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RT-PCR Detection of dsRNA Mycoviruses Infecting *Pleurotus ostreatus* and *Agaricus blazei* Murrill

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The partial nucleotide sequences of the genomic dsRNA mycoviruses infecting *Pleurotus ostreatus* (isolates ASI2596, ASI2597, and Bupyungbokhoe) and *Agaricus blazei* Murrill were determined and compared with those of the other dsRNA mycoviruses. Partial nucleotide sequences of the purified dsRNA from ASI2596 and ASI2597 revealed RNA-dependent RNA polymerase sequences that are closely related to *Oyster mushroom isometric virus 2*, while nucleotide sequences and the deduced amino acid sequence from dsRNA mycovirus infecting *Agaricus blazei* did not show any significant homology to the other dsRNA mycoviruses. Specific primers were designed for RT-PCR detection of these dsRNA viruses and were found to specifically detect each dsRNA virus. Northern blot analysis confirmed the homogeneity of RT-PCR products to each purified dsRNA. Altogether, our results suggest that these virus-specific primer sets can be employed for the specific detection of each dsRNA mycovirus in infected mushrooms.

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

Mycoviruses with double-stranded RNA (dsRNA) genomes have been reported in many fungi and yeasts (Burk, 1986; Castro et al., 1999; Huang and Ghabrial, 1996; Peery et al., 1987; Wickner, 1992). dsRNA viruses are classified into eight families based on their physicochemical properties: *Birnaviridae*, *Chrysoviridae*, *Cystoviridae*, *Endornaviridae*, *Hypoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae* (Mayo, 2002). Based on the sequences available in GenBank, the three most common genera of mycoviruses are *Mitovirus*, *Partitivirus* and *Totivirus*. In most cases, mycoviruses do not cause any distinguishable symptom in their host fungi. However, some dsRNA mycoviruses are related to hypovirulence of plant pathogenic fungi including *Cryphonectria parasitica* (Choi and Nuss, 1992), *Fusarium graminearum* (Chu et al., 2002), *Sclerotinia sclerotiorum* (Boland, 1992), and *Botrytis cinerea* (Vilches and Castillo, 1997).

Mycoviruses 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, 1950), and shown to be related to the presence of virus particles in 1962 (Hallings, 1962). It causes malformation, and this disease has been known as the La France disease or die-back disease (Revill et al., 1994; van der Lende et al., 1994). In *P. ostreatus*, *Oyster mushroom spherical virus* (OMSV) were isolated from malformed sporophores and their spawn (Go, 1992; Goodin, 1992) and *Oyster mushroom isometric virus 2* (OMIV2) were found recently (Lee, 2000). OMSV is single-strand RNA virus and causes a severe oyster mushroom die-back disease (Yu et al., 2003) caused by dsRNA-mediated infection processes (Lee, 2000; Revill and Wright, 1997).

Today, farmhouses of *A. bisporus* and *P. ostreatus* have suffered from viral disease of cultivated mushroom. Because mushrooms infected with virus form poor colonization of compost and total crop products decrease. In this study, we designed specific-primers for the detection of dsRNA viruses in SinRyung an isolate of *A. blazei* and in ASI2597, ASI2596, and Bupyungbokhoe isolates of *P. ostreatus*. Each specific primer specifically detected corresponding dsRNA virus by RT-PCR.

Materials and Methods

Mushroom strains and dsRNAs. dsRNA virus containing *Pleurotus ostreatus* isolates ASI2596, ASI2597, and Bupyungbokhoe, and *Agaricus blazei* were used. Each dsRNA samples were treated with DNase I and S1 nuclase at 37°C for 1-2 hrs and purified by acid phenol/chloroform extractions. Purified dsRNAs were checked by electrophoresis on 1% agarose gel and visualized by using ethidium bromide staining (Fig. 1).

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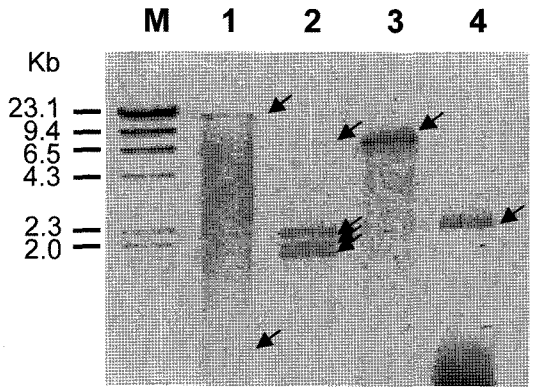

