Arch Virol (2003) 148: 1211–1218
DOI 10.1007/s00705-003-0009-9

Archives of
Virology
Printed in Austria

Molecular evidence that aphid-transmitted *Alpinia mosaic virus* is a tentative member of the genus *Macluravirus*

Brief Report

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Received October 17, 2002; accepted January 17, 2003
Published online March 21, 2003 © Springer-Verlag 2003

Summary. *Alpinia mosaic virus* (AlpMV), once assigned to the genus *Potyvirus*, infects primarily plants of the ginger family. To seek molecular evidence for correct classification of this virus, a cDNA clone corresponding to the 3′ portion of the AlpMV genome was obtained by reverse transcriptase-PCR and TA cloning. The authenticity of the cDNA clone was confirmed by expression of the coat protein (CP) in *E. coli* followed by immunoblot analysis. Sequence analysis indicated that, in contrast to its low identity with all the other genera of the family *Potyviridae*, the deduced amino acid sequence of AlpMV CP was 42.9 ∼ 61.9% identical to members of the genus *Macluravirus*. Phylogenetic analysis also demonstrated that the AlpMV CP clustered with those of *Cardamom mosaic virus* and Chinese yam necrotic mosaic virus. These results indicate that AlpMV should be classified as a tentative species within the genus *Macluravirus* rather than *Potyvirus* as proposed previously.

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Plants of the ginger family, *Zingiberaceae*, have long been used as condiments, dyes, perfumes, spices, and vegetables. Moreover, owing to their beautiful foliage and flowers, some species of the ginger family, especially *Curcuma alismatifolia*, have become floricultural crops of considerable economical importance in some countries in the past few years. Viruses that have been reported to infect plants of the ginger family include *Cardamom mosaic virus* (CdMV) [11], Cardamom chirke virus, Cardamom foorkey virus [4], *Cucumber mosaic virus* (CMV) [18], and Ginger chlorotic fleck virus (GCFV) [19]. Recently, a new virus known to infect primarily plants of the ginger family was described by Chen and Hong [5] in Taiwan and designated as *Alpinia mosaic virus* (AlpMV). It has flexuous filamentous particles with a modal length of 700 ∼ 750 nm and is transmissible only

by the banana aphid, *Pentalonia nigronervosa*, in a non-persistent manner. Plants which are known to be host of AlpMV include ginger (*Zingiber officinale*), *Alpinia formosana*, *Alpinia purpurata*, *Curcuma alismalifolia*, *Hedychium hybridum*, and *Phaeomeria magnifica*. After infection, numerous light green stripes appear on the leaves of infected plants, and pinwheel-shaped and laminated inclusion bodies can be detected in them. Accordingly, AlpMV has been classified under the family *Potyviridae* and the genus *Potyvirus* [5, 9]. Recently, the genus *Macluravirus* was established for species which, although aphid transmissible, contain coat protein genes significantly different from those of the genus *Potyvirus*. *Macluravirus* has two definitive members: *Maclura mosaic virus* (MacMV) and *Narcissus latent virus* (NLV) [3]. In addition, two viruses were recently proposed to be tentative members of *Macluravirus*, namely *Cardamom mosaic virus* (CdMV) [12] and Chinese yam necrotic mosaic virus (ChYNMV) [14], based essentially on the sequence data. To determine the classification of AlpMV, the nucleotide sequence of the 3′-terminal region of this virus was cloned and analyzed. The results obtained indicate that AlpMV is a tentative new member of the genus *Macluravirus* and not *Potyvirus* as proposed previously.

