Complete nucleotide sequence of the genome of an apple isolate of apple chlorotic leaf spot virus

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The complete nucleotide sequence of the genome of an apple isolate of apple chlorotic leaf spot virus (ACLSV-A) was determined. The genome is 7552 nucleotides excluding the poly(A) tail and contains three open reading frames (ORFs 1, 2 and 3), encoding proteins with M_r values of 216503 (216·5K), 50453 (50·4K) and 21394 (21·4K), respectively. Nucleotide sequence comparisons between ACLSV-A and the previously sequenced ACLSV from plum (ACLSV-P) showed that

the sequence identity at the nucleotide level was 79·8%. Amino acid sequence identities of ORFs 1 and 2 between both isolates were 88·4% and 79·9%, respectively. The 21·4K protein encoded by ORF 3 of ACLSV-A had an amino acid sequence identity of 88·6% with the 28·3K protein encoded by ORF 3 of ACLSV-P. Immunoblot analysis of the 21·4K protein expressed in *Escherichia coli* showed that this protein is the coat protein of ACLSV-A.

Apple chlorotic leaf spot virus (ACLSV) is distributed worldwide in fruit trees including apple, peach, pear, plum, cherry and apricot (Lister, 1970; Németh, 1986). In Japan, this virus is one of the causative agents of apple topworking disease and induces lethal decline in apple trees grown on Maruba kaido (*Malus prunifolia ringo*) rootstocks (Yanase, 1974). ACLSV has very flexuous filamentous particles, approximately 600 to 700 nm in length and contains a polyadenylated plus-sense, ssRNA with an M_r of $2·48 \times 10^6$ and a single coat protein of 22K (Yoshikawa & Takahashi, 1988). At present, this virus is classified into the closterovirus group based on its particle structure (Francki *et al.*, 1991). Recently, German *et al.* (1990) reported the nucleotide sequence of the genome of ACLSV isolated from plum (*Prunus domestica*) (ACLSV-P) in France.

In this paper, we report the complete nucleotide sequence of the genome of ACLSV isolated from apple (ACLSV-A) in Japan and the comparisons of the nucleotide sequence and the deduced amino acid sequence with those of ACLSV-P.

ACLSV-A (isolate P-205), originally isolated from an apple tree (Yanase, 1974), was propagated in *Chenopodium quinoa* and purified as described previously (Yoshikawa & Takahashi, 1988). Viral RNA was extracted from purified virus by dissociation in 2% SDS

for 15 min, followed by extraction with phenol-chloroform, precipitated from 70% ethanol and suspended in water. First and second strand cDNAs were prepared from 2 µg of ACLSV-A RNA according to Gubler & Hoffmann (1983) using oligo(dT) or random hexanucleotides as primers. The dsDNAs were blunt-ended using T4 DNA polymerase and ligated into the *EcoRV* site of Bluescript KS⁺ (BS) or dC-tailed and annealed to dG-tailed, *Pst*I-cut pUC9 (Yoshikawa *et al.*, 1988). The resulting plasmids were used to transform competent *Escherichia coli* DH5 α cells.

The cDNA clones or subclones produced after the ligation of restriction fragments into BS were digested with exonuclease III and mung bean nuclease (Henikoff, 1984) and the resulting deletion mutants were used for sequencing by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 (United States Biochemicals). Each base was determined by sequencing at least two independent clones. More than 80% of the genome was sequenced in both directions and dITP was used to resolve ambiguities resulting from compression.

The sequence of the 5'-terminal region of ACLSV-A RNA was determined by extending a synthetic primer (5' AGGCGTTACGTCAATCTG 3') complementary to nucleotides (nt) 38 to 55 of the viral RNA using reverse transcriptase and terminal deoxynucleotidyl transferase (Deborde *et al.*, 1986). All nucleotide sequence data were collected and analysed using the program GENETYX version 8.0 (Software Development Co. Ltd).

The genome of ACLSV-A consists of 7552 nt ex-

The nucleotide sequence reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number D14996.

