Molecular Plant Pathology

Hybridization Analysis of the Single-Stranded RNA Bacilliform Virus Associated with La France Disease of *Agaricus bisporus*

C. Peter Romaine and B. Schlagnhaufer

Associate professor and research assistant, respectively, Department of Plant Pathology, The Pennsylvania State University, University Park 16802.

This work was partially supported by the Biotechnology Grants Program, School of Agriculture, The Pennsylvania State University. Contribution 1824, Department of Plant Pathology.

Accepted for publication 28 June 1991 (submitted for electronic processing).

ABSTRACT

Romaine, C. P., and Schlagnhaufer, B. 1991. Hybridization analysis of the single-stranded RNA bacilliform virus associated with La France disease of *Agaricus bisporus*. Phytopathology 81:1336-1340.

We investigated the relationship between the 19- × 50-nm single-stranded RNA (ssRNA) mushroom bacilliform virus (MBV) and the double-stranded RNAs (dsRNAs) associated with La France disease of *Agaricus bisporus*. Agarose gel electrophoresis under formaldehyde-denaturing conditions revealed that the MBV genome was composed of a single RNA molecule of 4.4 kb. In northern analyses, a 1.4-kb cloned complementary DNA (cDNA) to MBV RNA hybridized to the full-length genomic RNA in purified virus preparations and total RNA fractions from diseased mushrooms. A minor 1.8-kb RNA, presumably a subgenomic component, also was detected in diseased tissues. No cross hybridization occurred between this MBV cDNA clone and total RNA of healthy mushrooms. When we used either the cDNA or a combination of the nine La France disease-related dsRNAs (0.8 to 3.8 kb) as a hybridization probe, no sequence homology existed between MBV RNA and the dsRNAs found in diseased as well as healthy tissues. Our findings are consistent with the hypothesis that a viral complex involving an ssRNA virus and an unrelated dsRNA virus(es) plays a role in the etiology of La France disease.

Additional keywords: button mushroom, mycovirus.

Our understanding of the prevalence and biological consequence of viral infection in fungi began with the discovery of viruslike particles in commercial mushrooms (*Agaricus bisporus* (Lange) Imbach) affected by an infectious disorder of unknown etiology referred to as La France disease (14,26). Today we recognize that viruses are widely distributed in fungi and that these virus-host interactions, although often highly productive in terms of virus replication, seldom culminate in a pathology (4). In the case of La France disease of *A. bisporus,* viral infection is associated with slow mycelial growth, the development of abnormal mushrooms, and a reduced yield (31). More compelling evidence exists for the involvement of viruses in a decreased virulence phenotype in the fungal pathogens *Cryphonectria parasitica* (27) and *Ophiostoma ulmi* (24), as well as in the killer trait of *Saccharomyces cerevisiae* (35) and *Ustilago maydis* (9).

After almost 30 yr of investigation, the viral etiology of La France disease has not been conclusively determined. Efforts to test the hypothesized viral etiology of this disease have been confounded by low virus titers, difficulty in purifying the viruses, the presence of viruses in healthy tissues, and lack of a reliable infectivity assay (8). Electron microscope studies have shown that mixtures of 25- and 35-nm isometric particles and a 19- × 50-nm bacilliform particle (mushroom bacilliform virus, MBV) are associated with the disease as it occurs worldwide (4,30). MBV has been purified and shown to have a viral composition consisting of a positive sense single-stranded RNA (ssRNA) genome of 1.4 $\times 10^6$ Da and a single capsid polypeptide of 24,500 Da (28,29). In addition to the three particle types, a conserved electrophoretic pattern of nine double-stranded RNAs (dsRNAs) correlates positively with the disease (13,25,32). The origin and nature of the dsRNAs found in diseased tissues, as well as those encountered in healthy tissues (13,25), is not understood. In this study, we show that no sequence homology can be detected by hybridization between the genomic RNA of MBV and the dsRNAs found in diseased and healthy mushrooms. The evidence in hand suggests that La France disease is associated with a virus complex involving an ssRNA virus and one or more unrelated dsRNA viruses.

MATERIALS AND METHODS

Source of tissue. Healthy and diseased hybrid white and off-white mushrooms were collected at commercial sites, washed, and stored at −20 C.

