Apple Stem Grooving and Citrus Tatter Leaf Capilloviruses Obtained from a Single Shoot of Japanese Pear (*Pyrus serotina*)

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Abstract

By means of polymerase chain reactions, nucleotide sequence analyses of the 3'-terminal regions, and amino acid sequence analyses, two capillovirus isolates, apple stem grooving (ASGV) and citrus tatter leaf virus (CTLV) were found from virus culture V3 obtained from a single shoot of Japanese pear (*Pyrus serotina*). V3 was originally presumed to be a pure culture of ASGV. The two isolates that were eventually determined to be different capilloviruses were designated V3-1 and V3-2, respectively. V3-1 differed from ASGV reference isolate P-209 from apple by only 3 base substitutions in the 3'-terminal 2611 nucleotides. V3-2 was closer to CTLV reference isolates Li-23 (95.9% homology) and L (95.6% homology) from lilies in the sequence of its 3'-terminal 1669 nucleotides than it was to that of ASGV isolate P-209 (89.1% homology). In the comparison of ORF2 proteins (313 amino acids, aa) the similarity between aa sequences of V3-2 and P-209 was 94.8%, while that between V3-2 and CTLV isolates (Li-23 and L) was 97.4%. In the case of the aa sequence homology of the putative coat protein regions (237 aa), equivalent to the C-terminal region of ORF1 protein, the similarity between V3-2 and P-209 was 97.5% and was 99.2% between V3-2 and CTLV isolates (Li-23 and L). The most striking difference in aa sequence homologies was found in the ORF1 protein (277 aa) encoding ORF2 in the other frame. The similarity between V3-1 and P-209 was 99.3%, while that between V3-2 and P-209 was 61.0%. Isolate V3-2 shared 85.6% and 93.8% aa sequence homologies with Li-23 and L, respectively. Solely on the basis of comparison of nucleotide and amino acid sequences of Japanese pear isolates V3-1 and V3-2 with reference isolates, we conclude that V3-1 is almost identical to an ASGV reference isolate from apple, while V3-2 is similar to CTLV reference isolates from lilies. We recommend that CTLV be considered as a strain of ASGV.

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Key words : capillovirus, apple stem grooving virus, citrus tatter leaf virus, Japanese pear.

INTRODUCTION

Apple stem grooving capillovirus (ASGV) is the type member of genus *Capillovirus*⁵⁾ and distributed worldwide in apple trees⁴⁾. In Japan, ASGV has been reported to be isolated from apple¹⁴⁾, pear⁹⁾, Japanese pear¹²⁾ and Japanese apricot imported from China¹¹⁾. ASGV has a coat protein of M_r 27,000 (27K) and a ssRNA of M_r 2.30×10^6 ¹⁴⁾. The complete nucleotide sequence of the ASGV genome was reported for an isolate (P-209) from an apple tree¹⁷⁾. The genome consists of 6496 nucleotides excluding the 3' poly(A) tail and contains two overlapping open reading frames (ORFs). ORF1 encodes a 241 K polyprotein containing consensus motifs of methyltransferase, helicase, RNA polymerase and a coat protein in the C-terminal region¹⁷⁾. ORF2 encodes a 36 K protein which may act as a cell to cell movement protein¹⁶⁾.

Recently, the sequences of the 3'-terminal region of RNA of citrus tatter leaf virus (CTLV), another member of genus *Capillovirus*, were reported for two isolates (Li-23 and L) from lilies^{7,16)}. The genome organization in the 3'-terminal region of CTLV-RNA is in perfect agreement with that of the ASGV genome. The sequence of the 3'-terminal 2956 nucleotides, excluding poly(A) tail, of the CTLV (isolate Li-23) genome shows 86.1% identity to that of the ASGV¹⁶⁾. Amino acid sequence identities of ORFs 1 and 2 between both viruses are 86.1% and 97.3%, respectively¹⁶⁾. Both viruses also have similar biological and serological properties. The results imply that CTLV may be regarded as a strain of ASGV.

