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Characterization and RT-PCR Detection of dsRNA Mycoviruses from the Oyster Mushroom, *Pleurotus ostreatus*

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The partial nucleotide sequences of the genomic dsRNA mycovirus infecting *Pleurotus ostreatus* isolates ASI2223 and Suhan were determined and compared with those of mycoviruses belonging to partitiviruses and totiviruses. Partial nucleotide sequences of the purified dsRNA from ASI2223 and Suhan showed RNA-dependent RNA polymerase sequences that are closely related to those of partitiviruses, including *Fusarium poae virus* 1, *Fusarium solani virus*, *Rhizoctonia solani virus*, *Discula destructiva virus* 2, and *Oyster mushroom isometric virus* 2. Specific primers were designed for RT-PCR detection of dsRNA viruses from the *P. ostreatus* isolate ASI2223 and Suhan. Two virus specific primer sets were found to specifically detect each virus among six sets of designed oligonucleotide primers. Collectively, these results suggest that dsRNA mycoviruses from *P. ostreatus* isolates ASI2223 and Suhan belong to the family *Partitiviridae*, although, they are not the same virus species. Our results also suggest that these virus-specific primer sets can be employed for the specific detection of each viral sequence in infected tissues.

Keywords : dsRNA mycovirus, partitivirus, *Pleurotus ostreatus*, RT-PCR detection

Farmhouses have recently suffered from economic losses on account of viral disease of the cultivated mushroom (Ross et al., 1986). Because mushrooms infected with virus form poor colonization of compost, misshapen fruiting bodies and small globular caps, total crop products abruptly decrease (Dieleman-Van Zaayen, 1969; Schisler et al., 1967; Sinden and Hauser, 1950).

Particularly, the malformation accompanied with virus particles, is one of the most serious diseases in oyster mushroom. Research on cultivated mushroom disease was first described as the wilt disease, or the *Agaricus bisporus* (Hollings, 1962). Since then, this disease has been known as the La France disease (Sinden and Hauser, 1950; Wach et al., 1987).

Today, this disease is considered as an important limiting factor in the commercial cultivation of *A. bisporus*. Since the first report of the viral nature of La France disease, the etiology, epidemiology, and molecular biology of 'La France' disease have been conducted extensively (Dieleman-Van Zaayen, 1972; Goodin et al., 1992; Hicks and Haughton, 1986; Hollings, 1962; Koons et al., 1989; Schisler et al., 1967; van der Lende et al., 1994, 1996).

Double-stranded (ds) RNA mycoviruses have been reported in many fungi including mushroom. The evidence that debilitation and malformation are associated with dsRNAs of viral origin in some fungi and mushroom has been reported continuously: La France isometric virus of *A. bisporus* (Goodin et al., 1997), the hypovirus CHV-1 infecting *Cryphonectria parasitica* (Choi and Nuss, 1992; Chen et al., 1994), the Hv190S virus and Hv145S virus co-infecting *Helminthosporium victroriae* (Ghabrial, 1986). Also, the same has been reported in the following: *Diarporthe ambigua RNA virus* (Preisig et al., 2000), dsRNA in *Leucostoma persoonii* (Hammar et al., 1989), dsRNA in *Sclerotinia sclerotiorum* (Boland, 1992), and dsRNA mycovirus infecting *Fusarium graminearum* (Chu et al., 2002). Most dsRNA viruses, however, have been reported to be cryptic symptom to their host.

DsRNAs are likewise observed in malformed oyster mushroom. It consists of several segments (Go et al., 1992; Lee, 2000; van der Lende et al., 1995), with reported sizes of 23, 24, 25, 30 and 50nm viral particles isolated from oyster mushroom (Go et al., 1992; Lee, 2000; Liang et al., 1990). The type of virus infecting the oyster mushroom is presumed to be up to 4~10, and out of these types only 5 types were clarified, including *oyster mushroom spherical virus* (OMSV), *oyster mushroom isometric virus*-1 and -II (OMIV-I and OMIV-II; Lee, 2000).

Recently, the single-stranded RNA genome of OMSV infecting oyster mushroom has been characterized (Yu et al., 2003). The viral disease pathology of the oyster mush-

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room is similar to that of 'La France' disease, such as dsRNA-mediated, disease symptom, and infection mechanism (Lee, 2000; Revill et al., 1997).

