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The nucleotide sequence and genome organization of *Sclerophthora macrospora* virus A☆

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Abstract

Sclerophthora macrospora virus A (SmV A) found in *S. macrospora*, the pathogenic fungus responsible for downy mildew of gramineous plants, is a small icosahedral virus containing three segments (RNAs 1, 2, and 3) of the positive-strand ssRNA genome. In the present study we report the complete nucleotide sequence of the SmV A genome. The viral genome RNA 1 consists of 2928 nucleotides (nt) and has two open reading frames (ORFs 1a and 1b). ORF 1a contains the motifs of RNA-directed RNA polymerase (RdRp). The function of ORF 1b is unknown. RNA 2 consists of 1981 nt and single ORF (ORF 2). ORF 2 encodes a capsid protein. RNA 3 consists of 977 nt but not any ORFs, suggesting it as a satellite RNA. The deduced amino acid sequence of ORF 1a shows some similarity to those of RdRp of certain positive-strand RNA viruses, especially to the members of the family *Nodaviridae*, and that of ORF 2 to CP of the members in the family *Tombusviridae*. The nucleotide sequence of RNA 3 shows a 40-nucleotide length of partial similarity to *S. macrospora* virus B (SmV B) RNA. The capsid of SmV A is composed of two capsid proteins, CP 1 (p43) and CP 2 (p39), both encoded in ORF 2. CP 2 is apparently derived from CP 1 via proteolytic cleavage at the N-terminus. The genome organization of SmV A is characteristic and distinct from those of other known fungal RNA viruses, including SmV B. These results suggest that SmV A should be classified into a new group of mycoviruses.

Introduction

In *Sclerophthora macrospora*, the pathogenic fungus responsible for downy mildew of gramineous plants, including rice, wheat, etc., two species of virus, *S. macrospora* viruses A (SmV A) and B (SmV B), have been described previously (Hibi et al., 1976; Honkura et al., 1983; Shirako and Ehara, 1985). SmV A is a 30-nm icosahedral virion with spikes of 4 nm on the periphery, composed of three segments of plus sense single-stranded (ss) RNA genome of approximately 3 kb (RNA 1), 2 kb (RNA 2), and 1 kb (RNA 3) and two capsid proteins of approximately 43 kDa (p43) and 39 kDa (p39) (Honkura et al., 1983; Shirako and Ehara, 1985). SmV B is a 32-nm icosahedral smooth virion, composed of one segment of plus sense ssRNA genome of 5.5 kb and one capsid protein of 41 kDa and is serologically distinct from SmV A (Honkura et al., 1983; Shirako and Ehara, 1985). One or both viruses are frequently detected in downy mildew-diseased gramineous plants, but not in all cases (Shirako and Ehara, 1985). These viruses exist abundantly in the cytoplasm of fungal oospores and mycelia, and the amount of the virus in the cells was found to decrease with increased vacuolation in the cytoplasm as the cells aged (Ehara, 1989). The role of these viruses in downy mildew disease and the phenotypic changes evoked in *S. macrospora* are unclear.

The single-stranded nature of the SmV A and B genome is exceptional, as most mycoviruses have double-stranded RNA genomes (Buck, 1986; Hollings, 1978). The positive sense ssRNA mycoviruses have been classified into the two families *Barnaviridae* (Romaine, 2000) and *Narnaviridae* (Wickner, 2000). However, SmV A and B differ from those