In this study, we investigated the nucleotide sequence of the 3'-terminal region of ASGV (V-3) from Japanese pear and compared the nucleotide sequence and the amino acid sequence coded by ORFs 1 and 2 with those of ASGV (P-209) from apple and two isolates of CTLV

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from lilies.

MATERIALS AND METHODS

Virus. ASGV (culture V-3), originally isolated from Japanese pear¹²⁾, ASGV (isolate P-209) from apple¹⁴⁾ and CTLV (isolate Li-23) from lily¹⁾ were propagated in *Chenopodium quinoa*. Virus (V-3) was purified from infected *C. quinoa* leaves and viral RNA was extracted from purified virus by the SDS-phenol method as described previously¹⁵⁾.

Polymerase chain reaction. Total RNAs were extracted from 100 mg of *C. quinoa* leaves inoculated with ASGV (V-3), ASGV (P-209) or CTLV (Li-23) according to the method of Verwoerd *et al.* (1989)¹³⁾. RNA was also prepared directly from freeze-dried leaves (10 mg) of *C. quinoa* infected with ASGV (V-3) which had been stored at the Fruit Tree Research Station. RNA was finally dissolved in 0.1 ml of sterile distilled water.

Several primers were designed for amplifications of the 3'-terminal regions of ASGV (V-3) genome on the basis of the sequences of ASGV (P-209)- and CTLV (Li-23)-genomes^{16,17)}. The locations and sequences of primers used in this study are shown in Fig. 1 and Table 1. First strand cDNA synthesis and polymerase chain reaction (PCR) was conducted as follows: a reaction mixture (50 μ l) containing 10 μ l of 5 $\times$ first strand buffer (250 mM Tris, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT), 10 μ l of 2.5 mM dNTPs, 2 μ l of primers [ASCT-3'(-) or ASCT-PC(-); 10 μ M] (Table 1), 5 μ l of total RNA, 18.5 μ l of distilled water and 2.5 μ l of M-MLV reverse transcriptase (200 units/ μ l, United State Biochemical Corporation) was incubated at 37°C for 1 hr. Aliquots (10 μ l) of the resulting cDNA solution were transferred to a tube containing 26.8 μ l of distilled water, 5 μ l of 10 $\times$ Taq buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), 4 μ l of 2.5 mM dNTPs, 2 μ l of primer 1 [ASCT-3'(-) or ASCT-PC(-); 10 μ M], 2 μ l of primer 2 [ASGV-ON2(+), CTLV-ON(+), ASGV-CP2(+) or ASGV-ORF2P(+); 10 μ M] (Table 1) and 0.25 μ l of Taq DNA polymerase (5 units/ μ l, Takara). PCRs were performed for 30 cycles with the program (95°C, 1 min; 52°C, 1 min; 72°C, 1 min) using ZYMOREACTOR II (ATTO). Aliquots (5 μ l) of the reaction mixture were analyzed on a 1% agarose gel containing 0.5 μ g/ml of ethidium bromide. The remnants were extracted with an equal volume of phenol/chloroform (1:1) and the amplified fragments were precipitated with ethanol, washed with 70% ethanol and dissolved in water.

cDNA cloning. The PCR products described above were ligated to pT7 blue T-Vector (Novagen) and used to transform *Escherichia coli* JM109. In the case of V-3, first and second strand cDNAs were prepared from purified viral RNA using oligo (dT) primer described before¹⁷⁾. The dsDNAs were ligated to the *Sma*I site of Bluescript KS⁺ and used to transform competent *E. coli* JM109.

Nucleotide sequencing. The nucleotide sequences of cDNA clones were determined with an Applied Biosystems 373A or a Hitachi 5500 DNA sequencer using the dideoxynucleotide method⁸⁾ and Taq polymerase. For nucleotide sequencing of cDNA clones derived from PCR products, at least 3 clones were used for the determination of the nucleotide sequence of a given region. For nucleotide sequencing of isolate V-3, 9 overlapping cDNA clones (nucleotide sizes 0.5 to 2.6 kbp) were used to determine the 3'-terminal 2.6 kb nucleotide sequence. Every base was determined by sequencing at least two independent clones or by sequencing twice from a single clone. Sequence data were collected, assembled and analyzed using the program GENETYX (Software Development Co., Ltd.).

