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PCR Analysis of the Viral Complex Associated with La France Disease of *Agaricus bisporus*

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Reverse transcription PCR analysis was used to investigate the involvement of two RNA-genome viruses, La France isometric virus (LIV) and mushroom bacilliform virus (MBV), in the etiology of La France disease of the cultivated mushroom *Agaricus bisporus*. Reverse transcription PCR amplification of sequences targeted to the genomes of LIV and MBV, with a sensitivity of detection of <10 fg of viral RNA, showed diseased mushrooms to be either singly infected by LIV or doubly infected by LIV and MBV. Of 70 geographically diverse diseased mushroom isolates, 100% were infected by LIV, whereas almost 60% of these isolates were coinfected by MBV. Of 58 mushroom isolates determined to be free of infection by LIV, 3 were found to be infected by MBV. This represents the first documented report of the independent replication of these two viruses. Our data support the hypothesis that La France disease is associated with infection by two autonomously replicating viruses in which LIV is the primary causal agent and MBV, although possibly pathogenic and capable of modulating symptoms, is not required for pathogenesis.

La France disease, also known as X-disease, dieback, watery stipe, and brown disease, is among the most serious infectious pathologies of the common cultivated mushroom, *Agaricus bisporus* (Lange) Imbach (1, 4, 23). The malady is characterized by slow and aberrant mycelial growth, reduced yield, and the development of mushrooms that are malformed and display a premature maturation and an accelerated postharvest deterioration. Since its initial description more than 40 years ago following an episode in southeastern Pennsylvania (18), La France disease has come to be considered an important limiting factor in commercially grown mushrooms throughout the world. Because of the lack of effective resistance within *A. bisporus*, management of the disease is limited to the performance of hygienic practices directed at the elimination of infected fungal propagules from the production areas (3, 17).

There is a wealth of circumstantial evidence implicating a double-stranded-RNA (dsRNA)-genome, 36-nm-diameter isometric virus (La France isometric virus [LIV]) in the etiology of La France disease (5). The nine virion-associated dsRNAs (3.8 to 0.8 kb) of LIV are present in mushrooms and mycelial cultures manifesting symptoms of the disease (7–9, 13, 24). Radiolabeling studies have shown that the LIV dsRNAs are the only duplex RNA molecules actively replicating in diseased tissues (9). All nine dsRNAs are gained and lost in concert from mycelial cultures with an accompanying conversion from a symptomatic to an asymptomatic phenotype in the fungal host (9). LIV is transmitted efficiently through sexual spores of *A. bisporus* (16), and this mode of transmission agrees with the recognized mode of transmission of the causal agent (17). Finally, the role of LIV in the disease also is supported by electron microscopic evidence identifying a ca. 36-nm-diameter isometric virus-like particle in diseased tissues (10, 22, 23).

In addition to LIV, a positive-sense, single-stranded-RNA (ssRNA)-genome, bacilliform virus (19 by 50 nm) (mushroom bacilliform virus [MBV]), is associated with some outbreaks of the disease (19). The possible participation of this virus in the disease has not been examined as extensively as that of LIV because of the unavailability of a rapid and specific assay to allow large-scale etiological studies. Herein, we report the findings of a study in which we have used reverse transcription PCR (RT-PCR) analysis to investigate the involvement of LIV and MBV in the etiology of La France disease.

MATERIALS AND METHODS

Source of tissue. Mushroom isolates were collected from 1981 to 1994 at commercial production sites located in the United States (110 isolates), Canada (11 isolates), Australia (3 isolates), England (3 isolates), and South Africa (1 isolate) and stored at −80°C. All mushroom isolates were taken from crops that were judged to be abnormal by growers who were questioning whether La France disease could be implicated by a clinical diagnosis. Isolates were scored as LIV positive (La France disease) or LIV negative (non-La France disease) on the basis of gel electrophoretic analysis for the diagnostic dsRNA fingerprint of LIV (13).

Isolation and quantitation of RNA. Total cellular RNA was isolated from frozen mushroom tissue according to the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (2) except that the final RNA pellet was resuspended in a solution containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 1 mM EDTA. Genomic RNA of LIV and MBV was extracted from purified virus as described previously (5, 14). The proportion of the nine dsRNAs of LIV representing the 1.3-kb segment, which was targeted for RT-PCR amplification, was determined by laser densitometry (Ultroscan XL; Pharmacia-LKB Biotechnology Inc., Piscataway, N.J.) with the photographic negatives of ethidium bromide-stained gels. Quantitative measurements of RNA were made by spectrophotometry, assuming that 1.0 A_{260} unit was equal to 44 μg of ssRNA and 50 μg of dsRNA.

