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CHARACTERIZATION OF A NOVEL VIRUS
ASSOCIATED WITH THE MVX DISEASE OF *AGARICUS BISPORUS*

Cranfield Health in collaboration with
Warwick HRI (Wellesbourne)

PhD THESIS

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Characterization of a novel virus
associated with the MVX disease of
Agaricus bisporus

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DEDICATION

To Maurizio
Thanks for being there and having lots of patience!

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I would like to thank...

My supervisors Dr Mike Challen, Prof Phil Warner and Prof Peter Mills for their invaluable support and guidance during my studies

All my colleagues for their support and advice in particular
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Finally, my family, particularly my husband Maurizio without whose love, support and encouragement I could not have achieved this

ABSTRACT

‘Mushroom Virus X’ (MVX) disease of the cultivated mushroom *Agaricus bisporus* first arose in UK during the 1990’s. This disease resulted in devastating crop losses in the UK and gradually became more widespread (e.g. Netherlands and Eire). Up to twenty-six, non-encapsidated, double stranded RNA (dsRNA) elements have been found to be associated with diseased mushrooms, and these are believed to be the result of a complex of viruses. Although considerable data has accumulated on the symptoms of infection, aetiological sources, epidemiology and molecular characterization of the MVX dsRNA elements are limited. Research described in this thesis focused principally on sequence characterization of a frequently occurring dsRNA element (^{MVX}14.4), which was shown to be a novel *Endornavirus*. Assigned ‘*Agaricus bisporus* endornavirus 1’ (AbEV1), this represents the first endornavirus known to infect edible mushrooms. AbEV1 is the first MVX element to be fully sequenced. Putative domains for RNA-dependent RNA polymerase (RdRp), helicase and glycosyltransferase were identified and used in comparisons with other viruses. Characterization of an AbEV1-type dsRNA found in a culture sample derived from a wild *Agaricus bisporus* collection indicates a possible source of the MVX dsRNA infections. Epidemiological studies were used to demonstrate that the AbEV1 dsRNA was transmissible both vertically through spores and horizontally by mycelial anastomosis between infected donor and MVX free acceptor strains. As a first step in the effort to understand the role of AbEV1 in MVX infections and to investigate possible host defence mechanisms, dsRNA hairpin sequences were introduced into *A. bisporus* by *Agrobacterium*-mediated transformation. Both helicase and RdRp sequences were able to confer resistance to the uptake of MVX dsRNA elements in transformants. These observations suggest that homology-dependent gene silencing pathway(s) may be present in *A. bisporus* and represent a residual antiviral defence mechanism. Advances and approaches developed in this project open new opportunities to characterize the other dsRNA elements from the MVX complex, to further our understanding of mycovirus infections and host responses, and to investigate the origins of infectious dsRNA elements.

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ABBREVIATIONS

(-) strand RNA	negative strand RNA
(+) strand RNA	positive strand RNA
°C	degrees centigrade
µg	microgram(s)
µl	microlitre(s)
µm	micron
(NH ₄) ₂ SO ₄	ammonium sulphate
aa	amino acid(s)
Ab	<i>Agaricus bisporus</i>
AbEV1	<i>Agaricus bisporus</i> endornavirus 1
An	<i>Aspergillus nidulans</i>
ARP	<i>Agaricus</i> Resource Programme
bp	base pair
CaCl ₂ •2H ₂ O	calcium chloride
cAMP	3':5'-cyclic adenosine monophosphate
cat. N.	Catalogue number
cm	centimetre(s)
Conc ⁿ	concentration
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dsRNA	double stranded RNA
FeSO ₄	iron sulphate
g	gram(s)
GFP	green fluorescent protein
<i>gpd</i>	glyceraldehydes-3-phosphate II
GT	glycosyltransferase
GUS	β-glucuronidase
h	hour(s)

HDGS	homology-dependent gene silencing
<i>hph</i>	hygromycin B phosphotransferase
ITS	intergenic spacer
K_2HPO_4	potassium phosphate dibasic
kbp	kilobase(s)
KCl	potassium chloride
KH_2PO_4	potassium phosphate
M	molar
MΩ	Megaohms
MES	2-(N-morpholino)ethanesulfonic acid
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
$MgSO_4 \cdot 7H_2O$	magnesium sulphate heptahydrate
min	minute
ml	millilitre(s)
mM	millimolar
ms	millisecond(s)
^{MVX} dsRNA	dsRNAs in mushrooms affected by MVX disease
MW	molecular weight
NaCl	sodium chloride
ng	nanogram(s)
nm	nanometre(s)
ORF	open reading frame
PCR	polymerase chain reaction
pM	picomolar
pmol	picomole(s)
ppm	part(s) per million
psi	pounds per square inch
PTGS	post-transcriptional gene silencing
RdRp	RNA-dependent RNA polymerase

RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
rpm	revolution(s) per minute
RT-PCR	reverse transcription- polymerase chain reaction
s	second(s)
ssRNA	single stranded RNA
ter	terminator
TGS	transcriptional gene silencing
tRNA	transfer RNA
trpC	tryptophan synthetase
U	unit(s)
UP water	ultra pure water
UTR	untranslated region
UV	ultraviolet
V	volt(s)
v/v	volume per unit volume
w/v	weight per volume
xg	rotation centrifugal force

Chapter 1

GENERAL INTRODUCTION

1. Summary

The work described in this thesis was designed to gain a better understanding of a viral dsRNA element associated with the Mushroom Virus X disease, affecting the cultivated mushroom *Agaricus bisporus*. An overview of mushroom biology and fungal virology will provide the background to this study.

1.1 The basidiomycete

Agaricus bisporus (Lange) Imbach

According to fossil records, fungi have existed since the lower cretaceous period, approximately 130 million years ago (Chang *et al.*, 1993).

In nature, fungi play an important role in the cycling of carbon and other elements. They live either as saprophytic organisms feeding from dead or decaying materials, as parasites or in symbiosis with other living organisms. All fungi, except yeasts, grow as microscopic filaments called hyphae which extend and branch to form a mycelium. The latter is the vegetative phase of fungal growth, while the sexual phase is represented by spore-bearing structures called sporophores.