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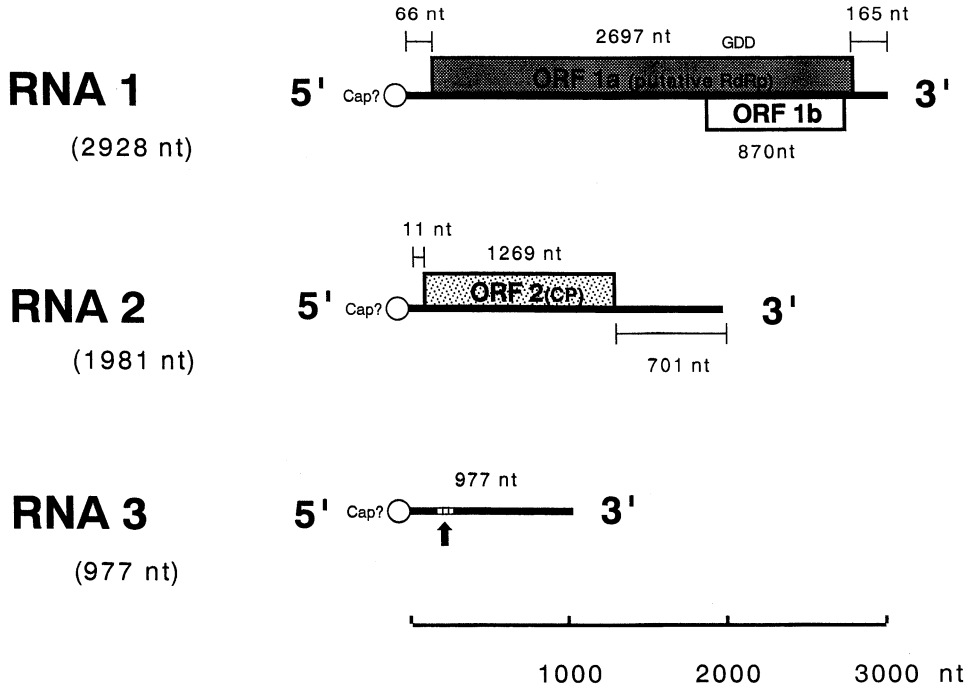

Fig. 1. The genome organization of SmV A. The number of nucleotides is indicated by the scale at the bottom of the figure. The homologous region to SmV B RNA is the hatched box indicated by the arrow within RNA 3.

viruses in particle shape. A member in the family *Barnaviridae* is a bullet-shaped particle (Hollings, 1962) and members in the family *Narnaviridae* do not display particle formation. *Botrytis* virus flexuous (BVF) is also a ssRNA virus, and its genome has been sequenced recently (Howitt et al., 2001). BVF, however, is a rod-shaped particle (Howitt et al., 1995).

Previously, we reported the complete nucleotide sequence of SmV B and indicated that this virus should be classified into a new group of mycoviruses because its genome organization is characteristic and distinct from those of other known fungal RNA viruses (Yokoi et al., 1999).

In this study, the complete nucleotide sequence and genome organization of SmV A RNA were determined and compared with those of some other positive-strand ssRNA viruses.

Results and discussion

Genome organization

RNA 1

Analysis of the determined sequence of SmV A RNA 1 (2928 nt) revealed the existence of two ORFs (ORFs 1a and 1b; Fig. 1). The calculated molecular masses of the proteins encoded by ORF 1a and ORF 1b are 100 and 33 kDa, respectively. Analysis of the predicted amino acid sequence of ORF 1a revealed the presence of the putative RNA-directed RNA polymerase (RdRp) domain containing the motif (Dx3[FYWLCA]x0-1-Dxn[STM]Gx3Tx3[NE]xn[GS]DD), known to occur in other positive-strand RNA viruses (Koonin and Dolja, 1993) near the C-terminus of the ORF 1a protein. Domains such as protease or helicase domains were not detected. The function of the ORF 1b product is unknown since it showed no significant amino acid homology to any other viral proteins. RNA 1 has a 5′-nontranslated region (5′-NTR) of 66 nucleotides and a 3′-nontranslated region (3′-NTR) of 165 nucleotides.

RNA 2

The RNA 2 (1981 nt) sequence revealed the existence of a single ORF (ORF 2; Fig. 1). The calculated molecular mass of the protein encoded by ORF 2 is 45 kDa. The 5′-NTR is 11 nucleotides and the 3′-NTR is 701 nucleotides.

RNA 3

There are no ORFs considered as encoding a protein in RNA 3 (977 nt). RNA 3 differed in relative abundance among the isolates (Shirako et al., 1985). Therefore, RNA 3 is probably a satellite RNA.

Termini of viral RNAs

The presence of a cap structure at 5′-termini of the three viral RNAs was indicated by the oligo-capping method (Maruyama et al., 1994) (data not shown). Poly(A) tails are lacking at the 3′-termini of the three viral RNAs. SmV A

RNA 1 showed low efficiency of 3′-anchor ligation compared with RNA 2 and RNA 3. The predicted amino acid sequence of RdRp encoded by SmV A RNA 1 shows significant similarity to that encoded by *Nodaviridae* RNA 1. Since this *Nodaviridae* RNA 1 is known to be blocked at the 3′-terminus (Ball and Johnson, 1999), this may also be the case for SmV A RNA 1.

