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PCR Analysis of Three RNA Genetic Elements in *Agaricus bisporus*

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ABSTRACT: We have described three different RNA genetic elements in the button mushroom *Agaricus bisporus*. One element consists of nine double-stranded (ds) RNAs (0.8 to 3.8 kb) encapsidated in 36 nm isometric particles (La France isometric virus, LIV). The second genetic element is a 4.4 kb single-stranded RNA packaged in 19 x 50 nm bacilliform-shaped particles (mushroom bacilliform virus, MBV). The third element consists of three dsRNAs (2.4, 5.2, and >13 kb) associated with 75 nm fungal vesicles (vesicle-associated dsRNA, VA-2.4). Reverse transcription (RT)-PCR using primers targeting RNA sequences within the genetic elements showed LIV is invariably associated with La France disease, MBV accompanies LIV in ca. 60% of the episodes of the disease, and VA-2.4 occurs in both healthy and diseased tissues. RT-PCR analysis also revealed that LIV and MBV are autonomously replicating viruses, each capable of independently and stably infecting *A. bisporus*. PCR amplification failed to reveal sequences corresponding to LIV, MBV, and VA-2.4 RNA in the genomic DNA of *A. bisporus*. The evidence supports a primary etiologic role for LIV in La France disease and suggests that LIV, MBV, and VA-2.4 are separate, extrachromosomally-replicating viral elements.

1 INTRODUCTION

In 1950, Sinden and Hauser described an infectious malady of the button mushroom *Agaricus bisporus* (Lange) Imbach as it occurred at a commercial farm in southeastern Pennsylvania. Today this disease, most commonly referred to as La France disease, has been reported throughout the world and is considered an important limiting factor in commercially-cultivated mushrooms (Schisler *et al.* 1967, van Zaayen 1979, Buck 1986).

Symptoms of the disease range from an insidious infection involving an almost imperceptible yield loss to acute outbreaks associated with slow mycelial growth, malformed basidiocarps, and total crop failure.

A wealth of circumstantial evidence implicates a double-stranded (ds) RNA, 36 nm isometric virus (La France isometric virus, LIV) in the etiology of La France disease (van Zaayen 1972, Wach et *al.* 1987, Koons *et al.* 1989, Romaine *et al.* 1989, 1993, and 1995). A single-stranded RNA, 19 x 50 nm bacilliform-shaped virus (mushroom bacilliform virus, MBV) is associated with some outbreaks of the disease, although its biological importance is not well understood (Tavantzis *et al.* 1980 and 1983; Romaine and Schlagnhaufer 1991, Revill *et al.* 1994, Romaine and Schlagnhaufer 1995).

In addition to the two RNA genetic elements associated with a pathology of *A. bisporus*, a third RNA element is prevalent in commercial mushroom strains. This element (vesicle-associated dsRNA, VA-2.4) consists of three naked dsRNA molecules of 2.4, 5.2, and >13 kb packaged by ca. 75 nm membrane vesicles (Romaine *et al.* 1993). VA-2.4 has not been linked to a specific phenotype; it can be detected in normal- and abnormal-appearing basidiocarps sampled from low- to high-yielding crops, both early and late in the production cycle (Romaine and Schlagnhaufer 1989).

In this paper, we present the findings of a study in which we have used PCR analysis to advance our understanding of the molecular biology of the three RNA genetic elements.

2 MATERIALS AND METHODS

2.1 *Source of tissue*

Basidiocarps were collected at commercial farms and stored at -80 C. Isolates were scored as LIV-positive (La France disease) or LIV-negative based on of gel electrophoretic analysis for LIV dsRNA (Wach *et al.* 1987, Romaine and Schlagnhaufer 1989). Mycelial cultures were derived from basidiocarps and maintained on potato dextrose agar. Basidiocarps were grown experimentally at The Pennsylvania State University Mushroom Research Center (Romaine and Schlagnhaufer 1992).

