MOLECULAR VARIABILITY OF *APPLE CHLOROTIC LEAF SPOT VIRUS* IN DIFFERENT HOSTS AND GEOGRAPHICAL REGIONS

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SUMMARY

A fragment of 500 nt corresponding to 85% of the coat protein (CP) gene of 35 isolates of *Apple chlorotic leaf spot virus* (ACLSV) from different hosts and geographical areas were sequenced and the results were compared with those already available. Sequence alignments at the amino acid level showed that most of the variability was present in the N-terminal part of the CP cistron (overlapping with the movement protein) whereas the C-terminus was significantly less divergent. Four isolates (APR-EA5, PE 150, PE 154 and PE 297) differed in showing high variability throughout the CP gene. Phylogenetic analysis at the nucleotide and amino acid level clustered the isolates in two groups: A, containing the great majority of the isolates, and B, containing the above four diverging isolates. A few subclusters could be identified in group A: pome fruit isolates clustered together in two different groups, one being close to the two almond isolates and one subcluster containing all the Spanish isolates. One subgroup was composed of three similar sequences each obtained from different hosts (peach, apricot, plum) originating from three different countries, respectively (Italy, Lebanon and Jordan). In Western blot analysis, three different migration rates were found for the CPs of ten representative isolates. No correlation was observed between the electrophoretic mobility of CPs and the phylogenetic groups, indicating that other factors besides the primary structure must account for the different electrophoretic mobilities observed.

Keywords: ACLSV, RT-PCR, western blot, fruit trees.

INTRODUCTION

Apple chlorotic leaf spot virus (ACLSV) is the type species of the genus *Trichovirus* (Martelli *et al.*, 1994). The severity of symptoms elicited by ACLSV depends largely on plant species and virus strains (Németh, 1986). Some virulent strains cause symptoms on fruits of apricot ("butteratura" or "viruela"), peach, and cherry (Ragozzino and Pugliano, 1974; Peña-Iglesias and Ayuso Gonzales, 1975; Cañizares *et al.*, 2001), bark split and pseudopox in some plum cultivars (Dunez *et al.*, 1972), and graft incompatibility in apricot (Desvignes and Boyé, 1989). The virus is symptomless in most apple cultivars, but in sensitive varieties malformation and reduction in leaf size, and chlorotic rings or line patterns are common (Németh, 1986). ACLSV virions are flexuous filaments, 640-760 x 12 nm in size. The complete nucleotide sequences of ACLSV isolates from plum, apple and cherry have been determined (German *et al.*, 1990; Sato *et al.*, 1993; Jelkmann, 1996; German *et al.*, 1997). The genome consists of one positive-sense, single-stranded RNA 7545-7555 nt in size excluding the poly-A tail. It contains three open reading frames encoding, respectively, a protein with molecular mass of 216.5 kDa (ORF1) involved in genome replication, a 50.4 kDa movement protein (ORF2), and the 21.4 kDa coat protein (CP) (ORF3).

Previous reports have shown that ACLSV genomes show high variability, differing by from 10 to 20% among different isolates (Candresse *et al.*, 1995; Pasquini *et al.*, 1998; Krizbai *et al.*, 2001) originating from Italy, France, Poland, Germany and Hungary. Thus it was interesting to know if this situation was maintained in other Mediterranean countries (Albania, Jordan, Lebanon, Turkey, Spain and Italy), and the Far East (China), from where no information regarding genetic diversity of the virus was available. In addition, Pasquini *et al.* (1998), on the basis of the electrophoretic mobility of the CPs, classified Italian ACLSV isolates in three main groups with apparent Mr of 22.7 kDa, 21.5 kDa and 19.7 kDa, respectively. Here, we investigated the phylogenetic relationships between 35 ACLSV isolates from different host species and geographical origins, and the electrophoretic mobility of the CPs of ten representative isolates was determined.