Selection of primers. LIV RNA-specific primers, LIVP1 (5′-CAGGGTGTG GTAACAGAGGTAAAC-3′; bp 313 to 336) and LIVP2 (5′-CTTGCGTTC -GGAGTCCTCTCTC-3′; antisense of bp 419 to 440), were designed on the basis of published sequence data for the 1.3-kb dsRNA (6). Primers specific for MBV RNA, MBVP1 (5′-AACAAGAGCCACACAGTCTTGA-3′) and MBVP2 (5′-GTTTAAGCCCATACCAGGAGC-3′), were tailored from sequence data derived from a 1.4-kb cDNA clone, pBv11 (14), and corresponded to bp 2258 to 2279 and the antisense of bp 2404 to 2424, respectively, of the putative replicase gene (11).

RT-PCR amplification. A standard RT reaction mixture contained the following in a final volume of 10 μl: 10 mM Tris-HCl (pH 8.3); 90 mM KCl; 1 mM $MnCl_2$; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 2.5 μM (each) either the LIV or the MBV primers; 2.5 U of *Tth* DNA polymerase (Epicentre Technologies, Madison, Wis.); and 25 to 200 ng of total cellular RNA. Just prior to addition, RNA was boiled for 10 min and transferred to an ice bath. The RT reaction mixture was overlaid with 50 μl of mineral oil, incubated at 70°C for 15 min, and transferred to an ice bath. For PCR amplification, 40 μl of a mixture

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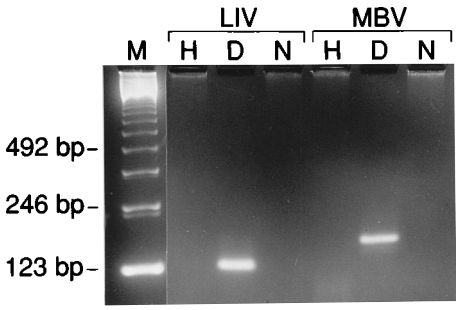

FIG. 1. Specificity of the RT-PCR targeting the genomic RNA of LIV and MBV. Total cellular RNA (200 ng) from healthy (H) or diseased (D) mushrooms was used as a template in a standard reaction mixture. Shown are the DNA amplification products with expected sizes of 128 and 167 bp for LIV RNA and MBV RNA, respectively. A negative control treatment in which RNA template was omitted from the reaction mixture was included (N). Numbers on the left refer to the sizes of the DNA markers (M).

containing 50% (vol/vol) glycerol, 100 mM Tris-HCl (pH 8.3), 1.875 mM $MgCl_2$, 1 M KCl, 7.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], and 0.5% Tween 20 was added to the RT reaction mixture. Amplification of DNA was carried out in a Perkin-Elmer Model 480 Thermocycler (Norwalk, Conn.) as follows: 1 cycle at 94°C for 2 min, 50 cycles at 94°C for 1 min and 60°C for 1 min, and 1 cycle at 60°C for 7 min. Each experiment included a negative control treatment consisting of a standard RT reaction mixture without added RNA and a positive control treatment in which the reaction mixture was amended with the targeted viral RNA.

Gel electrophoresis. Following amplification, 20 μl of the reaction mixture was added to 5 μl of a mixture containing 35% sucrose and 0.125% bromophenol. The entire sample was subjected to electrophoresis at 70 V for 2.5 to 3 h through a 4% composite gel consisting of NuSieve GTG agarose and SeaKem GTG agarose (3:1 [wt/wt]) (FMC BioProducts, Rockland, Maine). The electrophoresis buffer was 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA at pH 8.0 containing 50 ng of ethidium bromide per ml. DNA was visualized by transillumination with UV light and photographed with type 55 Polaroid film (Polaroid Corporation, Cambridge, Mass.). A 123-bp DNA ladder (Life Technologies, Inc., Gaithersburg, Md.) was used as a size standard.