[illegible]

4681 TGTTCCTCAATAAAGTGTGAAGAGATCAGAGACTTCTTGATCAATGCAGACAAGAGTTTGAAGAGACCAAAATGTCAAAAAGTGTGCAACCATTTGGAGCGCACTCACAAAGGTCGTGCTC
V P I R K C G R D Q R L L D Q C R Q E F E E T K L S K S A A T T I G A H S Q R S D S

4801 TGATTGGCCATTGGACAAAATCTTTTCATGAAGTCACAAATATGCACCAAGTTTCGAGAGAGAGTTTCACAGAAAGCCAAAGCGGGTCAGACTTTGGCCTGCTTTCCGCATAAGATACT
D W P L D K I F L F M K S Q L C T K F E K R F T E A K A G Q T L A C F P H K I L

4921 TGTGAGTTTTCAGCCCATGTCAGGTACACAGAGAAAGTACTCTAGTCAAAACCTTCCAGATAATATTACATTCATCAAAAGGAAGAACTTCAGTGAACCTTGAAGATTTTGAAGAGGTT
V E F S P W C R Y T E K V L T A N L P D N Y Y I H Q R K N F S E L E D F A K R F

5041 CTCAAATGGCTCAATGTGTGGGAATCGGATTACACAGCTTTCGATGTCTCGCAGGATCATACCATTTTACGCTTTGAAGTGGAGTTGCTCAAGCATTTTGGTGGGATGACAAGGTATT
S N G S M C V E S D Y T A F D V S Q D H T I L A F E V E L L K H F G W D D K V L

5161 ACAGAGTTTACATCAAAATGAAATGCACCTAGGATGCGAGATTGGGAGGTTTTCGCTTATGAGGTTTCACTGGAGAGTTCTCAACATTCCTGTTCAACACACTCGCAATATGGTATTTAC
Q S Y I K M K C T L G C R L G G F A I M R F T G E F S T F L F N T L A N M V F T

5281 CTTTTCAGGATGAAGTTCCAGCGGCACTCCAATTTGCTTTCAGGAGATGATATGTGCTCCCTCAGGAACCTTGAGAGAAATTGACACCATGAATGCATTTTGAAGTTGAGCCT
F C R Y E V P D G T P I C F A G G D D M C A L R N L R E I D T H E C I L S K L S L

5401 CAAAGCAAAAGTGAACAGAAACAAAGTACCGATGTTTGTGGATGGAGTTGTGTTTCAGACGGACTTATAAAGGAACCTTGTGTTGATTACGAAAGATTGCAAGTTGCAATGAAAAATGG
K A K V N R T K V P M F C G W R L C F D G L I K E P C L I Y E R L Q V A I E N G

5521 AAGGTTGATGGATGATAGACATATTTCTGGAATTTTCATTGCGCTACAATAGTGGAGGCTCTATTACATCTTGAATAGAACCACTGAATTACCATCAAGTTTGGACAAG
R L M D V I D S Y F L E F S F A Y K L G E R L Y S H L E I E Q L N Y H L T R

5641 ATTCTTCAATAAGAACCAAGCATTTGTTAAGGGGTGATTCAAGGCACAACTCTCAGAGCTTGAAGTGGCTTTCAGACGAGGATAGCGATGATGATAAGGGGTGACAAATGAAGATCGCAG
F F I R N K H L L R G D S R H N I S E L E W L S D E D S D D D K G S Q I E R R
ORF2 M M I R G H X L K I A E

5761 AAGGGGATATTCCTCAATTCCTGGGGTGAAGGCTCCAGAATTTATCTGATGTGAGTCTCTTCAGGAAGGCTCTGACCTTATGATTCATTGGAATGAATTGATTCAGGTTATGCCAG
R G Y S N C W G E K L Q N L F
G D I P I A G V K S S R I Y S D V S P F R K A S D L M I H W N E F V F K V M P E

5881 AGGATATTGACGGTGTGATTTTATAGATTGGCATCCATACCTGTTATACCTTCTCAGAGGTTCAAGCGGTGCTGAGAAAGAGAGAGTACCAACTACGTGATTTGGGAGCACTATCAA
D I A G D G F R L A S I P V I P S S E V Q A V L R K R E S T N Y V H W G A L S I

6001 TATCCATTGACGCACTGTTTCAGAAAGAAATGCCGGGTTTCAGGGTGTGTTATGTGTACGACAACAGATGGGAAACTTTTGAGCAGGCGATGCTACAAAAGTTTCGGTTCAACCTGGATA
S I D A L F R K N A G V S G W C Y V Y D N R W E T F E Q A M L Q K F R F N L D S