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MATERIALS AND METHODS

Virus isolates. The ACLSV isolates used in this study were from stone and pome fruit accessions from collections of the University of Bari and the Mediterranean Agronomic Institute - Bari (Italy) (Table 1). All isolates were mechanically transmitted to *Chenopodium quinoa* and/or *Nicotiana occidentalis* by using an extract of infected leaf tissue made in 0.05 M phosphate buffer pH 7.4 containing 2.5% nicotine.

Table 1. ACLSV isolates used in the study, and their EMBL Database accession numbers.

Isolate	Host	Origin	Accession no.
AL 19	Almond	Italy	AJ586621
ALF5	Almond	Italy	AJ586628
APR103	Apricot	Spain	AJ586622
APR109	Apricot	Spain	AJ586623
APR110	Apricot	Spain	AJ586629
APR60	Apricot	Spain	AJ586632
APR61	Apricot	Spain	AJ586633
APR62	Apricot	Spain	AJ586634
APR63	Apricot	Italy	AJ586635
APR20	Apricot	Italy	AJ586630
APR3	Apricot	Jordan	AJ586631
APR-EA5	Apricot	Turkey	AJ586636
AP119	Apple	Albania	AJ586637
AP139	Apple	Albania	AJ586638
AP54	Apple	Albania	AJ586624
AP62	Apple	Albania	AJ586639
AP76	Apple	Albania	AJ586625
AP93	Apple	Albania	AJ586626
AP-2	Apple	Italy	AJ586642
AP-CI	Apple	China	AJ586640
AP-T	Apple	Turkey	AJ586641
PEA-CAL	Pear	Italy	AJ586643
PE154	Peach	Hungary	AJ586650
PE118D	Peach	Hungary	AJ586646
PE 56	Peach	Italy	AJ586652
PE-FC	Peach	Italy	AJ586644
PE 150	Peach	Italy	AJ586645
PE 151	Peach	Lebanon	AJ586647
PE 152	Peach	Lebanon	AJ586648
PE153	Peach	Lebanon	AJ586649
PE297	Peach	Jordan	AJ586651
PL111	Plum	Jordan	AJ586654
PL110	Plum	Italy	AJ586653
PL112	Plum	Italy	AJ586655
PL45	Plum	Italy	AJ586627

Primers. All isolates were analysed by RT-PCR using a pair of primers described by Menzel *et al.* (2002) that amplify a 677 bp fragment overlapping the movement and CP genes, between nt 6860 (primer ACLSV-s 5'TTCATGGAAAGACGGGGCAA3') and 7536 (primer ACLSV-as 5'AAGTCTACAGGCTATTTATTA TAAGTCTAA3') (on ACLSV with accession number D 14996).

Nucleic acid extraction. Total nucleic acids were extracted as described by Menzel *et al.* (2002) from 0.5 g of leaf tissue homogenized in 1 ml grinding buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate pH 5.2, 25 mM EDTA, 1 M potassium acetate and 2.5% PVP-40). Five hundred µl of the homogenate were transferred to a sterile Eppendorf tube to which 100 µl of 10% SDS were added. The mixture was incubated at 70ºC for 10 min with intermittent shaking, and placed on ice for 5 min. After centrifugation at 13,000 rpm for 10 min, 300 µl of the supernatant fraction were transferred to a new tube and 300 µl of 6 M sodium iodide solution, stabilized by 0.15 M sodium sulphate, 150 µl ethanol (99.6%) and 25 µl silica-suspension (pH 2) were added. The mixture was incubated at room temperature for 10 min with intermittent shaking and then centrifuged at 6,000 rpm for 1 min. The pellet was washed twice with 500 µl wash buffer (10 mM Tris HCl, pH 7.5, 0.05 mM EDTA, 50 mM NaCl, and 50% ethanol), left to dry at room temperature, resuspended in 150 µl TE buffer (10 mM Tris HCl, pH 7.5, 0.1 mM EDTA), and incubated at 70°C for 4 min. After a final centrifugation for 5 min at 13,000 rpm, the supernatant fraction was transferred to a new tube and stored at -20ºC.

