Characterization of Apricot pseudo-chlorotic leaf spot virus, A Novel Trichovirus Isolated from Stone Fruit Trees

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ABSTRACT

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A trichovirus closely related to *Apple chlorotic leaf spot virus* (ACLSV) was detected in symptomatic apricot and Japanese plum from Italy. The Sus2 isolate of this agent cross-reacted with anti-ACLSV polyclonal reagents but was not detected by broad-specificity anti-ACLSV monoclonal antibodies. It had particles with typical trichovirus morphology but, contrary to ACLSV, was unable to infect *Chenopodium quinoa* and *C. amaranticolor*. The sequence of its genome (7,494 nucleotides [nt], missing only ≈ 30 to 40 nt of the 5' terminal sequence) and the

partial sequence of another isolate were determined. The new virus has a genomic organization similar to that of ACLSV, with three open reading frames coding for a replication-associated protein (RNA-dependent RNA polymerase), a movement protein, and a capsid protein, respectively. However, it had only ≈ 65 to 67% nucleotide identity with sequenced isolates of ACLSV. The differences in serology, host range, genome sequence, and phylogenetic reconstructions for all viral proteins support the idea that this agent should be considered a new virus, for which the name Apricot pseudo-chlorotic leaf spot virus (APCLSV) is proposed. APCLSV shows substantial sequence variability and has been recovered from various *Prunus* sources coming from seven countries, an indication that it is likely to have a wide geographical distribution.

The genus *Trichovirus* (27) comprises four species: the type member *Apple chlorotic leaf spot virus* (ACLSV), *Cherry mottle leaf virus* (CMLV), *Peach mosaic virus* (PcMV), and *Grapevine berry inner necrosis virus* (GINV). These viruses have elongated, highly flexuous particles 640 to 760 nm long and 10 to 12 nm wide. Their genome consists of a monopartite single-stranded (ss)RNA of positive polarity, 7.6 to 8 kb long, which harbors three overlapping open reading frames (ORFs) that encode an RNA-dependent RNA polymerase (RdRp), a movement protein (MP) belonging to the 30K superfamily (28), and a 22- to 28-kDa coat protein (CP) (1). The genomes of CMLV and PcMV also encode an additional small ORF of unknown function. Trichoviruses can be transmitted by mechanical inoculation, grafting, and plant propagation. CMLV, PcMV, and probably GINV are transmitted in nature by eriophyid mites (15,17), whereas no vector is known for ACLSV. The genus *Trichovirus* is included in the recently created family *Flexiviridae* (1), together with other viruses with flexuous, elongated viral particles and phylogenetically related, undivided ssRNA genomes belonging to the genera *Allexivirus*, *Capillovirus*, *Carlavirus*, *Foveavirus*, *Mandarivirus*, *Potexvirus*, and *Vitivirus*, as well as some viruses unassigned to any genus

(1). The recent creation of the family *Flexiviridae* was accompanied by a complete re-evaluation of the taxonomic criteria and, in particular, of the molecular criteria used for species discrimination. Extensive intraspecies and interspecies sequence comparison has led to the definition of species cut-off values of 72% nucleotide identity and 80% amino acid identity in the sequences of the complete CP or RdRp (1).

ACLSV, the type member of the genus *Trichovirus*, infects most of the fruit species belonging to the families *Prunoidae* and *Maloidae* (30). Being frequently symptomless in pome fruit, it can be responsible for serious diseases in stone fruit, including false plum pox (19), plum bark split (8), cherry fruit necrosis (6,7), and "viruela" and "butteratura" diseases in apricot (32,34). ACLSV also may cause tissue incompatibility and bud necrosis if infected material is used for grafting in nurseries (6,7). Complete nucleotide sequences have been reported for the P863 (plum bark split), P205 (apple topworking disease), PMB1 (false plum pox), and Balaton1 (cherry necrosis) isolates (11,12,18,36).

PcMV and CMLV naturally infect peach and cherry, respectively, causing distinct diseases in these hosts. They are transmitted by distinct eriophyid mite species but appear to be closely related in genome structure and sequence, CP size, double-stranded (ds)RNA profiles, and serological cross-reactivity (15,16).