Virus was purified directly from diseased *Alpinia formosana* showing the characteristic mosaic pattern according to Gonsalves et al. [11] and Chen and Hong [5]. Viral RNA was isolated from purified virus particles by proteinase K digestion, phenol chloroform extraction and ethanol precipitation [7]. Following reverse transcription primed with oligo(dT), viral cDNA was amplified by PCR using oligo(dT) and a potyvirus universal primer, which was provided by Dr. Y. C. Chang (Department of Plant Pathology, National Taiwan University), as the downstream and upstream primers, respectively. Each reaction (20 μl) consisted of 10 ng of viral cDNA, 1.25 μM of oligonucleotide primers, 0.2 mM dNTP, 1 × PCR buffer, and 1 U of DyNazyme™ II DNA polymerase (Finnzymes, Finland). PCR was performed by denaturation at 94 °C for 5 min, followed by 25 cycles of 95 °C/45 sec, 55 °C/1 min, 72°C/2 min, and a final 10-min extension at 72 °C in a thermocycler (GeneAmp PCR System 2400, Perkin elmer). After separation of the amplified product on a 1% agarose gel, DNA bands of expected size were collected from the gel using the Geneclean III kit (Bio101) and cloned into pGem T-easy (Promega). The nucleotide sequence was determined on both strands of DNA using the BigDye terminator cycle sequencing ready reaction kit and an autosequencer (Applied Biosystems, model 310). Sequence was analyzed using programs in the GCG software package (Genetics Computer Group, Wisconsin Package Version 10.0).

The coat protein (CP) of AlpMV was expressed using the pQE31 bacterial expression system (Qiagen). A DNA fragment containing the entire putative CP was amplified from the cDNA clone (alpcp-1) using the T7 promoter primer (5′-TAA TAC GAC TCA CTA TAG GG-3′) and alpexp1 (5′-CCA AGC TTG GTT AAT GTA GCG TTG CAC GCG-3′), which comprised the complement of the last 20 nucleotides of the open reading frame (ORF), including the termination codon and, immediately downstream, a *Hin*dIII site for the purpose of cloning. The amplified DNA fragment was gel purified, digested with *Bam*HI and *Hin*dIII, and cloned into

the expression vector pQE31 to generate the recombinant plasmid clone pExcp12. The *Bam*HI site was located at position 531 to 536 of the AlpMV cDNA and used to facilitate in frame ligation of the CP ORF with the translation initiation codon ATG in pQE31. For expression of the CP, pExcp12 was transformed into *E. coli* strain M15[pREP4]. Expression of the recombinant protein by *E. coli* was induced by addition of 1 mM IPTG (isopropyl-thio-β-D-galactopyranoside).

After IPTG induction, *E. coli* cells were harvested by centrifugation, and the pellet was mixed with a sample buffer [final concentration: 0.06 M Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol, 5% β-mercaptoethanol (v/v), and 0.1% bromophenol blue], and the proteins were analyzed by 12% SDS polyacrylamide gel electrophoresis (PAGE). The separated proteins were blotted onto PVDF membrane (Osmonics) using a Multiphor II Electrophoresis Unit (Amersham Pharmacia) as the transfer apparatus. The immunoblot was blocked for 1 h in a blocking solution (Roche), followed by incubation with polyclonal antibody raised against AlpMV in a rabbit, which was a gift from Dr. T. H. Chen [5]. To visualize antibody-specific proteins, the immunoblot was incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, Ohio) at a 1:1000 dilution, and then exposed to a solution containing NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo- 4-chloro-3-indolyl phosphate, toluidine salt) (Roche) in the AP substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl).

Sequence alignments were performed using Clustal W [20]. Phylogenetic analyses were carried out using programs in PHYLIP version 3.57c [10]. Genetic distances between pairs of amino acid sequences were calculated using PROTDIST (Dayhoff PAM). Phylogenetic trees were constructed by a distance method (FITCH) using the original data set and 1000 bootstrap data sets generated by the program SEQBOOT from the original set. The program TREEVIEW [16] was used to draw the phylogenetic tree.