DNA sequencing. Amplified DNA was electrophoretically purified through agarose gel and cloned by using a pCR-Script SK (+) kit as described by the manufacturer (Stratagene, La Jolla, Calif.). Bidirectional sequencing of dsDNA templates was carried out with Sequenase Version 2.0 according to the manufacturer's procedure (Amersham Life Science, Inc., Arlington Heights, Ill.).

RESULTS

RT-PCR amplification. With either the LIV RNA- or MBV RNA-specific primers, RT-PCR amplification of total cellular RNA from La France disease-affected mushrooms yielded a single major DNA product (Fig. 1, lanes D). The amplicons had estimated sizes of 128 and 167 bp for LIV RNA and MBV RNA, respectively, which agreed with the predicted sizes of the PCR products. These products were identical in size to those obtained by direct amplification of genomic RNA isolated from purified virus (see Fig. 3 and 4, lanes P). No DNA products were observed for amplifications either with total cellular RNA from healthy tissues (Fig. 1, lanes H) or without an exogenous source of viral RNA template (Fig. 1, lanes N). For control experiments, RT-PCR amplification of the targeted viral sequences was found to depend on an initial RT step and on the addition of 0.2 to 400 ng of total cellular RNA from diseased tissues, magnesium, and both primers within a pair.

Identity of the reaction products. The virus-specific amplification products shared 98 to 99% sequence homology with the expected sequence of the viral genomes. The 128-bp amplicon of LIV RNA contained two base substitutions of A→G at bp 342 and U→C at bp 387. The MBV RNA-specific 167-bp amplicon had a single base substitution of C→U at bp 2328.

Sensitivity. To determine the sensitivity of the RT-PCR-based assays, amplifications were carried out with purified viral

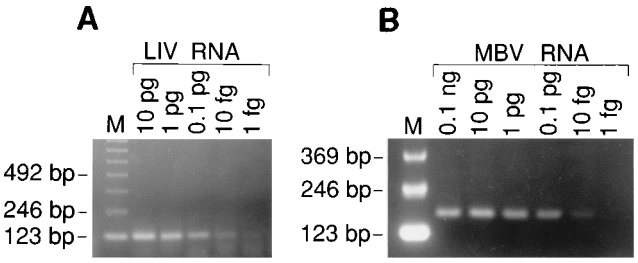

FIG. 2. Sensitivity of RT-PCR for the detection of genomic RNA sequences of LIV (A) and MBV (B). Purified genomic RNA was diluted with a total cellular RNA preparation of healthy mushrooms to provide a final concentration of 0.1 ng to 1 fg as indicated in a standard RT reaction mixture. Numbers on the left of the gels refer to the sizes of the DNA markers (M).

RNA that had been serially diluted in a total cellular RNA fraction from healthy mushrooms. In three separate experiments, 1 to 10 fg of the targeted viral RNA, i.e., the 4-kb ssRNA of MBV or the 1.3-kb dsRNA of LIV, was the lowest concentration of template that generated a detectable DNA product on agarose gels upon staining with ethidium bromide (Fig. 2).

Analysis of mushroom isolates. A total of 128 mushroom isolates collected over a 14-year period at commercial sites located primarily in the United States, but also representing Canada, Australia, England, and South Africa, were analyzed by RT-PCR amplification for single and double infection by LIV and MBV. For one category of 70 LIV-positive (La France disease) mushroom isolates, which was based on gel electrophoretic analysis of the LIV-specific dsRNAs, RT-PCR amplification confirmed that 100% of the isolates were infected by LIV (Table 1). Of these 70 isolates, 41 (59%) were doubly infected by LIV and MBV and the remaining 29 (41%) were singly infected with LIV. Figure 3 shows the typical amplification products, the diagnostic 128- and 167-bp amplicons for LIV and MBV, respectively, obtained with 16 arbitrarily selected La France disease-affected mushroom isolates.

For a second category of 58 LIV-negative (non-La France disease) mushroom isolates, those samples in which infection by LIV could not be established by electrophoretic analysis of dsRNA, one isolate was found by RT-PCR to be infected by LIV (Table 1). Additionally, three mushroom isolates with origins in Canada (isolate 13), the United States (isolate 30), and Australia (isolate 43) were found to be infected solely by MBV (Table 1). RT-PCR amplification of total cellular RNA fractions of these isolates yielded the diagnostic 167-bp DNA product for MBV but not the 128-bp DNA indicative of infection by LIV (Fig. 4). In a subsequent study, we found infection by MBV to persist through at least four serial transfers of a mycelial culture derived from infected mushroom tissue (15).