Amplification. One-step RT-PCR was done as follows: RT-PCR mixture, made of 2.5 µl 10xPCR buffer [10 mM Tris HCl, pH 8.9, 50 mM KCl, 0.3% Triton X-100 (w/v) (Promega, Madison, WI, USA], 2 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM each), 1 µl of 10 mM of upstream and downstream primers, 0.25 units of AMV-RT (Promega, Madison, WI, USA), 0.5 units of Taq DNA polymerase (Promega, Madison, WI, USA) and sterile water to a final volume of 23 µl, was added directly to tubes containing 2 µl RNA sample. cDNA synthesis and amplification were carried out at 42°C for 50 min followed by a denaturation step at 93°C for 2 min, 35 cycles of amplification (94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min), and a final extension for 7 min at 72°C. Ten µl of the PCR products were analysed by electrophoresis in 1.2% agarose gels in TAE buffer, and stained with ethidium bromide.

Cloning and sequence analysis. Amplified products were purified with QIAquick PCR purification Kit (Invitrogen Life Technologies, Carlsbad, CA, USA), and cloned in pGEMT Easy vector (Promega, Madison, WI,

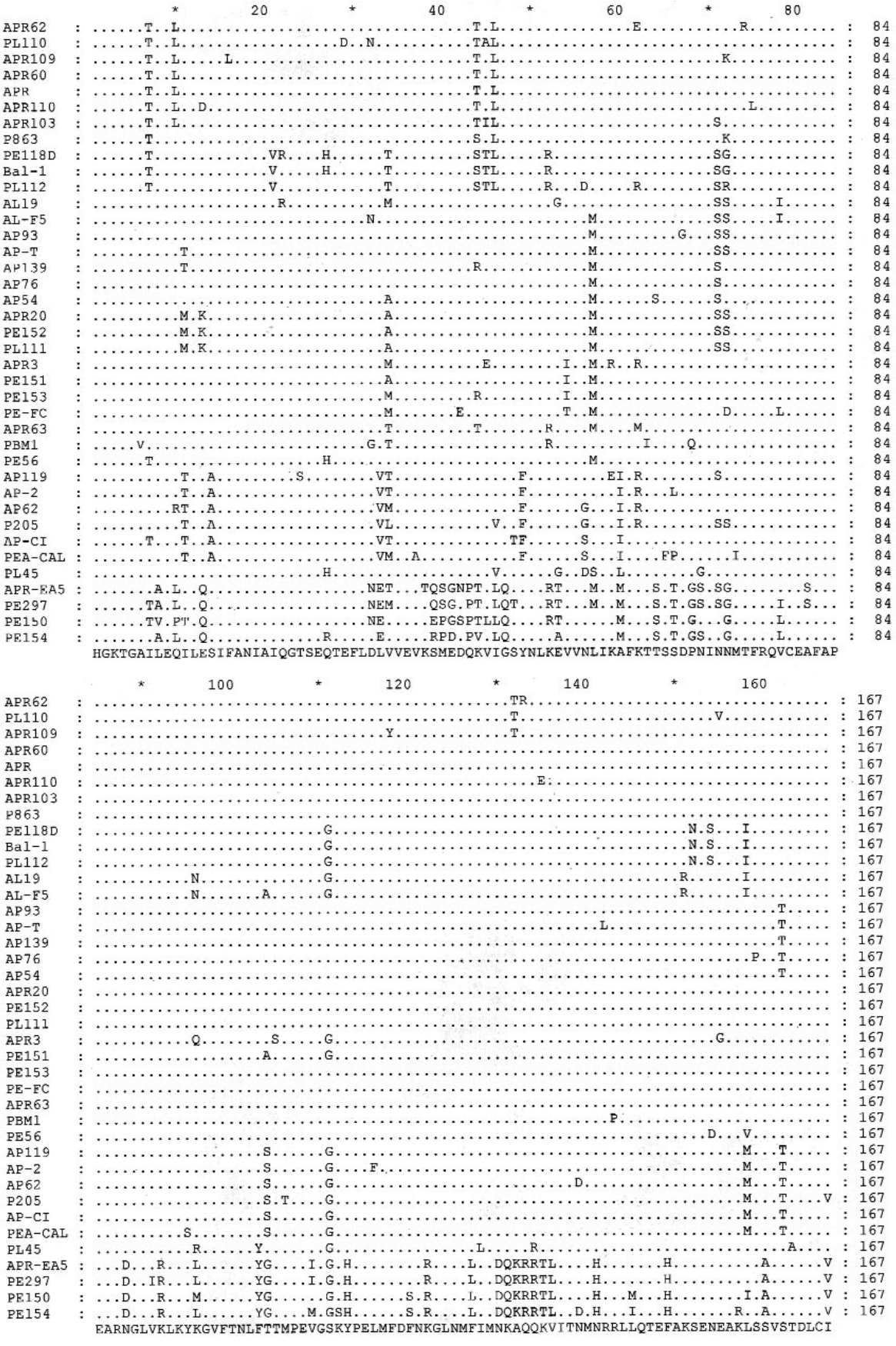

Fig. 1. Amino acid sequence alignments for the sequenced part of the CP gene of different ACLSV isolates. Previously published sequences are included: PBM1, P-863, P205 and Bal-1 (accession numbers AJ243438, M58152, D14996 and X99752, respectively).

USA). cDNA sequences of the recombinant plasmid were obtained by automatic sequencing (MWG Biotech, Ebersberg, Germany). Multiple alignments of nucleotide and amino acid sequences of ACLSV CP were obtained using the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment programme. Phylogenetic analysis was done using the minimum evolution method of phylogenetic inference (Rzhetsky and Nei, 1993) with 10,000 bootstrap replicates. The version 2.1 of the Molecular Evolutionary Genetics Analysis software MEGA version 2.1 was utilized (Kumar *et al.*, 2001).

Western blot. About 0.1 g leaf tissue from systemically infected *C. quinoa* or *N. occidentalis* was ground in 10 vol. of extraction buffer (Berger *et al.*, 1989) (0.5 M Tris HCl pH 8, 0.2% SDS, 40% sucrose, and 4% 2-mercaptoethanol). The homogenate was clarified by centrifugation at 3,000 rpm for 5 min. The supernatant fraction was mixed with an equal volume of Laemmli buffer (Laemmli, 1970), boiled for 5 min, and then placed on ice. Samples were analysed on 10% SDS-polyacrylamide gels (Laemmli, 1970) and electro-blotted onto nitrocellulose membranes. ACLSV CP was detected serologically using commercial ACLSV antibodies

