

Detection of Apple Chlorotic Leaf Spot and Apple Stem Grooving Viruses Using RT-PCR

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ABSTRACT

Kinard, G. R., Scott, S. W., and Barnett, O. W. 1996. Detection of apple chlorotic leaf spot and apple stem grooving viruses using RT-PCR. *Plant Dis.* 80:616-621.

Reverse transcriptase polymerase chain reaction (RT-PCR) procedures were used to amplify fragments specific for apple chlorotic leaf spot virus (ACLSV) and apple stem grooving virus (ASGV). Products visible in agarose gels were produced using templates consisting of as little as 5 pg of total nucleic acid extracted from infected apple leaves. Both viruses were also detected in fruit, bark, and root tissues of infected apples. These results suggest the viruses are distributed throughout actively growing trees. The systems were further validated by sequencing fragments amplified from leaves of an apple, cherry, and Asian pear trees growing in South Carolina. Comparisons with published sequences indicate that the fragments from the three ACLSV isolates have very high ($\geq 97.6\%$) percent identity values to the corresponding region of a plum isolate and moderately high ($\geq 81.4\%$) identities to an apple isolate from Japan. The fragments amplified from the ASGV isolates were found to be similar ($\geq 88.5\%$ identities) to the corresponding region of an apple ASGV isolate from Japan.

Additional keywords: capillivirus, trichovirus

Apple chlorotic leaf spot virus (ACLSV) and apple stem grooving virus (ASGV) both occur frequently in rosaceous fruit trees. Infection is often latent, although several economically important diseases are associated with the viruses (15). In the Orient, where topworking is performed to change production varieties in established orchards, several commonly used rootstocks are sensitive to latent viruses. If infected scions are grafted onto trees with these rootstocks, the trees decline and suffer a concomitant loss in productivity, followed by death within a few years. ACLSV, ASGV, and possibly apple stem pitting virus (ASPV), are causal agents of this decline, although the severity of the disease appears to be influenced by both the clones of rootstock and the strains of the viruses involved (28,29). Isolates of ACLSV are associated with bark splitting in plum, ring pattern mosaic disease and fruit malformations in pear, and green

sunken mottle disease in peach (3,7,15). Lesions on plum fruit caused by an isolate of ACLSV can be confused with those caused by plum pox virus (15). Other than probable involvement in topworking disease, ASGV has been associated only with symptomless infections of apple, pear, and apricot (15,21,23).

Graft bioassays with sensitive woody indicator host species and enzyme-linked immunosorbent assay (ELISA) are the techniques currently used for detecting ACLSV and ASGV. Both assays have significant limitations. The two viruses are known to occur in low concentrations, below the limits of detection in some instances. Variations occur in the responsiveness of indicator hosts to different isolates of ACLSV (18,22). Symptom expression is also dependent on environmental conditions, and considerable delay is possible before unambiguous symptoms appear. As long as 2 years may be required for conclusive diagnosis of ASGV infection in Virginia crab apple (*Malus pumila* Mill.) indicators (22). Diversity among virus isolates has caused problems with the detection of ACLSV using ELISA (6,12). In general, ELISA has proven most reliable when using floral tissue with double antibody sandwich (DAS)-ELISA (9), thereby restricting accurate detection to only a few weeks during each growing season.

Reliable detection of latent viruses is important in the implementation of programs to establish, maintain, and disseminate virus-free germ plasm in crops that are

vegetatively propagated. Because of the problems with detection and the need for rapid and sensitive systems to screen for the presence of latent apple viruses, we have investigated the potential of using the reverse transcriptase polymerase chain reaction (RT-PCR) for detecting ACLSV and ASGV. RT-PCR has become an important method for the detection of low-titer, RNA-containing viruses and generally has been found to be more sensitive than any of the previously used protein or nucleic acid based assays (25). In fruit trees, RT-PCR has been applied to detection of plum pox, cherry leaf roll, and prune dwarf viruses (2,17,25,26). Here, we report the development and application of RT-PCR procedures for the detection of ACLSV and ASGV in fruit tree hosts.

