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Short Communication

Amaranthus leaf mottle virus: 3′-end RNA sequence proves classification as distinct virus and reveals affinities within the genus *Potyvirus*

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Abstract

Amaranthus leaf mottle virus (AmLMV) was classified as a member of the genus *Potyvirus* on the basis of its particle morphology, serology, and biological properties (Casetta et al., 1986). Based on these properties, an *Amaranthus viridis*-infecting virus isolated in Spain, causing mottle and leaf blistering as well as reduced growth has been identified as AmLMV. The 3′ terminal genomic region of this and a reference isolate from Italy has been sequenced and reveals a 95% nucleotide identity between the two isolates. The sequenced part comprises the coat protein with 281 amino acids and 315 nucleotides of the 3′ untranslated region (UTR) preceding a polyadenylated tail. Pairwise comparisons and phylogenetic analysis of the nucleotide and deduced amino acid sequences of the CP and 3′ UTR of the cloned cDNAs with those of other potyviruses shows that AmLMV is a distinct potyvirus closely related to *Potato virus Y*.

Amaranthus leaf mottle virus (AmLMV) was described as a potyvirus (genus *Potyvirus*, family *Potyviridae*), serologically related to *Bean yellow mosaic virus* and *Plum pox virus*, and distantly related to *Peanut mottle virus* and *Zucchini yellow mosaic virus* (Rajeshwari et al., 1983; Lisa and Lecoq, 1984). Isolates of the virus from Italy, Spain, and Morocco have been described (Lovisolo and Lisa, 1976, 1979; Casetta et al., 1986) and have been found so far only in *Amaranthus deflexus* and *Cirsium arvense*. AmLMV has been transmitted by *Myzus persicae* and *Macrosiphum euphorbiae* (Lisa and Lecoq, 1984; Casetta et al., 1986).

In this paper we describe a new isolate of AmLMV from the host plant *Amaranthus viridis* in Almeria, Spain (AmLMV-AL). It was experimentally transmitted by *Aphis gossypii* and identified according to its biological and serological properties. Its taxonomic relationships were confirmed by comparing the CP coding regions and the entire 3′-UTR of the genomic RNA with those of the Italian AmLMV isolate. This is the first report based on the nucleotide sequence to establish that this virus is a distinct species, elucidating its phylogenetic relationship with other potyviruses.

Symptomatic wild *A. viridis* plants collected in the south-east of Spain exhibited leaf mottle, leaf blistering and growth reduction. Two diseased *A. viridis* plants isolated from different fields reacted positive using commercial monoclonal antibodies against general potyviruses by plate trapped antigen (PTA) ELISA (AGDIA PSA 272) and were negative with antisera against viruses known to occur in the region: *Potato virus Y* (PVY), *Turnip mosaic virus* (TuMV), *Southern bean mosaic virus*

(SBMV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato mosaic virus* (ToMV) and *Tomato spotted wilt virus* (TSWV). A local-lesion isolate was obtained by three serial passages on *Chenopodium amaranticolor* and was maintained in *Chenopodium quinoa* for subsequent analyses.

The host range of the Spanish isolate was determined by mechanical inoculation using potassium phosphate buffer (0.03 mM, pH 8.0). Inoculated plants of *A. viridis* resembled disease of field plants. *Chenopodium quinoa* exhibited chlorotic local lesions as well as systemic severe mosaic, leaf deformation, wilting, stunting and finally collapse of the plants. *Chenopodium amaranticolor* showed initial large local chlorotic lesions that became necrotic and were surrounded by red tissue. *Amaranthus deflexus*, *Capsicum annuum*, *Citrullus lanatus*, *Cucumis melo*, *Cucurbita pepo*, *Datura stramonium*, *Gomphrena globosa*, *Lactuca sativa*, *Lycopersicon esculentum*, *Nicotiana benthamiana*, *N. clevelandii*, *N. tabacum* cv. Samsun, *N. tabacum* cv. Xanthi, *Phaseolus vulgaris* cv. Donna, *P. vulgaris* cv. Pinto, *Pisum sativum*, *Solanum demissum*, *S. melongena*, *S. muricatum* and *Vicia faba* developed no symptoms. Leaves of healthy controls and inoculated plants were tested by PTA-ELISA. *Amaranthus viridis* and *C. quinoa* were positives and only in *G. globosa* and *A. deflexus* were latent potyviral infections detected by serological tests.