2.2 *Isolation of nucleic acid*

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987) as modified by Romaine

and Schlagnhaufer (1995). Genomic DNA was isolated by the method of Zolan and Pukkila (1986).

2.3 *Primers*

LIV RNA- and MBV RNA-specific primers were identical to those described previously (Romaine and Schlagnhaufer 1995). Primers targeting the 2.4 kb dsRNA of VA-2.4: 5'-CGGGTCTTGGGTAAC TTGCC-TA-3' and 5'-TTCGTGGGAGTTTCCAATTCGC-3' were designed based on sequence data (Romaine and Schlagnhaufer, unpublished results). Primers for laccase lcc1 gene: 5'-ATTTCTTGCAGGCCGTTC TTCACC-3' and 5'-TGGCATGCAGAATAACCGAGACAC-3' were designed based on published sequence data (Perry *et al.* 1993).

2.4 *RT-PCR*

The conditions for reverse transcription (RT)-PCR were after the method of Romaine and Schlagnhaufer (1995). Amplification of DNA was carried out in a Perkin Elmer Model 480 Thermocycler (Norwalk, CT) as follows: one cycle at 94 C for 2 min and 30 to 50 cycles at 94 C for 1 min and 60 C for 1 min.

2.5 *PCR*

PCR amplification was carried out as described for RT-PCR except that the initial RT step was omitted and 50 to 200 ng of genomic DNA was substituted for total RNA as template (Romaine and Schlagnhaufer 1995).

2.6 *Gel electrophoresis*

Amplified DNA was subjected to electrophoresis in 2% agarose gels or 4% NuSieve GTG-Seakem GTG composite agarose gels prepared in Tris-acetate-EDTA buffer (Romaine and Schlagnhaufer 1995).

2.7 *DNA sequencing*

Bi-directional sequencing of was carried out with Sequenase Version 2.0 according to the manufacturer's procedure (Amersham Life Science, Inc., Arlington Heights, IL).

3 RESULTS

3.1 *RT-PCR analysis of the RNA genetic elements*

RT-PCR amplification of total RNA from La France disease-affected basidiocarps using primers targeting either LIV RNA and MBV RNA yielded a single major DNA product (Figure 1, lanes D). The DNA products had estimated sizes of 128 bp and 167 bp for LIV RNA and MBV RNA, respectively, and corresponded to the predicted sizes of the products based on sequence data. The diagnostic 128 bp DNA product for LIV also was obtained using a dsRNA-enriched fraction from diseased tissues as template (Figure 1, lane Ds). No DNA products were observed from amplifications with total RNA from healthy basidiocarps (Figure 1, lanes H). The expected size 137 bp DNA for VA-2.4 was detected by amplification of total RNA from either healthy or diseased basidiocarps (Figure 1, lanes H and D). In all cases, amplification did not yield an observable DNA product when an exogenous source of the targeted RNA was omitted from the reaction mixture (Fig. 1, lanes N).