A total of 1,731 nucleotides were determined from the representative cDNA clone alpcp-1 (GenBank accession number AF499025). Preliminary analysis of the sequence revealed the presence of an open reading frame ranging from nucleotide 1 to 1545, followed by an untranslated region of 186 nucleotides. A blast search of the databases found a match with the NIb and coat protein sequences characteristic of the family *Potyviridae*. To confirm that the alpcp-1 cDNA clone was derived from AlpMV, sequence encompassing the CP was amplified by PCR and cloned in pQE31 expression vector. Analysis of the recombinant proteins obtained from pExcp12 transformants by SDS-PAGE demonstrated that a protein band with molecular mass of the expected size appeared 1.5 h after induction with IPTG (Fig. 1A, lane 2), and still persisted 3 h after induction (Fig. 1A, lane 3). The identity of the expressed protein was confirmed by immunoblot analysis using the polyclonal antibody against AlpMV (Fig. 1B, lane 2).

A database search using the deduced amino acid sequence of AlpMV exhibited the highest identity to the corresponding region of CdMV (AF189125), followed by ChYNMV (AB044386), NLV (U58770), and MacMV (U58771); all of them belong to the genus *Macluravirus*. In order to find out sequences which are highly

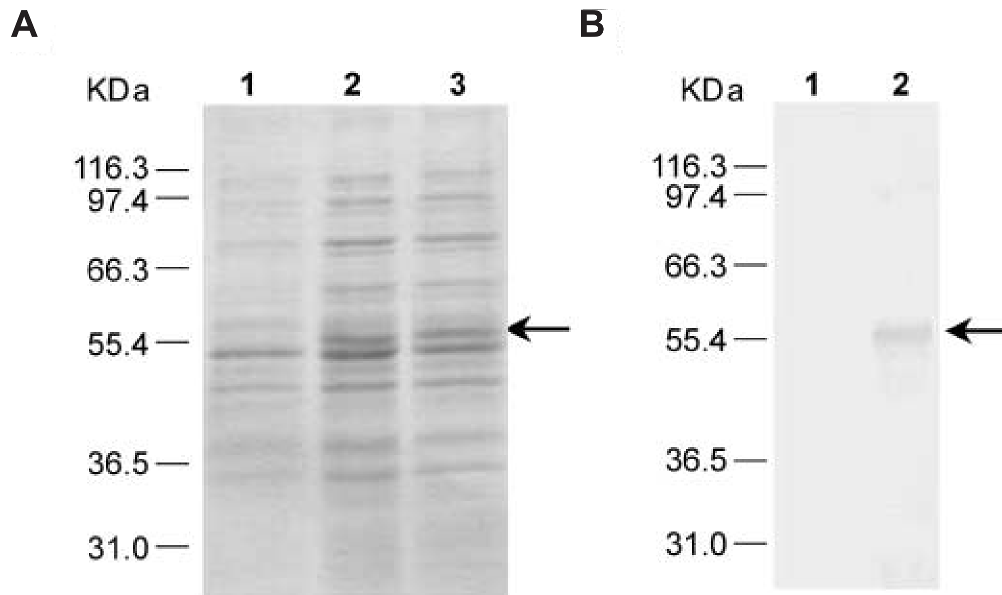

Fig. 1. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of AlpMV coat protein expressed in *E. coli*. **A** Expression of the recombinant protein by M15[pREP4] cells harboring pExcp12 were induced with IPTG for indicated period of time and cell extract was analyzed by SDS-PAGE, followed by staining with coomassie blue. *1*: no induction, *2*: 1.5 h after induction, *3*: 3 h after induction. **B** Immunoblot analysis using polyclonal antibodies against AlpMV. *1*: no induction, *2*: 1.5 h after induction with IPTG. The position of the recombinant AlpMV CP is marked with an arrow. Positions of the molecular weight size marker are indicated on the left

Table 1. Coat protein amino acid sequence identity between AlpMV and selected members from the *Potyviridae*

