CHAPTER VIII

SUMMARY AND GENERAL DISCUSSION

The characteristic symptoms of "La France" disease of the cultivated mushroom are locally dying mycelium and, if formed at all, fruit bodies with elongated stipes and small, early maturing caps or fruit bodies with thickened, barrel-shaped stipes. The disease is highly infectious in the sense that, when initiated, it is rapidly transmitted throughout shelves of mushrooms and can infect entire mushroom farms, resulting in complete loss of crop. Rapid spread of the disease is due to the fact that spores from diseased mushrooms (that can survive long periods without germination) transmit the disease by anastomosis with healthy mycelium. Sonnenberg and Van Griensven (1991) have shown that during this anastomosis only dsRNA and no nuclear genes are transmitted.

As reported first by Hollings (1962) and by others since, a number of virus particles of different sizes were found in diseased mushrooms, but also in apparently healthy mushrooms. Despite an inability to relate one or more specific virions to La France disease, the presence of virions (as detected by electron microscopy) was long used to diagnose La France disease. After it was found that the symptoms specific for La France disease were always associated with the presence of a specific set of nine dsRNAs (Marino *et al.*, 1976; Hicks and Haugton, 1986; Ross *et al.*, 1986; Deahl *et al.*, 1987; Koons *et al.*, 1987; Wach *et al.*, 1987; Harmsen *et al.*, 1989; Morten and Hicks, 1992), a diagnostic test based on these dsRNAs was developed (Harmsen, 1990). The main advantage in this approach is that detection is possible at a very early stage in the cultivation process before fruiting, thus enabling termination of the cultivation process at an early time and preventing spread of the disease by spores. Eventually, when practiced in a systematic way, this could lead to complete eradication of the disease in a certain area.

Because of the presence of virions in *A. bisporus* it was suspected that the disease, and consequently the dsRNAs, are of viral origin. A major aim of the research described in this thesis was to identify the virion(s) associated with the dsRNAs and to study the associated molecules. Molecular-genetic information on the virus is crucial for engineering a virus-resistant mushroom by transformation.

Development of an effective virus isolation procedure is described in Chapter

II. By screening for the presence of disease-specific dsRNAs it was found that these dsRNAs co-purify with 34 nm isometric virions, confirming results by Goodin *et al.* (1992). However, we showed that their analysis of the protein composition of the virions was wrong because proteolysis of the capsid proteins occurred after picking the mushrooms. Induction of protease activity after picking has been documented by Burton *et al.* (1993). By eliminating proteolytic events during the whole isolation process, from the picking of the mushrooms onwards, it was found (Chapter II) that the 34 nm particles contain at least two major proteins of M_r 120K and 90K and a minor protein of M_r 115K, all antigenically unrelated.

The relationship between the dsRNAs and proteins of the 34 nm virion is described in Chapter III and IV and summarised in Fig. 1. The nucleic acid sequence of L1 dsRNA and its deduced amino acid sequence (Chapter III), indicate that this sequence codes for the RNA-dependent RNA polymerase of the virion. Also partial amino acid sequence of the virion-associated protein of M_r 115K matches the deduced amino acid sequence of L1 dsRNA, thus identifying this dsRNA as the gene for the M_r 115K capsid protein and its function as the viral polymerase (Chapter IV).

In vitro translation of the L2 dsRNA resulted in a protein of M_r 120K which indicated that L2 dsRNA presumably encodes the largest virion associated protein of M_r 120K. The L3 dsRNA of which the nucleic acid sequence had been previously determined (Harmsen *et al.*, 1991) proved to encode a virion-associated protein of M_r 90K as shown by amino acid sequencing of this protein (Chapter IV). The L5 dsRNA, of which the nucleic acid and deduced amino acid sequences are given in Chapter III, also encodes a protein of 90K that can be visualised after *in vitro* translation of the L5 dsRNA (Chapter IV). Moreover, antibodies raised against whole 34 nm virions reacted with products obtained by *in vitro* transcription and translation of L5 dsRNA-specific cDNA clones. This suggests that the virion-associated protein band running at the position of M_r 90K in an SDS-PAGE gel (Chapter II) actually contains two proteins, one encoded by L3 dsRNA and another by L5 dsRNAs.