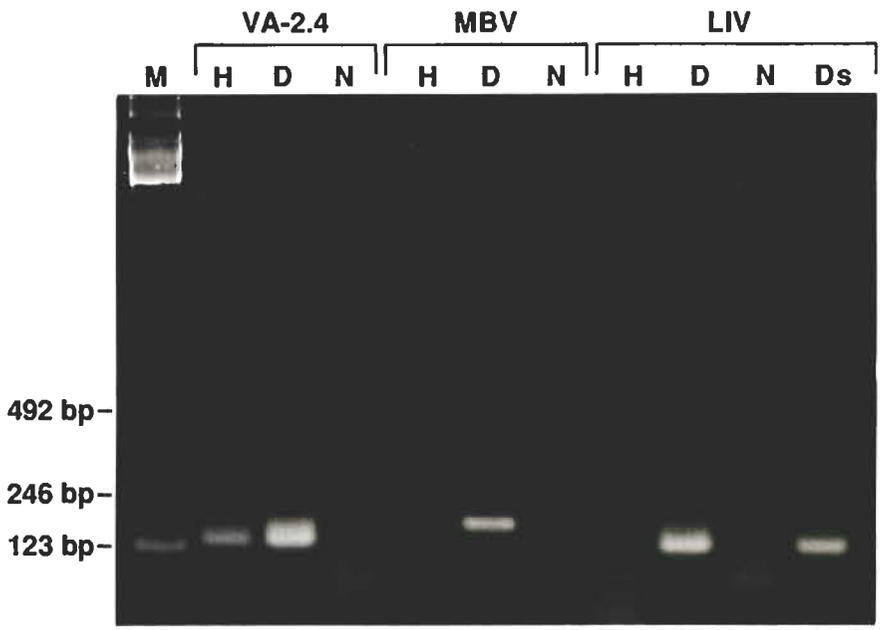

Fig. 1. RT-PCR targeting La France isometric virus (LIV), mushroom bacilliform virus (MBV), and vesicle-associated dsRNA (VA-2.4). Total RNA from healthy (H) and diseased (D) basidiocarps and a dsRNA fraction from diseased basidiocarps (Ds) were used as template. Shown are the DNA amplification products with expected sizes of 128 bp, 167 bp, and 136 bp for LIV, MBV, and VA-2.4, respectively. A negative control treatment in which RNA template was omitted from the reaction mixture was included (N). Numbers in the left margin refer to the size in bp of the DNA markers (M).

In other experiments, the sensitivity of the RT-PCR-based assays for LIV and MBV was between 1 and 10 fg of the targeted RNA on ethidium bromide-stained agarose gels (Romaine and Schlagnhaufer 1995). Also, RT-PCR amplification of the targeted sequences was dependent on the initial RT step, the addition of 0.2 to 400 ng of total RNA as template, magnesium ions, and both primers within a pair.

3.2 *Incidence of LIV and MBV in La France disease*

A total of 128 basidiocarp isolates collected over a 14-year period at commercial sites were analyzed by RT-PCR amplification for infection by LIV and MBV. Of 70 LIV-positive (La France disease) mushroom isolates, which was based on gel electrophoretic analysis for LIV dsRNA, RT-PCR amplification confirmed that all isolates were infected by LIV with 41 (59%) isolates being doubly-infected by LIV and MBV and the remaining 29 isolates (41%) being singly-infected by LIV (Table 1).

Table 1. RT-PCR analysis for the incidence of LIV and MBV in basidiocarps of commercially-cultivated *Agaricus bisporus*.

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

[a]Infection by LIV was previously determined by electrophoretic analysis of dsRNA on ethidium bromide-stained gels.
[b]No. of positive isolates (% positive).

One isolate among 58 LIV-negative basidiocarp isolates, those indexing negative for LIV dsRNA, was found by RT-PCR to be infected by LIV (Table 1). Three of the 58 LIV-negative isolates examined in the survey were infected solely by MBV (Table 1). RT-PCR amplification of total RNA from these isolates yielded the diagnostic 167 bp DNA product for MBV and not the LIV-specific 128 bp DNA (Fig. 2).

3.3 *MBV-infected cultures*

Mycelial cultures derived from MBV-infected basidiocarps remained infected after repeated serial transfer during an 8 month period. (Romaine and Schlagnhaufer, unpublished results). Moreover, the infection persisted in basidiocarps grown from spawn prepared from these cultures. In one