Recently, a survey of *Prunus* material from southern Italy affected by diseases of unclear etiology was carried out using a polyvalent polymerase chain reaction (PCR) assay allowing the detection of viruses belonging to the genera *Trichovirus*, *Capillovirus*, and *Foveavirus* (9). The presence, in a mixed infection, of ACLSV and of an uncharacterized filamentous virus possibly belonging to the genus *Trichovirus* was detected in two independent sources, a Japanese plum source (Sus2) with severe stem pitting or grooving symptoms and an apricot source (ARPox1) with symptoms resembling those described for the Apricot ringpox

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains supplemental material not included in the print edition. The online supplement table contains primers used for the cDNA synthesis and polymerase chain reaction amplification of different regions of the Sus2 isolate of the Apricot pseudo-chlorotic leaf spot virus.

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disease (23,24,33). This report presents a detailed characterization of this new agent, including the complete sequencing of the genome of the Sus2 plum isolate and the partial sequencing of the ARPox1 apricot isolate. These results provide evidence that this new virus, for which the name Apricot pseudo-chlorotic leaf spot virus is proposed, is a member of the genus *Trichovirus* closely related to ACLSV.

MATERIALS AND METHODS

Virus isolates and samples. The Sus2 and ARPox1 isolates were obtained from plum (*Prunus salicina* cv. Shiro) and apricot (*P. armeniaca* cv. Bulida) trees, respectively, from Southern Italy (23,24) and were propagated under greenhouse conditions by grafting on GF305 peach seedlings. The ACLSV *Prunus* isolates used are part of the collection maintained at INRA Bordeaux (France). Samples of two plum (*P. salicina*) plants, cvs. Black Beaut and Angeleno, showing severe stem grooving symptoms, and of one apricot plant, cv. Baracca, showing butterburn (34) symptoms on fruit, were collected from commercial orchards in Campania (southern Italy). The plum sample Tr1 (*P. domestica* var. Satsuma), showing severe symptoms of decline and stem grooving of the Myrobalan 29C rootstock, was collected in a commercial orchard in the Goulburn Valley (Australia) (35).

Determination of the herbaceous host range. The Sus2 isolate and the P863, PMB1, and Balaton1 ACLSV isolates were mechanically transmitted to *Nicotiana occidentalis* 37B using young, symptomatic GF305 leaves as the inoculum. Leaves were ground (1/4, wt/vol) in 30 mM potassium phosphate buffer, pH 7.0, as described (17). Before transmission, activated charcoal (VWR, Fontenay-sous-Bois, France) at 90 mg/ml and 2.5% (vol/vol) nicotine hemisulfate (Sigma-Aldrich, St. Louis) were added to the homogenates. Carborundum (400 mesh) was used as the abrasive. The following plants were evaluated as potential hosts: *Chenopodium album*, *C. amaranticolor*, *C. foetidum*, *C. murale*, *C. quinoa*, *N. clelandii*, *N. debnei*, *N. glutinosa*, *N. tabacum* cvs. Samsun and Xanthi, *N. rustica*, *Phaseolus vulgaris* cv. Vedette, and *Lycopersicon esculentum* cv. Rutgers. The appearance of symptoms was monitored over a 3-week period.

Serological analyses. The Sus2 isolate was compared serologically with ACLSV-P863, ACLSV-PMB1, and ACLSV-Balaton1 isolates. For the sake of comparison, all viruses were propagated in *N. occidentalis* 37B. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) tests were performed using immunoglobulins from the P2 polyclonal antiserum against the P863 ACLSV isolate (INRA, Bordeaux, France) or using a commercial kit (Bioreba AG, Reinach, Germany). Triple-antibody sandwich (TAS)-ELISA assays were carried out using the anti-P863 antiserum as trapping antibody and the 29.13 and 8.20 monoclonal antibodies (MAbs) developed by INRA against the P863 ACLSV isolate as secondary antibodies. All ELISA procedures were according to Alioto et al. (2). For the TAS-ELISA, the purified 29.13 and 8.20 MAbs were diluted to final concentrations of 1.5 and 1 µg/ml, respectively. The secondary antibody, goat anti-mouse immunoglobulin alkaline G (IgG) phosphatase conjugate, was used at a 1:5000 dilution. The optical densities (ODs) were measured at 405 nm with a Bio-Rad 3550 Microplate Reader (Richmond, CA) at intervals from 20 min to 2 h. Values above three times the average obtained for healthy samples were assumed to indicate infected samples. Each test was repeated at least twice.