Fig. 1. dsRNA profile. Electrophoreses of RNAs extracted from purified dsRNAs from virus-infected *Pleurotus ostreatus* isolates ASI2596, ASI2597, and Bupyungbokhoe, and *Agaricus blazei* isolate SinRyung. Each dsRNA was separated in a 0.8% agarose gel and stained with ethidium bromide. Lanes 1-4 represent dsRNAs from *A. blazei* and *P. ostreatus* isolates ASI2597, Bupyungbokhoe, and ASI2596, respectively. Lane M denotes λ DNA/*Hin*dIII marker. Arrowheads indicate the dsRNAs extracted from each isolate.

cDNA cloning and sequencing. In analyzing unknown sequence of dsRNA samples, we prepared random fragment sequences using DNA shearing by Nebulization (Surzycki, 1990), conducted RT-PCR by primer ligation using primer-1 (5'-CCCGTCGACGAATTCTTTAAAAA-AAAAAAAAAAAAA-3', modified 5': phosphate and 3' amine). The strategy for synthesis of the cDNA from dsRNA segments and the PCR amplifications were carried out as described previously (Chu et al., 2002; Chu et al., 2004). The PCR products were then purified and cloned into *Sma*I-linearized pGEM-3Zf(+) vector. Inserts were sequenced by the dideoxynucleotide chain termination method as previously described (Jung, 2002; Kim, 2003) using the ABI prism™ Terminator Cycle Sequencing Ready Reaction Kit and and ABI prism 3700 Genetic Analyzer (Perkin Elmer, USA) located at the NICEM(SNU). Partial sequences were identified by using the BLAST search program of GenBank (Zhang and Madden, 1997). The partial genome sequence of the dsRNA (SinRyung) from *A. blazei* was compared with OSIM2 (GenBank accession number AY308801.1).

dsRNA-specific primer design and RT-PCR detection. Based on the determined sequences of dsRNAs, dsRNA-specific primers for RT-PCR detection for each virual dsRNA were designed. RT-PCR using one cycle of RT reaction at 40°C for 1hr and 35 cycles of PCR amplification using the step program (94°C for 30 sec, 48°C or 55.3°C for 1 min, 72°C for 2 min) followed by a 7 min final extension at 72°C was conducted.

Northern blot analysis. RNA blot analysis was carried out as described previously (Chu et al., 2002; Chu et al., 2004). Purified dsRNAs were treated with DNase I and S1 nuclease, separated on 0.8%agarose gel in TAE buffer, chemical denatured by soaking the gel in 50 mM NaOH for 20 min, and neutralized with 1.5 M Tris-HCl(pH 7.5) containg 0.5 M NaCl for 20 min. dsRNAs were then transferred to a positively charged nylon membrane (Hybond N+, Amersham Biosciences) with 2SSC buffer. Membrane was blocked by pre-hybridization with salmon sperm DNA and hybridized with prepared DNA probes. DNA probes specific for each dsRNA region were prepared by PCR amplification with biotin labeled dCTP. Viral dsRNA bands were detected by BAS program or auto-radiography.

Results and Discussion

Sequence analysis. Nucleotide sequences of a 451bp, 459bp, 384bp cDNA clone (pASI2597-14, pASI2597-24, pBupyungbokhoe, respectively) were obtained from the *P. ostreatus* isolates ASI2597 and Bupyungbokhoe (data not shown). The cDNA clone containing 768bp insert (pSin-Ryung) was obtained from the SinRyung isolate of *A. blazei*. A comparative analysis of the nucleotide sequence from the pSinRyung, pASI2597-14 and -24, pBupyung-bokhoe was conducted with sequence in the databases using BLASTn programs (Zhang and Madden, 1997). pASI2597-14 revealed sequence homology to RNA-dependent RNA polymerase of OMIV2 (Fig. 2), while nucleotide sequences and the deduced amino acid sequence from dsRNA mycovirus infecting *A. blazei* did not show any sequence homology to the other dsRNA mycoviruses. Nucleotide sequence alignment of each dsRNA with OMIV2 is shown in Fig. 2.

Many dsRNA mycoviruses have been found in the same host species (Cole, 1998; Lemke, 1979). Our results also indicate that different dsRNA mycoviruses can infect *P. ostreatus* isolate. dsRNAs detected from the *P. ostreatus* isolate ASI2597 and Bupyungbokhoe were different from that detected in ASI2596 (Seo, 2004). These results suggested that at least three different dsRNA mycoviruses infected in *P. ostreatus* and that there are the genetic diversity of the dsRNA mycoviruses found in *P. ostreatus* isolates.