6121 GTGGCTCGGCCACATTTGGTGACCTCACCGAATTTCCAGITTCATTGGATGATCCTGGTTTGTCCAATTTCGATCAGTGTAGCGGTGATGTTTGAGAATCTGAACCTCAAGCTTGAAGCTY
G S A T L V T S P N F P V S L D D P G L S N S I S V A V M P E N L N F R L E S Y

6241 ACCCTATAAGTGTAGGGTAGGGAACATGTGTAGATTCTTCGACAGCTCTTGAAGTTCGCTCAAGAATAAAGTGGACTCCAACCTTTTGGCTTAGGCGCGCTAATGCAGATCCGCTTGGCG
P I S V R V G N M C R F F D S F L S C V K N K V D S N F L L E A A N A D P L G A

6361 CGGGTGTCTTCGGGTTTGAACAGGACGATCAGGTCTCTGAATTTGTTCAATATATTACAGACTGTCCCAACCCAGGCAATTAATCCAGGGAGCATGAAGTACCGAGGGGTTTGTTCGGTA
G A F G F E Q D D Q V S E L F N Y I Q T V P T Q A I K S R E H E V P R G L F G M

6481 TGATGGGAAAAAAGAGTCAAACTTTTGAAGTTTGCCTCTGTTCAAGAATCTGGGAAGAAGAAACCGCAACGCGGAAGACCTTTGGAAAGATCTGCTAGCCTGAGAGTTGCTCCTG
M G K K K V K S F E F A S G S R N L G R R K P Q R G R P L E R S A S L R V A P G

6601 GATTTAGTCCCAGGATGAGAGAGTTGAACATCAAGGATTATCGACTGACTCTGATTTTGAATAATTTCTGAGGAAGGCAAGGAAAGCTGGAATGAATCCATTACTTCAGAAAGGT
P R S Q D E R V E H Q G L S T D S D F E N F L R K G K G K A G T E S I T S E G S

6721 CATCCTTCGATAACATTTCTGCAAGAGAGTTTCAGTTTGTAGACAAGATCAGAAGGCGAAGGATGGCGGAGTGTGAACCTCCAATTAAGTGGACGAGATCTGAAGCGTTCTCTG
S F D N I S A R E F Q F A R Q D Q K A K D G G S A E P P I K S G R R S E S V P G
ORF3 N A A V L N L Q L K V D A D L K A F L

6841 GCCCGAGAAGCGAGACCCCTTCATGGAAGAGACAGGGGCAATTCGGAACAGACACTGGAGGCCATCTTCGCAACATAGCGATACAGGGGACCTCGGAACAAACAGAGTTCTCTGAGCGTG
R R R Q T P S W K D R G N S G T D T G G H L R E H S D T G D L G T N R V P G R A
A A E G R P L H G K T G A I L E Q T L E A I F A N I A I Q G T S E Q T E F L D V

6961 CTGTGGAGGTTGAATCCATGGAGGATCAGAAGGTGGTGGGTCATTCAATCTGAAGAGGTAGTCGGTTGATCAAAATATTACAGACTACATCTTCGACCCGAATAAAGCAGCATG
G G G E I H G G S E G G V I Q S E G S R F D Q N I Q D Y I F G P E Y K Q H D
L V E V K S M E D Q K V V G S F N L K E V V G L I K I F R T T S S D P N I S S M

7081 ACCTTCGCGCAAGTGTGTGAGGCAATTTGCCCGGAGGCAAGAAATGGGCTCGTAAAGCTAAAGTACAAGGGGTCTTCACAAACCTCTTTTCTACTACGCTGAAGTGGGGGGGAGTAT
L P P S V
T F R Q V C E A F A P E A R N G L V K L K Y K G V F T N L F S T T P E V G G K Y

7201 CGGAGCTCATGTTTGACTTCAATAAGGCGCTAAATATGTTCAATAATGAACAAAGCCAGCAGAAAGTGATAACTAATATGAACCGCGCTCTTTTACAGACTGAATTTGCAAGAGCGAA
P E L M F D F N K G L N M F I N N K A Q Q K V I T N M N R R L L Q T E F A K S E

7321 AATGAGGCGAAATGTCTGTCTGTTACAATGATCTTTGCGTTTAGTCTCATAAGGAAGTTTGGTTTAAATAAAGTTAATAAATAGTGTGCAATTTGGTTAATGTCAATTTGTGTAT
N E A K M S S V T T D L C V