Isolation of virus and viral RNA. MBV was purified from diseased mushrooms by polyethylene glycol-NaCl precipitation, differential centrifugation, rate-zonal centrifugation in sucrose, and equilibrium centrifugation in cesium sulphate (28). To obtain MBV RNA, the purified virus was dissolved in 10 mM Tris-HCl, 0.5 mM EDTA, and 0.5% SDS, pH 7.8, and incubated with 200 μg/ml of Proteinase K (Bethesda Research Laboratories, Gaithersburg, MD) for 30 min at 37 C. The RNA was extracted twice with a phenol/chloroform mixture and precipitated twice with ethanol (20). The final RNA pellet was dried in vacuo,

© 1991 The American Phytopathological Society

resuspended in 10 mM Tris-HCl, pH 8.0 (Tris buffer), and stored at −135 C.

Isolation of dsRNA. The dsRNA was isolated by phenol extraction and cellulose chromatography (22). To remove possible contaminating nucleic acids, the purified dsRNA in 50 mM Tris-HCl, 10 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.2, was incubated with 10 μg/ml of DNase I (RNase-free, Boehringer Mannheim Inc., Indianapolis, IN) for 30 min at 33 C, and then it was adjusted to 0.3 M NaCl and 1 μg/ml of RNase (Type IA, Sigma Chemical Co., St. Louis, MO) and incubated for an additional 30 min at 37 C. After nuclease treatment, dsRNA was treated with Proteinase K, phenol extracted and ethanol precipitated as before, and then precipitated with cetyltrimethylammonium bromide (23). The final dsRNA pellet was dried, dissolved in Tris buffer, and stored at −135 C.

Isolation of total RNA. The acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (5) was used. Aliquots of RNA were stored at −135 C.

Synthesis and cloning of cDNA to MBV RNA. MBV RNA, polyadenylated in vitro (1), served as template in an oligo (dT)-primed reverse transcription reaction (cDNA synthesis system; Amersham Inc., Arlington Heights, IL). Complementary DNA (cDNA) was inserted at the *Pst*I site in plasmid pBR322 by homopolymeric tailing (C-tailing and cloning system; Dupont Co., Boston) and the recombinant plasmids were used to transform *Escherichia coli* (DH5 alpha; Bethesda Research Laboratories) by standard procedures (11). Putative transformants were screened for a DNA insert at the *Pst*I site in the plasmid (3). Plasmid pBV11, which contains a virus-specific 1.4-kb cDNA representing about 30% of the MBV genome, was used as a source of the hybridization probe.

Radiolabeling of nucleic acid probes. The insert of pBV11, representing the cDNA to MBV RNA, was excised from the plasmid, electrophoretically purified by the DEAE-cellulose paper procedure (application update 364, 1989, Schleicher & Schuell, Inc., Keene, NH), and labeled (random primed DNA labeling kit; Boehringer Mannheim Inc.) with (alpha-^{32}P) dATP (3,000 Ci/mmol; ICN Radiochemicals, Irvine, CA). DsRNA was incubated in 25 mM glycine and 5 mM $MgCl_2$, pH 9.0, for 1 h at 60 C, ethanol precipitated, dissolved in sterile water, heated at 100 C for 5 min followed by quenching in liquid nitrogen, and 5′ end-labeled with (gamma-^{32}P) ATP (3,000 Ci/mmol; Dupont Co.) as described by Maniatis et al (19).

Gel electrophoresis and northern analysis. DsRNA was electrophoresed in 0.9% agarose gels and stained with ethidium bromide (32). For denaturing conditions, RNA was electrophoresed in formaldehyde-containing 1.2% agarose minigels for 2 h at 75 V (18). A *Bst*EII restriction endonuclease digest of lambda DNA (New England BioLabs Inc., Beverly, MA) and an RNA ladder (Bethesda Research Laboratories) were used as size standards for nondenaturing and denaturing gel electrophoresis, respectively. For hybridization analysis, RNA was capillary-blotted from formaldehyde gels to Nytran nylon membrane (Schleicher & Schuell, Inc.) according to the method of Fourney et al (7) and probed as described by Church and Gilbert (6). After hybridization, membranes were washed with 40 mM sodium phosphate, 1 mM EDTA, and 5% SDS, pH 7.2, for 5 min at 65 C and twice with the same buffer containing 1% SDS for 15 min each time at 65 C. Membranes were exposed to Kodak X-Omat RP film at −20 C. In rehybridization studies using a second probe, membranes were stripped of the initial probe by washing with 3 mM sodium acetate and 0.03 M NaCl, pH 7.2, for 15 min at 95 C.