RESULTS

Amplifications of the 3'-terminal region of ASGV (culture V-3) genome by polymerase chain reaction

At first we tried to amplify genome fragments of ASGV (V-3) in RNA samples extracted from infected *C. quinoa* by PCR using several primers (Fig. 1 and Table 1). Primers, ASGV-ON2(+) and CTLV-ON(+), were designed for specific amplifications of ASGV- and CTLV-genomes, respectively. We anticipated that the other primers would anneal with both virus genomes. As expected, use of the primer pair [ASGV-ON2(+) and ASCT-3'(-)] allowed the fragments with an expected size (*ca.* 0.6 kbp) to amplify in samples from ASGV (P-209)-infected leaves, but not from CTLV (Li-23)-infected ones (Fig. 2, lanes 1 and 5). Moreover, by use of the primer pair [CTLV-ON(+) and ASCT-3'(-)], fragments with an expected size (*ca.* 1.1 kbp) were amplified in samples from CTLV-infected leaves, but not from ASGV-infected ones (Fig. 2, lanes 2 and 6). When RNA samples from ASGV (V-3)-infected leaves were used as templates, the amplification of fragments with expected sizes of 0.6 kbp occurred for a primer pair [ASGV-ON2(+) and ASCT-3'(-)] and 1.1 kbp for a primer pair [CTLV-ON(+) and ASCT-3'(-)] (Fig. 2, lanes 3 and 4).

Fig. 1. Locations of primers used in this study for the PCR amplification of ASGV- and CTLV-genomes.

Table 1. Primers used for the PCR amplification of the 3'-terminal regions of apple stem grooving virus (culture V-3) genome

Primers ^{a)}	Sequences (5'-3') ^{b)}
forward	
ASGV-ON2(+)	⁵⁹²⁹ GAAGGGGAGACTGACGAAGG ⁵⁹⁴⁸
ASGV-CP2(+)	⁵⁶⁴¹ ATGAGTTTGGGAAGACGTGCT ⁵⁶⁶⁰
CTLV-ON(+)	⁵³⁴⁷ ATTCAGCTCGTTTCTTGAG ⁵³⁶⁶
ASGV-ORF2P(+)	⁴⁷⁸⁸ ATGGCTATCGTCAACGTCAA ⁴⁸⁰⁷
reverse	
ASCT-3'(-)	⁶⁴⁹⁶ AGAGTGGACAACTATAGAC ⁶⁴⁷⁷
ASCT-PC(-)	⁵⁷³⁴ TCAGAAGTTCCTTTGCCGGG ⁵⁷¹⁵

- a) Primers were designed on the basis of the nucleotide sequences of the genomes of ASGV (P-209)¹⁶⁾ and CTLV (Li-23)¹⁵⁾.
 b) Figures indicate the nucleotide positions in the genome of ASGV (P-209)¹⁶⁾.

Evidence for the presence of two capilloviruses in culture V-3 of ASGV

We determined the nucleotide sequences of cDNA clones from purified ASGV (isolate V-3). The sequence of the 3'-terminal 2611 nucleotides, excluding the poly(A) tail, of the V-3 genome is almost identical to that of the corresponding region of P-209 genome from apple. The difference in the 3'-terminal 2611 nucleotide sequence between both isolates is only three base substitutions; C in nt position 5249 of P-209 changes to A in V-3, G (position 5641 of P-209) to A (V-3) and G (position 5968 of P-209) to A (V-3). All three substitutions lead to changes in their coded amino acids (proline to glutamine, alanine to threonine and glycine to glutamic acids, respectively) in ORF1. An isolate having this nucleotide sequence was designated V3-1.

In the following experiments, we determined the nucleotide sequences of cDNA clones derived from PCR products of V-3. Four clones of the 0.6 kbp products which were amplified by a primer pair [ASGV-ON2(+) and ASCT-3'(-)] were used for the determination of nucleotide sequences and all clones were shown to be identical. The nucleotide sequence of the 0.6 kbp products was found to be completely identical to the 3'-terminal 0.6 kb nucleotide sequence of V3-1.