Immunosorbent electron microscopy. Carbon-coated 200-mesh electron microscopy grids were coated for 15 min at 25°C with P2 antiserum diluted 1:1,000 in 0.1 M phosphate buffer, pH 7, and then rinsed with the same buffer. *N. occidentalis* 37B leaves infected by the Sus2 isolate were homogenized in 0.1 M phosphate buffer, pH 7 (1/5, wt/vol), and coated grids floated on the homogenate for 25 min at 25°C. The grids then were rinsed

with distilled water, stained with 2% sodium phosphotungstate (PTA), pH 7, and examined using a Philips EM301 electron microscope operating at 80 kV.

Nucleic acids extraction. Total RNAs were extracted from infected or healthy GF305 and *N. occidentalis* 37B leaves using the silica-capture procedure described by Foissac et al. (9). Viral dsRNAs were purified from the same plant tissues as described previously (10).

Determination of the complete nucleotide sequence of the Sus2 isolate. Short internal cDNA fragments were generated using the degenerated oligonucleotide primer (DOP) random PCR strategy (37) on purified viral dsRNAs from infected *N. occidentalis* 37B as described by Gentit et al. (10). The amplification products were purified using the Nucleotrap kit (Clontech, Erembodegem, Belgium), cloned in the pGEM-T Easy vector (Promega, Lyon, France) following the manufacturer's instructions, and finally electroporated into *Escherichia coli* XL1 cells (Stratagene, Amsterdam, the Netherlands). All cloned fragments were commercially sequenced (Genome Express, Grenoble, France) on both strands.

Primers were designed from the sequence of the short internal DOP cDNAs and used in long-distance reverse-transcription PCR (LD-RT-PCR) performed on purified viral dsRNAs with the Advantage polymerase mix as recommended by the supplier (Clontech). The 3' terminal region between the polyvalent PDO (9) PCR fragment and the polyA tail was amplified in a similar fashion using an LD-polyT primer with (dT)₂₅ at its 3' end. The 3' terminal region of the virus present in the ARPox1 source was amplified in a similar fashion. The various PCR products were purified, cloned, and finally sequenced as described above.

The 5' terminal region of the Sus2 isolate genome was cloned in three successive steps. The first two steps involved a 5' random amplification of cDNA ends (RACE) strategy using the LD-CAP primer and the Clontech commercial kit. The last step involved the GSP6 primer designed from the sequence of the 5'-most RACE clone (RC4) and a second, degenerate primer (NCR1), designed from a consensus sequence generated from the alignment of the 5' noncoding regions (5'NCR) of CMLV and of the P205, PMB1, P863, and Bal1 ACLSV genomes. This primer covers positions 33 to 51 of the CMLV genome and positions 15 to 34 of the ACLSV genome.

Sequence assembly and analysis. The full-length sequence of the Sus2 isolate genome was assembled from the various overlapping cDNA fragments and its genomic organization deduced using the suite of programs available at the Infobiogen website. Database searches were performed using the BLAST facility of NCBI. Pairwise sequence comparisons and genetic distance calculations were performed using the MEGA program (22).

Multiple alignments and phylogenetic reconstructions were performed using CLUSTAL W (38) and phylogenetic trees were displayed using the Treeview program (31). The following viral sequences were used for comparison: ACLSV-P863 (M58152), ACLSV-P205 (D14996), ACLSV-PMB1 (AJ243438), ACLSV-Balaton1 (X99752), ACLSV-SX2 (AF251275), ACLSV-MK9 (AB060962), CMLV (AF170028), and GINV (D88448).

RT-PCR detection of ACLSV and of the new virus. ACLSV-specific detection by RT-PCR was performed using the A52 and A53 primers, as described by Candresse et al. (4). Nested RT-PCR for the detection of the new virus was carried out using the primers and procedure described by Liberti et al. (23).

RESULTS

Comparison of Sus2 isolate and ACLSV symptoms in herbaceous host plants. Using the simple inoculation buffer suggested by James and Murkerji (17) for CMLV, the Sus2 isolate was transmitted by mechanical inoculation to *N. occidentalis* 37B. Despite the fact that the original *Prunus* samples were in

