Characterization of a novel single-stranded RNA mycovirus in
Pleurotus ostreatus

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Abstract

A mycovirus, named oyster mushroom spherical virus (OMSV), was isolated from cultivated oyster mushrooms with a severe epidemic of oyster mushroom Die-back disease. OMSV was a 27-nm spherical virus encapsidating a single-stranded RNA (ssRNA) of 5.784 kb with a coat protein of approximately 28.5 kDa. The nucleotide sequence of the virus revealed that its genomic RNA was positive strand, containing 5784 bases with seven open reading frames (ORF). ORF1 had the motifs of RNA-dependent RNA polymerases (RdRp) and helicase. ORF2 encoded a coat protein. ORF3 to 7 could encode putative polypeptides of approximately 12, 12.5, 21, 14.5, and 23 kDa, respectively, but none of them showed significant similarity to any other known polypeptides. The 5’ end of the viral RNA was uncapped and the 3’ end was polyadenylated with 74 bases. Genomic structure and organization and the derived amino acid sequence of RdRp and helicase domain were similar to those of tymoviruses, a plant virus group.

Keywords: ssRNA; Mycovirus; RNA-dependent RNA polymerase; Coat protein; Pleurotus ostreatus

Introduction

Mycoviruses are widespread in fungi, including plant-pathogenic fungi. In most cases, they have been reported to be cryptic or show few symptoms leading to latent infection in host cells (Buck, 1986). However, several mycoviruses associated with fungal diseases were recently reported in Nectria radicicola (Ahn and Lee, 2001), Sclerotinia sclerotiorum (Boland, 1992), Fusarium graminearum (Chu et al., 2002), Cryphonectria parasitica (Lee, 1992), and so on. Mycoviruses present in mushrooms were also found in Agaricus bisporus (white-button mushroom) and Pleurotus ostreatus (oyster mushroom). The viral disease of A. bisporus was first described in 1950 (Sinden and Hauser, 1950) and subsequently shown to be related to the presence of virus particles in 1962 (Hallings, 1962). Its main symptom was the malformation of fruiting bodies, followed by loss of yield. The disease was named La France disease or Die-back disease. In P. ostreatus, spherical viruses were isolated from malformed sporophores and their spawn (Go et al., 1992; Goodin et al., 1992) and a possible relationship between the presence of double-stranded RNA (dsRNA) virus and reduced mycelial growth was suggested (Van der Lende et al., 1995).

Most mycoviruses reported have dsRNA genomes, either encapsidated in particles (Buck, 1986; Go et al., 1992), present as unencapsidated forms in cytoplasm (Ahn and Lee, 2001; Hansen et al., 1985), or found in mitochondrial fractions (Hong et al., 1998; Lakshman et al., 1998). It is unusual in some ways that the genome of mycovirus is single-stranded RNA (ssRNA). Until now only three ssRNA mycoviruses have been reported. The first, mushroom bacilliform virus (MBV) was found in A. bisporus (Ta-vantzis et al., 1980; Revill et al., 1999). The second, Selerophthora macrospora Virus B (SnVB), was isolated in S. macrospora, the pathogenic fungus responsible for downy mildew in gramineous plants (Honkura et al., 1983; Shirako

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and Ehara, 1985; Yokoi et al., 1999). The third, Botrytis virus F (BVF), infects Botrytis cinerea, an important pathogen affecting a large number of economically important vegetables, flowers, and fruit crops (Coley-Smith et al., 1980; Robyn et al., 2001).

We isolated a new ssRNA mycovirus, named oyster mushroom spherical virus (OMSV), from a cultivated oyster mushroom, P. ostreatus. This virus was strongly associated with the epidemic called oyster mushroom Die-back disease. We detected the virus in all 102 samples collected from 102 different commercial farms with the epidemic. The symptoms are rather complex and the disease spreads fast. The outbreak of the disease in a commercial farm often leads to the complete loss of yield and it is difficult to control. OMSV was not detected in healthy mushrooms and when we cured OMSV from diseased mushrooms, the epidemic disappeared (Yu et al., in preparation). This strongly suggests that OMSV is a causative agent of the disease.