Researches for the virus infecting the oyster mushroom are progressing steadily, but there are few remedies for the disease yet. Moreover, it is difficult to know whether the mushroom is infected or not until the harvest time, because virus-infected mushroom spawns are the primary source of the disease. Therefore, the unique solution to eliminate viral disease from cultivated mushroom is for researchers to supply virus-free mushroom spawns.

Thus to achieve this, it is necessary for researchers to develop more efficient methods to detect virus. In recent years, RT-PCR assay has been used to detect virus with greater sensitivity and practicability. Detecting ability of RT-PCR depends on the specificity of primers mainly.

This study attempted to design specific primers that could detect dsRNA virus infecting the oyster mushroom.

Materials and Methods

Mushroom strains and dsRNA extraction. DsRNA virus-containing *Pleurotus ostreatus* isolates ASI2223, ASI2228, and Suhan were used to extract dsRNA and store this as suspension in 15% glycerol at -80°C. To extract dsRNA, the researchers grew the fungal mycelium in mushroom complete media (0.2 g peptone, 0.2 g yeast extract, 2 g dextrose, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g KH_2PO_4 per 1 liter) in a 5-L fermenter for 5 days; then they harvested and resuspended the mycelium with 0.1 M phosphate buffer (pH 7.0). Cells were homogenized with bead beater (Biospecs, USA) for 15 min, and cell debris was removed with centrifugation at 15 K for 10 min. Virus particles were pelleted with an ultracentrifugation at 40 K for 2 hrs in the presence of 20% sucrose cushion.

The virus was resuspended with the same buffer and dsRNA was extracted with an equal volume of phenol/chloroform. The dsRNA was precipitated with 2 volume of cold ethanol and resuspended with water. Each dsRNA sample was further purified by precipitating in LiCl solution as described by Cheng et al. (2003). The quality and concentration of dsRNA were checked by electrophoresis on 1% agarous gel and visualized by using ethidium bromide staining.

cDNA cloning and sequencing. In analyzing unknown sequence of dsRNA samples, researchers conducted RT-PCR by using random hexamers. Approximately 2 μg of dsRNA was mixed with 2 μl of 90% dimethyl sulfoxide (DMSO) in a total volume of 10 μl. First-, and second-strands of cDNA synthesis reactions and cDNA cloning procedures were performed as described by Gubler and Hoffman (1983) and Hillman et al. (1992). The PCR amplifications were carried out as described previously (Chu et al., 2002, 2004). Recombinant plasmids containing cDNA inserts were sequenced by the dideoxynucleotide chain termination method using the ABI prism™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 377 Genetic analyzer (Perkin Elmer, USA). Protein-related sequences were initially identified by using the BLAST search program of GenBank (Zhang and Madden, 1997). Multiple sequence alignment was performed with the CLUSTAL W program (Thompson et al., 1994). Based on obtained dsRNA sequences, viral dsRNA-specific primers were designed and used for RT-PCR (Fig. 1). The GenBank under accession number for the complete genome sequence of the dsRNA-1 from *P. ostreatus* isolate ASI2596 used for the comparison with ASI2223 was AY533038.

DsRNA-specific primer design and RT-PCR detection. Based on the determined sequences of dsRNAs, dsRNA-specific primers for RT-PCR detection for each viral dsRNA were designed and used for RT-PCR. Two sets of dsRNA-specific primers, pr2223-A (pr2223-AF, 5'-ACGACCTCTTCATACGACTTGA-3'; pr2223-AR, 5'-AGTGAAAGCTGAATTATCGTCA-3') and prSuhan-C (prSuhan-CF, 5'-TACCCTGACCTCCGTTACTA-3'; prSuhan-CR, 5'-AGACCAGTCGAGCGATAAGA-3') for dsRNAs from ASI2223 and Suhan, respectively, were selected. RT-PCR using one cycle of RT reaction at 42°C for 60 min and 35 cycles of PCR amplification using the step program (94°C, 30 sec; 58°C, 1 min; 72°C, 1 min) followed by a 10 min final extension at 72°C was conducted.

