Analysis of Double-stranded RNA in Tissues Infected with Apple Stem Grooving Capillovirus

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Abstract

Northern hybridization analyses using an RNA probe corresponding to the 3'-terminal region of apple stem grooving virus (ASGV) genome indicated that all dsRNA preparations from tissues infected with 10 ASGV isolates contained three virus-specific dsRNAs (6.5, 2.0 and 1.0 kbp). Using additional RNA probes corresponding to different parts of the genome, a dsRNA preparation from ASGV (isolate P-209)-infected tissues was found to contain five virus-specific dsRNAs of 6.5 kbp (G-ds), 5.5 kbp (ds1), 4.5 kbp (ds2), 2.0 kbp (SG-ds1) and 1.0 kbp (SG-ds2). G-ds was probably the replicative form of the ASGV-genome. Ds1 and ds2 were thought to be 5'-coterminal with the ASGV genome. In contrast, SG-ds1 and SG-ds2 were thought to be 3'-coterminal with the genome. Both the positions of these RNAs relative to the genome and their sizes suggest that SG-ds1 and SG-ds2 are ds forms of subgenomic RNAs for the ORF2-encoded protein and the coat protein (CP), respectively. The C-terminal regions of the ORF1-encoded protein including the CP were expressed in Escherichia coli. The size of the expressed protein, which starts with methionine (Met) at amino acid position 1869 agreed with that of ASGV-CP, suggesting this Met is the starting amino acid of the CP.

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Key words: apple stem grooving virus, capillovirus, dsRNA, coat protein.

INTRODUCTION

Apple stem grooving capillovirus (ASGV) has a flexuous, filamentous particle 650 nm long, which contains a ssRNA and a coat protein of Mr 27 kDa14). ASGV is the type species of the genus Capillovirus, which also includes citrus tatter leaf virus (CTLV, recently proved to be an isolate of ASGV)9,14), and possibly lilac chlorotic leaf spot and Nandina stem pitting viruses10). Recently, cherry virus A (CVA) was also reported to be a capillovirus10). The complete nucleotide sequences have been reported for the genome of two capilloviruses, ASGV (isolates P-209, L and Li-23)11,17) and CVA13). The ASGV genome consists of 6496 nucleotides (nt), excluding the poly (A) tail, and has two overlapping open reading frames (ORFs 1 and 2) encoding a 240-241 kDa polyprotein (2105 amino acids, aa) and a 36 kDa protein (320 aa), respectively. The ORF1-encoded protein contains the conserved motifs of methyltransferase, papain-like protease, helicase and RNA polymerase, and a coat protein (CP) in the C-terminal region11,17). A 36 kDa protein encoded by ORF2 is thought to be a movement protein11,16). An ORF1 region (V-region)9) between the RNA polymerase and the CP, overlapping ORF2 in another frame, does not contain any known functional motifs found in plant virus genomes11,14). The CVA genome (7383nt, excluding the 3' poly (A) tail), also encodes a 266 kDa polyprotein (ORF1) and a 52 kDa protein (encoded by ORF2 which is located within ORF1)13).

Although the sequence data indicated that the CP in the capillovirus genome is located in the C-terminal region of the ORF1-encoded polyprotein11,13,16), the exact beginning of the CP has not been determined. At present, whether the capillovirus CP is expressed by translation as a part of ORF1 protein followed by proteolytic processing or from subgenomic RNA produced in infected cells is unknown.

To elucidate the expression strategy of ASGV-CP, we analyzed virus-specific double-stranded (ds) RNAs in infected leaves using several RNA probes corresponding to different parts of the genome and expressed of the CP region in Escherichia coli.

MATERIALS AND METHODS

Virus ASGV (isolates P-209, AF38 and AF39 from apple; JP1, JP2, JP3 and JP4 from Japanese pear; EPSB from European pear and OKITSU-WASE and BUC-neg. No. 3 from citrus14) were propagated by sap inoculation in Chenopodium quinoa for use in this study.
**Extraction of dsRNAs from infected leaves**

DsRNAs were extracted from a 20 g leaf sample infected with ASGV as described previously. The dsRNAs eluted from cellulose columns (two cycles) were precipitated in ethanol, dried and dissolved in distilled water.

**Northern hybridization**

Five cDNA fragments, which cover the entire genome of ASGV (P-209), were prepared from the clone pSG 417 by restriction enzyme digestion and were subcloned into plBluescript KS+.