mixed infection with ACLSV (described below), some plants were recovered in which only the Sus2 isolate was present, as demonstrated by repeated failure to detect ACLSV in the *N. occidentalis* plants by ACLSV-specific RT-PCR (4). Therefore, these plants were used as a pure source of the Sus2 isolate and compared with three reference ACLSV isolates (P863, PMB1, and Balaton 1) which had been transmitted to *N. occidentalis*. In this host, all viruses induced comparable symptoms, differing from isolate to isolate essentially by their severity (vein clearing, chlorotic spotting and mottling, and leaf deformation and curling). All viral isolates were inoculated to a range of potential herbaceous indicator plants in order to compare their host range and symptomatology. No infection was detected for any of the viruses assayed in the following species: *C. foetidum*, *C. murale*, *N. clevelandii*, *N. debnei*, *N. glutinosa*, *N. tabacum* cvs. Samsun and Xanthi, *N. rustica*, *Phaseolus vulgaris*, and *L. esculentum* (data not shown). Failure of the viruses to accumulate in the inoculated leaves of these plants was verified by ELISA using polyvalent anti-ACLSV reagents which also detected the Sus2 isolate (described below). The Sus2 isolate also failed to accumulate in the other *Chenopodium* spp. assayed (*C. album*, *C. amaranticolor*, and *C. quinoa*). In contrast, all three ACLSV isolates induced necrotic local lesions on the inoculated leaves of all three *Chenopodium* spp. and systemic infection symptoms (chlorotic spots and rings, line patterns, leaf deformation, and so on) were observed on *C. quinoa* and *C. amaranticolor* (data not shown). Therefore, the herbaceous host range of the Sus2 isolate appears to be more restricted than that of ACLSV, for which *C. quinoa* and *C. amaranticolor* are well-known propagation hosts (25).

Cross-reactivity of the Sus2 isolate with ACLSV. The reactivity of the Sus2 isolate from *N. occidentalis* with serological reagents specific for ACLSV was evaluated by DAS- (for polyclonal antisera) and TAS- (for monoclonal antibodies) ELISA. Reference ACLSV isolates were used as controls and all viral isolates were propagated in *N. occidentalis* 37B to facilitate comparison. The results obtained are presented in Table 1 and demonstrate that the Sus2 isolate is efficiently recognized by both ACLSV-specific polyclonal antisera used but is not recognized by the two monoclonal antibodies used, despite their broad specificity toward ACLSV isolates.

Morphology of Sus2 isolate particles. Using the P2 polyclonal antiserum against ACLSV, particles of the Sus2 isolate from *N. occidentalis* were trapped in immunosorbent electron microscopy (ISEM) assays. The viral particles were readily detected from infected *N. occidentalis* 37B extracts but not from similar extracts prepared from healthy plants. The Sus2 isolate particles have an elongated, flexuous morphology similar to that of ACLSV or other members of the genus *Trichovirus* (Fig. 1).

Cloning and genome sequence analysis of the Sus2 isolate. In addition to the initial small cDNA fragment obtained by a polyvalent nested RT-PCR assay (9) which revealed the originality of the Sus2 isolate (Fig. 2B, PDO fragment), three other short internal cDNA fragments were amplified from purified viral

dsRNAs using the DOP random PCR technique (10,37). Significant homology with the genomic sequence of ACLSV isolates allowed the tentative positioning of these short cDNA fragments (Fig. 2B, DOP7, 8, and 12) on the Sus2 genome. The sequences of these internal fragments then were used to design PCR primers for bridging the various gaps and for cloning the 5' genomic regions RACE. For the 5' region, two successive RACE experiments failed to reach the 5' end of the genome (Fig. 2C). A degenerated PCR primer (NCR1) based on a short region of homology between known 5' sequences of trichoviruses and equivalent to positions 15 to 34 of the ACLSV genome allowed the generation of a last 5' PCR fragment (Fig. 2C, N1). The sequence of the Sus2 isolate genome finally was assembled from the various overlapping partial cDNAs (Fig. 2B and C). Overall, $\approx 27\%$ of the sequence was determined on more than one cDNA clone. Very few mismatches (nine in total) between independent cDNA clones were observed in the regions sequenced more than once and, whenever such mismatches were observed, sequence from a third independent clone was used to generate the consensus used for the final genome sequence. The sequence obtained is 7,494 nucleotides (nt) long, excluding the 3' polyA tail, and lacks a small (34 nt, including the NCR1 primer binding site if the Sus2 genome length is similar to that of ACLSV) but as yet unspecified number of 5' nucleotides. Further efforts to obtain the complete sequence of the 5' region of the Sus2 genome through 5' RACE experiments have been unsuccessful thus far. The nucleotide sequence of the Sus2 isolate was deposited as GenBank accession no. AY713379.

Fig. 1. Negatively stained particles of the Sus2 isolate observed after immunosorbent electron microscopy using an anti-Apple chlorotic leaf spot virus serum. The bar represents 100 nm.