Results and Discussion

DsRNAs purified from virus-infected mushrooms. DsRNAs were isolated and purified from purified virions of the cultivated mushroom. Gel electrophoresis of these dsRNAs revealed that dsRNA mycovirus-infected *P. ostreatus* isolates ASI2223 and ASI2596 contained 2 similarly sized dsRNAs of ca. 2.2 and 2.3-kbp. *P. ostreatus* isolates

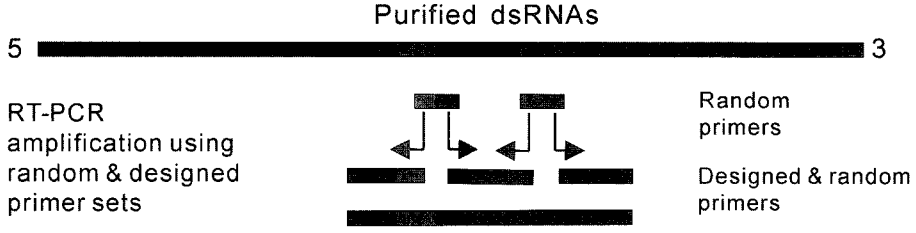

Fig. 1. Schematic representation of strategy used in cDNA cloning of dsRNA. Non-overlapping cDNA clones were synthesized by using random hexamer. Sequences of the region of the dsRNA that was not covered by these two cDNA clones were synthesized by using sequence specific primers designed based on the obtained sequences. Solid bars shown under dsRNA genome may not represent exact positions of obtained sequences since we did not complete dsRNA genome sequencing.

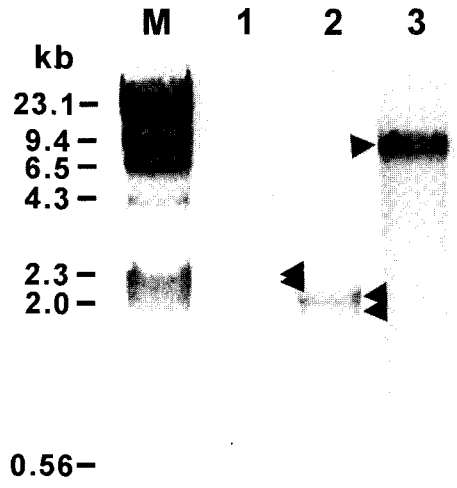

Fig. 2. Electrophoresis of dsRNAs extracted from purified virions of virus-infected *Pleurotus ostreatus* isolates ASI2223, ASI2228, and Suhan. Each dsRNA was separated in a 1% agarose gel and stained with ethidium bromide. Lanes 1-3 represent dsRNAs from *P. ostreatus* isolates ASI2223, Suhan, and ASI2228; respectively. Lane M denotes λ/*Hin*dIII marker. Arrowheads indicate the dsRNAs extracted from each isolate.

SI2228 and Suhan contained dsRNAs of ca. 2.0-kbp and 8.0-kbp, respectively (Fig. 2).

These results indicate that several different dsRNA mycoviruses are found in *P. ostreatus* isolates. Previously a 5.8-kb ssRNA virus, named oyster mushroom spherical virus (OMSV), was also isolated from cultivated *P. ostreatus* isolates showing a die-back disease (Yu et al., 2003). We concluded then that OSMV was associated with a severe epidemic of oyster mushroom die-back disease in Korea. We did not observe an apparent correlation between the presence of dsRNA mycoviruses and the hosts morphological and pathogenicity phenotypes in dsRNA-containing *P. ostreatus* isolates ASI2223 and Suahn (data not shown).

Based on the effect of dsRNA(s) on hosts morphology, we can divide *P. ostreatus* mycovirus into two groups: one that infects latently and another group that causes a die-back disease. The genetic diversity of the dsRNA mycoviruses found in *P. ostreatus* isolates was not surprising. Many dsRNA mycoviruses have been found in the same host species (Cole et al., 1998; Lemke, 1979).

Molecular characterization of dsRNAs. Nucleotide sequences of a 994 bp cDNA clone (p2223-1), obtained from the *P. ostreatus* isolate ASI2223, contained almost