MATERIALS AND METHODS

Virus sources and purification. Samples of ACLSV and ASGV (E-36 isolate), in *Chenopodium quinoa* Willd., were provided by G. I. Mink (Washington State University, Prosser). The *Prunus*-P863 isolate of ACLSV associated with bark splitting was provided by T. A. Candresse (Station de Pathologie Végétale, Bordeaux, France). Both viruses were purified from systemically infected *C. quinoa* 14 to 18 days postinoculation. Leaves were ground in 0.05 M Tris-Cl, pH 7.6, containing 0.01 M Mg_2SO_4 and 0.02 M mercaptoethanol, and the homogenate was filtered through cheesecloth. Clarification was achieved using a suspension of bentonite prepared according to the method of Dunn and Hitchborn (8). A small aliquot (≤ 5 ml) of bentonite (40 mg ml^{-1}) was slowly added to the filtrate, and the mixture was stirred for 15 min at 4°C and centrifuged ($7,800 \times g$) for 15 min. The supernatant was retained, and the cycle of bentonite addition, agitation, and centrifugation was repeated until the supernatant was straw-colored. Virions were then precipitated by the addition of PEG 6000 to 8% (wt/vol), with stirring for 1 h followed by centrifugation for 15 min ($10,400 \times g$). For ASGV only, NaCl was included at 0.02 M in the precipitation step. The pellets were resuspended in 0.05 M Tris-Cl, pH 7.6, and centrifuged through 10 to 40% linear (wt/vol) sucrose gradients, prepared in the same buffer, for 2.5 h at $115,820 \times g$. Bands were collected by fractionation, diluted with Tris-Cl buffer, and concen-

Technical contribution 4041 of the South Carolina Agricultural Experiment Station, Clemson University.

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Accepted for publication 27 February 1996.

Publication no. D-1996-0322-05R
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trated by centrifugation at $267,800 \times g$ for 1 h. Virus yields were estimated using an extinction coefficient of $2.02 \text{ cm}^2 \text{ mg}^{-1}$ (1). Purified virions were either stored at -70°C or lyophilized.

Template preparation from woody tissues. The method used to extract total nucleic acid from woody samples was slightly modified from the procedure of Rowhani et al. (19). Sample tissue was ground in liquid nitrogen, and extraction buffer was added at 5 ml of buffer per 1 g of tissue. The extraction buffer was 125 mM potassium phosphate, pH 7.6, containing 10% sucrose, 1.5% BSA (fraction V), and 20% PVP-10. The tissue was incubated on ice for 15 min, and the debris was removed by brief centrifugation ($16,000 \times g$) in a microfuge. The supernatant was centrifuged at $16,000 \times g$ for 15 min. The resulting pellet was resuspended in 50 mM Tris-Cl, 10 mM EDTA, pH 8.0, containing 0.1% mercaptoethanol and 2% sodium dodecyl sulfate (SDS), and incubated at 60°C for 10 min. After addition of 1/2 volume of 6 M potassium acetate, the tube was incubated on ice for 30 min followed by centrifugation ($16,000 \times g$) for 15 min. The total nucleic acid in the supernatant was precipitated by the addition of 1/10 volume of 3 M sodium acetate, pH 5.6, and 1 volume of isopropanol with incubation at -20°C for 1 h followed by centrifugation ($16,000 \times g$) for 20 min. The pellet was washed once with cold 70% ethanol and allowed to air-dry at room temperature. The pellet was resuspended in 10 mM Tris-Cl, 1 mM EDTA (TE), pH 8.0, and 1 to 2 μl were used in a reverse transcription reaction. The purity and quantity of the nucleic acid preparations were assessed by measuring the UV absorbance at 260 and 280 nm.

RT-PCR. PCR primers for ACLSV were selected based on the sequence of the *Prunus*-P863 isolate (10). The upstream primer (5'-GCAGCTCAAGGTGGACGCAAGTC-3') is located between nucleotides 6804 and 6826, while the downstream primer (5'-CGAGAGCAGACACAGCTGACTAG-3') is complementary to nucleotides 7332 to 7354, giving an expected amplification product of 551 bp. ASGV primer sites were selected based on the sequence of a *Malus* isolate from Japan (31). The upstream primer (5'-CCCCAAAGGAAGGGGAGACTGAC-3') is located between nucleotides 5921 and 5943, and the downstream primer (5'-CCGTCC-AAGCCTTTCATTGGACC-3') is complementary to nucleotides 6318 to 6340, providing an expected amplification product of 420 bp. For both viruses, priming sites are located in the putative coat protein encoding regions of the viral genomes. Primers were synthesized using an Applied Biosystems 394 synthesizer or were purchased from a commercial vendor (IDT, Coralville, IA).