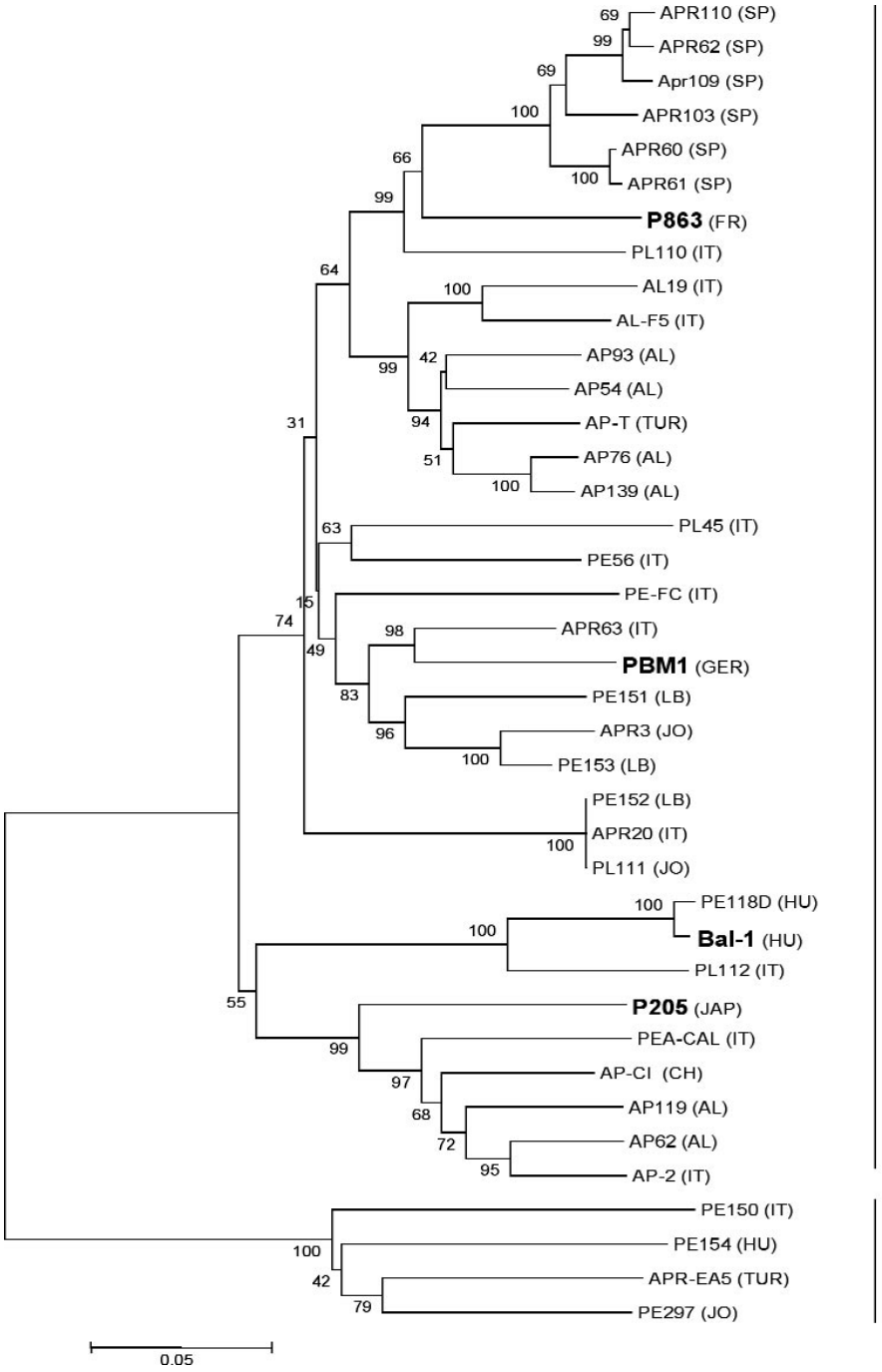

Fig. 2. Phylogenetic tree showing the relationships of the amplified product of ACLSV isolates at the nucleotide level reconstructed by the minimum evolution method with 10,000 bootstrap replicates. Previously published sequences (PBM1, P-863, P205 and Bal-1) are in bold; their corresponding accession numbers are: AJ243438, M58152, D14996 and X99752, respectively. Group A: the majority of isolates; group B: the four diverging isolates. The following abbreviations were used to indicate hosts and countries: AL, almond; APR, apricot; AP, apple; PE, peach; PEA, pear; PL, plum; AL, Albania; CH, China; FR, France; GER, Germany; HU, Hungary; IT, Italy; JAP, Japan; JO, Jordan; SP, Spain; TUR, Turkey.

(Loewe Biochemica, Sauerlach, Germany), followed by a goat anti-rabbit-AP conjugate. Alkaline phosphatase activity was detected by NBT/BCIP substrates.

RESULTS AND DISCUSSION

RT-PCR products using the primers described by Menzel *et al.* (2002) yielded the expected 677 bp fragment. One recombinant plasmid containing cDNA was selected from each isolate for sequence comparisons. Additional cDNA plasmids of some isolates were sequenced but only minor differences were found between clones from a single isolate, which did not change the amino acid sequence (data not shown). Thirty-five isolates were studied and compared with the sequences available in the GenBank database. Alignment of the amino acid sequences (Fig. 1) showed that, as already known for ACLSV (Candresse *et al.*, 1995), variability was higher in the N-terminal (overlapping movement protein) than the C-terminal part of the CP gene, except for isolates APR-EA5, PE 150, PE 154, and PE 297, which showed high variability throughout the whole CP gene.