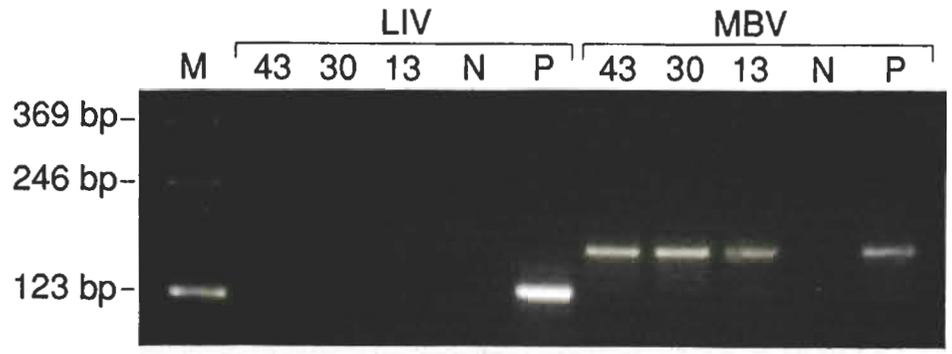

Fig. 2. Identification of MBV-infected basidiocarp isolates of *Agaricus bisporus*. Three basidiocarp isolates (43, 30, and 13) collected at commercial farms were analyzed by RT-PCR for RNA sequences of La France isometric virus (LIV) and mushroom bacilliform virus (MBV). A positive control treatment (P) in which the homologous RNA was added as template to the reaction mixture and a negative control treatment (N) in which RNA template was omitted are shown. Numbers in the left margin refer to the size in bp of the DNA markers (M).

experiment, each of 12 arbitrarily-selected basidiocarps harvested at first break in the cropping cycle was found to be infected solely by MBV, based on the detection of the diagnostic 167 bp amplified DNA and not the 128 bp DNA indicative of LIV (Fig. 3).

3.4 *PCR analysis of genomic DNA*

Sequences in the genomic DNA of *A. bisporus* corresponding to LIV, MBV, and VA-2.4 RNA could not be detected under conditions of PCR amplification that allowed detection of a 146 bp DNA sequence within the laccase lcc1 gene (Fig. 4). This was the case whether the source of genomic DNA was healthy or La France disease-affected basidiocarps.

4 DISCUSSION

We have used PCR analysis to gain additional insight into the pathogenicity, replication, and origin of three RNA genetic elements in *A. bisporus*. RT-PCR analysis of basidiocarp isolates collected at commercial farms showed that LIV was predominantly associated with La France disease. All of 70 diseased mushroom isolates examined in this study were infected with LIV, while only about 60% of the isolates were co-infected by MBV. These data implicate LIV as the primary etiologic agent and suggest MBV is not required for pathogenesis. Further research is needed to

