Nucleotide sequences of apple stem pitting virus and of the coat protein gene of a similar virus from pear associated with vein yellows disease and their relationship with potex- and carlaviruses

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The nucleotide sequence (9306 nucleotides) of cDNA clones of apple stem pitting virus (ASPV) obtained from a double-stranded RNA template, extracted from diseased plant tissue, was determined. The genome is composed of five open reading frames (ORFs) encoding putative proteins with Ms of 247083, 25147, 12832, 7429 and 43712, and has a poly(A) tail. Using two oligonucleotides designed from the ASPV sequence information a 1598bp fragment from near the 3' terminus of the viral RNA, containing the coat protein of Mr 43766, was amplified from vein yellows (VY)-infected pear plants by PCR. The sequence determined showed eight nucleotide changes resulting in five amino acid substitutions compared with the sequence of ASPV. When compared to potex-, carla-, clostero- and capilloviruses, the ASPV genome organization appeared to be most closely related to that of potexviruses, but with a larger coat protein of Mr 44K (ORF5). The predicted coat protein size was confirmed by immunoblot analysis. The results show that ASPV does not fall into subgroup A of the closteroviruses but that it probably belongs in an as yet undefined group of viruses. They also suggest that the virus associated with VY is a strain of ASPV.

Introduction

Apple stem pitting virus (ASPV) is a latent virus of apple, common in commercial cultivars. Symptoms occur only on woody indicators and some ornamental Malus species and comprise xylem pits in the stem of Virginia Crab and epinasty and decline of Spy 227 (Stouffer, 1989). Pear vein yellows (VY) is distributed worldwide in pear and is caused by a graft-transmissible viral agent, presumably closely related to ASPV (Refatti & Osler, 1975; Kegler et al., 1979; Yanase et al., 1989; Jelkmann et al., 1992a; Giunchedi & Poggi Pollini, 1992). Symptoms consist mainly of a narrow yellow banding of veins and a red mottling and flecking along the veins. Because infected cultivars often remain symptomless, sensitive indicators are used for VY indexing (Cameron, 1989). Until recently little was known about the viral agents causing these diseases. Koganezawa & Yanase (1990) transmitted a virus from stem pitting diseased apple to Nicotiana occidentalis and from this species purified a virus with flexuous, filamentous particles, 12 to 15 nm wide and 800 nm long, that readily formed end-to-end aggregates. The viral coat protein was estimated to be of Mr 48000 and the particles contained single-stranded RNA of Mr 3.1 x 10^6. These data and the lack of an obvious cross banding of particles suggested that the virus isolated does not belong to any of the established taxonomic groups of plant viruses.

Jelkmann et al. (1992a) reported the production of ASPV cDNA clones obtained from dsRNA. Based on preliminary nucleotide sequence information a portion of a coat protein gene clone was expressed in vitro and an antiserum was prepared that detected and decorated ASPV and VY-associated virus particles from woody and herbaceous hosts. The particle characteristics of both viruses were as described by Koganezawa & Yanase (1990). Currently ASPV is classified as a tentative member of the closterovirus subgroup A (Coffin & Coutts, 1993) which also contains apple chlorotic leafspot virus (ACLSV). Based on particle morphology and cross reaction with antiserum to ASPV Giunchedi & Poggi Pollini (1992) suggested that the VY-associated virus should be assigned to the closterovirus subgroup containing ACLSV.

This paper describes the genome organization of ASPV and a partial nucleotide sequence at the 3' terminus of the viral RNA, covering the coat protein of
a virus associated with VY of pear. The close relationship of the two viruses and an unexpected homology to potex- and carlaviruses is reported.

Methods

Preparation of viral dsRNA and cDNA cloning. ASPV (isolate PA66) and VY-associated virus (isolate PSA-H) were mechanically transmitted from apple rootstock M9 and pear cv. Williams, respectively, to N. occidentalis subsp. obliqua. Propagation of isolates was in N. occidentalis Wheeler '37B'. Extraction of ASPV dsRNA and cDNA synthesis was performed as described previously (Jelkmann et al., 1989, 1992a). Blunt-ended cDNA fragments were cloned into the EcoRV site of Bluescript M13+ (Stratagene). First-strand cDNA synthesis of the VY-associated dsRNA from pear, using the oligonucleotide (1') 5' CGGGGATCCGAGAGCTTTTTTTTTTTTTTTT 3' was basically similar to that used for ASPV. After removal of low M, materials from the cDNA on a Sepharose CL-4B column an aliquot was amplified by PCR using primer (1) and the ASPV primer (2) 5' GTACGAGTAACTCAGGACC 3'. The PCR product was purified on low melting point agarose and subcloned into the EcoRV site of Bluescript M13+ in which a single dTTP nucleotide had been added at the 3' end according to the method of Marchuk et al. (1990). Unless otherwise stated, molecular biology techniques were performed as described in Sambrook et al. (1989).