Here, we report the fourth ssRNA mycovirus, OMSV, and show the genome organization and the complete nucleotide sequence of the virus. The putative RNA-dependent RNA polymerase (RdRp) of OMSV was compared with that of other viruses to determine phylogenetic relationship. We also show that the 3' end of OMSV RNA is uncapped and the 5' end is polyadenylated.

**Results and discussion**

**Viral particle, genome, and coat protein**

The presence of OMSV in oyster mushrooms was strongly associated with the epidemic, oyster mushroom Die-back disease because the virus was detected in all 102 samples collected from 102 different commercial farms showing the epidemic; however, it was not detected in healthy control mushrooms. Since the disease spreads very fast and the symptoms are very complex, its outbreak in a commercial farm usually means the complete loss of yield. We extracted virus particles from diseased oyster mushrooms as described under Materials and methods. Using electron microscopes, virus particles were observed in the fractions having a buoyant density in CsCl of 1.585 g/cm³ (Fig. 1A). They exhibited a spherical shape with a diameter of about 27 nm and were thus named oyster mushroom spherical virus.

The viral genome extracted from the purified virus was electrophoresed in 1% agarose gel and showed a single band of about 5.8 kb. To investigate the nature of the viral nucleic acid, its resistance to nuclease in various conditions was examined. We treated the viral nucleic acid with ribonuclease A (RNase A) in 0.5 or 0.05 M NaCl, and DNase I in 10 mM MgCl₂. The viral nucleic acid was sensitive to RNase A under both high- and low-salt conditions, but not to DNase I (Fig. 1B). This result shows that the viral nucleic acid is ssRNA. Electrophoresis in 12% SDS–polyacrylamide gel was performed to identify the viral protein. The electrophoregram showed a single polypeptide with an estimated size of about 28.5 kDa (Fig. 1C). Thus, coat protein of OMSV could be made of a single polypeptide of 28.5 kDa. So far, three mycoviruses, MBV, SmVB, and BVF, have been reported to have ssRNA genome. The particle shape of MBV (4009 bp) (Revill et al., 1999), SmVB (5533 bp) (Yokoi et al., 1999), and BVF (6826 bp) (Robyn et al., 2001) was known to be bullet-shaped, spherical, and flexous rod-shaped, respectively. The OMSV is different from these three mycoviruses in terms of the shape and the size of virus particle, the size of capsid protein, and the viral genome.
This implies that OMSV is a new ssRNA mycovirus. To our knowledge, this is also the first report on spherical ssRNA virus in mushrooms.

**Nucleotide sequence and genome organization**

To determine the primary structure of the OMSV genome, cDNA libraries of genomic ssRNA were made by priming with random nonamers, or by priming with specific oligonucleotides complementary to internal regions of viral genomic RNA. To be sure that the cDNA clones obtained were specific to the viral RNA, dot hybridization analysis with viral RNA was carried out (data not shown). About 30 cDNA clones were sequenced in both directions. As cDNAs obtained by random priming could not cover the full length of the viral genome, clones covering the 5' or 3' end were constructed by oligoligation to both ends followed by RT-PCR as described under Materials and methods. In this way we could obtain cDNA clones containing the 3' or 5' end of viral genome. Also, the ligation property revealed the end structure of the viral genome.

As shown in Fig. 2, a RT-PCR product of about 560 bp containing the 5' end of genomic RNA was produced from the oligoligated RNA without any pretreatment (Fig. 2, lane 1). The same product, however, was not obtained when the genomic RNA was treated with either bacterial alkaline phosphatase (BAP) or BAP and subsequent tobacco acid pyrophosphatase (TAP) prior to oligoligation (Fig. 2, lanes 2–3). This result showed that the 5' end of the viral RNA was not capped. In contrast, the capped control RNAs (Cucumber mosaic virus-Fny, CMV-Fny) which had been treated with BAP and subsequent TAP, were ligated with the oligonucleotide (Fig. 2, lane 6), while CMV-Fny genome untreated or treated only with BAP were not ligated with the oligonucleotide (Fig. 2, lanes 4–5). We observed that the nucleotide sequence of the RT-PCR product of about 490 bp was identical with 5'-end region of CMV-Fny RNA 3 (GenBank Accession No. NC001440) (data not shown). The nucleotide sequences obtained from several independent cDNA clones showed that the 3' end of the viral RNA had a poly(A) tail. To determine the exact number of adenine residues, we did direct ligation of oligonucleotides (SV3-E) to the 3' end and performed RT-PCR. The nucleotide sequence of this product showed the genomic RNA contained 74 bases of adenine residue at 3' end.