The fungal kingdom comprises five major groups: chytridiomycetes, zygomycetes, ascomycetes, deuteromycetes, and basidiomycetes. The latter represent one third of the fungal kingdom. Basidiomycetes are classified as either homo- or heterobasidiomycetes based on their basidial morphology (Elliott, 1985a). Heterobasidiomycetes produce septate basidia of various shapes during their life cycle. Most plant pathogenic fungi (e.g. smuts, rusts) belong to this group. Homobasidiomycetes have non-septate basidia on large and fleshy fruiting bodies (mushrooms and toadstools) and are further subdivided into gasteromycetes and hymenomycetes. Gasteromycetes, also called “stomach fungi”, produce spores inside their fruiting bodies. Spores are dispersed when fruiting bodies are damaged by animals, heavy rain, etc. Hymenomycetes produce spores from an open surface and comprise two orders: the *Agaricales* (gilled mushrooms) and the *Aphylllophorales* (polypores, toothed fungi, coral fungi) (Hibbett *et al.*, 1997). In gilled mushrooms, basidia are aligned in close proximity to each other

forming extensive lamellae of meiotically dividing cells. Gilled mushrooms include most cultivated genera: *Agaricus*, *Lentinus*, *Flammulina*, *Volvariella* and *Pleurotus* (Chang *et al.*, 1989).

1.2 *Agaricus bisporus* description

Agaricus bisporus var. *bisporus*, commonly named ‘white button mushroom’ (Fig 1.1), is the premier cultivated species worldwide and falls within the *Agaricus* section *Duploannulatae* (Challen *et al.*, 2003). Its name comes from: “Agaricon” which is an ancient Greek word for fungus, derived from Agara, a Greek city where mushrooms were abundant; while “bisporus” means bearing two spores (web site: www.mushroomexpert.com).

A. bisporus is comprised of a cap (pileus) and a stalk (stipe). The spore-bearing lamellae or gills are found at the underside of the cap. The gills are not attached to the stipe in *Agaricus* spp., in contrast to other genera, and are covered early in development by a veil which opens in the mature mushroom resulting in a distinct ring (annulus) on the stipe (Elliott, 1985a). *Agaricus* species produce dark-brown coloured spores, causing the brown coloration of gills seen in mature mushrooms. However, the distinguishing feature of the cultivated *A. bisporus* var. *bisporus* is microscopic; each basidium bears two spores, instead of the usual four observed in most homobasidiomycetes (Fig. 1.2). There are four clearly differentiated strains within button mushrooms (Fritsche & Sonnenberg, 1988): white, off-white, brown, and hybrid varieties. White strains are characterised by smooth white fruiting bodies without scales and produce relatively small mushrooms (average 4-8 grams/unit). Off-white strains, also called “intermediate”, arose spontaneously amongst Dutch mushroom farms. They have rough scaly off-white caps. Fruiting bodies tend to be larger and denser than those of white varieties. They are particularly suited for mechanical picking and very high yields can be obtained. The brown strains have a more natural cap colour compared to wild *A. bisporus* and are grown for the excellent flavour. Brown strains are used to produce specialty mushrooms such as “cremini” and “Portobello”.



Fig. 1.1- *Agaricus bisporus* var. *bisporus*. The white button mushroom is the premier cultivated species worldwide and falls within the *Agaricus* section *Duploannulatae* (image courtesy of Warwick HRI)

Crosses between ‘smooth’ and ‘off-white’ varieties have been used to produce several ‘hybrid’ strains with improved agronomic characteristics. The first commercial hybrid strains were Horst U1 and U3 (Fritsche & Sonnenberg, 1988) and most modern hybrids are derivatives of these early strains (Fritsche, 1983; Kerrigan *et al.*, 1995; Moore *et al.*, 2001).

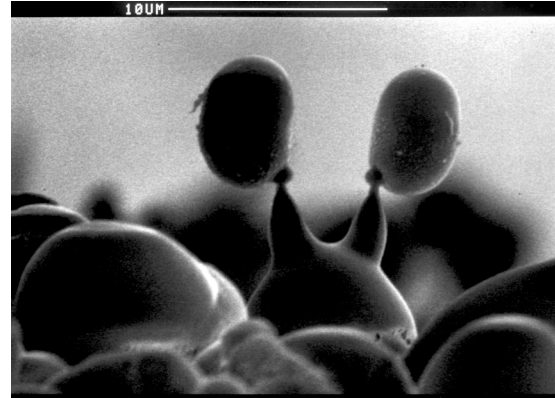


Fig. 1.2-*Agaricus bisporus* basidium bearing two spores (image courtesy of Warwick HRI)

1.3 Life-cycle of *Agaricus bisporus*

1.3.1 Homobasidiomycetes

The complete sexual life-cycle of homobasidiomycetes consists of three distinct phases: haplophase, dikaryonphase, and diplophase. A sexual spore (basidiospore) upon germination typically forms a non-self fertile mycelium which, when established, contains a single nucleus per cell and is referred to as a monokaryon. This uninucleate condition is maintained by a complex of intercellular septal apparatus (Giesy & Day, 1965) that allows cytoplasmic continuity, but restricts nuclei flow. In many species, further sexual development requires a mating interaction between compatible strains, leading to the formation of the fertile dikaryon (Koltin *et al.*, 1972) in which the two nuclei from compatible mating partners remain closely associated in each cell and divide in synchrony, but do not fuse (Casselton, 2002). The dikaryon phase is the predominant vegetative phase of basidiomycetes (Casselton, 2002) and can provide some advantages of diploidy (Bagueret *et al.*, 1994) such as the masking of recessive and deleterious alleles, and an increased ability to adapt to environmental change. It also provides an opportunity for somatic genetic recombination, which is particularly important in fungi that no longer have a sexual reproduction phase (Bagueret *et al.*, 1994). In many homobasidiomycete species characteristic lateral, bridge-like hyphal connections, termed clamp connections (Fig. 1.3) occur between adjacent cells. These

connections are diagnostic of dikaryon mycelium and play a role in the maintenance of the bi-nucleate cell (Casselton, 2002).

Unlike well-studied homobasidiomycete models (e.g. *Coprinus* and *Schizophyllum*), the fertile heterokaryotic mycelium of *A. bisporus* lacks clamp connections and there is no evidence of regulation of nuclear number (6-35 nuclei per cell, Jin *et al.*, 1992). The dikaryotic mycelia produce specialised cells, called basidia, in which nuclear fusion and meiosis occur (Koltin *et al.*, 1972). Generally these basidia are associated with well-organized structures, called fruiting bodies. Nuclear fusion occurs in the basidia and leads to the diplophase, which is limited to a single nuclear generation.