Vector transmission experiments were carried out using adult *Aphis gossypii*, which were starved for 8 h prior to a day of acquisition access period on infected *C. quinoa* plants. Then approximately 30 aphids were placed on three healthy *A. viridis* and *C. quinoa* plants for a 1-day inoculation access period, after which the insects were killed by an insecticide. The assay was repeated twice. All plants showed symptoms 2–3 weeks after transmission. The infection was confirmed by RT-PCR with the AmLMV specific primers Lta1 and Lts1 (see below).

Electron microscopy was applied for further detection and identification of the *A. viridis* isolate. Virus particles in crude plant extracts in 0.1 M phosphate buffer pH 7.0 (PB) were adsorbed to Pioloform-carbon coated grids floating on drops of extract for 5 min, and were visualized after negative staining with 1% (w/v) uranyl-acetate in a Zeiss EM 906 E electron microscope (LEO, Oberkochen, Germany). Negatively stained crude extracts of infected *C. quinoa* revealed a moderate concentration of flexuous filamentous particles, with a predominant length of about 800 nm and without an obvious cross-banding fine structure. This morphology is typical for potyviruses. Since the virus caused systemic infections in *C. quinoa*, immunoelectron microscopical decoration tests (Milne, 1984) were performed with antisera to detect those potyviruses known to infect this plant systemically: AmLMV, *Beet mosaic virus, Bidens mottle virus, Carnation vein mottle virus, Clover yellow vein virus, Lettuce mosaic virus* and *Watermelon mosaic virus*. Antisera to these potyviruses were available from the stock of the EM laboratory in BBA, Braunschweig, Germany. Antiserum to AmLMV was kindly donated by E. Luisoni (Istituto di Fitovirologia applicata CNR, Torino, Italy). The isolate was specifically and strongly decorated only by antiserum to AmLMV. As reference for comparisons, the Italian isolate of AmLMV PV-0363 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany.

The AmLMV antiserum with the homologous AmLMV isolate from Italy and with the Spanish isolate exhibited decoration titers of 1:400 and 1:200, respectively. This difference of one dilution step is considered to be within the range of variability. The titers thus indicate a close serological relation between the two isolates. For this reason the Spanish isolate has now been designated AmLMV-AL.

Ultrathin sections cut from leaf tissues (Koenig and Lesemann, 1985) of AmLMV-AL infected *C. quinoa* revealed cytopathological features in many cells. Large accumulations of cytoplasmic material often protruded into the vacuoles and contained mainly aggregated potyvirus-typical cylindrical cytoplasmic inclusions (CI) in modifications consisting of pinwheel (pw) and scroll (scr) structures if seen in cross-sections of CI (Figure 1). This pw modification of CI places AmLMV-AL in section I of CI-producing potyviruses as classified by Edwardson (1974), and closely resembles the pattern induced by an AmLMV isolate from *Cirsium* in Italy (Casetta et al., 1986). No other specific cytoplasmic or nuclear alterations could be recognized.