All reagents were, unless otherwise noted, from Perkin-Elmer (Norwalk, CT)

or Promega (Madison, WI). Total nucleic acid from tissue samples, as well as purified, untreated virions, was used as the template for cDNA synthesis. The reverse transcription cocktail (10 μl) contained template, 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 5 mM MgCl_2 , 1 mM each deoxynucleoside triphosphate, 2.5 μM oligo d(T)₁₆ (primer for first-strand synthesis), 1 U RNase inhibitor, and 2.5 U M-MLV reverse transcriptase. The mixture was incubated through a single cycle of 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. For PCR, an additional cocktail (40 μl) containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 2 mM MgCl_2 , 2.5 U *Taq* DNA polymerase, 0.2 μM each primer, and 0.8 $\mu\text{g} \mu\text{l}^{-1}$ BSA (New England BioLabs, Beverly, MA) was added to the reaction mixture. All reactions were performed in a DNA Thermal Cycler (Perkin-Elmer). The PCR was performed with a regime of an initial cycle of 94°C for 1 min; 35 cycles of 1 min at 94°C , 2 min at 64°C , and 2 min at 72°C ; and a final cycle of 10 min at 72°C . The amplification products were analyzed by electrophoresis through 1.2% agarose gels, submerged in $0.5 \times$ TBE, that were subsequently stained in a solution of $0.5 \mu\text{g} \text{ml}^{-1}$ ethidium bromide.

Limits of detection. To estimate the limits of detection of ACLSV and ASGV in leaf tissue, total nucleic acid was extracted from leaves of a red delicious apple tree growing in an orchard in South Carolina. The orchard contained approximately 30-year-old red delicious trees growing on domestic seedling rootstock. The tree had displayed no symptoms of viral infection, although DAS-ELISA using fresh bloom tissue had indicated the presence of ACLSV and ASGV (unpublished data). The nucleic acid was serially diluted in TE, and the RT-PCR reaction was performed as described.

Distribution and detection of viruses in different apple tissues. Samples (0.3 to 1.0 g) of young leaves, blooms, immature fruit, ripened fruit, bark, and root tips were processed and tested by RT-PCR to determine from which tissues the viruses could be detected. Bark and root tip samples were from a greenhouse-infected red delicious apple tree, and the other samples were from the same apple tree from the field that was used to determine the limits of detection. Young apical leaf tissue was also collected from six different locations corresponding to major branches of the tree, and these samples were processed and tested separately. Leaves growing from water sprouts that had emerged from the rootstock were also tested. Fruit tissue was collected by passing a no. 2 cork borer through the fruit; and the core, including skin and flesh, was used as the sample.

Cloning and sequencing. To further validate these PCR procedures, fragments of the appropriate sizes for both ACLSV and ASGV, which were amplified from

three different fruit tree hosts growing in South Carolina, were cloned and sequenced. These hosts were red delicious apple, sweet cherry (*Prunus avium* (L.) L.), and Asian pear cv. Shinsui (*Pyrus pyrifolia* Burm. f. Nakai (*P. serotina* Rehd.)). Leaf tissue from these trees had been assayed using the RT-PCR procedures on at least three separate occasions over two growing seasons. The Asian pear had displayed chlorotic line patterns similar to those described for pear ring pattern mosaic disease, whereas the apple and cherry had shown no symptoms of viral infection. To confirm their viral origin, the ACLSV- and ASGV-specific fragments were cloned using the TA cloning system (Invitrogen, San Diego, CA) and sequenced using the *Taq* DyeDeoxy Terminator Cycle Sequencing system (Applied Biosystems, Foster City, CA) with -21M13 forward, M13, and Sp6 primers. Alignments of nucleotide sequences were performed using GeneJockey (Biosoft, Cambridge, UK) software, and the percent identity values were calculated as the ratio of identical to total nucleotides, excluding primer sequences. As a control, the amplified fragment from the ACLSV *Prunus*-P863 isolate was cloned, sequenced, and compared to the corresponding published sequence for that isolate (10).