Nine clones of the 0.8 kbp products amplified by a primer pair [ASGV-CP2(+) and ASCT-3'(-)], four clones of the 1.1 kbp products by a primer pair [CTLV-ON(+) and ASCT-3'(-)] and three clones of the 1.0 kbp products by a primer pair [ASGV-ORF2P(+) and ASCT-PC(-)] were also sequenced. There were no sequence variations among clones of each PCR product, and overlapping regions among these three products were found to have identical nucleotide sequences. Unexpectedly, the assembled 3'-terminal 1669 nucleotide sequence (this isolate was designated V3-2) is not consistent with that of the corresponding region of V3-1 and the similarity between V3-1 and V3-2 was 89.1% (Fig. 3

Fig. 2. Agarose gel electrophoresis of DNAs amplified by a polymerase chain reaction from *C. quinoa* leaves infected with ASGV (P-209), ASGV (V-3) or CTLV (Li-23). Lane M, lamda DNA digested with *Hind*III, lanes 1 and 2, ASGV (P-209), lanes 3 and 4, ASGV (V-3), lanes 5 and 6, CTLV (Li-23), lanes 7 and 8, healthy *C. quinoa* leaves. Lanes 1, 3, 5 and 7 and lanes 2, 4, 6 and 8 were the products amplified by using the primer pair [ASGV-ON2(+) and ASCT-3'(-)] and the primer pair [CTLV-ON(+) and ASCT-3'(-)], respectively.

and Table 2). The nucleotide sequence of V3-2 is rather similar to those of CTLV genomes and the homologies with those of isolates Li-23¹⁶⁾ and L⁷⁾ are 95.9% and 95.6%, respectively (Table 2). The results indicate that a virus culture V-3 of ASGV is a mixture of two capilloviruses, one is almost identical to ASGV (P-209) from apple and another is similar to CTLV from lily.

Comparisons of amino acid sequences encoded by ORFs 1 and 2 among isolates of ASGV and CTLV

The 3'-terminal nucleotide sequences of V3-1 and V3-2 have neither deletion or insertion of bases compared with the corresponding regions of ASGV (P-209) and CTLVs (Li-23 and L) and so their coding regions (a part of ORF1 and ORF2) are completely consistent with those of ASGV and CTLV. We compared the amino acid sequences of a 36 K protein (320 amino acids; aa) encoded by ORF2 (nt positions 4788 to 5750 in Fig. 3), a putative coat protein (237 aa) equivalent to the C-terminal region of ORF1 (nt positions 5641 to 6354 in Fig. 3) and a protein region of ORF1 (284 aa) which encodes ORF2 in the other frame (nt positions 4789 to 5640 in Fig. 3) among isolates of ASGV and CTLV. As shown in Fig. 3, most of the base substitutions found among isolates of ASGV and CTLV were nonsense mutations for the 36 K protein encoded by ORF2 and for a putative coat protein region. The amino acid sequence of a 36 K protein was highly conserved among isolates of ASGV and CTLV and the similarity was 94.8% between V3-2 and P-209 and 97.4% between V3-2 and CTLV isolates (Table 3). The similarity of amino acid sequences of the putative coat protein was also high among isolates of ASGV and CTLV (Table 4). The amino acid sequence of V3-2 coat protein is closer to

Table 2. Percentage of sequence similarity of the 3'-terminal 1709 nucleotides among apple stem grooving virus- and citrus tatter leaf virus-genomes^{a)}

	ASGV (P-209)	ASGV (V3-1)	ASGV (V3-2)	CTLV (Li-23)	CTLV (L)
ASGV (P-209) ^{b)}	100.0	99.8	89.0	88.5	88.2
ASGV (V3-1)		100.0	89.1	88.7	88.4
ASGV (V3-2)			100.0	95.9	95.6
CTLV (Li-23)				100.0	99.1
CTLV (L)					100.0

a) For ASGV (V3-2), the sequence of 1669 nucleotides excluding two primer sequences at the 5'- and 3'-terminal regions was compared.

b) The nucleotide sequences compared had been presented in the papers by Yoshikawa *et al.*¹⁷⁾ for ASGV (P-209), Yoshikawa *et al.*¹⁶⁾ for CTLV (Li-23) and Ohira *et al.*⁷⁾ for CTLV (L).