7441 TTAATTAAGTCAAACTCTTGAACCCATGAGAGAGTATAAAGCAGTCATGGTATCTAATTTGGAGTGTTTAGACTTATAATAAAGCTGTAGACTTTTAAATATTTTACTAC poly(A)

Fig. 1. The complete nucleotide sequence of the ACLSV-A genome and the amino acid sequences of three ORFs. The amino acid sequences of the conserved motifs associated with the helicase and the RNA polymerase are underlined.

cluding the 3' poly(A) tail (Fig. 1), three nt fewer than that of ACLSV-P reported by German *et al.* (1990) (Table 1). Analysis of the putative open reading frames

(ORFs) of the nucleotide sequence in both positive and negative strands showed that the genome of ACLSV-A contains three ORFs in the positive strand (Fig. 1).

Table 1. Comparisons between ACLSV-A and ACLSV-P genomes

	ACLSV-A	ACLSV-P	Identity
Length excluding poly(A) tail			
Whole genome	7552 nt	7555 nt	79.8%
5' non-coding region	151 nt	151 nt	92.7%
3' non-coding region	187 nt	190 nt	81.2%
M_r of ORF products			
ORF 1	216503	216559	88.4%
ORF 2	50453	50831	79.9%
ORF 3	21394	28376	88.6%

ACLSV-A(5715) ACGAGGATAGCGATGATGATAA

 ACLSV-P(5712) ACGAAGATGGCGACAATGATAA

Fig. 2. The nucleotide sequences surrounding the ORF 2 initiation codons of ACLSV-A and ACLSV-P. Identical nucleotides are shown by asterisks. Second ATG codons within ORF 2 are underlined.

ACLSV-A(6604) TTAGGTCCGAGGATGAGAGAGTTGAACATCAAGGATTATC

 ACLSV-P(6601) TTAGGTCCGAGGATGAGAGAGTTGAACATCAAGGCTTATC

 ACLSV-A(6644) GACTGACTCTGATTTTGAATAATTTCTGAGGAA-AGGCAA

 ACLSV-P(6641) AACAGACAGCGATTTCGAGAATTTCTCAGAAACAAGAGG

 ACLSV-A(6683) AGGAA--AAGCTGGAAGTGAATCCATTACTTCAGAAGGGT

 ACLSV-P(6684) GGTAATAAAGCTGGAGTTAAATCCACTGCTTCAGAAGGGT

 ACLSV-A(6721) CATCCTTCGATAACATTTCTGCAAGAGAGTTTCAGTTTGC

 ACLSV-P(6721) CATCAGTTGATAACATTTCTTCAAGAGAATTTTCAGTTTGC

 ACLSV-A(6761) TAGACAAGATCAGAAGGCCAAGGATGCGCGCAGTGCTGAA

 ACLSV-P(6761) TCGACAGAACCAGGCCAAGGAGGATGCGCAGGTTCTGAA

Fig. 3. Nucleotide sequence alignment of the regions surrounding the ORF 3 initiation codons of ACLSV-A and ACLSV-P. Asterisks indicate identical nucleotides in both isolates. The stop codon between the ATG and second ATG of ACLSV-A is underlined.

ORF 1, preceded by a 151 base leader sequence, starts at AUG (positions 152 to 154) and ends at UGA (positions 5807 to 5809) and encodes a protein with an M_r of 216503 (216.5K). The ORF 1 protein contains two motifs associated with the helicase (GxxxGxGKS) and the RNA polymerase (GxxxTxxxNT, GDD) at positions 1060 to 1067 and 1694 to 1728, respectively (Fig. 1). The overall similarity of the ORF 1 protein between ACLSV-A and ACLSV-P is 88.4% (Table 1). However, the regions surrounding the above two motifs were highly conserved between both isolates and their identities were 97.6% (positions 1058 to 1305 for ACLSV-A and 1057 to 1304 for ACLSV-P) and 97.7% (positions 1637 to 1767 for ACLSV-A and 1636 to 1766 for ACLSV-P), respectively.