Subclones obtained were pBSG41E8EB (nucleotide positions 45 to 2290), pBSG41H18 (1822 to 3308), pBSG41H13 (3097 to 4749), pBSG41H2 (4750 to 5769) and pBSG41B3 (5138 to 6496). Positive-sense RNA probes (Fig. 2a) were transcribed from these clones by T7 RNA polymerase (BRL) in the presence of digoxigenin-UTP. DsRNAs from healthy and infected leaves were denatured with formamide, electrophoresed in 1% agarose gel in MOPS buffer and transferred to Hybond-N nylon membranes (Amersham). Prehybridization and hybridization were done at 68°C for 2hr and 16hr, respectively. The concentration of each RNA probe was ca. 200 ng/ml. Post-hybridization washing and immunological detection using CSPD™ as a chemiluminescent substrate followed protocols supplied with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

**Expression of coat protein regions in E. coli**

To construct the expression vector of ASGV-CP in E. coli, we used two forward primers, SGCP1Nde (5'-GAGACCATATGAGGAAAATCAAGATGTCGCT-3'), corresponding to nt positions 5430 to 5449 (underlined) of ASGV genome and containing a NdEl site) and SGCP2Nde (5'-GAGACATATGAGTTTTGGAAGAGGATCTAATTCTCCAGTCGCT-3'), corresponding to nt positions 5641 to 5660 and containing a NdEl site) and a reverse primer, SGCPBa (5'-AGAGGATCATGAGTTTGGAAGACGGCTTTTGAGGTCGCT-3'), complementary to nt positions 6337 to 6356 including the stop codon of ORF1 and containing a BamHI site). The CP regions were amplified by polymerase chain reaction (PCR) using pSG41 as a template and two primer pairs, SGCP1Nde+SGCPBa or SGCP2Nde+SGCPBa. The PCR products were digested with NdEl and BamHI and ligated to a pET3a expression vector (Novagen). The resulting plasmids, designed as pSGCP1 and pSGCP2, were expected to express the 35 kDa and 27 kDa proteins, respectively. We also constructed two plasmids, pSGCP2+1aa and pSGCP2-2aa by use of a forward primer, SGCP2Nde and two reverse primers, SGCP+1Ba (5'-TCTCTGGATCCTAACTTACCCCTACCTAGCTCAGGC-3') or SGCP-2Ba (5'-TCTCTGGATCCTACCCCTACCTAGCCTAGTTACT-3'). The reverse primer, SGCP+1Ba has a sequence to which a codon (TTT) was added in front of the ORF1 stop codon in SGCPBa. The reverse primer SGCP2-2Ba has a sequence in which two codons (5'-ACC CCC TC-3') in front of the ORF1 stop codon were deleted from SGCPBa. These plasmids were subsequently used to transform competent E. coli BL21(DE3)pLysS. The cells of E. coli containing each plasmid were grown at 37°C to a cell density of 0.6 (A600nm), with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM, and further grown for 2hr. Proteins were extracted from E. coli according to manufacturer's protocols.

**Immunoblot analysis**

In order to examine whether RNA transcribed from cDNA corresponding to the ASGV-CP region can direct the synthesis of the CP efficiently in an in-vitro translation system, a DNA fragment was amplified by PCR using the primer pair, SGCP2 (5'-GATTTAGGTCCCTCTCAGCT-3', corresponding to nt positions 5599 to 5618) and ASCT3' (5'-AGAGTGGACAAACTATAGAC3', corresponding to nt positions 6477 to 6496). A DNA product was ligated to pT7 blue T-vector (Novagen) and used to transform E. coli, we used two forward primers, SGCP1Nde (5'-GAGACCATATGAGGAAAATCAAGATGTCGCT-3'), corresponding to nt positions 5430 to 5449 (underlined) of ASGV genome and containing a NdeI site) and a reverse primer, SGCPBa (5'-AGAGGATCATGAGTTTGGAAGACGGCTTTTGAGGTCGCT-3'), complementary to nt positions 6337 to 6356 including the stop codon of ORF1 and containing a BamHI site). The CP regions were amplified by polymerase chain reaction (PCR) using pSG41 as a template and two primer pairs, SGCP1Nde+SGCPBa or SGCP2Nde+SGCPBa. The PCR products were digested with NdeI and BamHI and ligated to a pET3a expression vector (Novagen) and used to transform E. coli. The plasmid was digested with NdeI and SpAI, blunt-ended by T4 DNA polymerase and self-ligated to remove the multicloning site of pT7 blue. The RNA transcript synthesized from the construct by T7 RNA polymerase was translated in a rabbit reticulocyte system using the ECL in-vitro translation system (Amersham). The immunoprecipitation experiment was carried out as described previously.