RESULTS

Detection by RT-PCR. Using purified virus preparations (isolates from G. I. Mink) without additional treatment as templates for RT-PCR, products of the expected size of 551 bp and 420 bp were observed in agarose gels for ACLSV and ASGV, respectively. Nonspecific bands were rarely visible using the conditions described; furthermore, no amplification products were seen when ACLSV virions were used as the template in the presence of ASGV primers, or vice versa (Fig. 1). No amplification products were observed from reactions where the sample, reverse transcriptase, or DNA polymerase was omitted. The limit of detection of the assays using purified ACLSV or ASGV virions was 10 pg (not shown); for ACLSV, this corresponds to 520 fg of RNA, as the particles contain approximately 5.2% RNA (1). The procedures detected virus-specific nucleic acids in numerous leaf samples collected from pome and stone fruit trees, including apple trees in the field that were sampled on at least three occasions over two growing seasons. In addition to these source plants, both ACLSV and ASGV-specific fragments were amplified from leaves of doubly-infected *Malus* cultivars Emma Lepperman, Golden Delicious, Hopa, Mutsu, and Starking Delicious, and from the *Pyrus* cultivars Ayers and Twentieth Century that were doubly-infected with the two viruses. Appropriate fragments were also amplified from apple leaf tissues infected with an array of ASGV

isolates, provided by G. I. Mink, that originated from China, Israel, Italy, Japan, Nepal, and South Africa (not shown).

Amplification products were observed on agarose gels using as little of 5 pg of

total nucleic acid from infected apple leaves (not shown). Both virus-specific fragments were visible in reactions where the templates were nucleic acid extracts from apple leaves, blooms, fruit, bark, or

root tips from trees infected with both ACLSV and ASGV (Fig. 2). Amplification was also observed from each of six leaf samples collected from different locations of the same apple tree, as well as from water sprouts of that tree (not shown).

Sequence analysis. Determination of the nucleotide sequences of the fragments amplified from the leaves of the apple, sweet cherry, and Asian pear trees confirmed their relationships to ACLSV and ASGV. As a control, the fragment amplified from the *Prunus*-P863 isolate was sequenced and found to have identities of 99.8 and 100% at the nucleotide and amino acid levels, respectively, when compared to the published sequence (10). The ACLSV fragments from all three hosts exhibited $\geq 97.6\%$ identity in nucleotide sequence compared to the plum isolate (Fig. 3). Translations of the putative proteins encoded by these fragments (made assuming that the fragments would translate in the same context as the sequences from which the primers were made) showed that $\geq 94.6\%$ of the amino acids were identical to the plum isolate, and in the case of the fragment from Asian pear, complete amino acid identity was revealed (Table 1). Nucleotide and amino acid sequence identities were always less (81.0 to 82.4% and 85.6 to 89.8%, respectively) in comparisons with the apple isolate of ACLSV from Japan (Table 1).

ASGV fragments that were amplified from the apple, sweet cherry, and Asian pear hosts exhibited between 89.3 and 97.9% nucleotide sequence identities to one another and to the apple isolate from Japan (Fig. 4), and the amino acid identities were $\geq 95.1\%$ (Table 2).