According to the phylogenetic analysis generated using nucleotide sequences (Fig. 2) or amino acid sequences (not shown), the isolates clustered into two groups: A, containing most isolates, and B, containing the four diverging isolates. Several sub-clusters, with relatively high bootstrap values were identified in group A; one of them contained all the Spanish isolates. The high level of sequence homology observed among Spanish isolates (94-98%) can be explained by considering the source of infected plants used; all these isolates came from apricot trees grafted on 'Pollizo' plum, which is practically 100% infected by ACLSV in the Murcia Region (Llàcer *et al.*, 1985). This result is consistent with the fact that ACLSV spreads by propagation of infected material (no natural vector is known). Pome fruit isolates clustered together in two different groups, one being close to the two almond isolates. Interestingly, one of the phylogenetic subgroups was composed of three similar sequences obtained from three different hosts (peach, apricot, plum) originating from three different countries, respectively (Italy, Lebanon and Jordan).

Group B isolates were clearly separated, with a high bootstrap value, from all the others. These isolates were from different geographical areas (Turkey, Italy, Hungary and Jordan) and hosts, three from peach, and one (APR-EA5) from apricot, suggesting that this very different sequence may be restricted to stone fruits. However, more pome fruit isolates should be investigated to confirm this possibility. Due to their high level of divergence (30%) from ACLSV isolates of group A and high identity level (89-97% homology) with the virus recently described by Liberti *et al.* (2003), and proposed as a new trichovirus species, group B isolates could belong to this latter species. However, other parameters used for identifying Trichovirus species (Martelli *et al.*, 2000), in particular the presence of serological interrelationships, do not support this hypothesis (unpublished information). In any case, further work is needed to establish whether our divergent isolates belong to the proposed novel species.

Western blot analysis of ten representative isolates showed that ACLSV CPs have different migration rates, as already found by Pasquini *et al.* (1998), who noted three different CPs groups with sizes of 22.7 kDa (Group 1), 21.5 kDa (Group 2) and 19.7 kDa (Group 3). Malinowski *et al.* (1998) also reported an unusual migration rate of the CP of an isolate (SX/2) of ACLSV but were unable to correlate migration rate with differences in the amino acid sequences. It is possible, however, that differences in the migration rates are caused by differences in amino acid composition rather than size. This will determine different ratios of SDS binding to the CPs, an explanation previously put forward by Koenig *et al.* (1978) for PVX isolates. Our analysis confirms the finding of Krizbai *et al.* (2001) since the PEA-CAL isolate from pear shows a CP size of about 19.7 kDa, placing it in group 3 (see Fig. 3, lane 2).

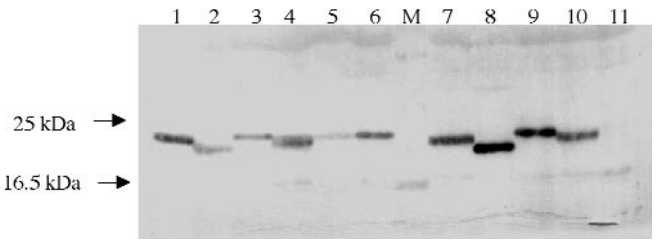

Fig. 3. Western blot analysis of total protein extracts from 10 representative ACLSV isolates. Lane 1, APR61; lane 2, PEA-CAL; lane 3, PL112; lane 4, APR3; lane 5, APR-EA5; lane 6, AP-CI; lane 7, PE150; lane 8, APR103; lane 9, AL-F5; lane 10, PE151; lane 11, healthy *Chenopodium quinoa*; lane M, prestained molecular weight marker.

The present analysis confirms the existence of variability among ACLSV isolates and provides more data supporting the importance of infected plant material in virus dissemination. The interesting clustering of pome fruit isolates having different geographic origins needs to be substantiated by additional studies.

The nucleotide sequences here reported have been assigned the GenBank accession numbers AJ586621 to AJ586655.

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