We further examined the 29 singly LIV-infected mushroom

TABLE 1. RT-PCR analysis for the incidence of single and double infection of commercially cultivated *A. bisporus* by LIV and MBV

Category of mushroom sample[a]	No. of isolates	Incidence of infection[b]			
		LIV alone	MBV alone	LIV plus MBV	Neither virus
LIV positive	70	29 (41)	0 (0)	41 (59)	0 (0)
LIV negative	58	1 (2)	3 (5)	0 (0)	54 (93)

[a] Infection by LIV was determined by electrophoretic analysis of dsRNA on ethidium bromide-stained gels.

[b] Number of positive isolates (percent positive).

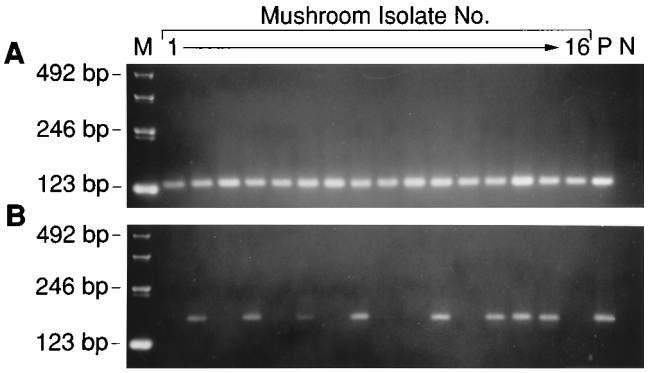

FIG. 3. Analysis of La France disease-affected mushroom isolates for single and double infection by LIV and MBV. Sixteen arbitrarily selected diseased mushroom isolates (1 to 16), which were previously determined to be infected with LIV by dsRNA analysis, were analyzed by RT-PCR amplification for infection by LIV (A) and MBV (B). For each virus, a positive control treatment (P) in which the homologous viral RNA (0.5 ng) was added as a template to the reaction mixture and a negative control treatment (N) in which RNA template was omitted were included. Numbers on the left refer to the sizes of the DNA markers (M).

isolates and the 3 singly MBV-infected isolates to determine if our inability to detect double infections was related to some feature of our procedure for RT-PCR. We were unable to establish a coinfection of these mushroom isolates by lowering the temperature of annealing for the primers from 60 to 45°C during PCR, in case the lack of amplification was related to a critical mismatching of sequences between the targeted viral RNA and the primers. In yet another study, strong amplification signals were obtained for both of the targeted viral RNAs with templates consisting of total cellular RNA preparations of singly infected mushroom isolates that had been supplemented with the undetected viral RNA, thereby ruling out the involvement of specific inhibitors of RT-PCR. We also failed to disclose infection by LIV in the three singly MBV-infected mushroom isolates by electrophoretic analysis of dsRNA on ethidium bromide-stained gels and RT-PCR carried out with a dsRNA-enriched fraction as a template.

DISCUSSION

All lines of evidence support the role of LIV, a 36-nm-diameter isometric dsRNA-genome virus, in the etiology of La France disease of *A. bisporus* (5, 7–9, 10, 13, 16, 22, 24). A bacilliform, ssRNA-genome virus (19 by 50 nm) known as MBV accompanies LIV in some episodes of the disease, although its relationship to a pathology is less clear (19). Herein, we have developed PCR-based assays targeting the genomic RNAs of LIV and MBV to clarify the nature of the viral complex associated with this disease. The high sensitivity of our RT-PCR-based assays, with limits of detection in the range of 1 to 10 fg of targeted viral RNA, provided a level of resolution in our analysis of the viral complex exceeding that of prior methods such as electron microscopy (10, 23) and electrophoretic analysis of dsRNA in ethidium bromide-stained gels (8, 9, 13, 24).