A universal PCR primer pair (Poty-1 and Poty-2), derived from conserved regions of the 3′-end region of the genome of the *Potyviridae* family (Gibbs and Mackenzie, 1997), was used to deter-

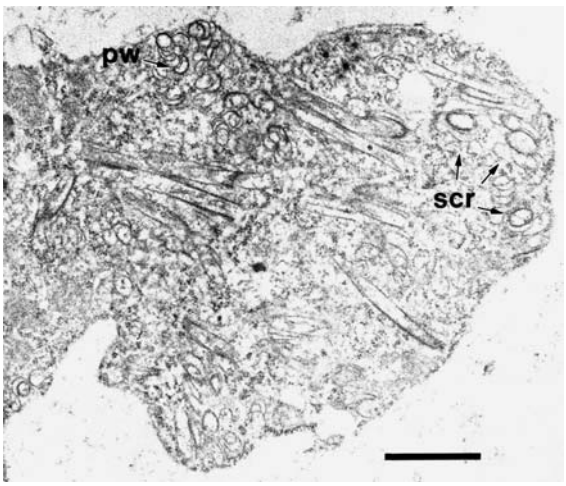

Figure 1. Ultra-thin section of AmLMV-infected *Chenopodium quinoa* leaf parenchyma cell showing a large cytoplasmic inclusion body protruding into the cell vacuole. The inclusion represents an aggregate of many cylindrical inclusions cut in various orientations. Cross-wise cut pinwheel inclusions (pw) and scroll structures (scr) allow us to classify AmLMV as a member of subdivision-I of cylindrical inclusion morphology. The bar represents 1 μm.

mine the first nucleotide sequence of AmLMV. Total RNA was extracted with chloroform–phenol (1:1) and nucleic acids precipitated by ethanol (Sambrook et al., 1989) from leaf homogenates in Tris-EDTA (pH 8.0) (0.1 g ml^{-1}) AmLMV PV-0363 and AmLMV-AL infected plants. First-strand cDNA was synthesised from total plant nucleic acid extract using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and primer Poty-1. PCR for cDNA synthesis was carried out with primers Poty-1 and Poty-2. RT-PCR amplification produced two fragments of 1.8 kb and 0.8 kb (data not shown). Amplicons were purified with a Wizard PCR Purification Kit (Promega, Madison, WI), cloned into the pGEM-T Easy vector (Promega), and sequenced in both senses with an ABI PRISM 377 DNA Sequencer.

The nucleotide sequence of the 0.8 kb fragment was identical to a homologous region contained within the 1.8 kb amplicon. Subsequent analysis revealed that the oligo d(T) (Poty-1) primer, paired to an adenine-rich region within the coat protein coding sequence of the virus, generated a 0.8 kb fragment similar to what has previously been described for some potyviruses (Gibbs and Mackenzie, 1997; Monger et al., 2001). GenBank accession numbers AJ580021 and AJ580095 were assigned to the two nt sequence data reported in this paper, corresponding to AmLMV-AL and AmLMV PV0363, respectively.

The cDNAs amplified from AmLMV-AL and AmLMV PV0363 were 1777 nt long (excluding primers). The two isolates were 95% and 96% identical at nucleotide and amino acid levels, respectively. Sequence analysis of the two isolates revealed an uninterrupted reading frame of 1461 nt (487 aa) that included part of the NIb and the complete CP coding regions followed by a UTR of 315 nt. A CLUSTAL W multiple alignments (Thompson et al., 1994) of deduced amino acid sequences between AmLMV and other potyvirus sequences was made. The putative NIb-CP cleavage site was found between amino acid positions 206–207 which is the dipeptide Q:A, and the sequence surrounding the cleavage site (EVHHQ:A) was similar to the corresponding consensus motif found for potyviruses (Chen et al., 2001). The putative Core-CP region, targeted by trypsin-like protease digestion (Shukla and Ward, 1989; Kim et al., 1998), was identified between aa D^{253} and R^{468}. Consensus sequence motifs STVVDNS and GDD, the latter proposed as the active site of the RNA dependent RNA-polymerase (Colinet et al., 1998), were found on the putative NIb protein sequence between aa positions 1 and 36. The putative CP coding region contained the consensus sequence motifs MVWCIENGTSPNING (Jacob and Usha, 2001), AFDF and QMKAAAL (Dujovny et al., 2000) at aa positions 129, 213 and 233, all downstream from the cleavage site. Sequence analysis also revealed the presence of two amino acids, R^{377} and D^{421}, which are highly conserved among potyviruses in the coat protein core, and they are known to be involved in virion assembly (Jacquet et al., 1998).