TABLE 1. Results of double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using the polyclonal antisera P2 (INRA-Bordeaux) and 10/15 (Bioreba) and triple-antibody sandwich (TAS)-ELISA with two different monoclonal antibodies (MAbs 29.13 and 8.20)^a

Sample ^b	DAS-ELISA		TAS-ELISA	
	P2 (Pab) (INRA-Bordeaux)	10/15 (Pab) (Bioreba)	8.20 (Mab) (INRA-Bordeaux)	29.13 (Mab) (INRA-Bordeaux)
Healthy <i>Nicotiana occidentalis</i>	0.011 ± 0.007	<0.001	<0.001	<0.001
ACLSV-P863 in <i>N. occidentalis</i>	>2	0.59 ± 0.004	1.666 ± 0.011	0.914 ± 0.357
ACLSV-PMB1 in <i>N. occidentalis</i>	1.141 ± 0.105	>2	0.688 ± 0.037	0.502 ± 0.158
ACLSV-Bal1 in <i>N. occidentalis</i>	1.912 ± 0.175	0.809 ± 0.038	1.851 ± 0.057	>2
APCLSV-Sus2 in <i>N. occidentalis</i>	1.243 ± 0.095	>2	<0.001	<0.001

^a Pab = polyclonal antisera-based reagents; OD values = mean optical densities ± standard deviations (calculated for three wells, six wells for healthy controls). Standard deviations were not calculated for values >2 or <0.001.

^b ACLSV = Apple chlorotic leaf spot virus and APCLSV = Apricot pseudo-chlorotic leaf spot virus.

Genomic organization of the Sus2 isolate. A search for ORFs showed that the Sus2 genomic RNA harbors three potential ORFs in its positive sense and that it has NCRs of 107 and 158 nt at its 5' and 3' termini, respectively (Fig. 2A). However, the length of the 5'NCR is not known precisely due to the problems outlined above. No ORF of significant size was found on the negative-sense strand. The initiating AUG codon for the first ORF was identified at positions 108 to 110 and is in a context (UAC-UAUGGGC) matching the consensus sequence for translation initiation in plants identified by Lutcke et al. (26). Another AUG codon is present upstream at positions 51 to 53, but it is followed closely by two in-frame TAA stop codons. A similar situation was identified in ACLSV P863 (11) and in CMLV (16). The three predicted overlapping ORFs encode proteins of 1,892 amino acids (aa) (ORF1, calculated molecular weight 216.1 kDa, positions 108 to 5,786), 457 aa (ORF2, 51.3 kDa, positions 5,698 to 7,071), and 193 aa (ORF3, 21.5 kDa, positions 6,755 to 7,336) (Fig. 2A).

Databank searches and computer-assisted comparisons of the deduced amino acid sequences of the proteins encoded by these three ORFs identified them as a potential replication-associated protein (RdRp) for the ORF1, a 30K superfamily-type (28) virus movement protein (ORF2), and a capsid protein (ORF3). The 21.5-kDa calculated molecular mass for the CP is in good agreement with the $\approx$ 20.5-kDa value experimentally determined by Western blotting using anti-ACLSV immunoglobulins (data not shown). The RdRp showed all the conserved motives found in the corresponding proteins of the family *Flexiviridae* (1). These included a type I methyltransferase domain (4,21) (amino acid positions 59 to 233), a potential papain-related thiol protease domain (12,13) (amino acids 841 to 950), a helicase domain containing the seven conserved motifs characteristic of the superfamily I helicases (14) (amino acids 1,063 to 1,304), and finally the eight motifs characteristic of an RdRp of positive-stranded RNA viruses (20,21) (amino acids 1,645 to 1,738).

Therefore, the genomic organization of the Sus2 isolate is extremely close to those of the four completely sequenced ACLSV isolates, with a single potential difference. For three of these isolates (P863, PMB1, and Balaton 1), it has been reported that the CP ORF is included in frame within a larger ORF potentially initiated by an upstream ATG codon, resulting in an $\approx$ 28-kDa protein identical in its C-terminal three-fourths with the CP itself (12). In the case of the fourth sequenced isolate, P205, the up-

stream initiating codon was present but followed in frame by a TGA stop codon (36). The Sus2 isolate presents a similar situation, with an ATG at positions 6,554 to 6,556 but an in frame TAG at positions 6,635 to 6,637.