Alignment of the overlapping cDNA clones from random priming and the clones covering both termini was presented in Fig. 3. The complete nucleotide sequence of OMSV genome was made of 5784 nucleotides and its base composition was A (18.9%), C (30.9%), G (23.7%), and U (26.5%). There were 78 bases of 5' untranslated region (UTR) between 5' terminus and AUG codon to start ORF1 and 99 bases of 3' UTR including the poly(A) tail of 74 bases at the 3' end (Fig. 3).

The viral genome contains seven open reading frames which encode polypeptides of molecular weight greater than 10 kDa (Fig. 3). Open reading frame (ORF1), the largest gene with 1581 amino acids (about 174 kDa), starts at the first AUG positioned at nucleotide 79 and finishes at codon UAA positioned at nucleotide 4882, covering 83% of the total genome. ORF2, the second largest gene with 235 amino acids (about 28.5 kDa) located at the 3' part of the RNA, starts at AUG of nucleotide 4899 and terminates at UGA of nucleotide 5612. There are five other ORFs; ORF3, 4, 5, 6, and 7 encode the putative polypeptides of molecular weights of 12, 12.5, 21, 14.5, and 23 kDa, respectively.
ORF3 to 6 are located within ORF1 sequence with a +1 frameshift with a joint sequence of UGAUG (ORF3 and 4), UAAUG (ORF5), or GAAUG (ORF6). ORF7 is overlapped with ORF2, showing +1 reading frame with respect to ORF2 (Fig. 3).

**ORF analysis and comparison with ORFs of other viruses**

The databases were searched for the comparison of amino acid sequence homology to those encoded by the seven ORFs of OMSV. The deduced amino acid sequence of ORF1 showed homology to those of RNA-dependent RNA polymerase (OMSV) of other viruses and contained the highly conserved motif GDD of RDRP (Bruenn, 1993; Hong et al., 1998) (Fig. 4A). This indicates that the ORF1 encodes the putative RDRP. We also found the conserved helicase motif GXGKS(T) in ORF1 (Kadare and Haenni, 1997) (Fig. 4A). From BLAST search using the database, we found that the amino acid sequence of the entire RDRP of OMSV is similar to those of some plant viruses such as Erysimum latent virus (ErLV). White clover virus (WCMV), and Garlic virus C (GarVC), which belong to the Tymovirus, Potexvirus, and Allexivirus group, respectively. Fig. 4 shows amino acid sequence comparison between RDRP and helicase domains of OMSV and those of ErLV, WCMV, GarVC, and BVF, a ssRNA mycovirus.

Similar search with amino acid sequence of ORF2 to ORF7 did not show any homology with other gene products of the database. To find out which ORF encodes viral coat protein, N-terminal amino acid sequencing of OMSV coat protein was performed. However, no sequence data could be obtained by Edman degradation, implying that the N-terminal of the viral coat protein might be blocked. Therefore, internal amino acid sequencing of OMSV coat protein was carried out by mass spectrometry. Among several fragments obtained from tryptic digestion of the coat protein, one fragment was sequenced using the Q-TOP-MS method. This sequence was IPVGSFTGTTFTSTSR, which is identical to the deduced amino acid sequence of ORF2 (data not shown). The molecular weight of the predicted protein encoded by ORF2 (26.5 kDa) is similar to that of the polypeptide observed in the polyacrylamide gel electrophoresis of viral coat protein (28.5 kDa). This result shows that the coat protein is encoded by ORF2.

The function of the putative polypeptides encoded by ORF3 to ORF7 within the cell is still unclear. In general, infection, replication, propagation, and transmission of viruses need at least three gene products: RdRp, coat protein (CP), and movement protein (MP). Though these three proteins are essential for the viral life cycle, we could identify only two genes (RDRP and CP) in the OMSV genome. This raises the possibility that at least one of the unassigned ORFs may encode the movement protein.

