were unsuccessful.

Transmission of AcLV by aphids from either naturally infected
A. napellus or mechanically inoculated *N. clelandii* to healthy
N. clelandii was unsuccessful.

EM. Flexuous, elongated virus particles were consistently ob-
served in negatively stained samples from leaves of naturally in-
fected *A. napellus* (Fig. 2A). The modal length of particles was
about 640 nm. The virus particles gave a distinct decoration with
the specific antisera produced against the virus. Decoration tests

Fig. 3. Ultrathin section of *Aconitum napellus* leaf cells showing *Aconitum latent virus* particles in the cytoplasm (particles are indicated by arrows). Bar = 500 nm.

with SDS-PB/ppV antisera resulted in a strong decoration of the
particles (Fig. 2B).

EM studies using ultrathin sections of infected *A. napellus* tis-
sues revealed the presence of flexuous, elongated rods resembling
virus particles in the mesophyll, epidermal, and vascular cyto-
plasm of parenchyma cells (Fig. 3). Some of the chloroplasts in
cells that contained these particles were vacuolated and disorga-
nized. Cytoplasmic inclusion bodies characteristic of potyvirus in-
fection were not observed.

Virus purification. Using the purification procedure outlined
above, virus particles were located approximately 20 mm from the
bottom of the cesium chloride gradient. Yields of purified virus from
naturally infected *A. napellus* leaves ranged from 30 to 40 mg/kg
of tissue. Preparations for EM of purified fractions contained large
numbers of virus particles with relatively little contamination. The
absorbance ratio of 260 nm to 280 nm ($A_{260/280}$) was found to be
1.235, uncorrected for light scattering. The acceptable $A_{260/280}$ ratio
for carlaviruses is 1.37 (18).

SDS-PAGE and serology. PAGE of SDS-disrupted purified vi-
rus preparations revealed one major and two minor polypeptide
bands (Fig. 4A, lane 2). The M_r of the major protein was estimated
to be 35,000 Da. This band was identified as the virus CP. It is
uncertain whether minor bands were derived from virus particles
or degradation products of the CP.

Immunoblots with SDS-PB/ppV and ppV/ppV of AcLV gave a
clear and strong reaction with the polypeptides corresponding to
CP, as identified by their M_r (Fig. 4B, lanes 3 and 4). Both AcLV

TABLE 1. Percent identity between the partial amino acid sequence of *Aconitum latent virus* open reading frame 1 and sequences of related carlaviruses

Virus	Amino acid identity (%)
Potato virus M	64.1
Potato virus M Russian isolate	63.6
Blueberry scorch virus	47.8
Grapevine rupestris stem pitting associated virus	42.9
Rupestris stem pitting associated virus	42.9
Garlic latent virus	41.3
Apple stem pitting virus	37.1
Sour cherry green ring mottle virus	36.7

Fig. 4. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of purified *Aconitum latent virus* (AcLV). **A and C,** Gels stained with Coomassie bril-
liant blue. Lane 1, Molecular weight markers: phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), aldolase (45 kDa), carbonic anhydrase (31 kDa),
trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); and lane 2, purified AcLV. **C,** Lanes 5 to 7, Purified preparation of *Potato virus S*, *Potato virus M*, and
AcLV, respectively. **B,** Gels were blotted on membranes and probed with AcLV polyclonal antibodies (diluted 1:2000) obtained after primary injections with
SDS-denatured protein bands of AcLV (SDS-PB) (lane 3) or partially purified viral (ppV) antigen boosted with ppV virus (lane 4). **D,** Lanes 5 to 7, Gels were
blotted on membranes and probed with AcLV polyclonal antibodies (diluted 1:2000) obtained after primary injections with SDS-denatured protein bands of AcLV
(SDS-PB).

antisera specifically reacted with the CP. In comparing the mobility of AcLV CP in SDS-PAGE with that of other carlaviruses, the estimated size of PVS, PVM, and AcLV CPs was found to be 33, 34, and 35 kDa, respectively (Fig. 4C). In reciprocal tests with AcLV, PVS, and PVM antisera in immunoblots, only homologous reactions were obtained (Fig. 4D, AcLV).

In ELISA tests, considerably higher titers were obtained with the SDS-PB/ppV than with ppV/ppV antisera (data not shown). The reactions of SDS-PB/ppV antisera were highly specific and more efficient in detecting AcLV, even when infected plant material was used at a dilution of 1:10,000, whereas the ppV/ppV antisera failed to detect the virus at dilutions beyond 1:1,000 when ELISA was used.