Capsid protein

The N-termini of SmV A capsid proteins were sequenced directly and compared with the deduced amino acid sequences of the ORFs. The result of the N-terminal amino acid sequence obtained by Edman degradation is that the major coat protein, CP 1 (p43), is AKKRKTKP?R and that the minor coat protein, CP 2 (p39), is DYKVSQNSLV. Both sequences match the parts of ORF 2, but the sequence of CP 1 matches at the N-terminus, whereas CP 2 matches at the internal sequence (from the 71st residue). The molecular mass of the predicted proteins encoded by whole ORF 2 is 45 kDa and that of the downward 71st residue is 38 kDa, which are similar to those obtained by polyacrylamide gel electrophoresis analysis of the protein in a purified virion preparation. Therefore, it appears that CP 2 is derived from CP 1 via proteolytic cleavage at its N-terminus. Similar proteolytic processing of the capsid protein is also reported in *Helminthosporium victriae 190S totivirus*, but near the C-terminus (Huang et al., 1997). The capsid protein of *Giardiavirus* is also cleaved by cellular protease during the maturation of the virus (Yu et al., 1995). In *Nodaviridae,* the capsid protein undergoes autolytic maturational cleavage to produce two proteins (Hosur et al., 1987). The particle shape of SmV A, with many spikes on the periphery, might be associated with this proteolytic procession of the CP.

Comparisons of ORFs and genome organization with those of other viruses

The predicted amino acid sequence of ORF 1a shows similarity to those of RdRp of viruses of the "sobemovirus lineage in supergroup 1" among the positive-strand RNA viruses classified by Koonin et al (1993), especially to members in the family *Nodaviridae* (about 23% identities). Multiple alignment with those viruses of the sobemovirus lineage shows that the eight RdRp motifs identified by Koonin and Dolja (1993) are highly conserved (Fig. 2A).

The amino acid sequence of ORF 1b does not show any significant similarity to proteins of any other viruses, as previously described.

The amino acid sequence of ORF 2 encoding the virus capsid protein shows a similarity to those of viruses belonging to the family *Tombusviridae*. After pairwise alignment for maximum matching, sequence identities of 17–22% were observed between the CP of SmV A and those of *Tombusviridae* viruses (*Tomato bushy stunt virus*, TBSV; *Carnation mottle virus*, CarMV). Multiple alignment at the putative S domain is shown in Fig. 2B

No ORFs encoding a protein exist in RNA 3, but the part of the nucleotide sequence from 213 to 252 shows identity to SmV B 5236 to 5275. Since SmV A and SmV B are often detected together in *S. macrospora* (Shirako and Ehara, 1985), it is possible that RNA recombination has occurred between these two viruses.

The sequences of three SmV A RNAs show no similarity to each other except the 5′-terminal eight-nucleotide sequence. Each of the three RNA molecules of this virus might originate from different ancestor viruses.

The whole genome structure of SmV A is more similar to that of *Nodaviridae* than those of other ssRNA fungal viruses (Fig. 3), while those of SmV B, MBV, and BVF resemble *Sobemovirus, Luteovirus,* and *Potexvirus,* respectively.

In conclusion, SmV A differs from the other known mycoviruses in terms of particle shape and the characteristics of its genome. Moreover, the genome organization of SmV A does not resemble that of any other mycoviruses, including SmV B. These results suggest that SmV A should be classified into a new group of mycoviruses.

Further investigation is required to understand the replication strategy of SmV A and its role in downy mildew disease also remains to be elucidated.

Materials and methods

Virus purification and nucleic acid extraction

Rice plants which exhibited typical downy mildew symptoms, such as stunting and chlorotic flecks on the foliage, were collected from Okayama Prefecture, Japan, in 1999. SmV A was purified from these diseased leaves by differential centrifugation followed by sucrose density gradient centrifugation as described previously (Honkura et al., 1983). Immunoelectron microscopy was employed for detection and identification of the virus. Nu-