There are many observations that though the amino acid sequence of RdRp between two different viruses showed strong homology, no significant similarity was found between the sequences of CP. For example, the amino acid sequences of the putative RdRp of tymoviruses had the closest relationship to those of potexviruses (Rozanov et al., 1990; Murphy et al., 1995), but no sequence homology was found between the coat proteins of two different virus groups. Thus, it is not unusual that the amino acid sequence of RDRP and helicase domain of OMSV showed a strong similarity with those of most viruses belonging to tymoviruses, though the coat protein of OMSV did not have any homology between them. Phylogenetic analysis and alignment of amino acid sequence of entire RdRp also showed that amino acid sequence of OMSV is most similar to that of tymoviruses (Fig. 4). There was also amino acid homology between OMSV and tymoviruses in the N-terminal domain as well as RdRp and helicase domain of ORF1.
comparison of amino acid 1 to 350 between OMSV and tymoviruses showed that 21% of amino acid is identical and 35% of amino acid is similar. Among mycoviruses with ssRNA genome, MBV and SmVB did not show a significant relationship to OMSV in terms of genomes organization and sequence homology, while BVF is more related to OMSV (Fig. 4). It has been reported that amino acid sequence of BVF is closely related to that of both “lymoto-” and “potex-like” virus (Robyn et al., 2001). Therefore, we conclude that among known virus groups, OMSV has the closest relationship with the tymoviruses.

In short, OMSV is a 27-nm spherical mycovirus consisting of a plus-sense single-stranded RNA of 5.784 kb and a coat protein of approximately 28.5 kDa. Comparison of ORFs and amino acid sequence analysis with other viruses showed that OMSV has the closest relationship with tymoviruses than any other mycovirus. OMSV differs from other mycoviruses according to genome sequences and ORF analysis, suggesting that OMSV might be a new group of mycovirus. The OMSV genome has the potential to encode seven proteins. Sequence analysis of the OMSV genome implies that the virus uses a variety of translational control mechanisms for expression of seven ORFs. Further work is required to express the OMSV ORFs, especially ORF3 to 7, to identify the expressed proteins and to elucidate their role in the viral life cycle. These studies could provide more insight into the pathogenicity of OMSV.

Materials and methods

Purification and electron microscopy of OMSV

Abnormal and healthy fruiting bodies of *P. ostreatus* were collected from oyster mushroom farms in Southeastern Korea. Control healthy mushrooms were obtained from Gyeongnam Provincial Agricultural Research and Extension Services (GNARES).

The frozen sporophores of *P. ostreatus* (100 g) were homogenized in 2 volumes (w/v) of extraction buffer consisting of 50 mM Tris–Cl, 1 mM EDTA, pH 8.0 (TE) in a Waring Blender (Waring commercial) for 3 min at high speed. The mixture was centrifuged at 10,000 g for 20 min and cell debris was removed. To the aqueous phase, polyethylene glycol (PEG) 6000 and NaCl were added to a final concentration of 10% and 0.6 M, respectively. After centrifugation at 10,000 g for 20 min, the supernatant was discarded and the pellet was resuspended in TE buffer. The suspension was clarified by centrifugation at 10,000 g for 20 min and then centrifuged at 105,000 g for 90 min. The pellet was resuspended in 1 ml of TE buffer and clarified by low-speed centrifugation. The supernatant was used as crude virus preparation.

One milliliter of the crude virus preparation was layered onto 1.585 g/cm² cesium chloride (CsCl) solution prepared in TE buffer and isopycnic centrifugation was performed in a Beckman Type 90Ti rotor at 130,000 g for 20 h. Gradients were fractionated in 750 µl. Fractions were dialyzed for 24–36 h against 3 L of TE buffer with two changes and virus particles were precipitated by centrifugation at 130,000 g for 90 min. The pellet was resuspended in TE and preserved frozen at −20°C. Each fraction was analyzed for virus particle, viral RNA, and coat protein.

For transmission electron microscopy, purified virus was incubated on formbar-coated 400-mesh copper grids and stained with 2% (w/v) uranyl acetate, pH 4.5. Grids were examined with a JEOL model 200 transmission electron microscope.