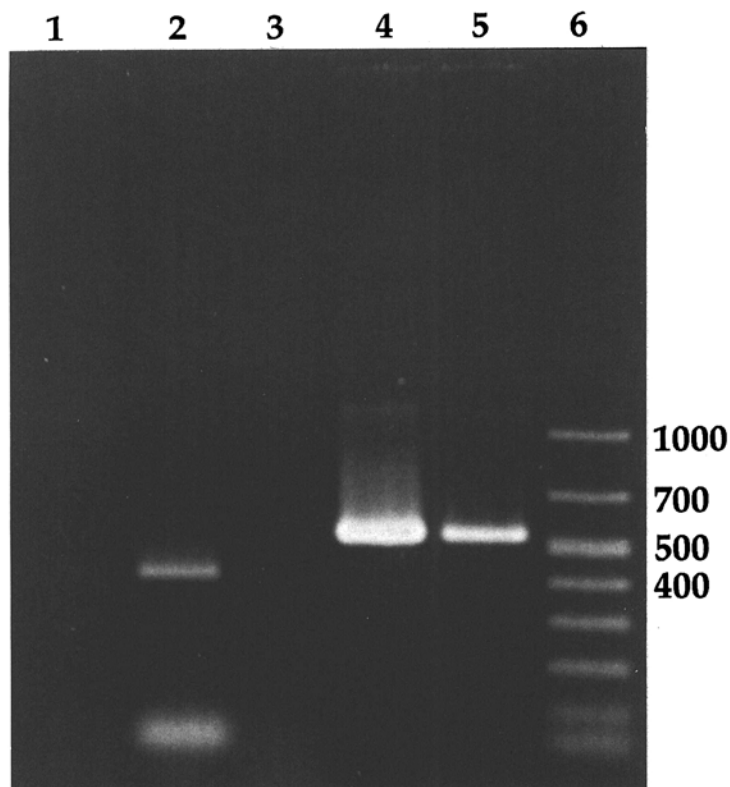


Fig. 1. Agarose gel of products amplified from purified (1 μ g) apple chlorotic leaf spot virus (ACLSV) and apple stem grooving virus (ASGV) preparations using reverse transcriptase polymerase chain reaction (RT-PCR). Lane 1, purified ASGV with ACLSV-specific primers; lane 2, ASGV with ASGV-specific primers; lane 3, ACLSV *Prunus*-P863 isolate with ASGV-specific primers; lanes 4 and 5, ACLSV *Prunus*-P863 and ACLSV (apple-US), respectively, with ACLSV-specific primers. Lane 6, molecular size markers in bp.

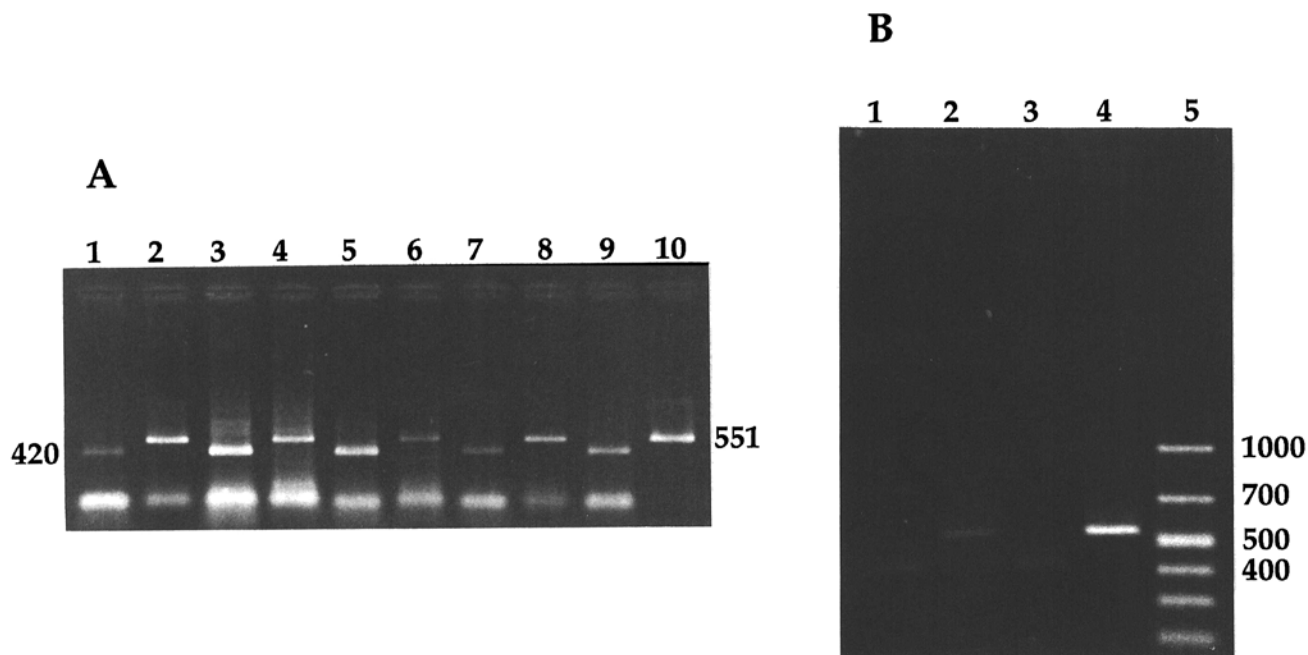


Fig. 2. Agarose gel of products amplified from apple chlorotic leaf spot virus (ACLSV)- and apple stem grooving virus (ASGV)-infected apple tissues. (A) ACLSV- and ASGV-specific products, respectively, from leaves (lanes 1 and 2), immature fruit (3 and 4), blooms (5 and 6), bark (7 and 8), and purified virus (9 and 10); (B) mature fruit (lanes 1 and 2), root tips (3 and 4). Lane 5, molecular size markers in bp.