Partial sequencing of the genome of the isolate present in the ARPox1 source. The 3' terminal region of the genome of the isolate of the new virus present in the ARPox1 apricot source was amplified by LD-PCR using an LD-polyT primer and a primer designed from the sequence information of the PDO nested RT-PCR fragment. The sequence deduced from this PCR product then was merged with that of the PDO fragment to generate a final contig of 2,591 nt, excluding the polyA tail (accession no. AY713380). As evaluated from the complete genome of the Sus2 isolate, this sequence represents approximately the 3' terminal third of the complete genomic sequence of this second isolate.

The sequence from the ARPox1 source has the same genetic organization as that of the Sus2 isolate (data not shown) and shows an overall nucleotide sequence identity of 84.6%. It is strictly collinear with the Sus2 sequence, with two exceptions affecting the 3' NCR: a 7-nt deletion corresponding to nucleotides 7,367 to 7,373 of the Sus2 sequence, and a 4-nt insertion just before the polyA tail, at the extreme 3' end of the genome.

Sequence comparisons with other members of the genus *Trichovirus* and determination of the phylogenetic affinities of the new virus. Preliminary analyses and database searches clearly positioned the new virus with the genus *Trichovirus*; therefore, all further detailed comparisons were limited to members of this genus. The overall nucleotide sequence identity levels calculated for the complete genomes were 66.7, 66.4, 65.9, and 65.8% for the P205, PMB1, P863, and Balaton1 isolates, respectively, of ACLSV; and only 59.8% for CMLV and 55.1% for GINV. Surprisingly, values lower than these averages were observed for the 5' and 3' NCR (Table 2). In the 5' NCR, the Sus2 isolate showed an average of only $\approx$ 49.4% identity with ACLSV isolates and 47.7% with CMLV. The corresponding values for the 3' NCR were 49 and 60.8% for ACLSV isolates and CMLV, respectively. Thus, it appears that, despite having an overall genome slightly closer to ACLSV than to CMLV, the Sus2 isolate has a 3' NCR that is significantly more homologous to CMLV than to ACLSV.

Detailed comparisons also were performed on all three ORFs of the genome, both at the nucleotide and amino acid sequence levels (Table 2). In each case, a closer relationship was observed

Fig. 2. Genomic organization of the Sus2 isolate and strategy used for the complete sequencing of the genome. **A**, Schematic representation of the genomic organization. RdRp: RNA-dependent RNA polymerase. MP: movement protein. CP: capsid protein. **B**, Position on the genome of the short initial PDO and degenerated oligonucleotide primer (DOP) polymerase chain reaction (PCR) cDNA fragments. **C**, Position on the genome of the long PCR and 5' random amplification of cDNA ends products generated to cover the genome. Shaded boxes represent the viral open reading frames (ORFs). The size of the encoded proteins is indicated above the boxes. The positions of the conserved domains in ORF1 are indicated by darker shading.

TABLE 2. Average intraspecific and interspecific homology (identity) levels for the coding and noncoding regions of trichoviruses^a

Calculations ^b	Nucleotide sequences (%)					Amino acid sequences (%)		
	5' NCR	ORF1 (RdRp)	ORF2 (MP)	ORF3 (CP)	3' NCR	ORF1 (RdRp)	ORF2 (MP)	ORF3 (CP)
Within ACLSV isolates	85.3 ± 2.2	77.6 ± 0.4	80.1 ± 0.8	83.8 ± 1.5	78.1 ± 2.4	86.6 ± 0.6	80.8 ± 1.4	91.2 ± 1.3
Within APCLSV isolates	NA	NA	83.6 ± 1	87.1 ± 1.3	89.4 ± 2.5	NA	86.0 ± 1.5	94.3 ± 1.5
Between APCLSV and ACLSV isolates	49.4 ± 1.2	65.0 ± 0.4	66.6 ± 1.1	69.0 ± 1.5	49.0 ± 3.3	66.2 ± 0.4	57.3 ± 2.1	70.9 ± 2.9
Between APCLSV isolates and CMLV	47.7	64.0 ± 0.3	61.6 ± 0.8	60.2 ± 0.2	60.8 ± 2.9	62.0 ± 1.1	45.6 ± 0.1	54.0 ± 0.1
Between APCLSV isolates and GINV	NA	NA	48.5 ± 0.7	47.0 ± 0.1	39.5 ± 0.7	NA	35.6 ± 0.2	36.0 ± 0.7

^a Average values ± standard deviation are given (when applicable). NCR = noncoding regions, ORF = open reading frame, RdRp = RNA-dependent RNA polymerase, MP = movement protein, CP = coat protein, and NA = not applicable (no sequence data available).