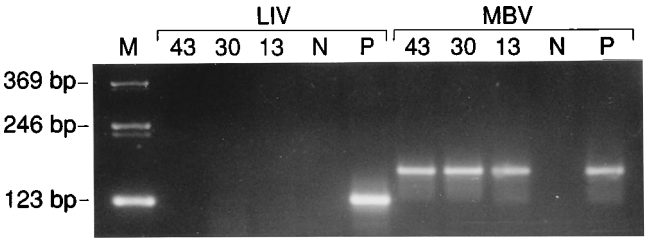

FIG. 4. Identification of singly MBV-infected mushroom isolates. Three mushroom isolates (43, 30, and 13) were analyzed by RT-PCR amplification for the targeted genomic RNA sequences of LIV and MBV. For each virus, a positive control treatment (P) in which the homologous viral RNA (0.5 ng) was added as a template to the reaction mixture and a negative control treatment (N) in which RNA template was omitted were included. Numbers on the left refer to the sizes of the DNA markers (M).

RT-PCR analysis of mushroom isolates collected from geographically dispersed regions over a 14-year period showed that of the two viruses, LIV was predominantly associated with La France disease. Of 70 diseased mushroom isolates, 100% were infected with LIV, while only about 60% were coinfected with MBV. These data strongly suggest that LIV is the primary etiologic agent and that MBV is not required for pathogenesis. Our findings confirm and extend the results of earlier etiological investigations implicating LIV as the causal agent on the basis of the disclosed positive correlation that exists between the presence of the conserved gel electrophoretic dsRNA fingerprint of LIV and the disease as it occurred in mushrooms (7, 13, 24) and mycelial cultures (8, 9). Because the majority of the mushroom isolates used in the present investigation originated in the United States, we do not know if our estimated rates of single and double infection by the two viruses reflect the worldwide pattern associated with the disease.

Considering the gross dissimilarities in morphology of the virion, structure of the capsid, and strandedness and nucleotide sequence of the genomic RNA (5–7, 11, 14, 19, 20), LIV and MBV must be considered two distinct viruses. In fact, MBV has recently been recognized as the sole member of a new family of viruses referred to as the *Barnaviridae* (12). Electron microscopic analysis suggested that LIV may infect mushrooms singly, whereas MBV occurs only as a mixed infection with isometric virus-like particles, particularly LIV (23). This pattern of infection implies a satellite-helper virus relationship in which MBV is a satellite virus whose replication is dependent on the helper virus LIV (4, 21). However, the fact that we now have identified mushroom isolates singly infected by these viruses establishes unequivocally that they do not rely on each other for replication. Moreover, we have found that single infections by LIV and MBV persist after infinite dilution through repeated serial transfer of mycelial cultures. Recent nucleotide sequence data suggesting that the genomic RNA of MBV encodes a replicase (11), a gene product unexpected of a satellite virus defective in replication, are consistent with our discovery of autonomous replication of this virus.

While our evidence indicates that MBV may not be essential to elicit La France disease, several questions regarding its replication and pathogenicity remain unanswered. For example, it is unclear if infection solely by MBV alters the phenotype of the fungal host and whether this virus attenuates or exacerbates symptoms associated with LIV. All 128 mushroom isolates used in this study were provided to us by growers who were questioning the involvement of La France disease in their cropping anomalies. Seventy of the isolates were from mushrooms with La France disease confirmed on the basis of the clinical diagnosis of the LIV dsRNAs (13). We do not know whether the remaining 58 isolates that typed negative for LIV were abnormal for reasons due to other viral infections, genetic disorders in the *A. bisporus* strains, adverse environmental factors, or cultural malpractices. For this reason, we are unable to speculate about the pathogenicity of MBV on the basis of our knowledge of the three singly infected mushroom isolates. Further research is needed to determine if MBV is a

second, albeit minor, causal agent of La France disease, the etiologic agent of an unrecognized pathology, or benign.

In the absence of evidence supporting the interdependency of LIV and MBV for replication, it is perhaps significant to note the dramatically higher incidence of MBV in doubly infected mushrooms. The rate of infection by MBV was almost 60% for LIV-positive mushroom isolates compared with 5% for LIV-negative isolates. Therefore, MBV may derive a survival advantage through its association with LIV. Our data do not support a reciprocal advantage for LIV, as the frequencies of this virus in single and double infections, 40% and 60%, respectively, were more comparable. We now seek to determine the individual and combined pathogenicities of LIV and MBV by using in vitro infection of spheroplasts, and ultimately, we will explore the possible interaction between the two viruses.

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