A

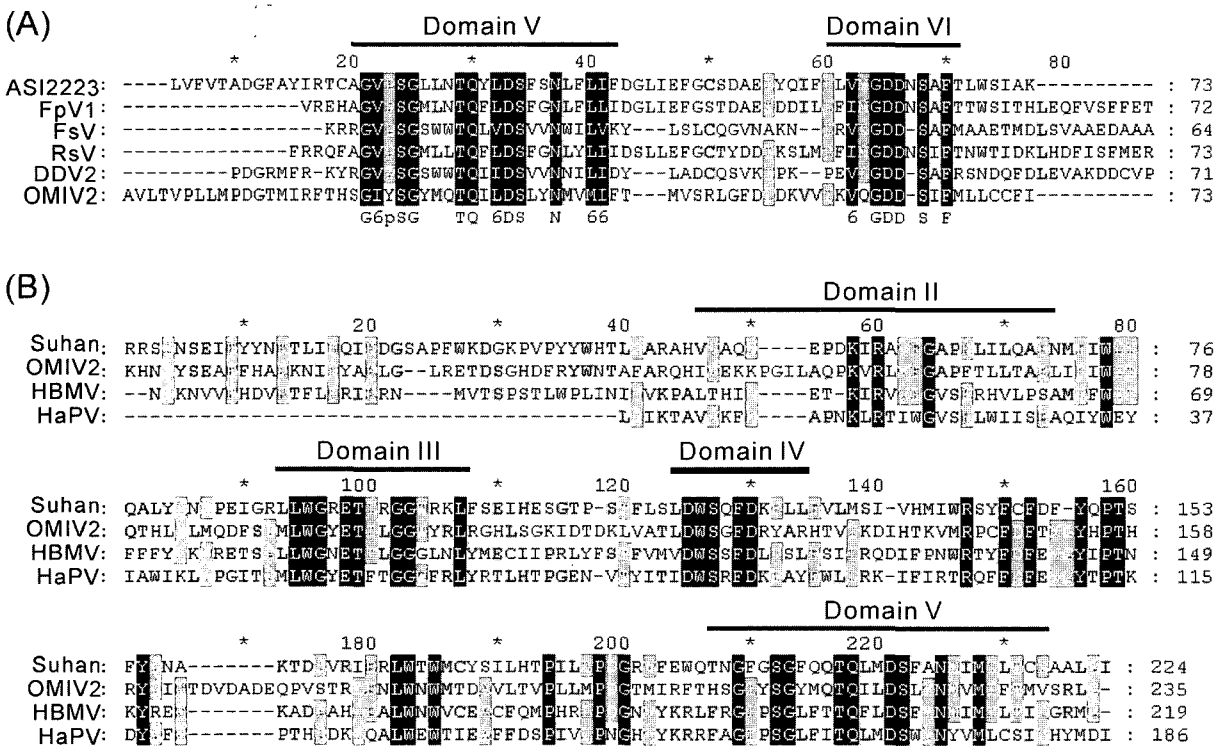

Fig. 3. Alignment of the RdRp conserved motifs of ASI2223 (**A**) and Suhan (**B**). Partitiviruses used in the analysis and their NCBI GenBank accession numbers are *Fusarium poae virus* 1 (FpV1; AF015924), *Fusarium solani virus* (FsV; BAA09520), *Rhizoctonia solani virus* (RsV; AF133290), *Discula destructiva virus* 2 (DDV2; AV033436), *oyster mushroom isometric virus* 2 (OMIV2; AY308801), *Heterobasidion annosum partitivirus* (HaPV; AF473549), and *Helicobasidium mompa dsRNA mycovirus* (HBMV; AB085814). Amino acids that are conserved are reverse highlighted. The tentative superpositions of the conserved motifs in the RdRp correspond to the motifs described by others (Koonin, 1991; Koonin and Dolja, 1993) and are indicated with solid lines.

100% nucleotide sequence identity similar to those of ASI2596 dsRNA-1 (GenBank accession number AY533038).

A comparative analysis of the putative amino acid sequences from the p2223-1 with sequences in the databases using BLAST programs (Zhang and Madden, 1997) revealed sequence homology to RNA-dependent RNA polymerase (RdRp) of other partitiviruses including *Fusarium poae* virus 1 (FpV1; Compel et al., 1999) and *Rhizoctonia solani virus* (RsV; Jian et al., 1998; Fig. 3A). Nucleotide sequences of 1119 bp cDNA clone (pSuhan-1) were obtained from the *P. ostreatus* isolate Suhan. Sequence analysis with GenBank search system of NCBI revealed that deduced amino acid sequences of pSuhan-1 also had high homology to the RdRps encoded by several partitiviruses especially to OMIV2 (GenBank accession number AY308801; Fig. 3B). The tentative superposition of the conserved motifs of RdRp is designated as suggested previously (Koonin, 1991; Koonin and Dolja, 1993).