Nucleotide sequencing. For nucleotide sequencing of double-stranded plasmid DNA, 37 randomly selected ASPV cDNA clones ranging from 800 to 3600 bp in length were analysed by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a commercial T7 DNA polymerase sequencing kit (Pharmacia) and Bluescript M13+ primers SK 5' TCTAGAAGTCGCGGCTC 3', KS 5' CCGAGCTCGAG- GTATCG 3', T7 5' AATACGACTCACTATAG 3' and T3 5' ATTAACCTCTAAAG 3'. Where convenient, subclones were generated by restriction enzyme digestion and subcloned into Bluescript M13+. Sequence data were obtained from both strands. The cloned VY-associated virus PCR fragment was sequenced as described above.

Direct dsRNA sequencing of the 5'-terminal region was performed by extending a synthetic primer 5' GATACCTTCACCAGCCTG 3' (nt 72 to 53) with dideoxynucleotides using Moloney murine leukaemia virus reverse transcriptase (BRL) in the presence of [α-32P]dATP (Deborde et al., 1986). Attempts were also made to amplify the cDNA by PCR using the 25 bp primer 5' CGGAATTCCTCCGGGGGGGGGGGGG 3' following dC-tailing of the ssDNA with terminal transferase (Boehringer) and the primer used in attempts to determine the sequence at the 5' terminus using direct sequencing.

The 5'-terminal nine nucleotides of ASPV were determined by direct sequencing in the presence of the oligonucleotide 5' CGGGGATCCGAGAGCTTTTTTTTTTTTTTTT 3' and PCR amplification with a second oligonucleotide 5' CTCTGAAAACGCTGATGGC 3' (ASPV nt 8993 to 9012). The sequence was then obtained from dsRNA after subcloning of the PCR fragment into the EcoRV site of Bluescript M13+. Sequence data were collected, assembled and analysed using the GCG program package (Devereux et al., 1984) and the sequence databases of the Heidelberg Unix Sequence Analysis Resources (HUSAR; German Cancer Research Centre Heidelberg).

Electro-blot immunoassay. Total phenol-soluble proteins were extracted from infected N. occidentalis leaves following the method of van Eten et al. (1979). A protein assay (Bradford, 1976) (Bio-Rad) was performed on an 1:10 diluted aliquot of the total preparation. Samples were boiled for 5 min and approximately 100 μg per lane was separated on a 12% SDS-polyacrylamide gel. The gels were semi-dry transferred to nitrocellulose membranes at 100 mA for 45 min (Hoefer TE 70). After blocking with Tris-buffered saline (TBS; 30 mM-Tris–HCl pH 7.4, 200 mM-NaCl) containing 0.5 ml/l Tween 20 (TBS-T) and 30 g/l gelatin for 1 h, the membranes were probed overnight on a shaker at 4°C with specific antisera diluted in TBS-T, and 0.2 g/l Na2SO4. The ASPV antiserum used were ASPV 403 fusion protein antiserum (Jelkmann et al., 1992a) and ASPV Yanase (obtained via A. N. Adams, East Malling, U.K.).

Results and Discussion

Nucleotide sequences of ASPV RNA and the coat protein gene of the VY-associated virus

To determine the nucleotide sequence of ASPV RNA, 37 cDNA clones with inserts ranging from 800 to 3600 bp in length were partially sequenced. The sequence determined from subclones and from the 3'-terminal region totalled 9306 nucleotides, excluding the 3' poly(A) tail which was previously unreported (Fig. 1). The experiment designed to determine the sequence of the 3' terminus was conducted twice and revealed nine nucleotides in addition to those found from clone pASPV206 (nt 7064 to 9297). The two cloned PCR fragments pASPV1 and 2 possessed poly(A) stretches of 32 and 17 nt, respectively. The PCR product from positions 8993 to 9306 was sequenced completely and no differences from the sequence obtained from cDNA clone pASPV206 were found. The 3'-terminal non-translated region was found to be 135 nucleotides long. At the 5' terminus 33 non-coding nucleotides were identified but attempts to determine the exact 5' end from dsRNA using the method described failed repeatedly. Purification of virus particles from N. occidentalis was not successful, therefore genomic viral ssRNA was not available to allow determination of the sequence of the 5' end. In apple stem grooving capillovirus (ASGV) 36 5' non-coding nucleotides (Yoshikawa et al., 1992) were detected and in ACLSV 151 were detected (German et al., 1990). Five separate potexviruses revealed non-translated regions of 80 to 107 nucleotides (summarized in Jelkmann et al., 1992b). Shallot virus X (ShVX), a virus combining elements of carla- and potexviruses, has 98 5' non-coding nucleotides (Kanyuka et al., 1992). Therefore the number of nucleotides not determined at the 5' terminus of ASPV is probably less than 1% of the total sequence.

The ASPV sequence has a base composition of 27.6% A, 20.0% C, 23.4% G and 29.0% T. In the cDNA clones analysed six ambiguous nucleotides (denoted with capital letters above the sequence in Fig. 1) were found when sequencing overlapping cDNA clones which were resolved by sequencing at least one other clone. None of these ambiguities resulted in an amino acid change.