Genus	Virus	Identity (%)[a]	Accession number
Macluravirus	MacMV	42.9	U58771
	NLV	46.9	U58770
	ChYNMV	56.9	AB044386
	CdMV	61.9	AF189125
	AlpMV	100.0	AF499025
Bymovirus	BaYMV	26.7	X69757
	WSSMV	25.8	X73883
Ipomovirus	CVYV	24.8	AF233429
	SPMMV	24.7	Z48058
Potyvirus	PVY	30.3	X68222
	TEV	30.2	M15239
Rymovirus	AgMV	30.9	U30615
	RGMV	29.4	U27383
Tritimovirus	BrSMV	22.9	Z48506
	WSMV	22.5	AF057533

[a]Pairwise sequence identities were calculated using GAP of GCG

conserved among these viruses, the amino acid sequences of partial NIb and full-length CP from NLV, MacMV, ChYNMV, CdMV, and AlpMV (Table 1) were analyzed by Clustal W. Alignments of the sequences demonstrated that NIb is highly conserved in several regions among these viruses (Fig. 2). The consensus motifs (T/S)GX_3-TX_3-N(T/S) and GDD, proposed to be the active site for the RNA-dependent RNA polymerase of positive-strand RNA viruses [8, 15], were found at positions 4–14 and 47–49 of AlpMV, respectively. The possible NIa cleavage site between NIb and CP of NLV and MacMV has been suggested to be LQ/M by Badge et al [3]. As shown in Fig. 2, 'LQ/M' also exists in ChYNMV, but not in AlpMV and CdMV, which contained instead FQM at the corresponding position. If NIa cleavage indeed occurs after the glutamine residue (FQ/M), the N-terminal residue of AlpMV CP will be methionine, the same as those of NLV, MacMV, and ChYNMV. Besides, it will generate a coat protein with a predicted molecular mass of 33.4 KDa, close to the size estimated for NLV (32.8 KDa) and MacMV (34.1 KDa) [3]. Attempts to determine the N-terminal sequence of the AlpMV CP by the Edman degradation method failed (data not shown), however, perhaps because methionine is relatively resistant to degradation compared to