In Chapter IV the product of the M2 dsRNA is identified as a M_r 38K protein cytoplasmic protein of unknown function. This was done using an antibody raised against a protein expressed in *E. coli* from a M2 dsRNA derived cDNA clone. As indicated in Fig. 1 no proteins could be related to dsRNAs L4, M1, S1 and S2. The latter three dsRNAs which are sometimes absent, when La France disease symptoms are detected (Harmsen *et al.*, 1989).

In Chapter III some speculations were given on the etiology of La France

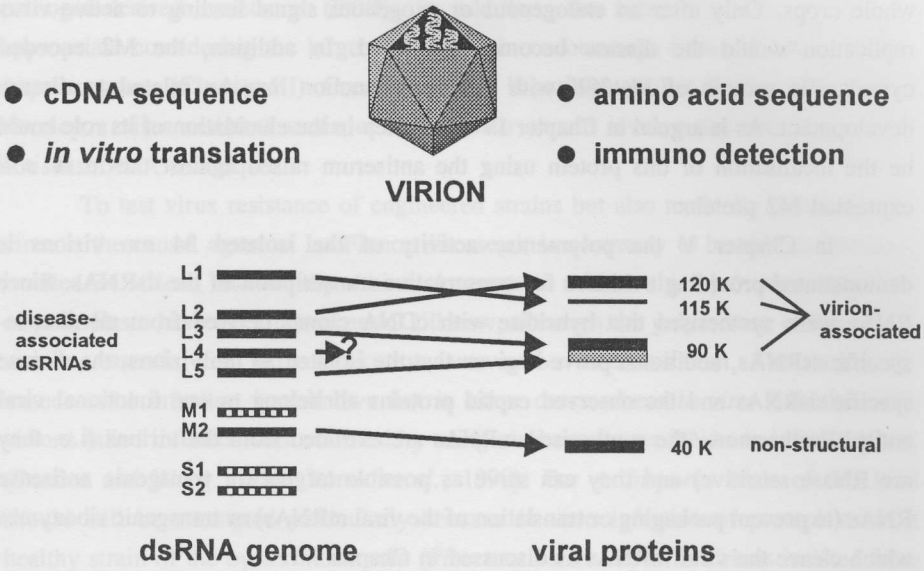

Figure 1. *Schematic diagram of the relationship between the dsRNAs and proteins as separated by gel electrophoresis encoded by the 34 nm virion. Description in the text.*

disease based on the striking difference in codon usage between ABV1 genes and genes of the host *A. bisporus*. We suspect that virus replication might interfere with translation of highly expressed host genes as a result of a diminished concentration of rare amino-acyl tRNAs. Among the translation products may be proteins needed in high quantities during fruiting and substrate colonisation, such as hydrophobins implicated in fungal emergent growth (Wessels, 1994). Such hydrophobins were recently identified in *A. bisporus* (Lugones, Bosscher, Scholtmeyer, de Vries and Wessels, Symposium on Genetics and Cellular biology of Basidiomycetes III London, 1995, Book of abstracts). ABH1p hydrophobin is particularly abundant in the surface of fruit bodies, ABH3p hydrophobin is found in colonising strands of *A. bisporus*. Limitation of synthesis of these and other hydrophobins could very well cause the decreased ability to colonise the substrate and the failure to form abundant and normal-shaped fruit bodies as observed in La France disease. This would imply that it is not so much the mere presence of the virus that causes the disease but rather the rapid replication of the virus that debilitates growth and fruiting. The virus could be present in a latent form without causing disease. This would explain the presence of 34 nm particles in healthy mushrooms or

whole crops. Only after an endogenous or exogenous signal leading to active virus replication would the disease become manifested. In addition, the M2 encoded cytoplasmic protein of M_r 38K with unknown function may be related to disease development. As is argued in Chapter IV a first step in the elucidation of its role could be the localisation of this protein using the antiserum raised against the in *E. coli* expressed M2 protein.