DISCUSSION

We report here the development of RT-PCR procedures for the detection of ACLSV and ASGV. Using the conditions described, we amplified a 551-bp fragment specific for ACLSV and a 420-bp fragment specific for ASGV from the leaves, bark, roots, and fruit of rosaceous fruit tree hosts. The primers were used to detect isolates of ACLSV and ASGV that originated from both pome and stone fruit species. Moreover, ASGV isolates of widely separated geographical origin were detected using these procedures. Thus, it appears that RT-PCR is not subject to some of the limitations that have been reported for the biological and serological assays presently used to detect these viruses (4,6,7,12,18,22). The rapidity of RT-PCR offers a considerable advantage over the use of a bioassay. Furthermore, the abilities of this technique to detect a diverse range of isolates of the two viruses and to detect the viruses in a range of tissues (thereby permitting detection of these viruses throughout the year) are clearly advantages over ELISA. In addition, RT-PCR is capable of detecting ACLSV and ASGV at concentrations below those normally detected by ELISA. Although it is possible to prepare numerous samples for RT-PCR, for example, by using GeneReleaser matrix (G. R. Kinard, *unpublished*), the cost of reagents involved has always given ELISA (even though less sensitive) a cost advantage over RT-PCR. However, recent publication of an improved method for purifying *Taq* (5) may change this item from an expensive enzyme available only from commercial suppliers to one that can be produced in large quantities in any laboratory. This will probably enhance the cost effectiveness of PCR.

Amplification of specific fragments from different apple tissues and from several leaf samples collected at different locations in the same tree suggests that these viruses are distributed throughout all tissues (Fig. 2). As the quantities of total nucleic acid added to the reactions varied from 0.08 µg (ripened fruit) to 1.4 µg (immature fruit), it is not possible to use the intensities of the bands to arrive at any conclusion about the relative concentrations of the viruses in the tissues. However, when testing leaf samples collected from different locations in the apple tree, an equal quantity (0.5 µg) of nucleic acid was added to each reaction, and bands of uniform intensities were observed in agarose gels stained with ethidium bromide (not shown). Although not strictly a quantitative assay, this does suggest that concentrations of the viruses in the leaf canopy of actively growing trees are nearly uniform. Root grafting has long been considered a general means by which viruses are transmitted among trees (15), and natural spread of ACLSV in the field by underground contact among roots has been re-

ported (11,14). The detection of both ACLSV and ASGV in root tips gives support to the idea that viruses of trees may be spread by root contact; indeed, spread in this way might constitute the only means

of natural transmission of these two viruses for which no vector has yet been identified.

Determining the nucleotide sequences of products amplified from apple, sweet cherry, and Asian pear hosts confirmed

	6827				
Plum	TAAAGGCATT	TCTGGGCGCA	GAAACAGAC	CTCTTCATGG	AAAGACAGGG
Apple-USC.....
CherryT.....
PearT.....
Apple-Japan	.G..A..G..	C.T..C....	..GG.....	.C.....
	6877				
Plum	GCAACCTGG	AACAGATACT	GGAGTCCATC	TTCGCGAACA	TAGCGATCCA
Apple-US
Cherry
Pear
Apple-JapanTT....C....	..G.....A..
	6927				
Plum	GGGAACGTCA	GAGCAGACGG	AGTTTCTGGA	CCTAGTGGTG	GAAGTGAAT
Apple-USC.....
CherryT.....
PearT.....
Apple-Japan	..G..C..G	..A..A..A	..C.....	.GTGC....	.G.....
	6977				
Plum	CAATGGAGGA	TCAATCAGTC	CTCGGCTCTT	ACAACCTGAA	GGAAGTGGTC
Apple-US	..C.....	..C.....
Cherry	..A.....	..A.....
Pear	..A.....	..A.....
Apple-Japan	.CA.....	..GAAG..G	G.G.....	A.T...T....	..G..A...
	7027				
Plum	AACTTGATCA	AAGCTTTCAA	GACTACCTCT	TCGGACCCGA	ACATCAACAA
Apple-US	C.....T
Cherry	A.....A
Pear	A.....A
Apple-Japan	GGT.....	A.ATA...GA...T..A.G..G
	7077				
Plum	AATGACATTC	CGTCAGGTCT	GTGAAGCTTT	CGCACCAGAG	GCAAGAAATG
Apple-USC..
CherryT..
PearT..C.....
Apple-Japan	C.....CT..	..C..A..G.G..A..	T..C..G...
	7127				
Plum	GACTGGTCAA	ATTGAAATAC	AAAGGGGTTT	TTACAAACCT	CTTTACAAC
Apple-US
CherryC.....C
Pear
Apple-Japan	.G..C..A..	GC.A..G..C..	.C.....	..T..T...
	7177				
Plum	ATGCCGGAAG	TGGGGAGCAA	GTACCCGGAG	CTAATGTTTG	ATTTCAATAA
Apple-USA.....
CherryG.....
PearG.....
Apple-Japan	.C...T....	..G..G.G..	..T.....	..C.....	..C.....
	7227				
Plum	GGGTCTGAAC	ATGTTTCATCA	TGAACAAGGC	CCAGCAGAAA	GTTATAACCA
Apple-UST
CherryT
Pear
Apple-Japan	..C..A..TA.A..G.....T
	7277				
Plum	ATATGAATCG	GCGTCTTTTG	CAGACTGAAT	TCGCAAAGAG	CGAGAACGAG
Apple-USA
Cherry
Pear
Apple-JapanC..AT.....	..A..T...
	7327				
Plum	GCAAA				
Apple-US				
Cherry	...G				
Pear				
Apple-Japan	..G..				