ORF 2 begins at AUG (positions 5727 to 5729) and terminates at UGA (positions 7098 to 7100) to yield a protein with an M_r of 50453 (50.4K) (Fig. 1). The

Fig. 4. Immunoblot analysis of the proteins expressed in *E. coli* containing pTrc99A-CP. Lane 1, proteins from *E. coli* cells containing pTrc99A grown in the presence of IPTG; lane 2, proteins from *E. coli* containing pTrc99A-CP without IPTG; lane 3, proteins from *E. coli* containing pTrc99A-CP induced by IPTG; lane 4, coat protein from purified ACLSV-A; lane 5, proteins from *C. quinoa* leaves infected with ACLSV-A; lane 6, proteins from healthy *C. quinoa* leaves. An arrowhead indicates the position of the coat protein of ACLSV-A.

position of the initiation codon of the 50.4K protein is six nt downstream from the corresponding position in ACLSV-P (Fig. 2). This location of the initiation codon was the same in the three different cDNA clones sequenced. The 50.4K protein of ACLSV-A, as did that of ACLSV-P, showed an amino acid similarity of 22.4% with the gene I protein of cauliflower mosaic virus (Franck *et al.*, 1980), and the identity of the ORF 2 protein between ACLSV-A and ACLSV-P was 79.9% (Table 1).

ORF 3 contains an AUG (positions 6784 to 6786) and ends at UGA (positions 7363 to 7365) encoding a protein with an M_r of 21394 (21.4K) which is dissimilar to the 28.3K protein encoded by ORF 3 of ACLSV-P. This discrepancy is explained as follows. The initiation codon (AUG, positions 6616 to 6618) is located in the ACLSV-A genome at the almost same position as the AUG codon of the 28.3K protein of ACLSV-P, but the termination codon (UGA, positions 6670 to 6672) exists between the first AUG and the second AUG (positions 6784 to 6786) which is the initiation codon of the 21.4K protein (Fig. 3). The identity of ORF 3 proteins between both isolates was 88.6% (Table 1).

In *in vitro* translation experiments reported previously

(Yoshikawa & Takahashi, 1989), ACLSV-A RNA directed the synthesis of 105K and 51K proteins as major products and several minor proteins of which the 23K protein was immunoprecipitated with ACLSV-A antiserum. The products (51K and 23K proteins) detected in translation experiments may be synthesized from fragmented RNAs of ACLSV-A RNA because the proteins encoded by ORFs 2 and 3 are thought to be translated from subgenomic RNAs (German *et al.*, 1992).

The M_r (21·4K) of the protein encoded by ORF 3 of ACLSV-A agrees with that of the ACLSV-A coat protein M_r estimated by PAGE (Yoshikawa & Takahashi, 1988). To examine whether the 21·4K protein is indeed the coat protein, the ORF 3 region was amplified by PCR using pBCLS38 containing the 3·8 kb insert of the 3'-terminal region, a synthetic oligonucleotide (5' AAGAATTCAGGATGGCGGCAGTGCTG 3' corresponding to nt positions 6781 to 6798 and containing an *EcoRI* site) and a universal primer (5' AACAGCTATGACCATG 3') located in the multicloning site of the BS plasmid. The PCR product was digested with *EcoRI* and *KpnI* and ligated to a pTrc99A expression vector restricted with the same enzymes (designated pTrc99A-CP) and the product was used to transform *E. coli* JM105. Proteins were then prepared from the cultures grown in the presence of IPTG and analysed by immunoblotting using ACLSV-A antiserum as described previously (Yoshikawa *et al.*, 1992). Fig. 4 shows the result of immunoblot analysis, in which the protein expressed in *E. coli* containing pTrc99A-CP reacted with the antiserum against purified ACLSV-A. This protein is slightly larger than coat protein from purified virus and infected *C. quinoa* leaves because the expressed protein contains four additional amino acids derived from the vector plasmid and ACLSV-A cDNA. These results demonstrate that the 21·4K protein encoded by ORF 3 is the coat protein of ACLSV-A, supporting the results obtained before following the determination of the partial amino acid sequence of the ACLSV-P coat protein (German *et al.*, 1990).

At present, ACLSV is classified into the closterovirus group, based mainly on its particle structure (Francki *et al.*, 1991). This group is composed of heterogeneous virus species with different particle lengths, transmission modes and tissue localization of the virus particles in infected material. The genome organization of ACLSV is quite different from that of the 3'-terminal genomic region of beet yellows virus (Agranovsky *et al.*, 1991), the

type member of the closterovirus group, indicating that ACLSV should be classified into a new virus group. Further investigations on the genome organization of closteroviruses will provide valuable information on the classification of this group.

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