Fig. 1.3- Clamp connection. Bridge-like hyphal connection between adjacent cells, termed clamp connection (arrow; image courtesy of Warwick HRI)

Meiosis occurs immediately after fusion of the nuclei and the meiotic products enter the spores, which are borne on the basidia. These are then dispersed and establish monokaryotic mycelia. Instances of incomplete sexual progression are distributed throughout the homobasidiomycetes (Koltin *et al.*, 1972). For example, precocious fruiting can occur in the absence of a dikaryon phase. Fruiting bodies have been found on monokaryotic mycelia of *Peniophora ludoviciana* (Biggs, 1938), *Schizophyllum commune* (Raper & Krongelb, 1958), *Sistotrema brinkmanni* (Lemke, 1969), and *Fomes cajanderi* (Neuhauser & Gilbertson, 1971). Nevertheless, these species normally form their basidia on dikaryotic mycelia. Other homobasidiomycetes lack a conventional vegetative dikaryophase and mycelia contain an undefined number of nuclei per cell (Koltin *et al.*, 1972). *Thanatophorus cucumeris* (Mckenzie *et al.*, 1969), *Schizophyllum umbrinum* (Raper, 1959), and *A. bisporus* (Raper *et al.*, 1972) are species which exhibit sexual progression without a well-defined dikaryophase. In higher fungi the transition from the haplophase to dikaryosis follows two basic pathways (Koltin *et al.*, 1972): (i) transition in the absence of mating interaction with another mycelium; (ii) transition only after a mating interaction with another mycelium. The former is known as homothallism and the latter as heterothallism.

1.3.2 Heterothallism and Homothallism

Two principle breeding systems are recognised in the basidiomycetes: homothallism and heterothallism.

Most basidiomycetes (*ca* 90%) are heterothallic (Whitehouse, 1949; Elliott, 1985b). In heterothallic basidiomycetes such as *Coprinus cinereus*, each basidium usually bears four spores. Each basidiospore receives a single post-meiotic nucleus and germinates to form a primary, monokaryotic mycelium, which is septate with uninucleate cells. Two compatible mating-type monokaryons mate through anastomosis to form a dikaryon (Elliott, 1985b) capable of fruiting (Fig. 1.4 A).

In homothallic species, the individual mycelium mates with itself and produces a viable offspring. A consequence of this breeding system is a reduced gene flow into the genetic population, which may reduce genetic variation in subsequent generations (Raper, 1966). However, only 1% of basidiomycete species are primarily homothallic (Lemke, 1969). Many homothallic basidiomycetes are secondarily homothallic, converting an essentially outbreeding heterothallic system into habitual inbreeding (Skolko, 1944; Kemp, 1970; Raper *et al.*, 1972). These basidiomycetes produce self-fertile spores and some two-spored and four-spored species have been described (Elliott, 1986). In bisporic species, such as *A. bisporus* each basidiospore receives two nuclei and can germinate without requiring mating to form a heterokaryon (Fig. 1.4 B). *A. bisporus* exhibits low recombination during meiosis but an apparent non-random nuclear migration occurs that favours the pairing of non-sister nuclei in each single basidiospore (Raper *et al.*, 1972; Summerbell *et al.*, 1989). Each spore, therefore, may germinate forming a heterokaryon without going through the usual homokaryotic stage of basidiomycetes. Over 90% of *A. bisporus* basidiospores are heterokaryotic and self-fertile. Aberrant three- and four-spored basidia can also occur (*ca* 5%) because of occasional aberrant packaging of nuclei in bisporic basidia (Elliott, 1985b). Resulting spores germinate to produce homokaryotic mycelia that will need to mate with compatible strains in order to complete their cycle. In four-spored species such as *Mycocalia denudata* there is a precocious mitosis in the basidium, making eight nuclei available to migrate into four basidiospores (Challen, 1993).