**RESULTS**

**Analysis of dsRNA from ASGV-infected tissues by northern hybridization**

In preliminary experiments, RNA probes did not hybridize with any dsRNAs from healthy C. quinoa tissues under the hybridization conditions used. Then we analyzed dsRNAs from 10 isolates of ASGV using probe 41HB3(+), corresponding to the 3'-terminal region of the genome. The results indicated that all tissues infected with one of the 10 isolates contained three virus-specific dsRNAs of 6.5 kbp (G-ds), 2.0 kbp (SG-ds1) and 1.0 kbp (SG-ds2) (Fig. 1). We further analyzed the positions of dsRNAs relative to the genome by use of five RNA probes (Fig. 2a). Three positive-sense RNA probes, EB1(+), 41H18(+) or 41H13(+), gave the same hybridization signals, in which three dsRNAs were hybridized with these probes (Fig. 2c, lanes 1 to 3). The slowest migrating dsRNA (G-ds) has a size of 6.5 kbp equivalent to that of the entire ASGV genome. The others have ca. 5.5 kbp (ds1) and 4.5 kbp (ds2). Probe 41H21(+), which corresponds to the ORF2 region, hybridized with ca. 2.0 kbp dsRNA (SG-ds1) as well as G-ds and ds1, but not with ds2 (Fig. 2c, lane 4). Probe 41H31(+), corresponding to the 3'-terminal 1.4 kbp region of the genome hybridized with G-ds, SG-ds1 and an additional 1.0 kbp dsRNA (SG-ds2) (Fig. 2c, lane 5). A weak signal of ds2 was also detected by this probe. From these results, we determined the positions of ASGV (P-209)-specific dsRNAs relative to the ASGV genome (Fig. 2b). G-ds was thought...
Fig. 1. Analysis of dsRNAs from tissues infected with ten isolates of ASGV by northern hybridization using a probe [41HB3(+)] corresponding to the 3'-terminal region of the ASGV (P-209) genome. Lane 1, P-209; lane 2, AF38; lane 3, AF39; lane 4, JP1; lane 5, JP2; lane 6, JP3; lane 7, JP4; lane 8, EPSB; lane 9, OKITSU-WASE; lane 10, BUC-neg. No. 3.

Fig. 2. Analysis of dsRNAs from ASGV (P-209)-infected tissues by northern hybridization. (a) The relative positions of positive-sense RNA probes to ASGV genome. (b) The predicted positions of ASGV-specific dsRNAs from infected tissues relative to the ASGV genome. (c) Northern hybridization of dsRNAs, using positive-sense RNA probes EB(+) (lane 1), 41H18(+) (lane 2), 41H13(+) (lane 3), 41H2(+)(lane 4) and 41HB3(+) (lane 5). The positions of RNA size markers (transcripts from ASGV cDNA clones) are indicated on the right.

Attempts to detect subgenomic RNAs in total and poly(A)+ RNA preparations from infected tissues by northern hybridization were unsuccessful, probably due to low concentrations of subgenomic RNAs in infected tissues.

Expression of coat protein regions of ORF1 protein in E. coli

From the results of the northern hybridization experiments described above, we speculate that ASGV-CP may be expressed from a 1 kb subgenomic RNA in to be a replicative form of the ASGV genome because it hybridized with all probes and its size was the same as that of the entire genome. Both the positions of SG-ds1 and SG-ds2, which are thought to be 3'-coterminal with the genomic RNA, and their sizes suggested that these RNA species may be ds forms of subgenomic RNAs for the ORF2 protein and CP, respectively. On the other hand, ds1 and ds2, thought to be 5'-coterminal with the genomic RNA, have unknown functions, although the production of these dsRNAs is quite similar to that of dsRNAs in apple chlorotic leaf spot trichovirus (ACLSV)-infected tissues.

Attempts to detect subgenomic RNAs in total and poly(A)+ RNA preparations from infected tissues by northern hybridization were unsuccessful, probably due to low concentrations of subgenomic RNAs in infected tissues.
infected tissues. If so, methionine (Met) at aa position 1869 (AUG at nucleotide position 5641-5643) of this ORF1-encoded protein is a candidate for the starting amino acid of the CP because the calculated Mr (27041 Da) of this putative CP agrees with the Mr of ASGV-CP from purified particles. Several attempts to determine the N-terminal sequence of ASGV-CP from purified viruses were unsuccessful, probably due to blocking of the N-terminus of the ASGV-CP.

