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 the 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.
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.

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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’TTCATGGAAGACGGGGCAA3’) and 7536 (primer ACLSV-as 5’AAGTCTACAGGCTATTTATTA TAAGTCTAA3’) (on ACLSV with accession D14996).

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 70ºC 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 MgCl2, 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) 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 42ºC for 50 min followed by a denaturation step at 93ºC for 2 min, 35 cycles of amplification (94ºC for 30 sec, 62ºC for 30 sec and 72ºC for 1 min), and a final extension for 7 min at 72ºC. 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.

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**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 (Llacer 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 CP 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 PECAL isolate from pear shows a CP size of about 19.7 kDa, placing it in group 3 (see Fig. 3, lane 2).

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.
ACKNOWLEDGEMENTS

This work was supported by “Centro di eccellenza: Genomica comparata - geni coinvolti in processi fisiopatologici in campo biomedico ed agrario (C.E.G.B.A.)” and PRIN 2002 (Biodiversity of Citrus and Stone Fruit Viruses and Viroids Causing Economically Relevant Diseases Project). The authors gratefully thank Prof. G.P. Martelli for careful reading of the manuscript.

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Received 3 November 2003
Accepted 28 April 2004