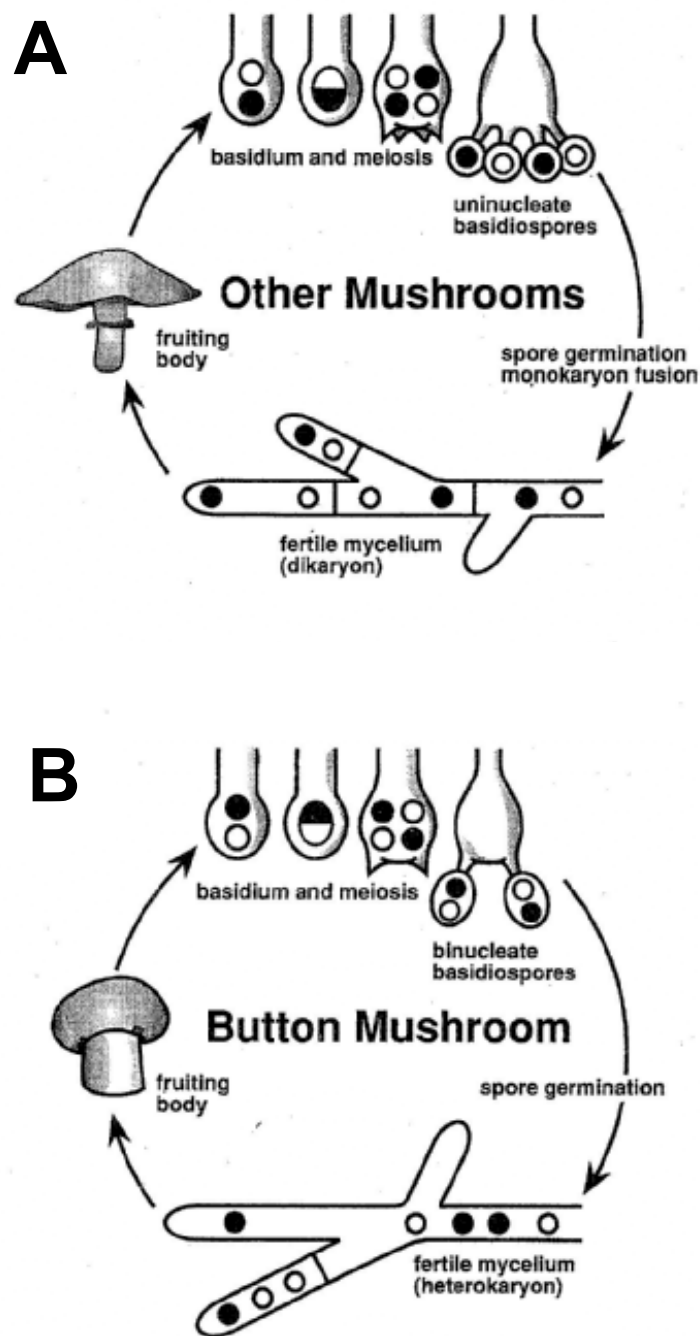


Fig. 1.4- Diagrammatic representation of mushroom life cycle. (A) A typical 4 spored heterothallic life history, representative of *A. bitorquis* and many other gilled basidiomycetes. (B) The secondarily homothallic life cycle of the button mushroom, *A. bisporus*. (from Horgen, P.A. *et al.*, 1991)

1.4 Cultivation of *Agaricus bisporus*

A. bisporus has been cultivated since the 17th century, firstly in open fields and later in caves, which provided a stable environment in terms of temperature and humidity. Nowadays they are grown in mushroom sheds or houses, where they are cropped in trays or plastic bags filled with a specific compost. This compost is made from a mixed chicken or horse manure, wheat straw, gypsum (hydrated calcium sulphate) and water. The straw provides a good carbon source in the form of cellulose, hemicelluloses and lignin; manure provides a nitrogen source, vitamins and carbohydrates, while gypsum ensures a better compost texture and a lower pH (Gerrits, 1988). Compost is prepared in different phases to allow fermentation to occur.

During phase I substrates are combined and allowed to ferment for 2-3 weeks. During this time the temperature rises to 76°C due to microbial activity, breaking down biochemical complexes and killing many potential pathogens and pests of mushrooms. The compost is usually turned to ensure aeration and maintain a constant temperature throughout.

During phase II the compost is incubated at 60°C for 8 h, to further reduce pathogen load and then cooled to 52°C for up to 9 days. At this stage carbohydrates are decomposed and the volatilisation of ammonia occurs reducing the toxic concentrations of this compound in the compost (Gerrits, 1988). This microbial conversion causes a change in the compost pH from alkaline to neutral at the time of spawning. Indeed on completion of phase II the compost is inoculated with *A. bisporus* mycelium (mushroom spawn) by mechanical mixing. Approximately 0.5% spawn (w/w) is added to the compost. Spawn produced by commercial suppliers is usually rye or millet grains colonised with a pure culture of *A. bisporus* mycelium (Elliott, 1985a). *A. bisporus* mycelium colonises the compost completely in approximately 2 weeks at 25°C, 84-86% relative humidity and 5000 ppm carbon dioxide. When the spawn run is complete, the compost is covered with *ca* 5 cm deep layer of 'casing' (peat mixed with lime and chalk) and the temperature and carbon dioxide levels are reduced to *ca* 20°C and 1100 ppm, respectively, to induce the fruiting bodies production. The casing acts as an anchor

for mushrooms to grow on and has excellent water retaining properties. The trays are incubated at 20-22°C and regularly watered over a three-week period, before the temperature is dropped to 16-18°C and watering stops. These conditions mimic the onset of autumn and trigger the fungus to produce mushrooms in three approximately synchronous intervals called flushes, each of 8-10 days in duration (van Gils, 1988).

Microflora play an important role in fruiting body production (reviewed by Flegg & Wood, 1985). The growth of *A. bisporus* mycelium through the casing and compost generates volatiles such as ethanol, ethanal, ethyl ethanoate and carbon dioxide. These metabolites build up in the casing and selectively promote the growth of a specialised microflora including the bacterium *Pseudomonas putida*. These biological stimuli are thought to induce the production of mushroom initials (pins), which will grow and differentiate into mushrooms. Visscher (1988) suggested that the role of *P. putida* is to remove or decrease the concentration of some inhibitors allowing the fructification of *A. bisporus*. According to Hammond & Wood (1985), mannitol is significantly higher in fruiting bodies (25-35%) than in mycelium (1.5-4.0%) and plays an important role, creating hydrostatic pressure for hyphal extension by attracting water into fruiting bodies. *A. bisporus* typically yields between 200-250 kg of mushrooms per tonne of compost (van Gils, 1988).

1.5 Nutritional and medicinal aspects of

Agaricus bisporus

Mushrooms have been treated as a special kind of food since earliest times. Chinese and Japanese chronicles recorded Shiitake (*Lentinula edodes*) mushrooms being offered to emperors. The Romans ate mushrooms on special occasions, and Mexican Indians used hallucinogenic mushrooms in religious ceremonies (van Griensven, 1988).

Mushrooms are considered to be 'healthy food' as they contain large amounts of essential amino acids, unsaturated fatty acids, vitamins and minerals. In particular, they are a good source of ascorbic acid (vitamin C) and B vitamins such as thiamine (B1), riboflavin (B2), niacin and biotin. Oei (1996) suggested that B vitamins may aid

treatment of stress, depression and fatigue. Mushrooms also contain significant amounts of selenium and potassium, important as antioxidant and controller of blood pressure, respectively (Oei, 1996).

Chen (2004) suggested that white button mushrooms may play a role in treating and preventing breast cancer: fresh white mushroom extracts contain chemicals which inhibit the activity of aromatase, an enzyme involved in estrogen production. It is known that estrogen has breast cancer-promoting effects especially in postmenopausal women. *A. bisporus* extracts also seem to suppress steroid 5-alpha-reductase, which plays an important role in the development of prostate cancer cells (Chen, 2004).

1.6 Economic importance of *Agaricus bisporus*

The history of commercial cultivation of mushrooms is extensive and dates back to the 17th century, when the industry first started in France (van Griensven, 1988). During the last 400 years, three major events have dramatically affected the mushroom industry: (i) the development of mushroom spawn in the late 1800s, (ii) improved composting technologies, and (iii) the breeding of new hybrid spawn (van Griensven, 1988).