Although we were able to transmit AmLMV-AL by aphids, we did not detect the DAG motif (also involved in vector transmission; Atreya et al., 1990; Harrison and Robinson, 1988) downstream of the putative NIb/CP cleavage site. It has been reported that the DAG sequence might be lost after mechanical transmission (Husted et al., 1994). So we tested for DAG in the sequences of several other isolates of AmLMV from naturally infected *A. viridis* plants and from the reference strain PV-0363. After producing new RT-PCRs fragments with primers Lts1, 5′-TAGACGA

CGACATGGAATGTGAACAA-3′ (nt 575–600) and Lta1 (5′-TCTGTGTTTTCCTCTTGTG-TACT-3′ (nt 1378–1400) we found no DAG in any of the isolates. Therefore we can discard the idea that mechanical transmission is the cause of the loss of this particular DAG motif. In contrast, the genomic region contained DAT- and QAG-like sequences, and there are several reports describing aphid-transmissible potyviruses that contain motifs other than DAG. Also, the molecular context is known to be important in aphid transmission efficiency, even with motifs different from DAG (Husted et al., 1994; Kimalov et al., 2004; Dombrovsky et al., 2005).

We used the complete nt and predicted aa sequences of the NIb-CP encoding regions and also the 3′-UTR sequence from AmLMV to make a BLASTN and BLASTP search (Altschul et al., 1997), which revealed that AmLMV has a close relationship with species belonging to the PVY cluster (Adams et al., 2005), i.e. *Potato virus Y* (PVY), *Pepper severe mosaic virus* (PepSMV), *Pepper mottle virus* (PepMoV), Sunflower chlorotic mottle virus (SuCMoV) (this virus has been listed as a strain of PVY or as PVY synonym in the 8th ICTV report; Berger et al., 2005), *Pepper yellow mosaic virus* (PepYMV), *Potato virus V* (PVV), *Wild potato mosaic virus* (WPMV), *Peru tomato virus* (PTV), *Alstroemeria mosaic virus* (AlsMV) and *Amazon lily mosaic virus* (ALiMV). After performing multiple sequence alignments, comparison of the coat protein amino acid sequence from these potyviruses revealed that the identity percentages among the closest potyvirus species varied from 66.7 to 71.0% (Table 1), and that the CP nt varied from 63.1 to 67.4. These comparisons show that all the closest potyvirus species belong to the PVY cluster. Analysis of the coat protein core showed that the amino acid identities of the closest viruses ranged between 76.8 and 83.3%, reflecting a highly divergent N-terminal region of the CP. Comparison of the aa sequence of the C-terminal extreme of NIb showed identity values similar to the CP (data not shown). However, comparison of the 3′-UTR nt sequence to the closely related viruses produced percentage identity values of 26.5–49.2%. Low identity values were also found with other less related potyvirus species used for comparison, such as *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus* (LMV) and *Potato virus A* (PVA) (Table 1).

Table 1. Percent identity between the nt and aa coat protein (CP), core-CP and the 3′ untranslated region (3′-UTR) of AmLMV-AL (AJ580021) and those of other potyviruses