Agarose gel electrophoresis of ssRNA

The viral nucleic acids were extracted from the purified virus with phenol and phenol/chloroformisoamyl alcohol (25:24:1, v/v/v) and then precipitated with ethanol. The nucleic acid pellet was washed with 70% ethanol, dried, and dissolved in DEPC-treated sterile water. The extracted nucleic acid was treated with RNase A (10 µg/ml) in high salt (0.5 M NaCl) or low salt (0.05 M NaCl) at 37°C for 30 min or with DNase I (5 unit) in 10 mM MgCl₂. For electrophoresis, the RNA samples were adjusted to 0.04% bromophenol blue, 0.04% xylene cyanole FF, and 5% glycerol and then separated on 1% agarose gel. Electrophoresis was carried out at a constant 50 V for 8 h. Electrophoresis buffer was 40 mM Tris–acetate and 1 mM EDTA, pH 7.5 and the gels were stained in ethidium bromide solution (0.5 µg/ml).

Cloning of cDNA from the viral genome

Synthesis of cDNA from OMSV RNA was carried out using the cDNA synthesis kit (TaKaRa) according to the manufacturer’s instructions. The first-strand cDNA was synthesized by priming with random nonamer and the second-strand DNA was synthesized with *Escherichia coli* DNA polymerase I in the presence of RNase H. The cDNA, blunt-ended with T₄ DNA polymerase, was ligated to EcoRI-NorI-BamHI adaptor and subsequently ligated into the EcoRI site of the plasmid vector pUC19.

To obtain cDNA clones representing the 5’ end of the viral RNA and to find out whether the 5’ end has a cap structure, an oligonucleotide SV5-E (5’-GAGCGAAT-TCACTGTGTTGGCCTACTGCGCCGG-3’) was ligated to the 5’ end of OMSV RNAs untreated, treated with only BAP, or treated with BAP and subsequent TAP using T₄ RNA ligase (Takemoto et al., 2000; Yokoi et al., 1999; Maruyama and Sugano, 1994). After this procedure, the nucleic acid containing the 5’ end was amplified by RT-PCR using SV5-E and SV5-I primer (5’-TACAGAATTTC-3’) (Fig. 5). The underlined sequence is complementary to the viral RNA sequence at nucleotide positions 528–505 and the PCR product was cloned into plasmid pUC19. The capped CMV-Fny RNAs were used as control RNAs. The control experiments were
performed as described in the case of OMSV RNA except that CY5-I primer (5'-TACAGAATTCC-3'); the underlined sequence is complementary to the CMV-Fny RNA at nucleotide positions 450–427) was used in RT-PCR.

To get the RT-PCR product corresponding to the 3' end of the viral RNA, an oligonucleotide SV3-E (5'-TCACAGGC-CATGTACAGGAAAGCC-3'; phosphorylated at the 5' end and blocked by amine group at the 3' end) was ligated to the 3' end of the viral RNA using T4 RNA ligase. The 3'-end sequence was amplified by RT-PCR using SV3-I primer (5'-TGTTGAATTCC-3'; the underlined sequence corresponds to the viral RNA sequence at nucleotide positions 5171–5194) and SV3-ER primer (5'-AGCCGAATTCC-3'; complementary to the oligonucleotide SV3-E). The PCR products were digested by EcoRI and cloned into pUC 19.

DNA sequencing and sequence analysis

From the cDNA clones obtained, about 30 clones were sequenced in both directions. DNA sequencing was performed with an Applied Biosystems Model 370 automated sequencer using a cycle-sequencing kit with AmpliTaq DNA polymerase. Multiple alignments for sequence comparison with other viruses were performed by the program DNA STAR or Clustal W and GenBank searches were done with the BLAST program.

Coat protein analysis

The virus coat protein was separated by SDS–polyacrylamide gel electrophoresis using the discontinuous buffer system (Laemmli, 1970). Before electrophoresis, the virus particles were denatured for 5 min at 95°C in 50 mM Tris–Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromophenol blue. The denatured viral proteins were separated in polyacrylamide gels with a 4% stacking gel and a 12% separating gel. Gels were stained with 0.25% Coomassie brilliant blue R-250. For N-terminal amino acid sequencing, the viral protein in the gel was blotted onto PVDF membrane (Bio-Rad) and the protein band was excised and sequenced directly by Edman degradation using protein sequencer (ABI 491, USA). Internal amino acid sequencing of virus coat protein was performed on Quadrupole/Time-of-flight mass spectrometer (Micromass, UK) after in-gel digestion (Stensballe et al., 2001; Ha et al., 2002).

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References