^b ACLSV = *Apple chlorotic leaf spot virus*. Calculations within Apricot pseudo-chlorotic leaf spot virus (APCLSV) isolates were performed using the Sus2 and ARPox1 sources sequences or only the Sus2 sequence in cases when the ARPox1 partial sequence did not cover the region considered. CMLV = *Cherry mottle leaf virus* and GINV = *Grapevine berry inner necrosis virus*.

Fig. 3. Phylogenetic trees reconstructed for the **A**, replication protein, **B**, movement protein, and **C**, coat protein amino acid sequences of known trichoviruses. Trees were constructed using the neighbor-joining method with 1,000 bootstrap replicates. Only bootstrap values above 70% are shown. The bar represents 0.1 substitutions per site. The following sequences were used: Apricot pseudo-chlorotic leaf spot virus (APCLSV)-Sus2 and ARPox1, this work; *Apple chlorotic leaf spot virus* (ACLSV) (P863: M58152; P205: D14996; PMB1: AJ243438; Balaton1: X99752; SX2: AF251275; MK9: AB060962); *Cherry mottle leaf virus* (CMLV, AF170028); and *Grapevine berry inner necrosis virus* (GINV, D88448).

with ACLSV isolates, but the average divergence level between the new virus isolate or isolates and ACLSV isolates always was significantly higher than the average divergence between ACLSV isolates (1.6- to 1.9-fold higher and 2.2- to 3.2-fold higher when comparing nucleotide and amino acid sequences, respectively) (Table 2). In particular, the Sus2 proteins were only, on average, 66.2% (ORF1, RdRp), 57.3% (ORF2, MP), and 70.9% (ORF3, CP) identical with those of ACLSV isolates.

This analysis is confirmed by the phylogenetic trees reconstructed for each of these proteins, which are shown in Figure 3. In each case (Fig. 3A to C), the Sus2 isolate (and, when available, the ARPox1 isolate) clustered separately from known ACLSV sequences with very high bootstrap support. For both the MP and the CP trees, the new virus was found to be closer to ACLSV than to CMLV and GINV, again with strongly supported branches. Thus, the new virus appears to be distinct from ACLSV but closely related to it.

Detection of the new virus in other *Prunus* material. As a first step to evaluate the prevalence and geographical distribution of the new virus, collections of *Prunus* spp.-infecting viruses held at INRA Bordeaux and field samples from the Campania region of Italy were evaluated for the presence of the new virus using a specific nested RT-PCR assay. This assay uses primers within the region targeted by the polyvalent PDO assay (9) and selected using the Sus2 and ARPox1 sequences so as to amplify isolates of

the new agent but not isolates of ACLSV (23). Among the Bordeaux collections, the new virus was detected in only three accessions: A337 and A4, two apricot sources from France, and Bulida-64, an apricot source from Spain (data not shown). In each case, it was found to be in mixed infection with ACLSV. In parallel, a total of 42 field and collection samples from the Campania region (Italy) were assayed and the virus was detected in samples of two Japanese plum (*P. salicina* Lindl) plants (cvs. Black Beaut and Angeleno), showing severe stem-grooving symptoms, and in one apricot plant (cv. Baracca), showing butteratura symptoms on fruit. These results were confirmed by sequence analysis of the 205-nt-long PCR products (accession nos. AY713381 to AY713385 and AF413932). Comparison of these new sequences among themselves and with the Sus2 and ARPox1 sequences showed nucleotide identity levels ranging from 79.1 to 98.2% and amino acid identity levels ranging from 92.5 to 100% (data not shown). The phylogenetic tree reconstructed from this limited sequence information is shown in Figure 4. It provides evidence for the existence of substantial sequence variability and of clustering of isolates within the populations of this new agent.

The virus recently was detected in Australia from a "Black Amber" Japanese plum tree showing distinct circular blotches on the skin of the ripening fruit and from declining Satsuma plum trees showing severe grooving of the Myroblan 29C rootstock (35). A high homology was observed between a PCR-amplified

Fig. 4. Phylogenetic tree of Apricot pseudo-chlorotic leaf spot virus (APCLSV) isolates reconstructed from the sequence of the APCLSV-specific nested polymerase chain reaction detection assay (23). Trees were constructed using the neighbor-joining method with 1,000 bootstrap replicates. Only bootstrap values above 70% are shown. The bar represents 0.1 substitutions per site.

sequence from the Satsuma plum source and corresponding to the 3' end of the genome (deposited under accession no. AY713386) and the Sus2 sequence (85.7% identity, data not shown).