Fig. 3. Nucleotide sequence comparisons of regions amplified from apple chlorotic leaf spot virus (ACLSV) isolates (apple-US, cherry, pear) to sequences of plum isolate (10) and apple isolate from Japan (20). Dots indicate identical nucleotides. Numbering is consistent with that of German et al. (10).

Table 1. Nucleotide and amino acid sequence comparisons among apple chlorotic leaf spot virus (ACLSV) fragments amplified using reverse transcriptase polymerase chain reaction (apple-US, cherry, Asian pear) with corresponding fragments of an apple isolate from Japan (20) and the plum isolate (10)^a

	Apple-J	Apple-US	Cherry	Asian pear	Plum
Apple-J		81.4	82.2	82.4	81.0
Apple-US	85.6		97.2	97.8	98.0
Cherry	88.6	94.6		99.0	97.6
Asian pear	89.8	95.2	99.4		98.2
Plum	89.9	95.2	99.4	100	

^a Values are expressed as percent identities, excluding primer sequences. Comparisons above the diagonal refer to nucleotide sequences; values below the diagonal are amino acid sequence comparisons.

	5944				
Apple-Japan	GAAGGAAGGA	AGGCCAGAGC	GGTTCGTCT	GCGAAAAAA	CAAAAAATTG
Apple-US	..GCA.....A
Cherry	..GCA..A..A	A..GA.....T.....
Pear	..GCA..A..A	A..GA.....T.....
	5994				
Apple-Japan	GAGGATCGAT	TTGTCAAATG	TTGTTCTCTGA	ATTGAAAACC	TTTGCTGCCA
Apple-US	..A.....C	C.....A..C..T
Cherry	..A.....CA..A..
Pear	..A.....CA..A..
	6044				
Apple-Japan	CTTCCAGGCA	GAACTCTTTG	AACGAATGTA	CGTTCAGAAA	GCTTTGCGAG
Apple-UST.....G.....T.....
CherryT.....G.....T.....
PearT.....G.....T.....
	6094				
Apple-Japan	CCATTTGCCG	ATTTGGCTCG	AGAATTTCTA	CATGAAAGGT	GGTCTAAGGG
Apple-UST.....	G.....A.....A.G.....
CherryT.....	..C.....T.....A.A.....
PearT.....	..C.....T.....G.A.....
	6144				
Apple-Japan	ATTGGCCACC	AATATTTACA	AGAAATGGCC	CAAAGCTTTC	GAAAAAAGTC
Apple-US	..C.T.....	..C.....T.T.....G..C.
CherryT.....	..C.....T.	A..AGC..T	CG.....
PearA...T	..C.....T.	A.....
	6194				
Apple-Japan	CATGGGTGGC	CTTTGATTTT	GCCACTGGTC	TGAAAAATGAA	TCGTCTAACA
Apple-US	..T.....	TC.....C.....T.....C.....G
Cherry	..T.....	A.....C.....C...T	A.....T.....
Pear	..T.....	A.....C.....C...T	A.....T.....
	6244				
Apple-Japan	CCTGATGAGA	AACAGGTGAT	TGATAGAATG	ACCAAAGAC	TTTTTCGTAC
Apple-USA.....A..	A..C.....T..G..G.
CherryA.....C..G.....G.....
PearA.....C..G.....G.....
	6294				
Apple-Japan	TGAAGGACAA	AAAGGGGTTT	TCGA		
Apple-USG.....		
Cherry		
Pear		