Specific detection of each dsRNA mycovirus by RT-PCR. Based on the partial nucleotide sequences of pASI2597, pSinRyung, pBupyungbokhoe, dsRNA-sepcific primers were designed for RT-PCR detection of each dsRNA from infected mushroom (Table 1). Five primer

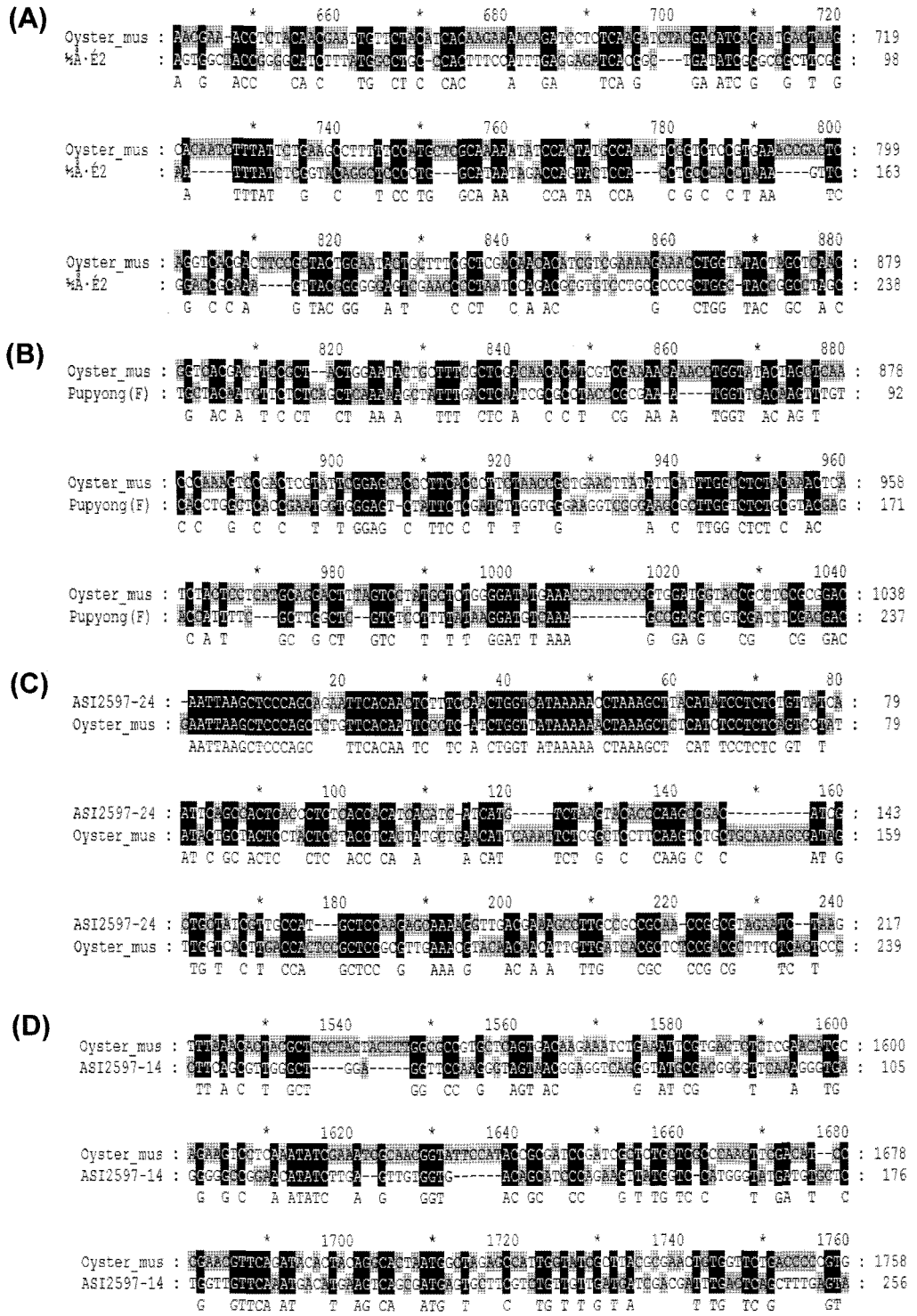

Fig. 2. Alignment of partial nucleotide sequence of pSinRyung (A), pASI2597-14 (B), pASI2597-24 (C), and pBupyungbokhoe (D). NCBI GenBank accession number for *Oyster mushroom isometric virus 2* (OMIV2) used in the analysis is AY308801.