In Chapter V the polymerase activity of the isolated 34 nm virions is demonstrated providing evidence for conservative transcription of the dsRNAs. Since RNAs were synthesised that hybridise with cDNA clones derived from all disease-specific dsRNAs, additional prove is given that the isolated 34 nm virions, the disease specific dsRNAs and the observed capsid proteins all belong to one functional viral entity. Furthermore, the synthesised mRNAs are extruded from the virions (i.e. they are RNase sensitive) and they can serve as possible targets for transgenic antisense RNAs (to prevent packaging or translation of the viral mRNAs) or transgenic ribozymes which cleave the viral mRNAs as discussed in Chapter VI.

With respect to antiviral strategies it is interesting to note that a stretch of 23 nt (GGCAACGGCUAGUUGGCCAAAUU), present in the 5' region of the (+) strand of the L5 dsRNA (this Thesis) is also present in the 5' region of the (+) strand of the M2 dsRNA (Harmsen *et al.*, 1991), whereas part of this sequence (AACGGCUAGUU) is also present in the (+) strand of L3 dsRNA (Harmsen *et al.*, 1991). Of the two other dsRNAs which have been sequenced, L1 dsRNA (this Thesis) and M1 dsRNA (Harmsen *et al.*, 1991), the 5' ends of the (+) strands were not determined. If present in the 5' region of the (+) strands of all dsRNAs this sequence may perform an important function, such as interaction with capsid proteins. In that case, it might serve as a preferable target for an antisense RNA strategy to impair its function.

In deciding the strategy for obtaining virus-resistant mushrooms it is important to consider the observation by Schuren and Wessels (to be published) that at least in the homobasidiomycete *Schizophyllum commune* transcription of transgenes is early terminated by the fact that some AT-rich regions are recognized by the host organism as transcription terminators. Probably because of this, attempts (Chapter IV) to develop a ribozyme-based antiviral modelsystem in *S. commune* were not yet successful. As discussed in Chapter VI, transcription termination induced by transgene stretches of DNA might also occur in *A. bisporus*. Therefore, a ribozyme-based system using short viral sequences only may be more appropriate than expressing entire viral genes from the nuclear genome of *A. bisporus*, as generally used in plant systems to obtain

pathogen-derived resistance (PDR). In addition, the latter approach seems less appropriate considering the large differences in codon usage between ABV1 and *A. bisporus* genes (Chapter III). Consequently, it is anticipated that the best approach to develop a virus resistant strain of *A. bisporus* should involve the ribozyme and/or antisense RNA techniques.

To test virus resistance of engineered strains but also to finally prove that the virus is the causal agent of La France disease, it is necessary to be able to introduce isolated 34 virions into healthy strains. Attempts to achieve this by *in vitro* infections of protoplasts have been unsuccessful till now, although the polymerase of the 34 nm virions, essential for virus replication, was active after isolation. The reason for unsuccessful infection is unknown but in other cases where *in vitro* infection with virions failed infection was achieved by using viral messenger RNAs to infect the host (Furfine and Wang, 1990; Roner *et al.*, 1990). For *A. bisporus* trials of this kind should still be attempted particularly because *in vitro* infection of protoplasts of a healthy strain of the oyster mushroom (*Pleurotus ostreatus*) with virions isolated from infected mycelium was achieved (Chapter VII). After infection one dsRNA of the initial seven dsRNA was stably maintained, in the infected mycelium. The different dsRNAs were shown to be independent of each other as far as maintenance was concerned.