RESULTS AND DISCUSSION

Electrophoretic analysis of MBV genomic RNA. Upon electrophoresis in formaldehyde-permeated agarose gels, the ssRNA isolated from purified 19- × 50-nm virions of MBV migrated as a single 4.4-kb RNA molecule (Fig. 1). Thus, the molecular size and complexity of the MBV genome, based on an electrophoretic analysis under denaturing conditions, agrees closely with

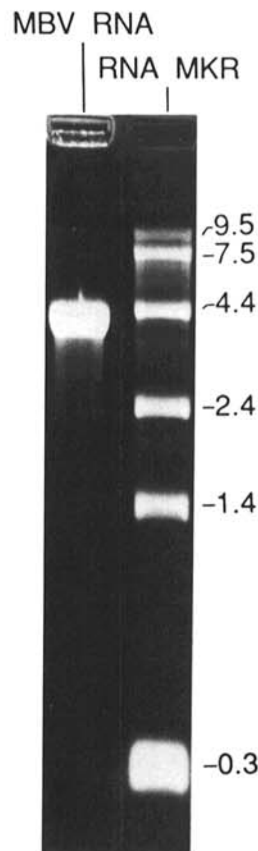

Fig. 1. Agarose-formaldehyde gel electrophoretic analysis of mushroom bacilliform virus (MBV) genomic RNA. The genomic RNA of MBV isolated from purified virions = MBV RNA; RNA ladder = RNA MKR. Numbers indicate the size (kb) of the RNA markers (Bethesda Research Laboratories). Electrophoresis was conducted in formaldehyde-containing 1.2% agarose gel at 75 V for 2 h.

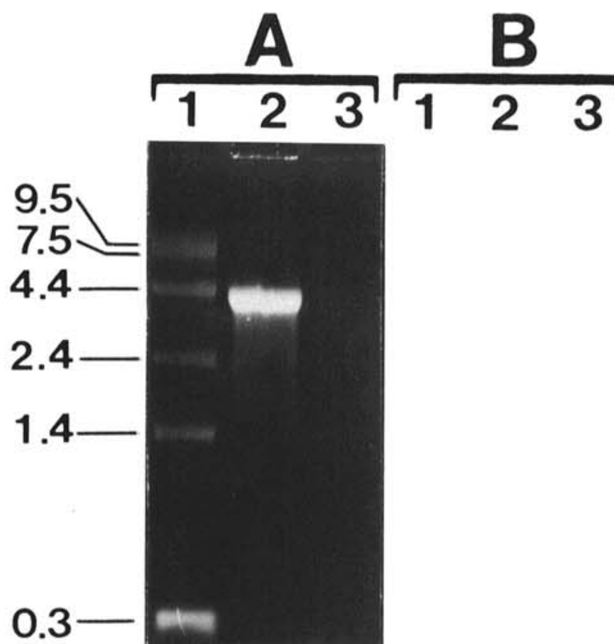

Fig. 2. Northern hybridization of the genomic RNA of mushroom bacilliform virus (MBV) using the 1.4-kb cDNA clone as a probe. After formaldehyde-agarose gel electrophoresis, the gel was **A,** stained with ethidium bromide, and **B,** capillary-blotted to Nytran nylon membrane and probed with the ^{32}P-labeled cDNA. Lane 1, RNA ladder; lane 2, MBV genomic RNA isolated from purified virions; lane 3, the comparable fraction from healthy mushrooms. Numbers refer to size (kb) of the RNA markers (Bethesda Research Laboratories). Electrophoresis was conducted in formaldehyde-containing 1.2% agarose gel at 75 V for 2 h.

our earlier findings, determined under nondenaturing conditions in polyacrylamide gels (28), which indicated the genome is composed of one ssRNA molecule of 1.4×10^6 Da.