Table 3. Percentage of amino acid sequence similarity of an ORF2 protein (320 amino acids) among isolates of apple stem grooving virus and citrus tatter leaf virus

	ASGV (P-209)	ASGV (V3-1)	ASGV ^{a)} (V3-2)	CTLV (Li-23)	CTLV (L)
ASGV (P-209)	100.0	99.7	94.8	94.7	94.7
ASGV (V3-1)		100.0	94.9	95.0	95.0
ASGV (V3-2) ^{a)}			100.0	97.4	97.4
CTLV (Li-23)				100.0	98.8
CTLV (L)					100.0

a) For ASGV (V3-2), the sequence of 313 amino acids (aa) excluding N-terminal 7 aa derived from a primer sequence was compared.

Table 4. Percentage of amino acid sequence similarity of a putative coat protein region (237 amino acids) of an ORF1 protein among isolates of apple stem grooving virus and citrus tatter leaf virus^{a)}

	ASGV (P-209)	ASGV (V3-1)	ASGV (V3-2)	CTLV (Li-23)	CTLV (L)
ASGV (P-209)	100.0	99.6	97.5	98.3	98.3
ASGV (V3-1)		100.0	97.5	98.3	98.3
ASGV (V3-2)			100.0	99.2	99.2
CTLV (Li-23)				100.0	100.0
CTLV (L)					100.0

a) The putative coat protein region is equivalent to the amino acid positions 1869-2105 of ASGV ORF1 protein¹⁶⁾.

Table 5. Percentage of amino acid sequence similarity of an ORF1 protein region (284 amino acids) encoding ORF2 in the other frame among isolates of apple stem grooving virus and citrus tatter leaf virus^{a)}

	ASGV (P-209)	ASGV (V3-1)	ASGV ^{b)} (V3-2)	CTLV (Li-23)	CTLV (L)
ASGV (P-209)	100.0	99.3	61.0	58.5	56.7
ASGV (V3-1)		100.0	61.0	59.5	56.7
ASGV (V3-2) ^{b)}			100.0	85.6	93.8
CTLV (Li-23)				100.0	97.5
CTLV (L)					100.0

a) The protein region compared is equivalent to the amino acid positions 1585-1868 of ASGV ORF1 protein¹⁶⁾.

b) For ASGV (V3-2), the sequence of 277 aa excluding N-terminal 7 aa derived from a primer sequence was compared.

that of CTLV (99.2%) than to ASGV (P-209) (97.5%). The most striking difference that we observed among isolates of ASGV and CTLV was in the amino acid sequence of an ORF1 protein region encoding ORF2 in the other frame (Table 5). The similarity was 99.3% between P-209 and V3-1, 61.0% between P-209 and V3-2 and 85.6% between V3-2 and Li-23. Comparison of amino acid sequences described above also indicate that V3-1 is almost identical to ASGV (P-209) from apple and V3-2 is closer to CTLV than to ASGV.

DISCUSSION

The results of PCR amplifications and nucleotide and amino acid sequence analyses of the genome of ASGV (culture V-3) described above indicate that V-3 contains two capilloviruses (designated V3-1 and V3-2). Fragments of both V3-1 and V3-2 were amplified from RNA samples directly extracted from freeze-dried materials stored at the Fruit Tree Research Station, Tsukuba, excluding the possibility of contamination in the greenhouse during this study. It is very probable that V-3 is a

mixture of two capilloviruses because the virus has been latently infecting fruit trees such as apple and pear for a long time and so several isolates and/or strains may possibly exist in a single tree.