It is estimated that approximately 3.2 million metric tonnes (mt) of edible mushrooms were produced during the year 2004 (FAOSTAT data)¹. China leads world mushroom production (1,359,335 mt in 2004), followed by the USA (391,000 mt in 2004). In Asia mushroom production is dominated by shiitake (*Lentinus edodes*) and oyster mushrooms (*Pleurotus* species), while the American and European industries predominantly produce button mushrooms. *A. bisporus* is the most broadly cultivated mushroom worldwide, with an annual production in the region of five million tonnes (Kües & Liu, 2000) and an estimated commercial value of £ 3000 million (Scrase & Elliott, 1998). In Europe, button mushroom production is estimated at around 1 million mt (Gaze, *pers. comm.*). The Netherlands is the major European mushroom producer

source: ¹ <http://faostat.fao.org/faostat>

(260,000 mt in 2004)¹, followed by France, Poland, Spain, Italy, UK, Ireland, and Germany. In the UK, *A. bisporus* mushrooms account for around 10% of total horticultural production (reviewed by Burns *et al.*, 2005) with an annual production of approximately 80,000 tonnes in 2004 (FAOSTAT data)¹.

1.7 Pests and diseases of *Agaricus bisporus*

Mushroom crops can be affected by a wide range of pests and diseases. Susceptibility to disease is exacerbated by the fact that the crop is a virtual monoculture and the controlled environment in which *A. bisporus* mushrooms are grown is ideal for the survival of pests and pathogens (Milgroom, 1999).

Few genetic differences exist in mushroom crops throughout the world as most commercial mushrooms are derived from a few hybrid strains (Section 1.2). This lack of genetic diversity greatly increases the chances of pathogens and pests attacking mushroom crops (Milgroom, 1999; Moore, 2001). Moreover, mushroom cropping involves a network of labour intensive activities not all of which take place on site, including emptying tunnels, compost production, transport and filling of full growth compost, spawn production (Section 1.4). Many of these are critical steps with regards to the infection. Hygiene measures to minimise development of pest or diseases are important and the following procedures are often implemented by the industry (Geels *et al.*, 1988): use of spore filters; disinfection of equipment, footwear, etc.; wearing clean and disinfected clothing; controlling insects; using disinfected packing containers; and through ‘cooking out’ of the growing room at the end of the harvest.

1.7.1 Pests of *Agaricus bisporus*

Insects and mites infections usually occur after the pasteurisation process, as the majority of these cannot survive through the compost peak-heating reached during phase II. The main mushroom pests are phorid flies and sciarid. Phorid flies (*Phoridae*) belong to the *Diptera* family. *Megaselia halterata* and *M. nigra* are found in UK and

source: ¹ <http://faostat.fao.org/faostat>

both species are very active in the vicinity of light sources. *M. halterata* larvae feed on mushroom mycelium in compost and casing soil, while *M. nigra* dig tunnels in the mushroom fruiting bodies from the top of the cap down to the stem (Fletcher *et al.*, 1986). Sciarid flies (*Lycoriidae*) also belong to the *Diptera* family and the main species is *Lycoriella auripila*. The larvae not only feed on the mycelium in the compost and/or casing soil, but also on fruiting bodies. Sciarid flies are also known to have a role in transmission of fungal diseases, mites, bacteria and nematodes (Geels *et al.*, 1988).

1.7.2 Bacterial Pathogens of *Agaricus bisporus*

Numerous bacteria, most of them from the genus *Pseudomonas*, are capable of infecting *A. bisporus* mushrooms.

‘Brown blotch disease’ is caused by *Pseudomonas tolaasii* and characterised by the formation of brown spots on the caps. *P. tolaasii* occurs together with other Pseudomonads (including *P. fluorescens* and *P. putida*) in the casing soil. During mushroom fruiting, pinheads, primordia and sporophores can be colonised by the bacterium. Excessive watering and/or humidity can establish optimal conditions for *P. tolaasii* (Geels *et al.*, 1988; Soler-Rivas *et al.*, 1999).

“Ginger blotch” is caused by *P. gingeri* and symptoms include pale yellow to yellowish-brown blotches on young fruiting bodies, generally only at the edge of the cap (Wong *et al.*, 1982).

“Mummy disease” is thought to be caused by another Pseudomonad bacterium, whose species has not been determined (Fletcher *et al.*, 1986). Two different types of symptoms can be observed. Firstly, after casing it is possible to have bare patches on the bed because pinheads are stuck in the casing soil without further growth. Secondly, mushrooms can turn greyish in colour, stems become crooked and often thickened at the basis surrounded by a fluffy edge of mycelium, while caps become tough, spongy, dry, and mummified (Geels *et al.* 1988; Dyki *et al.*, 1993).

“Drippy gill” is another important disease of *A. bisporus* (Gill & Cole, 2000) caused by *P. agarici*, which infects the mushroom gills before the veil opens; small brown patches with creamy white bacterial slime appear on the gills, which consequently do not develop properly (Young, 1970; Fletcher *et al.*, 1986).

1.7.3 Fungal Diseases of *Agaricus bisporus*

Fungi affecting mushroom crops can be categorised as competitive or parasitic fungi. Competitive fungi are often described as “weed moulds” acknowledging the fact that they are undesirable and can quickly spread on a massive scale. They usually affect mushroom mycelium during the spawn run phase by competing for CO₂, nutrients, water and substrate. Their presence is often associated with the preparation of poor mushroom compost and/or ineffective peak-heating. Some examples of competitive fungi are, ink caps (*Coprinus* spp.) and various green moulds (*Trichoderma*, *Aspergillus* and *Penicillium* species) (Geels *et al.*, 1988).

Parasitic fungi can damage the mycelium or fruiting bodies of mushrooms. ‘Dry bubble disease’ is caused by *Verticillium fungicola* var. *fungicola*, a major fungal disease of *A. bisporus*. Various symptoms can be observed, depending on the stage of infection. Mushrooms, infected at the pinhead stage, form undifferentiated mass of bodies (dry bubble). At a later stage of infection mushrooms appear with crooked and often split stipes (stipe blowout). A lesser symptom develops on fully formed mushrooms, cap spotting or lesions. *V. fungicola* grows in the casing and can be rapidly spread by spores, splashing/aerosols, but also by phorid and sciarid flies, dirty equipment, hands and clothing (Geels *et al.*, 1988).

‘Wet bubble’ is caused by *Mycogone pernicioso* (Fletcher *et al.*, 1986). Symptoms include misshapen and/or brown mushrooms, swollen stems and caps and in later stages of the disease, a rotten mass appears on the deformed mushrooms (Geels *et al.*, 1988). The disease is spread via infected spores and mycelium.