Fig. 4. Nucleotide sequence comparisons of regions amplified from apple stem grooving virus (ASGV) isolates (apple-US, cherry, Asian pear) to sequence of apple isolate from Japan (31). Dots indicate identical nucleotides. Numbering is consistent with that of Yoshikawa et al. (31).

Table 2. Nucleotide and amino acid sequence comparisons among apple stem grooving virus (ASGV) fragments amplified using reverse transcriptase polymerase chain reaction (apple-US, cherry, Asian pear) with corresponding fragments of an apple isolate from Japan (31) and citrus tatter leaf virus (30)^a

	Apple-J	Apple-US	Cherry	Asian pear	CTLV
Apple-J		89.3	88.5	89.8	90.9
Apple-US	96.8		89.3	89.8	91.4
Cherry	95.1	95.1		97.9	93.9
Asian pear	96.0	96.0	97.6		94.4
CTLV	97.6	97.6	97.6	98.4	

^a Values are expressed as percent identities, excluding primer sequences. Comparisons above the diagonal refer to nucleotide sequences; values below the diagonal are amino acid sequence comparisons.

their viral origin (Figs. 3 and 4). This report also represents, as far as we can determine, the first report of ASGV infection in a cherry species, although the virus has been detected in apricot (23). The ACLSV fragments from all three hosts exhibited very strong nucleotide identities to the corresponding region of the plum isolate from France (10) and moderately strong identities to that of an apple isolate from Japan (20). The percent identity value between the entire nucleotide sequences of the plum and apple-Japan isolates is 79.8% (20), which would suggest, based on the criteria used with potyviruses (24), the occurrence of at least two strains of ACLSV. Chairez and Lister (4) and Paunovic (18) distinguished isolates originating from *Malus* and *Prunus* as strains based on biological and serological properties, although Marenaud et al. (13) were unable to classify isolates based on the hosts in which they were found. Our sequence information supports the latter idea that it may not be possible to delineate ACLSV isolates as strains based solely on the genera of the hosts from which they are isolated.

Among ASGV fragments isolated from apple, cherry, and Asian pear, all exhibited strong nucleotide identities ($\geq 89.3\%$) to the corresponding region from the apple isolate from Japan. The ASGV fragments also exhibited strong identities to citrus tatter leaf virus (CTLV), a virus that can cause graft incompatibility reactions in citrus and is classified in the same genus as ASGV (*Capillovirus*) (16,30). Amplification of a fragment that co-migrated with the ASGV PCR product was observed using the ASGV-specific primer pair and a template of total nucleic acid from CTLV-infected *C. quinoa* (CTLV-infected tissue provided by S. M. Garnsey, USDA, Orlando, FL). However, a reduction of the annealing temperature to 58°C was necessary to achieve amplification products visible on agarose gels (not shown).

The amplification observed by using purified but otherwise untreated virions was surprising. We cannot exclude the possibility that the preparations contained disrupted particles, although the integrity of several was confirmed by electron microscopy using uranyl acetate staining. cDNA can be transcribed directly from potyvirus particles destabilized by freezing and thawing (27), an observation that may be applicable to ours, as purified preparations were repeatedly thawed and frozen. Another interesting possibility is that the highly flexuous, open architecture of these viruses may make them inherently accessible to reverse transcription, after which particles would have been denatured by the 99°C incubation.

The simplicity, rapidity, and sensitivity of the methods presented here supports their use for detection of ACLSV and ASGV in certification schemes. The pro-

cedures should also prove useful in advancing research related to the epidemiology of the viruses. The ability to detect ACLSV and ASGV simultaneously is of particular interest because it appears that these viruses usually occur together in mixed infections.

ACKNOWLEDGMENTS

We thank T. A. Candresse, S. M. Garnsey, W. E. Howell, and G. I. Mink for virus isolates used in this work, and M. T. Zimmerman and R. E. Baker for technical assistance.

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