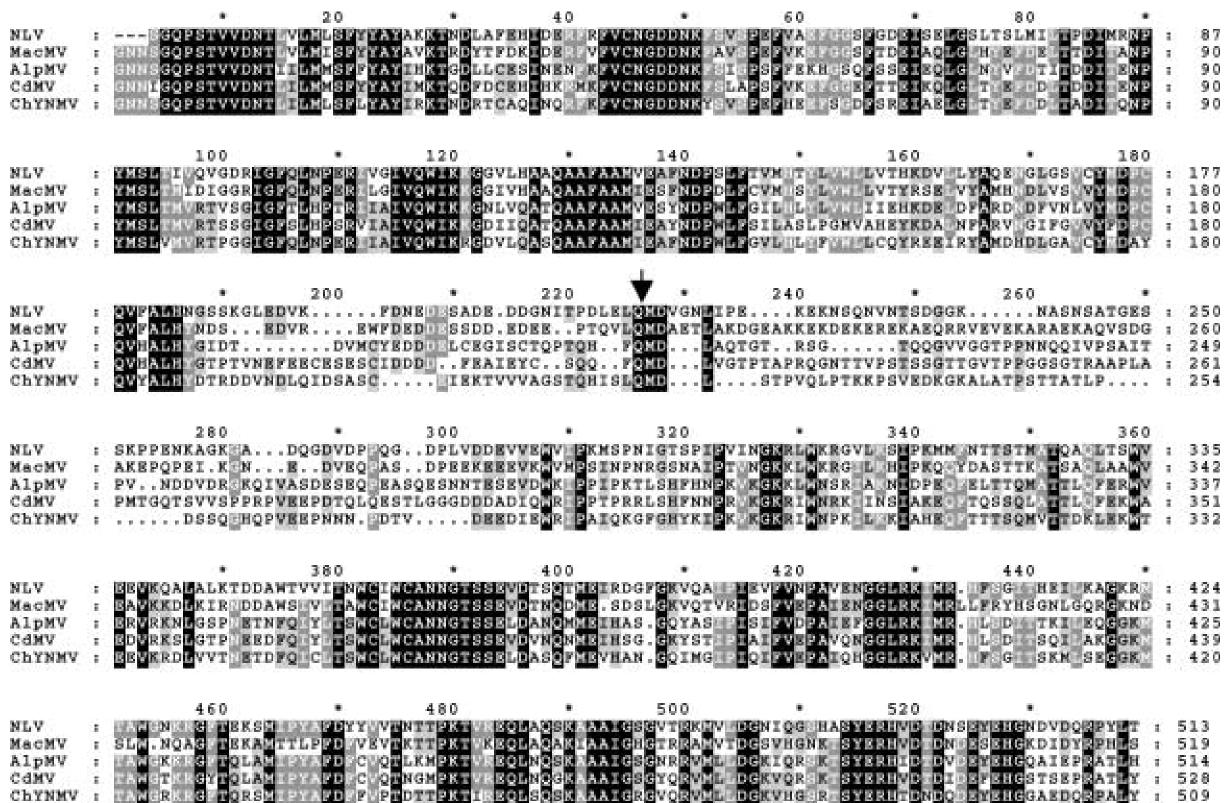

Fig. 2. Alignment of the partial ORFs of NLV (U58770), MacMV (U58771), CdMV (AF189125), ChYNMV (AB044386), and AlpMV (AF499025) by CLUSTAL W. The predicted NIa cleavage site is indicated with an arrow