DISCUSSION

Initial evidence based on a short amplification product obtained using a polyvalent nested RT-PCR technique (9) indicated that the Sus2 and ARPOx1 sources were infected by a viral agent belonging to the genus *Trichovirus* but distinct from ACLSV (23). The detailed characterization of the Sus2 isolate and the determination of the partial genome sequence for the ARPOx1 isolate confirm and extend these first results. For both the RdRp gene and the CP gene, the nucleotide identity observed between the Sus2 or ARPOx1 sequences and ACLSV isolates are below the 72% cut-off value recently proposed for distinct species in the family *Flexiviridae* (1). A similar situation applies to the amino acid sequences of these two proteins, which show identity values in the range of 65 to 73% compared with ACLSV isolates, whereas the cut-off value for species discrimination is 80% (1). Together with the phylogenetic reconstructions based on all three viral proteins (Fig. 3), these results strongly support the idea that the Sus2 and ARPOx1 sequences represent a new *Trichovirus* sp. distinct from ACLSV, for which the name Apricot pseudo-chlorotic leaf spot virus (APCLSV) is proposed, in order to highlight the close proximity of the new virus to ACLSV.

The differences in herbaceous host range and serology reported here further support this analysis. If verified on more APCLSV isolates, the inability to infect *C. quinoa* and *C. amaranticolor* may provide a useful discrimination technique and a way to separate ACLSV from APCLSV when the two viruses are encountered in mixed infection. Results with two polyclonal antisera raised against different ACLSV isolates clearly indicate that ACLSV and APCLSV are serologically related. This situation is similar to that encountered with CMLV and PcMV, which currently are regarded as two distinct but serologically related species in the genus *Trichovirus* (15). This cross-reactivity may explain, at least in part, why APCLSV escaped recognition for so long because any symptoms observed would have been ascribed to ACLSV. At present, the discrimination of the two viruses directly in *Prunus* samples can be achieved reliably only by ACLSV- and APCLSV-specific PCR assays (4,23). The two ACLSV-specific MAb 8.20 and 29.13 may be used to discriminate between these two agents. However, the availability of APCLSV-specific serological reagents would provide a very useful diagnostic tool and greatly assist in

determining the prevalence, geographical distribution, and epidemiology of this new virus.

So far, APCLSV has been recovered from *Prunus* samples from Italy, Spain, France, and Australia. In addition, APCLSV appears to be present in other countries because comparisons of sequence data obtained by Al Rwahnih et al. (3) in a recent study of the genetic variability of ACLSV, using the ACLSV primers designed by Menzel et al. (29), indicated that four of the isolates tested (APR-EA5, an apricot source from Turkey [AJ586636]; PE154, a peach source from Hungary [AJ586650]; PE150, a peach source from Italy [AJ586645]; and PE297, a peach source from Jordan [AJ586651]) appear to represent, in fact, APCLSV isolates, with partial CP amino acid sequences 88 to 97% identical to the Sus2 and ARPOx1 sequences reported here (data not shown).

APCLSV has been recovered so far from only a limited number of *Prunus* spp., including apricot, plum, Japanese plum, and peach, which are all known natural hosts of ACLSV. It will be interesting to see whether APCLSV has a broader natural host range, including cherry and species in the family *Maloidae* (apple, pear, quince, and so on), as is the case for ACLSV.

A key question, of course, is that of the symptoms that may be caused by APCLSV infection in its hosts. In a number of cases, APCLSV has been retrieved from source plants with severe symptoms on the wood (severe stem pitting or grooving on the Japanese plum Sus2 source [23], on the Italian Black Beaut and Angeleno Japanese plum, and on the Myrobalan 29C rootstock of the Satsuma plum from Australia [35]) or on the fruit (apricot ringpox-like symptoms of the ARPOx1 source [23], butterarura symptoms on the apricot cv. Baracca from Italy, and circular blotches on the skin of the Black Amber Japanese plum from Australia). In each case, APCLSV appears to be in mixed infection in these hosts, in particular with ACLSV. Therefore, it is not possible to ascertain which of the viruses present are responsible for the symptoms observed or even whether these symptoms are a consequence of the mixed infections. Further work is urgently needed, possibly involving the use of infectious cDNA clones to separate the various agents, before the pathogenicity of APCLSV in *Prunus* spp. can be understood.

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