Detection of MBV RNA in healthy and diseased tissues. The 1.4-kb cDNA clone to MBV RNA hybridized specifically to the 4.4-kb genomic RNA of MBV isolated from purified virions (Fig. 2A and B, lane 2). In contrast, no hybridization signal was obtained with the comparable fraction from healthy mushrooms that lacked MBV RNA on ethidium bromide gels (Fig. 2A and B, lane 3). Similarly, the cDNA probe detected MBV RNA sequences in dot blots of total RNA preparations from diseased mushrooms, but not from that of healthy mushrooms (data not shown). Northern analysis of total RNA fractions showed that the cDNA hybridized to a major 4.4-kb RNA present in diseased tissue (Fig. 3, lane 2), which was identified as the genomic RNA because it comigrated with authentic genomic RNA extracted from purified virions (Fig. 3, lane 3). A minor 1.8-kb RNA (Fig. 3, lane 2) was consistently detected in our RNA preparations from diseased mushrooms. For this reason, we tentatively consider it to be a subgenomic RNA. No MBV RNA-related sequences were detected in total RNA fractions of healthy mushrooms (Fig. 3, lane 1).

Hybridization of MBV RNA and the dsRNAs. We previously reported (25,32) that nine dsRNAs of 3.8, 3.1, 3.0, 2.8, 2.6, 1.7, 1.3, 0.9, and 0.8 kb are associated with mushrooms affected by La France disease (Fig. 4, lane 2), whereas a major 2.4-kb dsRNA could be detected in healthy mushrooms (Fig. 4, lane 1). Upon electrophoresis in 1.2% agarose-formaldehyde gels, the ssRNA molecules of the dsRNAs from both healthy and diseased mushrooms migrated faster than MBV RNA (Fig. 5A, lanes 1–3). In northern analyses using the 1.4-kb cDNA clone as a probe, a hybridization signal was obtained with MBV RNA, but not with the disease-specific or nondisease-specific dsRNAs (Fig. 5B, lanes

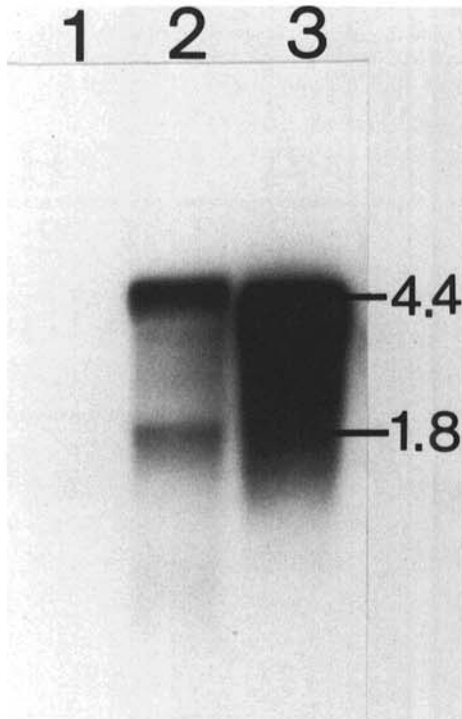

Fig. 3. Northern hybridization of total RNA isolated from healthy and diseased mushrooms. Lane 1, total RNA (15 μg) from healthy mushrooms; lane 2, total RNA (15 μg) from La France disease-affected mushrooms; lane 3, the genomic RNA of mushroom bacilliform virus (MBV) (10 ng) isolated from purified virus. Total RNA was isolated from mushrooms with acid guanidinium thiocyanate-phenol-chloroform, electrophoresed in formaldehyde-containing 1.2% agarose gel at 75 V for 2 h, and capillary-blotted to Nytran nylon membrane. The hybridization probe was the ^{32}P-labeled 1.4-kb cDNA to MBV RNA. Numbers indicate the position of the 4.4-kb MBV genomic RNA (4.4) and the putative 1.8-kb subgenomic RNA (1.8).