sets were designed and tested to specifically detect each dsRNA virus. As might be suspected, prSinRyung-2, prASI2597-14, prASI2597-24, prBupyungbokhoe, and pr2596det primer sets specifically amplified DNA fragments from dsRNAs of SinRyung, ASI2597, Bupyungbokhoe, and ASI2596, respectively (Fig. 3). prASI2597-14 and prBupyungbokhoe primers also less specifically amplified DNAs with the other dsRNAs. Besides prBupyung-

Table 1. Nucleotide sequences of oligonucleotide primers used RT-PCR assays for the detection of dsRNAs from the SinRyung, ASI2597, ASI2596, and Bupyungbokhoe

Virus/Primers	Sequence (5' → 3')	Length (bp)	Tm (°C)
SinRyung			
prSinRyung2-F	GGGCTTTGTACTTTAATTG	766	44.0
prSinRyung2-R	GAATAATAACCGGCCTG		44.7
ASI2597			
prASI2597 No14-F	GGTCGGAATCGGAA	451	42.8
prASI2597 No14-R	AGAAAGAGTATCCTGAAAC		38.9
prASI2597 No.24-F	AATTAAGCTCCCAGC	459	38.4
prASI2597 No.24-R	ATGCGGAAGGCAGA		43.8
Bupyungbokhoe			
prBupyungbokhoe-F	CCCTGCTACAATGTTCTCTCA	368	51.6
prBupyungbokhoe-R	AGAATTCGTCGACGGGC		54.0
ASI2596			
2596det-for[a]	ACGACCTCTTCATACGACTTGA	550	52.8
2596det-rev[a]	AGTGAAAGCTGAATTATCGTCA		50.8

[a] Oligonucleotide primers used for the detection of ASI2596 were adapted from Seo et al.(2004).

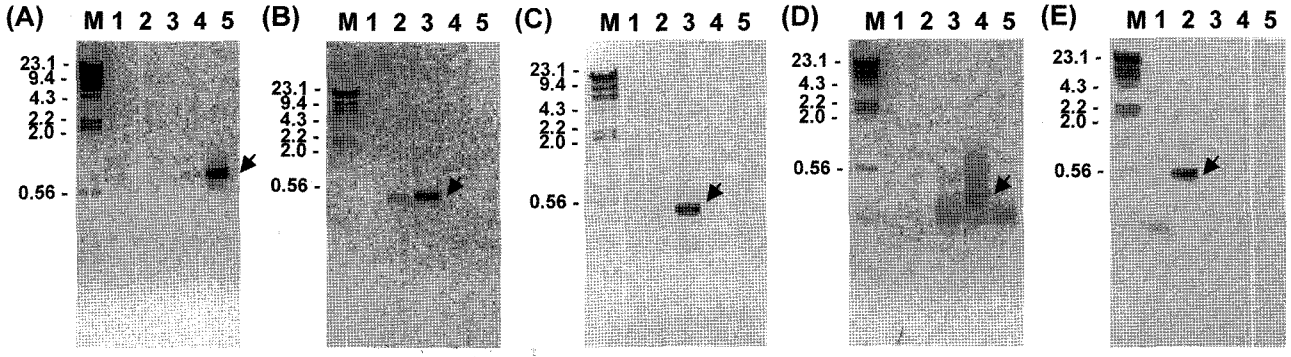

Fig. 3. RT-PCR detection of dsRNA from SinRyung (A), ASI2597-14 (B), ASI2597-24 (C), Bupyungbokhoe (D), and ASI2596 (E) using designed primer sets. cDNAs were synthesized using sheared dsRNAs extracted from virus-infected SinRyung, ASI2597, Bupyungbokhoe, and ASI2596. Lane M denotes λ DNA/*Hin*dIII marker. Lane 1, (−) control; lane 2, ASI2596; lane 3, ASI2597; lane 4, Bupyungbokhoe; lane 5, SinRyung.

bokhoe primer, non-specific bands were also detected, but the size and amount of amplified DNAs were significantly different. We used previously reported specific primers (pr2596det primer set) for ASI2596 for comparison of specificity with other primer sets (Seo, 2004). Gradient (temperature gradient: 45°C-60°C) RT-PCR amplification of each dsRNA with the corresponding primer sets revealed PCR melting temperature of 48 for SinRyung, Bupyungbokhoe, and ASI2596 detection and of 55.3°C for ASI2597-14 and -24 detection. Although dsRNA extraction technique using affinity to CF11 cellulose has been well investigated (Morris and Dodds, 1979; Valverde et al., 1986), the total amount of dsRNA was reduced due to strict washing conditions. This procedure, however, increased the purity of dsRNAs and thus reduced nonspecific annealing during RT-PCR assay.