Fig. 2. Amino acid alignment of the putative RdRp domains of SmV A, SmV B (Yokoi et al., 1999), *Mushroom bacilliform virus* (MBV) (Revill et al., 1994), *Black beetle virus* (BBV) (Dasgupta et al., 1984), *Southern bean mosaic virus* (SBMV) (Wo et al., 1987), and *Potato leafroll virus* (PLRV) (Mayo et al., 1989) (A) and the putative S domains of capsid protein of SmV A, SmV B, *Tomato bushy stunt virus* (TBSV) (Hillman et al., 1989), *Carnation mottle virus* (CarMV) (Guilley et al., 1985), SBMV, and PLRV (B). The conserved motifs of RdRp according to Koonin and Dolja (1993) are indicated. Asterisks show the residues strictly conserved in CP sequences of the small spherical positive-strand RNA viruses (Dolja and Koonin 1991). Gaps are introduced for better alignment. Numbers on the top and bottom refer to the amino acid positions in the ORF and the distances from the protein termini, respectively. Those between the motifs show the numbers of amino acid residues. Residues conserved among at least four of the viruses are in bold.

A)

```
                   I                          II
SmV A   480  FGKAEA   8  NISQYNDA-DKL-DMATFALALSEHMKQFK  2
SmV B   929  FLKIEP  12  CVQAVGLDTQLYFRCHFGALADVASANYRK 12
MBV     216  FNKNEP  12  IISGFPDILFIL-KVSRYTLAYSDIVLHAE  5
BBV     512  FIKQEP   7  IIAAVGLVDQIVTRLLCMKQNNAEIDCWES  7
SBMV    618  FVKQEP  12  LISSVSIVDQLVERMLFGAQNELEIAEWQS  5
PLRV    326  FVKGEP  12  LIMSVSLVDQLVARVLFQNQNKREISLWRS  5
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            III               IV                      V
SmV A   GPGKTPLEIANR 13  SDYHRMDGTIT  44  GSSHGSGCSATSLFQTLRAAFNAYLGFR  19
SmV B   GDGHYLYNVITR  8  YDGKAFEYVAH  58  PFVLSSGRWDTFLRNSLTGY-YWLIIGL  15
MBV     GMGLNDEGLRTL 15  TDISGWDWSVQ  51  PGGQLSGDYNTSSSNSRMR--VIATMFA  14
BBV     YPGRNPTEIADG 14  TDFSNLDGRVS  47  GVGVKSGSSTTTPHNTQYNGCVEFTALT  15
SBMV    GMGLSVIHQADA 16  ADISGFDWSVQ  49  PGIMKSGSYCTSSTNSRIR--CLMAELI   4
PLRV    GFGLSTDTQTAE 27  TDCSGFDWSVA  50  PGVQKSGSYNTSSSNSRIR--VMAAYHC   4
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                  VI               VII              VIII
SmV A         GIHLGDDGLD  28  RGVNFLARY   13  CDFKRQISKF  158
SmV B         IKVGGDDVIL  31  DDFEFFSWK   13  TRFSKHLENF   87
MBV           IKAMGDDSFE  25  VGFEFCSQV    9  VDFSKTLYRF   51
BBV           -PKCGDDGLS  27  IGLCFLSRV   10  IQDPLRTLRK  145
SBMV          CIAMGDDSVE  31  YAVEFCSHV   15  TSWPKTLYRF  100
PLRV          AMAMGDDALE  19  -ELEFCSHI    9  VNTNKMLYKL   51
```

B)