In our experiments, 9 cDNA clones derived from purified viral RNA of V-3 all have the nucleotide sequence of V3-1. This result is not consistent with those obtained by PCR amplifications in which fragments representing the nucleotide sequences of either V3-1 or V3-2 were amplified from the same RNA sample. One possible explanation of this discrepancy is that the titer of V3-1 was higher than that of V3-2 in infected leaves used for virus purification. Two forward primers [ASGV-ON(+) and CTLV-ON(+)] were designed for specific amplifications of ASGV (P-209) and CTLV (Li-23), respectively. As shown in Fig. 1, use of the primer pair [ASGV-ON2(+) and ASCT-3'(-)] allowed the amplification of the expected fragments (0.6 kbp) of P-209 and V3-1, while the primer pair [CTLV-ON(+) and ASCT-3'(-)] amplified the fragments (1.1 kbp) of Li-23 and V3-2. Two other forward primers [ASGV-CP2(+) and ASGV-ORF2P(+)] were expected to an-

neal to both V3-1 and V3-2 genomes. However, 9 cDNA clones from fragments (0.8 kbp) amplified by a primer pair [ASGV-CP2(+) and ASCT-3'(-)] and three clones from fragments (1.0 kbp) by a primer pair [ASGV-ORF2P(+) and ASCT-PC(-)] had the nucleotide sequence of V3-2. We do not know why these primer pairs only allowed amplification of the V3-2 fragments, although clones containing V3-1 fragments may be found if more clones are sequenced.

The data presented in this paper have some implications for the classification of capillovirus. ASGV and CTLV are presently regarded as distinct viruses. In fact, ASGV has been naturally isolated from *Malus*¹⁴⁾, *Pyrus*^{9,12)} and *Prunus* spp.¹¹⁾ and, on the other hand, CTLV from *Citrus* spp.^{6,10)} and lily^{1,7)}. However, both viruses have similar particle structures and biological (experimental host ranges and symptoms on indicator plants) and serological properties^{1,3,6,14,16)}. Recently, Iwanami *et al.*²⁾ demonstrated that ASGV (V-3) could infect Rusk citrange and induce symptoms similar to those induced by CTLV infection. From the results of our present study, we propose that CTLV be regarded as a strain of ASGV.

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和 文 摘 要

吉川信幸・笹本和稔・桜田 学・高橋 壮・柳瀬春夫：ニホンナシから分離されたリンゴステムグルービングウイルスとカンキツタターリーフウイルス

ニホンナシから分離され、リンゴステムグルービングウイルス (ASGV) の V-3 株と呼ばれていたウイルスに関して、ゲノムの 3'末端領域の PCR による増幅、塩基配列およびコードされるタンパク質のアミノ酸配列の解析を行った結果、V-3 株には 2 種類のカピロウイルス (分離株 V3-1 と V3-2) が含まれていることが明らかになった。V3-1 は、ゲノムの 3'末端 2661 塩基において、ASGV のリンゴ分離株 (P-209) と 3 塩基の置換が認められるのみであった。一方、V3-2 は、3'末端 1669 塩基において、P-209 とは 89.1% の相同性であるのに対し、ユリから分離された CTLV の 2 分離株、Li-23 と L とは、それぞれ 95.9% と 95.6% の相同性であった。ゲノムがコードする ORF2 のアミノ酸 (313 アミノ酸; aa) 配列の比較では、V3-2 と P-209 間で 94.8%、V3-2 と CTLV (Li-23 と L) 間で 97.4% の相同性が認められ、ORF1 の外被タンパク質 (CP) 領域 (237aa) のアミノ酸配列では、V3-2 と P-209 間で 97.5%、V3-2 と CTLV (Li-23 と L) 間で 99.2% の相同性であった。また ORF1 の CP 領域の上流側 277aa (別のフレームで ORF2 をコードしている領域) のアミノ酸配列の比較では、V3-1 と P-209 間で 99.3% の相同性であるのに対して、V3-2 と P-209 間では 61.0% で、V3-2 と CTLV の 2 分離株 Li-23 と L 間では、それぞれ 85.6% と 93.8% の相同性が認められた。以上から、V3-1 は ASGV のリンゴ分離株とほとんど同一であり、V3-2 はユリから分離された CTLV に類似していることが明らかになった。現在、ASGV と CTLV は、主に分離される原宿主の違いから別種のウイルスとして扱われているが、両ウイルス間でゲノムの塩基配列やコードされるタンパク質のアミノ酸配列に高い相同性が認められること、また生物学的および血清学的にも区別できないことから、CTLV は ASGV の系統であると提唱したい。