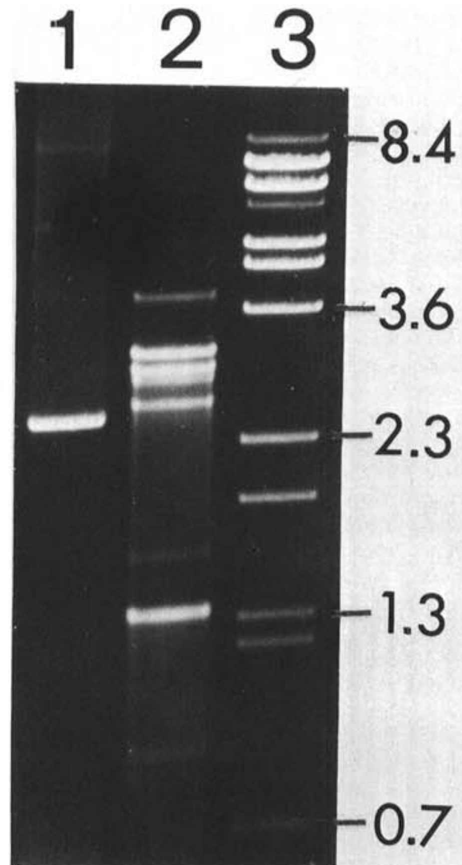

Fig. 4. Nondenaturing agarose gel electrophoresis of the dsRNAs in healthy and diseased mushrooms. Lane 1, the dsRNA pattern of healthy mushrooms; lane 2, the dsRNA pattern of La France disease-affected mushrooms; lane 3, the *Bst*EII restriction endonuclease digestion fragments of lambda DNA (New England BioLabs). Numbers indicate size (kb) of the DNA markers. Electrophoresis was carried out in 0.9% agarose gel at 100 V for 2 h.

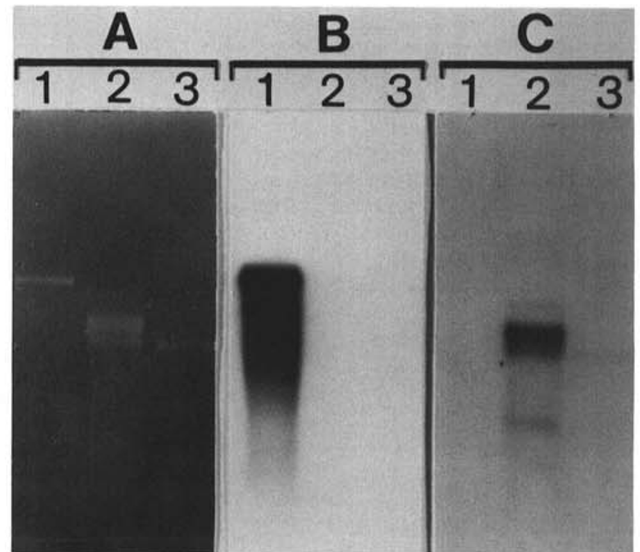

Fig. 5. Northern hybridization of MBV RNA and the dsRNAs. After formaldehyde-agarose gel electrophoresis, the gel was **A,** stained with ethidium bromide and then capillary-blotted to Nytran nylon membrane. The membrane was **B,** incubated with the ^{32}P-labeled MBV RNA-specific cDNA probe, and **C,** stripped and incubated a second time with the ^{32}P-labeled disease-related dsRNA probe. Lane 1, the genomic RNA of MBV extracted from purified virus; lane 2, the dsRNAs isolated from diseased mushrooms; lane 3, the dsRNA isolated from healthy mushrooms. Electrophoresis was conducted in formaldehyde-containing 1.2% agarose gel at 75 V for 2 h.

1–3). Because the dsRNA sequences might have corresponded to the two-thirds portion of the MBV genome not represented by pBV11 cDNA, we performed the reciprocal hybridization using end-labeled disease-specific dsRNAs as the probe. Again, no sequence homology was revealed between the dsRNAs and MBV RNA (Fig. 5C, lane 1). Hybridization was detected, however, between the disease-specific dsRNAs and the 2.4-kb dsRNA in healthy tissues (Fig. 5C, lane 3), but this was attributed to the presence of the latter dsRNA in the dsRNA probe rather than being interpreted as evidence for sequence relatedness. This conclusion is substantiated by the finding of Harmsen et al (13), who used the individual dsRNAs as probes to show that they have unique sequences.