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SmV A    96  KIMVR-HREFVMEVRSAIN--FSVQR-----TFPLNPGMSQSFPWLAKLASSFQQYS
SmV B    92  DLVVL-PSAFHHAVSPRNNIPSGVTWST---LSGATAANALVFTSPGQLANSLVNYR
TBSV    103  SVTVT-HREYLSQVNNSTG--FQVNGGIVGNLLQLNPLNGTLFSWLPAIASNFDQYT
CarMV    82  SMTMS-KTELLSTVKGTTGVIPSFE------DWVVSPRNVAVFPQLSLLATNFNKYR
SBMV     84  DVTILSHCE-LSTELA-VTD-TIVV------TSELVMPFTVG-TWLRGVAQNWSKYA
PLRV     63  PRGRGSSETFVFTKDNLVG--NTQG------SFTFGPSLSDCPAFKDGILKAYHEYK
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```
SmV A   IKGMVFHYVPTSG-SAISGTNNALGSIMLQTSYRANDSTPQSKVECLNEYWACESV-PSETF-
SmV B   IVGYGVRIFGVASMTNTAGR---AVIATVPIASWINDKTASVGGQVSNAVNAAANVAGTLVAY
TBSV    FNSVVLHYVPLCS-TTEVGR------VAIYFDKDSEDPEPADRVELANYSVLKETA-PWA---
CarMV   ITALTVKYSPACS-FETNGR------VALGFNDDASDTPPTTKVGFYDLGKHVETA-AQT---
SBMV    WVAIRYTYLPSCP-TTTSGA------IHMGFQYDMADTLPVSVNQLSNLKGYVTGPVWEGQSG
PLRV    ITSILLQFVSEAS-STSSGS------IA--YELDPHCKVSSLQSYVNKFQITKGGA-------
                          *                 *
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SmV A   ---------------------AHPIECNPKENPFQVQYIRTGAVPAGDNVLLYDLGLTSIA
SmV B   GVPVT--GAYVDIASLPSLPNTVGVSMMNLSERQMIITPKITGPEAFVFNETTDNAIGFNI
TBSV    ---------------------EAMLR---VPTDKIKRFCDDSSTSDH--KLIDLGQLGIAT
CarMV   ---------------------AKDLV---IPVDGKTRFIRDSASDDA--KLVDFGRIVLST
SBMV    LCFVNNTKCPDTSRAITIALDTNEVSEKRYPFKTATDYATAVGVNANIGNILVPARLVIAM
PLRV    ---------------------KTYQA---------RMINGVE-------WHDSSEDQCRIL
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SmV A   VSGCQVDGVTLGDLWVTYEVEL  156
SmV B   VDQTSVSFVASGDASYLRVAGF   85
TBSV    YGGAGT--NAVGDIFISYSVTL  125
CarMV   YGFDKA-DTVVGELFIQYTIVL  109
SBMV    EGGSSKTAVNTGRLYASYTIRL   10
PLRV    WKGNGKSSDSAGSFRVTIKVAL    4
                   *
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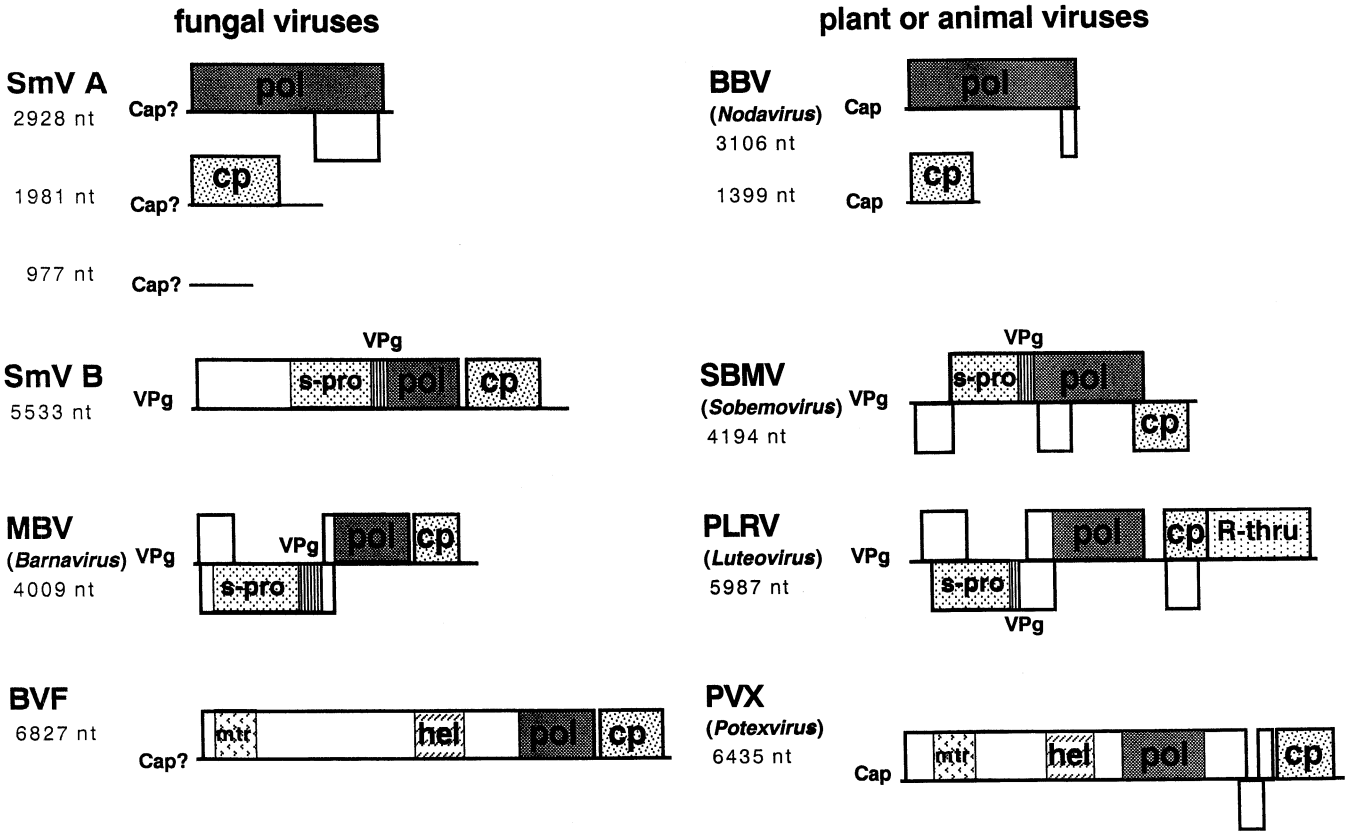