Virus species	Accession numbers	CP		Core CP		3′-UTR
		aa	nt	aa	nt	nt
AmLMV PV-0363	AJ580095	96.0	94.4	96.0	95.5	95.8
SuCMoV	AF255677	69.6	66.0	81.0	72.9	45.5
PVY	D00441	69.2	66.9	80.5	75.3	49.2
PepSMV	X66027	71.0	67.4	83.3	75.7	28.6
PepMoV	M96425	68.9	66.3	79.6	73.3	33.1
PTV	AJ437280	68.7	64.9	80.0	73.6	27.0
PVV	X61279	69.1	65.1	79.1	72.8	27.1
WPMV	AJ437279	68.0	63.8	79.1	72.0	26.9
PepYMV	AF348610	69.0	64.4	78.7	71.6	36.0
ALiMV	AB158523	67.1	63.8	76.8	70.8	34.7
AlsMV	AB158522	66.7	63.1	77.7	71.6	26.5
PVA	Z21670	54.7	53.9	65.2	61.0	19.2
LMV	X65652	59.2	61.7	69.9	64.5	22.7
TuMV	AF185963	57.0	58.4	71.7	65.8	21.3

Potato virus Y (PVY), *Pepper severe mosaic virus* (PepSMV), *Sunflower chlorotic mottle virus* (SuCMoV), *Pepper mottle virus* (PepMoV), *Pepper yellow mosaic virus* (PepYMV), *Potato virus V* (PVV), *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus* (LMV), *Potato virus A* (PVA), *Amazon lily mosaic virus* (ALiMV), *Alstroemeria mosaic virus* (AlsMV), *Wild potato mosaic virus* (WPMV), *Peru tomato virus* (PTV). The identities were calculated with the BioEdit 5.0.9 programme on multiple alignments carried out with Clustal W.

Although pairwise identity comparisons (Table 1) of the CP and UTR sequences of AmLMV with the other 13 potyviruses exhibit relatively high values, AmLMV should be considered a different species. This conclusion is based on the range of identity percentage found by Adams et al. (2005) (from 13.2% to 88.6% for aa CP, 35.6–81.1% for nt CP and 30.9–84% for UTR), and on the criterion for species demarcation proposed: 76–77% nt identity. For further discrimination between virus and strain, CP-core amino acid sequence identities of 55–75% are believed to indicate differences in potyvirus species; 74–88% identities reflect groups of closely related species, while 90 to 99% identities correspond to strains of a given potyvirus (Ward and Shukla, 1991; Shukla et al., 1994). Therefore our data (Table 1) indicate that AmLMV is a species belonging to the PVY group.

The alignment was used to construct phylogenetic trees from the aligned whole CP aa sequences of the potyviruses, using the Neighbour-joining method (Saitou and Nei, 1987) and a bootstrap

with 1000 replicas in Mega 2.1 (Kumar et al., 2001). The alignment placed AmLMV in the PVY cluster, and established its differentiation from the subgroup composed by WPMV, PVV and PTV (Figure 2; Spetz et al., 2003). Trees constructed by Maximum Parsimony analysis and the analysis of CP nt and 3′-UTR gave the same position to AmLMV.

Viruses of the PVY cluster are closely phylogenetically related, and have a common ancestor (Spetz et al., 2003). Members from this aphid-transmitted virus group generally infect plants of the *Solanaceae* (order *Solanales*), but recently other natural hosts have been discovered, such as those belonging to the *Alstroemeriaceae (Liliales)* (Fuji et al., 2004), *Amaryllidaceae (Asparagales)* (Fuji et al., 2004), and *Asteraceae (Asterales)* (Dujovny et al., 1998). And now our research shows that the *Amaranthaceae (Caryophyllales)* is also a natural host. Interestingly, all these plants originate from South America. AmLMV, however, is widespread in the Mediterranean region (North-Africa and Europe) and was not detected in a preliminary survey in Brasil (Lovisolo and Lisa, 1979).