‘Cobweb’ is caused by *Cladobotryum dendroides* (Fletcher *et al.*, 1986). Symptoms include the appearance of *C. dendroides* mycelium patches, which form a white cotton-

like web over the mushrooms, causing discoloration and death. Symptoms generally occur in the late flushes, but if casing is contaminated with *C. dendroides* mycelium, symptoms can appear in the first flush. Spores and remains of casing soil can spread the disease, which is made worse by high temperature and relative air humidity.

1.8 Viral diseases of *Agaricus bisporus*

1.8.1 Viruses in fungi

Mycovirology is a relatively recent branch of virology. Interestingly the first mycovirus identified was observed in diseased mushrooms of *A. bisporus* (Hollings, 1962). Mycoviruses are widespread among fungi and in most cases their presence is symptomless (Buck, 1986). They typically possess dsRNA genomes (Ghabrial, 1998), which are either encapsidated by proteins forming virions of 20-50 nm in diameter (Buck, 1986), or are associated with membrane vesicles (Nuss & Koltin, 1990). Mycoviruses can be either monopartite (genome in one molecule), bipartite (genome split between two molecules packaged into two separate particles), or multipartite (genome divided into three or more molecules packaged separately). Because mycoviruses are often asymptomatic and unencapsidated, their detection must rely on demonstrating the presence of transmissible dsRNAs. During the last decade detection of mycoviruses by electron microscopy has been replaced by screening on ethidium-bromide stained gels for the presence of dsRNA banding pattern, typical for the disease (Sonnenberg *et al.*, 1995). Comparison of both methods has shown that the latter is more sensitive, faster and more reliable as a diagnostic tool (Franklin, 1966; Bishop & Koch, 1969; Bar-Joseph *et al.*, 1983; Yardimci & Korkmaz, 2004). This method exploits the fact that most fungal viruses have dsRNA genomes and that each virus has a characteristic dsRNA profile defined by the number and size of segments. In the early history of mycovirology scientists attempting to use electron microscopy as a diagnostic tool, often observed fungal tissues densely infected with various shaped-virus particles (Hollings, 1962; Buck, 1986). The discrimination and identification of the virus of interest was often extremely challenging. DsRNA analysis succeeded where electron microscopy failed at discriminating between morphologically similar, but genetically

distinct viruses, and between viruses and normal cellular components (Romaine & Goodin, 2002).

Mycoviruses can be transmitted horizontally via anastomoses between hyphae (Day & Anagnostakis, 1973; Sonnenberg & van Griensven, 1991; Ihrmark *et al.*, 2002), or vertically via infected spores (Romaine *et al.*, 1993; Ihrmark *et al.*, 2002; Chu *et al.*, 2003).

Mixed infections with two or more unrelated viruses are not uncommon (Ghabrial, 1998). Another feature of mycovirus infections is the accumulation of defective and satellite dsRNAs (Ghabrial, 1998). Defective RNAs are subgenomic RNAs generated from infectious virus genome by replicase error (Dhar and Bandyopadhyay, 1999), whereas satellites are sub-viral agents whose replication is dependent on co-infection with another virus (www.ncbi.nlm.nih.gov/ICTVdb).

1.8.2 Taxonomy of Mycoviruses

Mycoviruses are divided into the following families: *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Hypoviridae*, *Narnaviridae*, *Barnaviridae*, *Pseudoviridae*, *Metaviridae* and two unassigned genera, *Rhizidiovirus* and *Endornavirus* (Table 1.1). There are also several unassigned mycoviruses (www.ncbi.nlm.nih.gov/ICTVdb).

The *Totiviridae* family is the best characterised whose members have monopartite dsRNA genomes encompassing genes for RNA-dependent RNA polymerase (RdRp) gene and coat protein (CP). Three genera belong to this family: *Giardiavirus* and *Leishmanivirus* infect parasitic protozoa whilst *Totivirus* infect fungi. e.g. *Helminthosporium victoriae* 190S virus (Huang & Ghabrial, 1996), *Saccharomyces cerevisiae* virus L-A (L1) (Icho & Wickner, 1989) and *Ustilago maydis* virus HI (Kang *et al.*, 2001).

Partitiviridae have bipartite genomes with the CP and RdRp genes on separate dsRNA segments. This family includes the genus *Cryptovirus* infecting plants and the genus

Partitivirus infecting fungi. e.g. *Discula destructiva virus* (Rong *et al.*, 2002), and *Heterobasidion annosum virus* (Ihrmark *et al.*, 2002).

Chrysovirus is the only genus of the *Chrysoviridae* family. These viruses were previously classified as *Partitiviridae*, but sequence data showed that the two families are only distantly related. Chrysoviruses are multipartite viruses with the four linear dsRNA genomic segments encapsidated in isometric particles. *Helminthosporium victoriae 145S virus* is a chrysovirus containing dsRNAs ranging from 2.9 to 3.5 kbp (Soldevila, 2000).

Mycoviruses of the family *Hypoviridae* lack conventional capsids and their linear, monopartite dsRNA genome is enclosed in membranous host-encoded vesicles. They normally are associated with decreased virulence of fungal hosts. *Cryphonectria hypovirus* is an example of *Hypovirus*, the only genus belonging to this family (Griffin, 1986).

Narnaviridae mycoviruses have a ssRNA genome, ranging from 2.3 to 3.6 kbp, which is associated with the RdRp into ribonucleoprotein complex. No capsids have been found. This family comprises two genera both infecting fungi. *Mitovirus*, associated with mitochondria usually cause hypovirulence in the fungus host e.g. *Cryphonectria mitovirus 1* (Polashock & Hillman, 1994) and *Ophiostoma novo-ulmi mitovirus* (Hong *et al.*, 1998). *Narnavirus* are cytoplasmic parasites of yeasts with no phenotypic effects. e.g. *Saccharomyces cerevisiae narnavirus 20S* and *23S* (Esteban *et al.*, 1992).

Barnavirus, the only genus of the *Barnaviridae* family comprises ssRNA viruses encapsidated in bacilliform virions. *Mushroom bacilliform virus* (MBV) is the type species of the family and is often found in association with *Agaricus bisporus virus 1* (ABV1), the virus associated with 'La France' disease of mushrooms (Revill *et al.*, 1994).