Fig. 3. Comparison of the genome organization of fungal viruses (SmV A, SmV B, MBV, and BVF) and animal or plant viruses (BBV, SBMV, PLRV, and PVX). S-pro, putative chymotrypsin-related serine protease domain; pol, putative RNA-directed RNA polymerase domain; cp, capsid protein; R-thru, domain expressed by translational readthrough; mtr, putative methyrtransferase domain; hel, putative helicase domain.

cleic acid was extracted from purified virions by treatment with 1% sodium dodecyl sulfate (SDS) and 0.2 mg/ml of proteinase K followed by phenol/chloroform extraction, and the size was determined by electrophoresis through denaturing formaldehyde agarose gels (Sambrook et al., 1989).

cDNA synthesis and cloning

Synthesis of cDNA from SmV A RNA was carried out using the "cDNA synthesis module RPN 1256" (Amersham) according to the manufacturer's instructions. Briefly, first-strand cDNA was synthesized with random hexanucleotide primers and AMV reverse transcriptase, and second-strand DNA was synthesized with *Escherichia coli* DNA polymerase I in the presence of RNase H. After rendering the dsDNA blunt-ended with T4 DNA polymerase, the resulting DNA fragment was ligated into the *Hinc*II site of the plasmid vector pUC118. No clone was obtained from RNA 3 by the procedure described above. Therefore SmV A RNA was polyadenylated by poly(A) polymerase (BRL), followed by cDNA synthesis with oligo(dT) primer, dA or dC tailing with terminal nucleotidyl transferase, and amplification by PCR with oligo(dT) or (dG) primer.

DNA sequencing and sequence analysis

Both strands of the inserts from selected cDNA clones were sequenced. Sequence analysis was performed with an Applied Biosystems Model 377 and 3100 automated sequencer, employing a DNA sequencing kit (Applied Biosystems).

Multiple alignments for sequence comparison with other viruses were performed using CLUSTAL W (Thompson et al., 1994) as incorporated into DNASIS version 3.6 for Macintosh (Hitachi Software). GenBank searches were performed using the BLAST programs (Altschul et al., 1990).

Determination of the 5′- and 3′-terminal nucleotides

First, we clarified the existence of the cap structure at the 5′-termini of SmV A RNA by the same procedure as that for SmV B (Yokoi et al., 1999; Maruyama et al., 1994). For determining the 5′-terminal sequences, we employed the 5′ RACE system for rapid amplification of cDNA ends, version 2.0 (Gibco BRL).

The 3′-terminal sequence of SmV A RNA was determined by the procedure of Weng and Xiong (1995).

Amino acid sequencing

The purified virus was dissolved in 62.5 mM Tris–HCl (pH 6.8) containing 2.5% SDS, 5% 2-mercaptoethanol, and 10% glycerol and then heated at 95°C for 3 min. The viral protein was electrophoresed through a SDS–polyacrylamide gel (Laemmli, 1970; Hirano, 1989), blotted onto an Immobilon P membrane (Millipore), and visualized by staining with Coomassie brilliant blue (Matsudaira, 1987); the protein band was excised from the membrane and sequenced directly by sequential Edman degradation using a protein sequencer (Applied Biosystems 492).

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