Cloning the AcLV genome. DNA complementary to the 5'-terminal region of AcLV was generated by using the universal Riboclonc cDNA Synthesis System Kit with both random and oligo dT primers. A 620-bp clone derived from the 5'-terminal sequence of the viral RNA was selected for further study. This clone was sequenced, and the nucleotide sequence of the 5' nontranslated region (5' NTR) was established and deposited in the GenBank database under the accession number AF177225. It was comprised a 71-nucleotide, untranslated 5' terminus and 549 nucleotides encoding 183 amino acids in open reading frame (ORF) 1. The sequence exhibited significant identity (40 to 60%) to the N-terminal sequences of other carlaviruses (Table 1). The relatedness of AcLV to other carlaviruses based on partial polypeptide sequence homology is shown in a phylogenetic tree (Fig. 5). These results suggest that AcLV shares high identity with PVM and that both are clearly distinct from other carlaviruses.

DISCUSSION

AcLV seems to be similar to other carlaviruses in particle length and morphology, the apparent M_r of the CP, and the nucleotide and the predicted amino acid sequences of the 5' terminus. Like many carlaviruses, AcLV is symptomless in its infected natural host and has a narrow host range. The cytological properties of the virus are apparently different from those of potyviruses, and cytoplasmic inclusions typical of potyviruses were not found in infected *Aconitum* spp. (18).

All attempts to find alternative propagation hosts for AcLV failed. Although, the virus was transmitted to *N. clevelandii* from *A. napellus*, further attempts to pass the virus mechanically or by aphids to more healthy *N. clevelandii* were unsuccessful.

Fig. 5. Relationship of *Aconitum latent virus* (AcLV) with selected carlaviruses. The deduced amino acid sequence of open reading frame 1 was compared first by a BLASTP search of GenBank. Tree descriptions were generated using the neighbor-joining algorithm and based on calculations from pairwise amino acid sequence distances derived from the multiple alignment format (data not shown). Horizontal scale indicates sequence divergence and vertical scale is arbitrary. The data set was subjected to 1,000 bootstrap replicates. All nodes supported by >45% confidence values are shown.

AcLV can be purified directly from *A. napellus* leaves. Virus preparations purified by this procedure were infectious in *N. clevelandii* by mechanical inoculation. The M_r of 35 kDa observed in SDS-PAGE for the CP was in agreement with those published for other carlaviruses, but different in size from the CPs of PVS (33 kDa) and PVM (34 kDa), (18,19). The two minor PBs observed may be a breakdown product of the CP. Serological reaction in western blot and decoration test with SDS-PB/ppV and ppV/ppV of AcLV were very specific and gave clear and strong reactions with the polypeptide corresponding to the CP. Although both AcLV antisera reacted with the virus, the reactivity and specificity of the SDS-PB/ppV antisera were higher in ELISA and western blot. This confirms our previous results with antisera produced against *Potato virus Y* and *Iris yellow spot virus* (6). These antisera did not react with the carlaviruses PVM and PVS in either ELISA or western blots. Furthermore, antisera against PVM and PVS reacted with the homologous virus but not with AcLV, indicating that these viruses are serologically different.

The sequenced clone included 71 nucleotides from the 5' NTR and 549 nucleotides resembling part of the carlavirus ORF 1. A portion of the 5' NTR is conserved among carlaviruses (3,11,19), and nucleotide sequence comparison of this region showed 64.1% identity, while the amino acid sequences were 61.9% identical. The phylogenetic relationship between AcLV and other filamentous viruses, based on analysis of a small portion of the viral genome, showed that the members of each virus group clustered together as expected. Several features of the analysis indicate that PVM and AcLV are clearly distinct from other carlaviruses and that AcLV should be considered as a virus distinct from PVM. Guidelines for demarcation of virus species cannot be exactly defined and have to be established for each virus genus or family (17). Data are limited for the entire genome sequence of AcLV and other carlaviruses. However, in the *Geminiviridae* and *Potyviridae* plant virus families, isolates with less than 85 to 90% nucleotide identity in the entire genome would normally be regarded as different viruses (17). These criteria are exceeded substantially for the AcLV-PVM comparison (64.1%), indicating that the differences are of an order that separates distinct viruses.

In conclusion, based on particle morphology, the absence of inclusion bodies in ultrathin sections, the M_r of the CP, and the nucleotide sequence, it is suggested that this virus belongs to the carlavirus group. Moreover, the host range, the differences in M_r of the CP, the serological data, the nucleotide sequence, and predicted amino acid homology provide sufficient justification to consider AcLV a distinct entity and a new member of the carlavirus group. In considering a suitable designation for the *A. napellus* virus, we propose that it be called *Aconitum latent virus* (AcLV) in order to designate the natural host of the virus.

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