The *Pseudoviridae* family encompasses retrotransposons infecting invertebrates, plants and fungi. Structurally, they have long terminal repeat (LTR) sequences and coding regions with sequence motifs for reverse transcriptase (RT), ribonuclease H (RH),

**Table 1.1- Classification of mycovirus family according to
The Universal Virus Database of the International Committee on Taxonomy of Viruses***

FAMILY	GENUS	NUCLEIC ACID	MORPHOLOGY	HOST
<i>Totiviridae</i>	<i>Giardiavirus</i>	dsRNA (4-7 kbp)	isometric virions	Protozoa
	<i>Leishmaniavirus</i>			Protozoa
	<i>Totivirus</i>			Fungi
<i>Partitiviridae</i>	<i>Cryptovirus</i>	dsRNA (2-3 kbp)	isometric virions	Plant
	<i>Partivirus</i>			Fungi
<i>Chrysoviridae</i>	<i>Chrysovirus</i>	dsRNA (3-3.6 kbp)	isometric virions	Fungi
<i>Hypoviridae</i>	<i>Hypovirus</i>	dsRNA (9-13 kbp)	cytoplasmic vesicles	Fungi
-	<i>Endornavirus</i>	dsRNA (14-18 kbp)	cytoplasmic vesicles	Plants, Fungi ¹ , Protists ²
<i>Narnaviridae</i>	<i>Mitovirus</i>	ssRNA (2-3 kbp)	ribonucleoprotein complexes	Fungi
	<i>Narnavirus</i>			Fungi
<i>Barnaviridae</i>	<i>Barnavirus</i>	ssRNA (4 kbp)	bacilliform virion	Fungi
<i>Pseudoviridae</i>	<i>Pseudovirus</i>	ssRNA (5-8 kbp)	unknown	Fungi, Invertebrates, Plants
	<i>Hemivirus</i>			Fungi, Invertebrates, Plants
<i>Metaviridae</i>	<i>Metavirus</i>	ssRNA (4-10 kbp)	unknown	Fungi, Invertebrates
	<i>Errantivirus</i>			Invertebrates
-	<i>Rhizidiovirus</i>	dsDNA (27 kbp)	Isometric virions	Fungi

*Source: www.ncbi.nlm.nih.gov/ICTVdb/

¹ Source: Osaki *et al.*, 2006

² Source: Hacker *et al.*, 2005

protease and integrase activities. Their morphology is poorly characterised. This family comprises two genera, *Pseudovirus* and *Hemivirus* e.g. *Saccharomyces cerevisiae Ty1 virus* (Boeke *et al.*, 1988) and *Drosophila melanogaster copia virus* (Mount & Rubin, 1985).

The *Metaviridae* family comprises retrotransposons with similar features to those of the *Pseudoviridae*. They are divided according to the presence of the envelope gene (*Errantivirus*) or its absence (*Metavirus*) (Hull, 2001) e.g. *Saccharomyces cerevisiae Ty3 virus* (Peterson-Burch *et al.*, 2004) and *Drosophila melanogaster gypsy virus* (Harpen *et al.*, 2002).

Rhizidiovirus forms a genus not yet assigned to a family. They also infect fungi but have a dsDNA genome of 27 kbp encapsidated in 60 nm isometric particles e.g. *Rhizidiomyces virus* (Dawe & Kuhn, 1983).

The International Committee on Taxonomy of Viruses (ICTV) has recently accepted *Endornavirus* as a new genus (ICTVdB Management, 2006). Some authors have suggested the new family, *Endornaviridae*, to encompass the new genus (Horiuchi & Fukuhara, 2004; Fukuhara *et al.*, 2005). Endornaviruses have been mostly observed in plant hosts (Moriyama *et al.*, 1995; Pfeiffer, 1998; Coutts, 2005; ICTVdB Management, 2006), but also in some fungi (Osaki *et al.*, 2006) and protists (Hacker *et al.*, 2005). They have a monopartite linear dsRNA genome associated with RdRp activity (Lefebvre *et al.*, 1990; Pfeiffer *et al.*, 1993). The genome size has recently been estimated between 14-18 kbp (ICTVdB Management, 2006). However, smaller genomes have also been reported (Tuomivirta & Hantula, unpublished). Conventional virus-like structures have not been found, but the viral genome is encapsidated in cytoplasmic vesicles in the form of a nucleoprotein complex (Lefebvre *et al.*, 1990; ICTVdB Management, 2006). Although *Endornavirus* are mostly symptomless (Fukuhara, 1999), deleterious symptoms have been reported in some cases (Lefebvre *et al.*, 1990; Osaki *et al.*, 2006).

1.8.3 Mycovirus Replication Strategy

There is no universal life cycle for dsRNA viruses (Nemeroff & Bruenn, 1986). Nothing is known about the replication and packaging of *A. bisporus* viruses but studies of other dsRNA viruses have yielded information, which may be relevant.

Two important events have to take place during virus replication: the synthesis of messenger RNA (mRNA) for the expression of viral genes and the replication of the viral genome. The latter can take place in either a conservative or semi-conservative manner.

During conservative replication, the newly synthesized strand serves as mRNA and as template for the replication of the dsRNA molecule, while the old (+) strand remains attached to the old (-) strand (Goodin *et al.*, 1997). During semi-conservative replication, the old (+) strand is displaced by the newly synthesized strand. The displaced (+) strand then serves as mRNA and as template for the synthesis of (-) strand. Both mechanisms are found in fungal viruses (Goodin *et al.*, 1997). The viruses classified in the *Totiviridae* family use a conservative mode of replication (Sclafani & Fangman, 1984; Ghabrial & Havens, 1989; Wickner, 1993; Murphy *et al.*, 1995). The viruses belonging to the family of *Partitiviridae* replicate in a semi-conservative way (Buck, 1978; Buck *et al.*, 1981; Wickner, 1993; Murphy *et al.*, 1995), but the molecular mechanisms have not been studied in detail. The only difference is whether the template (-) strand remains annealed to the new (+) strand (semi-conservative) or repairs with the parental strand (conservative) (Wickner, 1993; Fig. 1.5).