To elucidate the coat protein sequence of the VY-associated virus, which from hybridization experiments with cDNA clone pASPV206 to dsRNA from infected pear (Jelkmann et al., 1992a) was suspected to be closely related to ASPV, a 1598 bp fragment was amplified by
PCR. The sequence, determined from the cloned PCR product is identical with the ASPV sequence from nt 7709 to 9306, except for eight nucleotides shown in Fig. 1 by lower case letters. These result in five amino acid substitutions by bold capital letters below the sequence.

A relationship between symptom expression and the coat protein gene composition has been demonstrated with tobacco mosaic virus (Dawson et al., 1988). Coat protein mutants induced a variety of different symptoms in the systemic host, N. tabacum L. cv Xanthi. Shintaku et al. (1992) showed that only a single amino acid substitution in the coat protein of cucumber mosaic virus was associated with the chlorotic phenotype.

Coding regions of ASPV

Computer-assisted translation of the ASPV nucleotide sequence (Fig. 1) revealed five ORFs reflecting a genome organization similar to that of potexviruses. Potato virus X (PVX) is shown for comparison in the schematic representation on Fig. 2. The putative polypeptides encoded by the different ORFs are: ORF1 (nt 34 to 3682), Mr 247083; ORF2 (nt 6685 to 7353), Mr 25147; ORF3 (nt 7358 to 7717), Mr 247083; ORF2 (nt 6685 to 7353), Mr 25147; ORF5 (nt 7930 to 9171), Mr 43712 (referred to hereafter by their approximate Mr). The ORF for the coat protein of VY-associated virus, equivalent to ORF5 of ASPV (nt 7930 to 9171), translates to a polypeptide of Mr 43766.

The 247K polypeptide encoded by ORF1 reflects the putative viral RNA-dependent RNA polymerase. The initiation codon of this ORF is located at nt 34 to 36; this was supported by a multiple alignment of the N-terminal region including the putative methyltransferase domain (Morozov et al., 1990) with the equivalent sequences of potato virus M (PVM), ASGV and ACLSV. Additionally the GAAATGG is in good context for functional initiation sites with a G in position -3 and a purine at +4 (Kozak, 1987). As presented by Kanyuka et al. (1992) in alignments of ShVX, potex-, carla- and tymoviruses and ACLSV, the motifs of the NTP-dependent helicase (Habili & Symons, 1989) and the RNA polymerase (Argos, 1988) can be observed (alignments not shown).

Homologies of ORFs 2 to 4 of ASPV to those of PVM (Zavriev et al., 1991) and PVX (Skryabin et al., 1988) (members of the carla- and potexvirus groups, respectively) were found by a multiple alignment. The identity and similarity scores are shown in Table 1. These ORFs represent the triple gene block typically found in potex- and carlaviruses. One proposed function of all triple gene block proteins is involvement in cell-to-cell spread, as suggested by mutation studies of infectious in vitro RNA transcripts of white clover mosaic virus (Beck et al., 1990). Other reported roles for the 12K polypeptide of PVM are binding nucleic acids and a regulatory function during virus replication (Gramstat et al., 1990) and for the 7K protein of PVM a membrane-binding function (Morozov et al., 1991).
The most interesting feature of the ASPV sequence is the size of the polypeptide encoded by ORF5. The alignment of Fig. 3 shows significant amino acid sequence similarities to the central and the C-terminal domains of the coat proteins of PVM and PVX. The nature of this protein was clearly identified by expression as a chimeric protein in *Escherichia coli* and the preparation of an antiserum which was able to trap and decorate virus particles in immunosorbent electron microscopy (Jelkmann et al., 1992a). This antiserum was used in immunoblot analysis and identified a 48K protein from ASPV-infected plant tissue (Fig. 4). In parallel experiments with the ASPV antiserum of Yanase a band of the same size was observed. The M,r estimated from the immunoblots is in accordance with the ASPV coat protein size estimated from SDS–PAGE by Koganezawa & Yanase (1990). The difference between the computer-predicted size and immunoblot analysis may be due to high hydrophilicity, as suggested by Kanyuka et al. (1992) for a similar discrepancy with ShVX coat protein.

Carlaviruses have flexuous filamentous particles, normally 610 to 700 nm long and 12 to 15 nm in diameter, composed of a single type of coat protein of M,r 31K to
properties with potex- and carlaviruses have been viruses with coat proteins of 27K to 28K which share the modal particle length of 800 nm and particle morphology by negative staining (Coffin & Coutts, 1993; Guinchedi & Poggi Pollini, 1992). Other unclassified viruses with coat proteins of 27K to 28K which share properties with potex- and carlaviruses have been reported from garlic (Sumi et al., 1993) and further nucleotide sequence data are awaited to clarify the taxonomic position of the viruses concerned.

The homology of the coat protein of ASPV with that of the virus associated with pear VY supports the assumed close relationship between the two viruses. It is proposed that the virus associated with VY be regarded as a strain of ASPV.

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References


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