The findings of our hybridization study indicate that the ssRNA genome of the 19- × 50-nm bacilliform virus associated with La France disease is unrelated in nucleotide sequence and therefore distinct from the putative dsRNA-containing virus(es) implicated in the etiology of this disease. Still, we recognize that at a level of resolution higher than hybridization analysis (i.e., DNA sequencing), some degree of homology may exist between the MBV genome and the dsRNAs. However, the fact that MBV RNA and the disease-specific and nondisease-specific dsRNAs do not share extensive homology precludes the idea that the dsRNAs are replicative forms of MBV subgenomic RNAs or that MBV RNA is a single-stranded molecule of a dsRNA. What remains unanswered is whether the 19- × 50-nm bacilliform particle is the sole component of this virus or only one of several components composing a multicomponent virus system similar to alfalfa mosaic virus (15). If in fact MBV has unaccounted for genomic RNAs, then the disease-specific dsRNAs might represent their replicative forms. Although the presence of dsRNA is generally regarded as a strong indication of viral infection, in some instances dsRNA appears to be host derived (21,30,33,34). Essential to our understanding of the etiology of La France disease will be a knowledge of how the dsRNAs reside in vivo. Perhaps the dsRNAs are packaged within the 25- and 35-nm isometric particles present in diseased tissues (4,31) or exist as naked molecules within a membraneous system (2,12,16,17,24). Recently, we have obtained preliminary data that suggest the nine disease-related dsRNAs copurify with a 36-nm isometric particle (10).

We found no evidence for the presence of MBV RNA sequences in healthy mushrooms. We do not know if this is generally true of all strains of *A. bisporus* because the scope of our study was limited to only two healthy mushroom isolates. Evidence from electron microscope studies (4,31) revealing the occurrence of MBV at low levels in healthy mushrooms and mycelial cultures suggests this may not be the case. These same studies also have shown that whereas the isometric particles occur in either single or multiple infections, MBV invariably exists as a coinfection with isometric particles. This is an intriguing observation because it raises the possibility that MBV is a satellite virus whose replication is dependent on a isometric helper virus. We are now using the polymerase chain reaction assay to survey healthy and diseased mushrooms and mycelial cultures for the incidence of MBV. These studies should provide an insight into the possible satellite nature of MBV as well as address the question of whether this virus plays an obligatory role in the etiology of La France disease.