To understand the relationship between dsRNAs from *P. ostreatus* isolate ASI2223 and Suhan with other dsRNA mycoviruses, comparative analysis was performed by using PAUP 4.0 program. The predicted amino acids of p222301 and pSuhan-1 were compared with those of the corresponding regions of RdRp sequences of partitiviruses and totiviruses. P2223-1 showed high sequence homology to FpV1 (69%), RsV (54%) and *Heterobasidium annosum partitivirus* (HaPV; 37%), while other dsRNA mycoviruses including OMIV2 and totiviruses had relatively low identity.

In contrast, the predicted amino acid sequence of the pSuhan-1 showed high homology to *Helicobasidium mompa virus* (HbMV; 40%), OMIV2 (39%), HaPV (37%), while showing relatively low sequence homology to RsV (27%). Although p2223-1 and pSuhan-1 contained different sizes of dsRNAs and did not show close sequence identity, both p2223-1 and pSuhan-1 formed distinct clade with partiticiruses, including FpV1, RsV and OMIV2 in phylogenetic analysis (data not shown).

Altogether, these results represent that dsRNA mycoviruses isolated from *P. ostreatus* isolates ASI2223 and Suhan are RdRp coding segments of dsRNA partitivirus. Although no attempt was made to determine full-length sequence of dsRNAs from ASI2223 and Suhan in this study, it is necessary to analyze the entire genome to better characterize and understand the biology of *P. ostreatus* dsRNA mycoviruses.

Specific detection of each dsRNA mycovirus. Based on the nucleotide sequences of p2223-1 and pSuhan-1, dsRNA-specific primers were designed for RT-PCR detection of each dsRNA from infected mushroom. Six primer sets were designed and tested to specifically detect each virus. To obtain suitable annealing temperature, gradient PCR reaction was conducted for each primer set. Although we designed

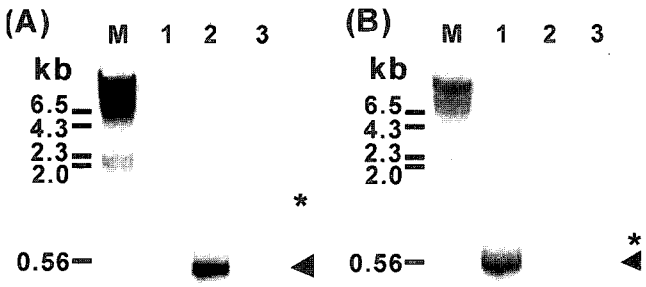

Fig. 4. RT-PCR detection of dsRNAs from *Pleurotus ostreatus* ASI2223 and Suhan with the use of the designed primer set. PCR amplified fragment(s) using p2223-A (panel A) and pSuhan-C (panel B) are separated on a 1% agarose gel. Lanes 1 to 3 in each panel represent PCR amplified product(s) using dsRNAs from Suhan, ASI2223, and ASI2228, respectively. The arrowhead indicates the RT-PCR amplified DNA band of 550 bp. In some cases, small-sized non-specific bands are also detected (asterisk). Lanes M indicate molecular size markers, λ DNA digested with *Hin*dIII. Numbers on the left of each gel indicate sizes of DNA digested with *Hin*dIII in kilobases.

and tested 6 primer sets, we obtained expected results from two sets of primers for dsRNA virus detection, including pr2223-A and prSuhan-C primer sets for dsRNAs from ASI2223 and Suhan, respectively.

As might be suspected, pr2223-A primer sets amplified DNA fragments only from dsRNAs of ASI2223, while prSuhan-C amplified DNAs from Suhan dsRNAs (Fig. 4). In some cases, non-specific bands were also detected, but the size and the amount of amplified DNAs were significantly different. The other primer sets did not effectively detect each virus.

Viral disease of cultivated mushroom has several characteristics: i) the source of infection is from infected mushroom spawn; ii) there are few remedies for viral infection; and iii) it is difficult to determine tt if its host is infected or not in the early stage. Therefore, to eliminate viral disease from cultivated mushroom, researchers must supply virus-free mushroom spawn.

Thus, in this study, we developed an efficient procedure to detect the virus. This procedure, described in this study, is relatively simple and applicable for many other dsRNA mycoviruses. RT-PCR using specific detection-primers can be applied for the detection of viral infection in the early stage, even if there is a little amount of virus in the mushroom spawn. Detection of virus in the early stage can prevent viral disease from replicating and spreading. It can also minimize the damage on cultivated products caused by the viral disease. Moreover, the use of RT-PCR using specific primers is expected to help researchers detect the specific type of virus infecting the host, and from this, they can design fitting disposal and treatment.

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