Northern blot analysis. To confirm the homogeneity of RT-PCR products to each purified dsRNA, Northern blotting was conducted. Each PCR product of SinRyung, ASI2597-14, -24, and ASI2596 were used as probes. SinRyung probe specifically hybridized with dsRNA from SinRyung isolate, while ASI 2597-14 and -24 probe specifically hybridized with ASI2597 dsRNA, respectively. In addition, ASI2596 probe specifically hybridized to ASI2596 dsRNA (Fig. 4). Altogether, these results confirmed the homogeneity and specificity of each amplified DNAs.

In this study, we showed that the designed primer sets specifically detected each dsRNA virus infecting both *A. blazei* and *P. Ostreatus*. RT-PCR can be applied for the detection of more than one distinct dsRNA virus with primer pairs, even if they are not closely related. Lack of

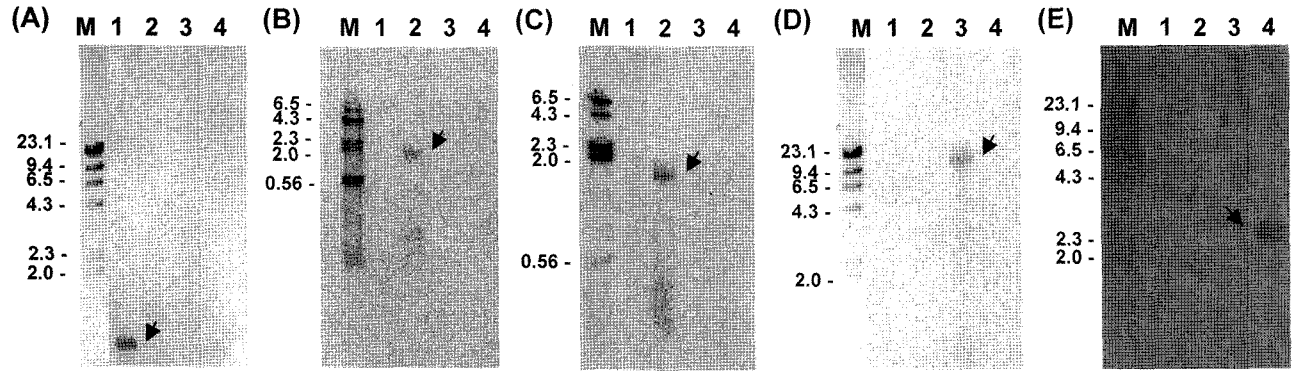

Fig. 4. Northern blot analysis. dsRNA were extracted from virus-infected *Agaricus blazei* and *Pleurotus ostreatus* isolates ASI2596, ASI2597, Bupyungbokhoe, and ASI2596. Each dsRNA was treated with DNase I and followed by S1 nuclease, seperated on a 0.8%agarose gel, stained with ethidium bromide, and blotted to a nylon membranes (panels from A to E). Panels A-E represent the RNA blot detection from the virus-infected SinRyung, ASI2597-14, ASI2597-24, Bupyungbokhoe, and ASI2596, respectively. DNA probes specific for each dsRNA region prepared by PCR amplification with biotin labeled dCTP were used. Lane M, λ DNA/*Hin*dIII marker; lane 1, SinRyung; lane 2, ASI2597; lane 3, Bupyungbokhoe; lane 4, ASI2596.

genome information among dsRNA mycoviruses infecting mushrooms makes it difficult to construct primers for specific detection. Therefore, RT-PCR protocols developed in this study can be employed for the detection and/or quarantine purposes of dsRNA mycoviruses infecting *A. blazei* and *P. ostreatus*. Since this procedure 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 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 diseases. Moreover, the use of RT-PCR using specific primers is expected to help researchers detect the dsRNA virus infecting the host fungi and further analysis of dsRNA mycovirus-host fungus interactions. However, the usefulness in a virus a virus indexing program has until now remained unproven since these techniques have not been tested using field samples. The requirement of time-consuming techniques for dsRNA or total RNA extraction is another obstacle for large-scale application of this method. The use of one-step RT-PCR kit is much simpler and less prone to contamination than the traditional two-step RT-PCR employed in this study. Therefore, the primer sets developed in this study would be useful for rapid detection and identification of several dsRNA viruses infecting mushrooms. Although RT-PCR assay has widely been applied for the detection of many RNA viruses (for review, see Khan et al., 1998), this is the first report of a RT-PCR assay that detect at least three unrelated dsRNA mycoviruses infecting mushrooms using specific primer sets.

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