LITERATURE CITED

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 1987. DNA-independent RNA polymerases. Section 3.9 in: Current Protocols in Molecular Biology. Greene Publishing Associates–Wiley-Interscience, New York.
2. Barroso, G., and Labarere, J. 1990. Evidence for viral and naked double-stranded RNAs in the basidiomycete *Agrocybe aegerita*. Curr. Genet. 18:231-237.
3. Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
4. Buck, K. W. 1986. Fungal virology—An overview. Pages 1-84 in: Fungal Virology. K. W. Buck, ed. CRC Press, Inc., Boca Raton, FL.
5. Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.
6. Church, G. M., and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81:1991-1995.
7. Fourney, R. M., Miyakoshi, J., Day, R. S., and Patterson, M. C. 1988. Northern blotting: Efficient RNA staining and transfer. BRL Focus 10:5-7.
8. Frost, R. R., and Passmore, E. L. 1980. Mushroom viruses: A reappraisal. Phytopathol. Z. 98:272-284.
9. Ganesa, C., Chang, Y., Flurkey, W. H., Randhawa, Z. I., and Bozarth, R. F. 1989. Purification and molecular properties of the toxin coded by *Ustilago maydis* virus P4. Biochem. Biophys. Res. Commun. 162:651-657.
10. Goodin, M. M., Schlagnhaufer, B., and Romaine, C. P. 1991. The La France disease-specific double-stranded RNAs are encapsidated in isometric virus-like particles. (Abstr.) Phytopathology 81:702.
11. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.
12. Hansen, D. R., Van Alfen, N. K., Gillies, K., and Powell, W. A. 1985. Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. J. Gen. Virol. 66:2605-2614.
13. Harmsen, M. C., van Griensven, L. J. L. D., Wessels, J. G. H. 1989. Molecular analysis of *Agaricus bisporus* double-stranded RNA. J. Gen. Virol. 70:1613-1616.
14. Hollings, M. 1962. Viruses associated with dieback disease of cultivated mushrooms. Nature (Lond.) 196:962-965.
15. Jaspars, E. M. J., and Bos, L. 1980. Alfalfa mosaic virus. No. 229 in: Descriptions of Plant Viruses. Commw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
16. Kim, W. K., and Klassen, G. R. 1989. Double-stranded RNAs in mitochondrial extracts of stem rusts and leafrusts of cereals. Curr. Genet. 15:161-166.
17. Lefebvre, A., Scalla, R., and Pfeiffer, P. 1990. The double-stranded RNA associated with '447' cytoplasmic male sterility in *Vicia faba* is packaged together with its replicase in cytoplasmic membraneous vesicles. Plant Mol. Biol. 14:477-490.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Electrophoresis of RNA through gels containing formaldehyde. Pages 202-203 in: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Labeling the 5′ ends of DNA with T4 polynucleotide kinase. Pages 122-127 in: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Purification of nucleic acids. Pages 458-460 in: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Meyers, C. J., Griffiths, A. J. F., Kraus, S. R., and Martin, R. R. 1988. Double-stranded RNA in natural isolates of *Neurospora*. Curr. Genet. 13:495-501.
22. Morris, T. J., and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology 69:854-858.
23. Poulson, R. 1973. Isolation, purification, and fractionation of RNA. Pages 241-261 in: The Ribonucleic Acids. P. R. Stewart and D. S. Letham, eds. Springer-Verlag, New York.
24. Rogers, H. J., Buck, K. W., and Brasier, C. M. 1987. A mitochondrial target for double-stranded RNA in diseased isolates of the fungus that causes dutch elm disease. Nature (Lond.) 329:558-560.
25. Romaine, C. P., and Schlagnhaufer, B. 1989. Prevalence of double-stranded RNAs in healthy and La France disease-affected basidiocarps of *Agaricus bisporus*. Mycologia 81:822-825.
26. Sinden, J. W., and Hauser, E. 1950. Report of two new mushroom diseases. Mushroom Sci. 1:96-100.
27. Tartaglia, J., Paul, C. P., Fulbright, D. W., and Nuss, D. L. 1986. Structural properties of double-stranded RNAs associated with biological control of chestnut blight. Proc. Natl. Acad. Sci. USA 83:9109-9113.
28. Tavantzis, S. M., Romaine, C. P., and Smith, S. H. 1980. Purification and partial characterization of a bacilliform virus from *Agaricus bisporus:* A single-stranded RNA mycovirus. Virology 105:94-102.
29. Tavantzis, S. M., Romaine, C. P., and Smith, S. H. 1983. Mechanism of genome expression in a single-stranded RNA virus from the cultivated mushroom, *Agaricus bisporus*. Phytopathol. Z. 106:45-50.
30. Valverde, R. A., Nameth, S. T., and Jordan, R. L. 1990. Analysis of double-stranded RNA for plant virus diagnosis. Plant Dis. 74:255-258.
31. van Zaayen, A. 1979. Mushroom viruses. Pages 239-324 in: Viruses

and Plasmids in Fungi. P. A. Lemke, ed. Marcel Dekker, Inc., New York.
32. Wach, M. P., Sriskantha, A., and Romaine, C. P. 1987. Double-stranded RNAs associated with La France disease of the commercial mushroom. Phytopathology 77:1321-1325.
33. Wakarchuk, D. A., and Hamilton, R. I. 1985. Cellular double-stranded RNA in *Phaseolus vulgaris*. Plant Mol. Biol. 5:55-63.
34. Wang, B., Li, Y. N., Zhang, X. W., Hu, L., and Wang, J. Z. 1990. Double-stranded RNA and male sterility in rice. Theor. Appl. Genet. 79:556-560.
35. Wickner, R. B. 1986. Double-stranded RNA replication in yeast: The killer system. Annu. Rev. Biochem. 55:373-395.