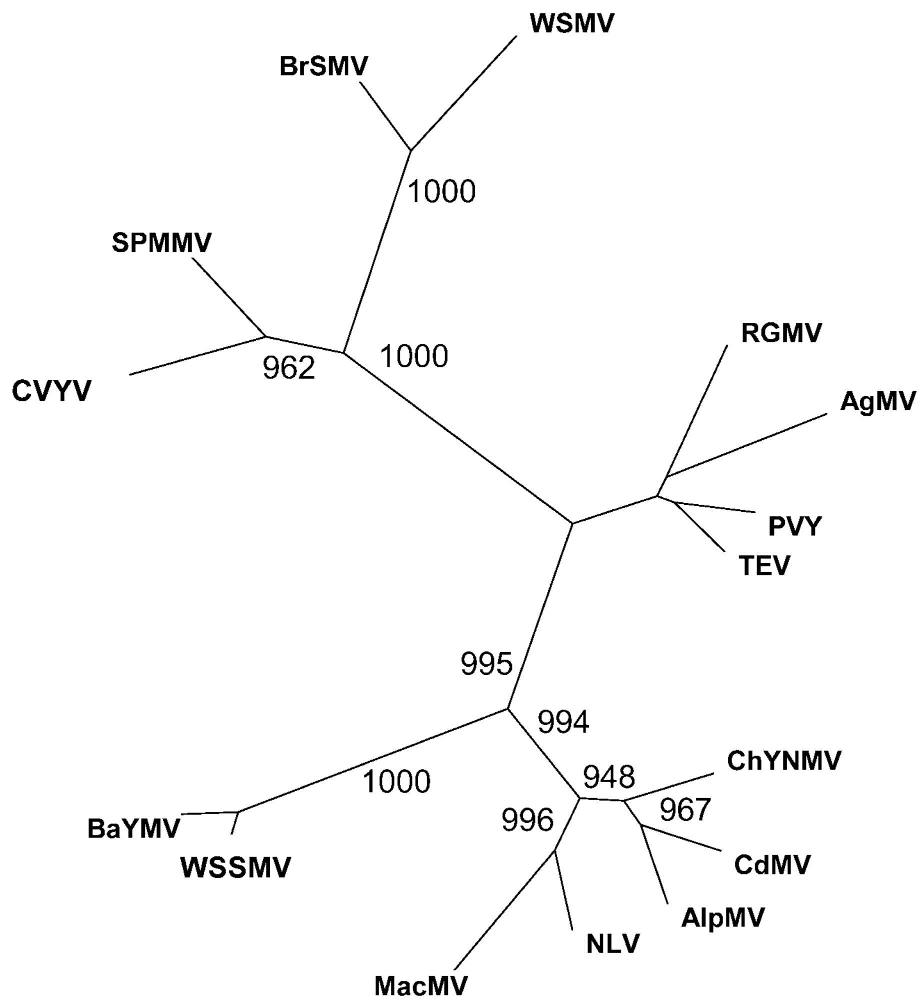

Fig. 3. Phylogenetic tree family for the amino acid sequences of the coat protein of AlpMV and selected members of the *Potyviridae* (Table 1). The tree was constructed using FITCH in PHYLIP. The values at the fork indicate the number of times out of 1000 trees that this grouping occurred after bootstrapping the data

other amino acid residues [6]. The deduced amino acid sequence of AlpMV CP had an estimated molecular mass of 33.4 KDa, whereas gel analysis of CP from purified virus suggested a molecular mass of 41 KDa [5]. A similar discrepancy has been observed in NLV (32.8 KDa *vs.* 39.5 KDa) and MacMV (34.1 KDa *vs.* 40 KDa), and may be an artifact of the SDS-PAGE procedure [3].

As shown in Fig. 2, the C-terminus of viral CP is comparatively more conserved than the N-terminal portion. A tripeptide motif, 'DAG', is highly conserved within a heptapeptide block at or near the N terminus of the coat protein of aphid-transmitted potyviruses [1, 2]. Neither 'DAG' nor motifs similar to 'DAG' were found near the N terminus of the viral CP analyzed, although a 'DKG' tripeptide was found further downstream in the ChYNMV CP [14], and a 'DRG' was found in the AlpMV CP. The sequence 'NGTS', highly conserved in almost all potyviruses [17], was found in the context of 'WCANNGTSSE' in all five viruses (position 362–371 of AlpMV) (Fig. 2). Two highly conserved amino acid residues known to participate in virion assembly in potyvirids [13], R and D, were found in the sequence of AlpMV (R^{405} and D^{445}) as well as other viral sequences analyzed. The sequence downstream of D^{445} of AlpMV is also highly conserved among the macluraviruses. The significance of this conservation awaits further investigation.

To investigate the relationship of AlpMV with other members of the *Potyviridae*, the deduced amino acid sequence of its coat protein was compared with those of 14 selected members of the family by GAP (GCG). The CP of AlpMV was $42.9 \sim 61.9\%$ identical to that of members of the genus *Macluravirus*, but only $22.5 \sim 30.9\%$ identical to members of other genera (Table 1). According to Shukla et al. [17], distinct species within the genus *Potyvirus* have coat protein sequence identity in the range of $38 \sim 71\%$. Therefore, it is not appropriate to classify AlpMV as a species of the genus *Potyvirus*. Phylogenetic analyses of the coat protein amino acid sequences showed six clusters corresponding to the recognized genera and placed AlpMV in the same cluster as CdMV and ChYNMV. This demonstrates clearly that AlpMV could be recognized as a tentative member within the genus *Macluravirus*. As shown above, the 'DAG' motif essential for aphid transmission in the genus *Potyvirus* is not found in the CPs of macluraviruses, although similar motifs may be found downstream. Mutational analysis of CP or sequencing of the viral genome may provide information in regard to the mechanism of aphid transmission in the genus *Macluravirus*.

GenBank accession number

The nucleotide sequence obtained was deposited in GenBank with the accession number AF499025.

Acknowledgement

We thank Dr. T. H. Chen for supplying the polyclonal antibody against AlpMV, and Dr. Y. C. Chang for the potyvirus universal primer.

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