The host range of the Almeria isolate (AmLMV-AL) appears to be more restricted than those of other reported AmLMV isolates (Lovisolo and Lisa, 1976; Casetta et al., 1986), since only 3 of 20 putative host species became infected after mechanical inoculation (i.e. AmLMV-AL could not be transmitted to *L. sativa*, *P. vulgaris*, *V. faba*, *N. clevelandii* and *N. tabacum*). It is not clear whether these differences in host range are due to the cultivars of test plants used in this and previous studies (Lovisolo and Lisa, 1976; Casetta et al., 1986), or to differences among virus isolates. Since the identity values of 95% at the nt level, and 96% at the aa level among the two isolates studied suggest possible CP differences, and since virion properties such as antigenicity and long distance movement are supposed to be influenced by their molecular determinants in the N-termini of the CP, the host range differences might be caused by genetic diversity of the CP (Ward and Shukla, 1991; Lehmann et al., 1997).

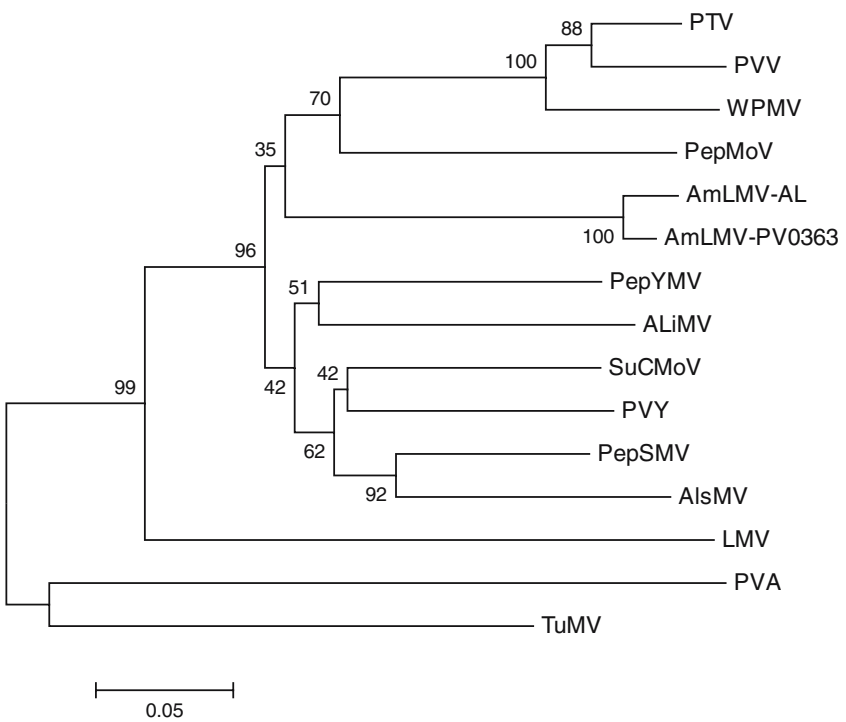

Figure 2. Phylogenetic relationships among PVY-related potyviruses, based on multiple alignment of the derived amino acid sequences of the complete CP coding region. The tree was obtained by the neighbour-joining method, with the potyviruses *Turnip mosaic virus* (TuMV), *Potato virus A* (PVA) and *Lettuce mosaic virus* (LMV) defined as the outgroups. The tree was bootstrapped using 1000 replications. The bar represents 0.05 amino acid changes per site.

Although AmLMV has been described only in wild plants, its host range and the transmission by aphids suggests that it may be a potential pathogen for susceptible crops, mainly in the families to which this virus is restricted, *Leguminosae* (*Lupinus albus* and *Trigonella foenum-graecum*), *Chenopodiaceae* (*Atriplex hortensis*, *Spinacea oleracea*, and *C. quinoa*), and *Amaranthaceae* (*Gomphrena globosa, Amaranthus caudatus*, *A. hybridus*, *A. tricolor*, *A. cruentus* and *A. hypochondriacus*) (Lovisolo and Lisa, 1979; Casetta et al., 1986). Some of these are ancient South-American crops that are now considered to be new crops (Sleugh et al., 2001; Bavec and Mlakac, 2002; Mason et al., 2005).

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