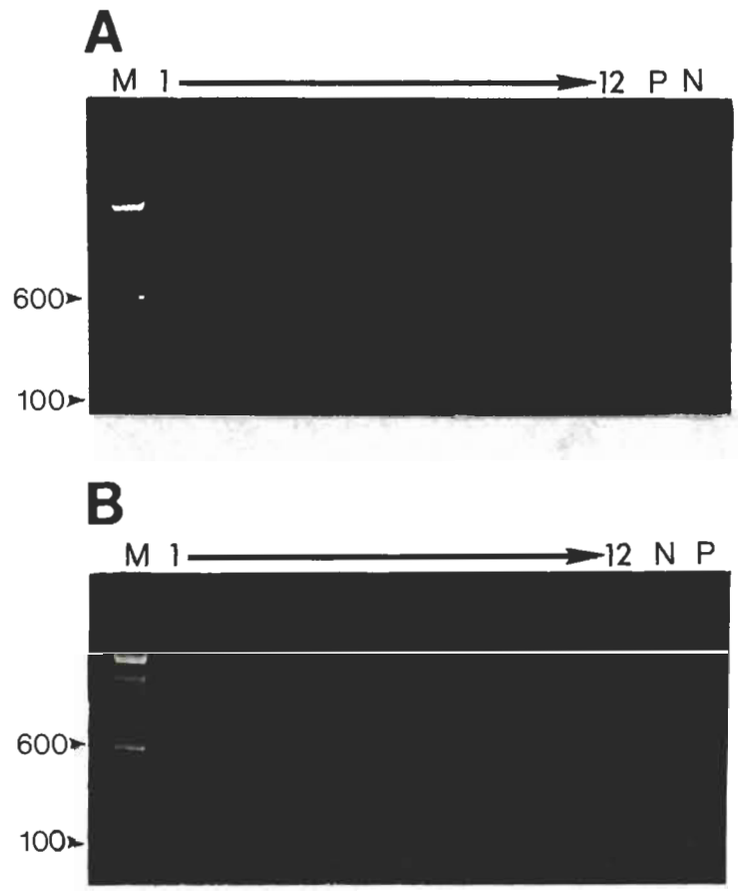

Fig. 3. RT-PCR analysis of basidiocarps grown experimentally from a MBV-infected culture of *Agaricus bisporus*. Spawn was prepared from a mycelial culture that was singly-infected by MBV and used to produce basidiocarps under experimental conditions. Twelve arbitrarily-selected basidiocarps (1-12) were examined for infection by MBV (A) and LIV (B). A positive control treatment (P) in which the homologous RNA was added as template to the reaction mixture and a negative control treatment (N) in which RNA template was omitted are shown. Numbers in the left margins refer to the size in bp of the DNA markers (M).

determine if MBV is a second causal agent of La France disease, the etiologic agent of another pathology, or benign. Our findings confirm and extend the results of other investigations identifying LIV as the causal agent based largely on a positive correlation between the presence of the LIV dsRNAs and the disease (Wach et *al.* 1987, Koons *et al.* 1989, Romaine *et al.* 1989, 1993, and 1995).

The fact that basidiocarps of *A. bisporus* may be singly-infected by LIV or MBV establishes unequivocally that these two RNA elements replicate independently. Similarly, VA-2.4 appears to be an independently-

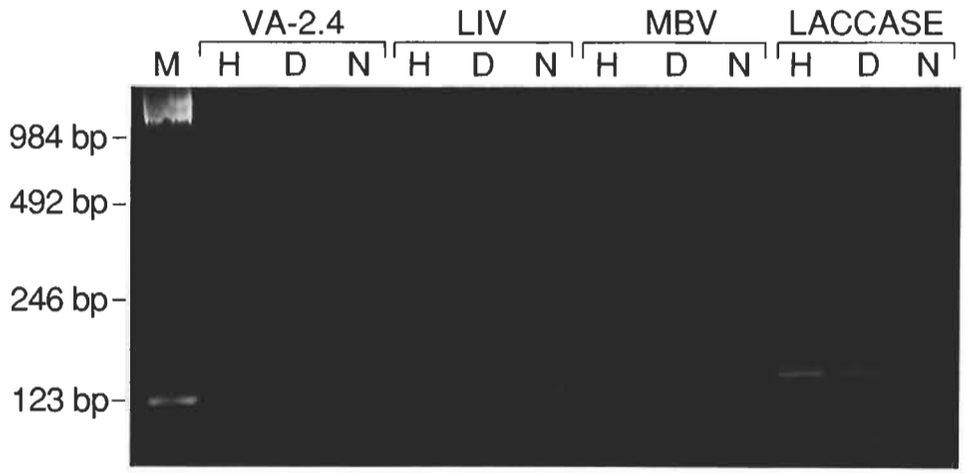

Fig. 4. PCR analysis of genomic DNA with primers targeting nucleotide sequences of La France isometric virus (LIV), mushroom bacilliform virus (MBV), vesicle-associated dsRNA (VA-2.4), and the laccase lcc1 gene (LACCASE). Genomic DNA of *A. bisporus* isolated from healthy (H) and La France disease-affected (D) basidiocarps was used as template. Shown is the expected 146 bp amplicon for the laccase lcc1 gene. A negative control treatment (N) in which DNA template was omitted was included. Numbers in the left margin refer to the size in bp of the DNA markers (M).

replicating element, because it can be found in healthy tissues in the absence of LIV and MBV. It remains to be determined if LIV or MBV require VA-2.4 for their replication since the latter is also associated with diseased tissues.

We found no evidence for the presence of sequences corresponding to the three RNA elements in the genomic DNA of *A. bisporus*. This finding is consistent with an extrachromosomal mode of replication. The absence of sequences imprinted in the genomic DNA also implies that the three RNA elements are foreign to the cell and, therefore, should be considered viral in nature.

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