Figure 3a shows that proteins of the expected sizes were expressed in E. coli containing plasmids pSGCP1 and pSGCP2. Immunoblot analysis also indicated that both proteins reacted with ASGV antiserum (Fig. 3b). The electrophoretic mobility of protein CP2 from pSGCP2 was almost the same as that of the ASGV-CP from infected C. quinoa tissues (Fig. 3b). The expression vectors pSGCP2+1aa and pSGCP2-2aa were expected to express CP2+1aa (CP2 plus an additional amino acid phenylalanine) and CP2-2aa (CP2 minus two amino acids threonine and leucine), respectively. The expressed proteins co-electrophoresed with CP2 and ASGV-CP from purified viruses in an SDS-12.5% polyacrylamide gel. Figure 4 shows that the electrophoretic mobility of ASGV-CP is identical to that of CP2 and can be distinguished from those of CP2+1aa or CP2-2aa. This result strongly suggests that the Met at aa position 1869 of ORF1-encoded protein is the starting amino acid of ASGV-CP. To investigate whether the AUG of this position can act as an initiation codon in an in vitro translation system, the RNA transcript (nt positions 5599 to 6496 of ASGV genome) corresponding to the CP2 region was tested in a rabbit reticulocyte lysate. RNA transcribed from pSGCP2R was a highly efficient messenger for the 27kDa protein, which comigrated with ASGV-CP and reacted with ASGV antiserum (data not shown).

DISCUSSION

The capillovirus CP is located in the C-terminal region of the ORF1-encoded polyprotein. Although the evidence is circumstantial, the following results obtained in this study suggest that the CP of ASGV is expressed from subgenomic RNA, not by translation, as a part of the ORF1-encoded protein followed by proteolytic processing in infected cells: (1) A dsRNA (SG-ds2) corresponding to the 3'-terminal region of ASGV genome was found in vivo, (2) The size of the E. coli-expressed protein corresponding to the C-terminal region of the ORF1-encoded protein which starts with the Met at the aa position 1869 agreed with that of ASGV-CP, and (3) In vitro translation of the RNA transcript from the cDNA clone showed that this Met (AUG) codon acts efficiently as an initiator for ASGV-CP. The Met at this position is conserved in all 21 isolates or sequence variants sequenced. Comparison of the preceding nucleotide sequence of the AUG (nt position 5641) of the putative coat protein with that of the ORF2-encoded protein from ASGV isolates P-209 and Li-23 shows some homologous regions (Fig. 5), suggesting that these
regions may function as promoters of subgenomic RNA synthesis and/or as recognition signals for translation. The determination of the 5′-terminal nucleotide sequences of putative subgenomic dsRNAs will confirm this hypothesis.

The capillovirus is phylogenetically related to trichoc-, carla- and tymoviruses in which CPs are expressed from this hypothesis. Sequences of putative subgenomic dsRNAs will confirm the determination of the 5′-terminal nucleotide synthesis and/or as recognition signals for translation.

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Literature cited


和文摘要

真髙 洋・寺内英貴・吉川信幸・高橋 努：リンゴシステムグルービングウィルス感染組織中の二本錠RNA解析

リングシステムグルービングウィルス（ASGV）の10分離株について、感染果から2本錠RNA（dsRNA）を抽出し、ASGV（P-209分離株）ゲノムの3′未端領域に対応するRNAブロープを用いてノーザンハイブライダイゼーション解析を行った。その結果、いずれの分離株でも3種類のウィルス特異的dsRNA（約6.5kbp、2.0kbp、1.0kbp）が検出された。続いて、P-209ゲノムの異なる領域に対応する5種類のプラス錠RNAブロープを用いて、P-209感染果からのdsRNAを分析したところ、5種類のウィルス特異的dsRNA（G-ds（6.5kbp）、ds1（5.5kbp）、ds2（4.5kbp）、SG-ds1（2.0kbp）、SG-ds1（1.0kbp））が検出された。これらdsRNAのうち、G-dsはゲノムの複製型と考えられた。また、SG-ds1とSG-ds2はゲノムの3′未端側に位置していることから、ORF2および外送タンパク質（CP）遺伝子のサブゲノムRNAに対する考えが示唆された。P-209ゲノムの3′未端側領域（CP領域）のcDNAを発現ベクター（pET3a）に組み込み、細胞内でCPを発現させた。ORF1タンパク質（2015アミノ酸）の1869番目のメチオニンからの発現タンパク質はASGV-CPとサイズが一致したことから、このメチオニンがCPの開始アミノ酸と推定された。