DsRNA replication occurs in the cytoplasm for all dsRNA viruses (Wickner, 1993). Transcription defined as the synthesis of viral (+) strand from a dsRNA template, takes place within viral particles or core particles. The exceptions are the viruses lacking a conventional protein coat and associated with membranous vesicles (Dodds, 1980). The (+) strands are generally extruded from the viral particles and then translated to make new viral proteins, which will package the (+) strands. Once the new particles or cores have formed, (-) strand synthesis on the (+) strand template completes the formation of new dsRNA (Wickner, 1993) as all dsRNA viruses have capsid-associated RNA

polymerase activities (Nemeroff & Bruenn, 1986). The mechanism by which multiple segmented-genome viruses manage to package one segment in each particle is less clear. A suggestion might come from the bacteriophage $\delta 6$, an enveloped dsRNA virus of *Pseudomonas syringae*. It has been shown that the *in vitro* (-) strand synthesis occurs only when all three (+) strands have been packaged (Gottlieb *et al.*, 1992; Frilander *et al.*, 1992). No single segment or pair of segments is sufficient for synthesis to proceed and data indicate that the procapsids have specific binding sites for each segment (+) strand. However, the mechanism might be different in other multisegmented viruses (Wickner, 1993).

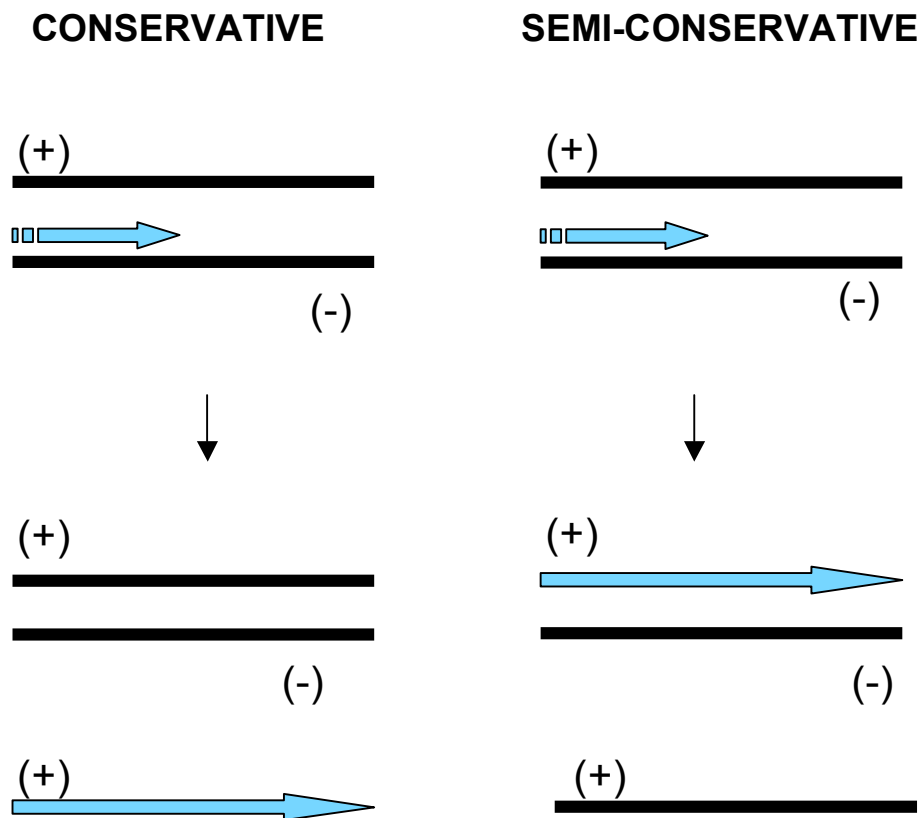


Fig. 1.5- Fungal virus replication modes. During conservative replication, the newly synthesized strand serves as mRNA and as template for the replication of the dsRNA molecule, while the old (+) strand remains attached to the old (-) strand. During semi-conservative replication, the old (+) strand is displaced by the newly synthesized strand. The displaced (+) strand then serves as mRNA and as template for the synthesis of (-) strand. Both mechanisms are found in fungal viruses

1.8.4 Genome Expression Strategies

RNA viruses exhibit wide variations in structure and organization of their genome. They may also have different terminal structures, such as a cap structure or a genome-linked protein (VPg) at the 5' end, and a poly(A)-tail or a tRNA-like structure at the 3' end of their RNA genome (reviewed by Goldbach *et al.*, 1991). RNA viruses appear to have evolved different strategies for genome expression overcoming constraints of different host DNA-based systems. The eukaryotic 80S ribosome is generally able only to translate monocistronic mRNAs starting from the 5' region according to the "scanning ribosome model" proposed by Kozak (1991). According to this model eukaryotic translation begins with the ribosomal recognition of the 5' cap (an additional GTP molecule forming a 5'-5' linkage with mRNA) at the 5' end of mRNA. Then the migrating 40S ribosomal subunit stalls at the first AUG codon, which is recognised in a large part by base pairing with the anticodon in Met-tRNA^{imet} (Kozak, 1991). The main strategies used by RNA viruses to allow protein synthesis in a eukaryotic system are discussed below.

Multipartite genome

Genome segmentation can result in monocistronic RNAs (reviewed by Bustamante & Hull, 1998). Each segment contains one or two open reading frames (ORFs). A segmented genome has additional benefits such as inter-virus recombination as easier way to exchange genetic information between two viruses (<http://jpkc.ynan.edu.cn>).

Subgenomic RNAs

The expression of internal genes is frequently mediated by subgenomic RNAs, which can be produced by two mechanisms: (i) during (-) RNA strand synthesis, premature termination can lead to truncated subgenomic RNAs; (ii) alternatively subgenomic RNA can result from an internal deletion event (Tartaglia *et al.*, 1985). The generation of internally deleted defective RNA molecules appears to be a common feature of hypovirulence-associated dsRNA replication. This event could be explained by the presence of bulky secondary structures such as stem-loop structures in the template strand leading to the interruption of the nascent strand. Therefore, the synthesis complex

dissociates and reassociates with the template to overcome the hurdle, completing the synthesis (Shapira *et al.*, 1991).

Polyprotein

In this strategy the viral genome contains a long ORF, which is translated and then cleaved into smaller, functional proteins by viral proteinases (reviewed by Bustamante & Hull, 1998). Hypoviruses undergo this strategy (reviewed by Ghabrial, 1994).

Protein read-through

In normal protein synthesis, a ribosome stops protein elongation when it encounters one of the three terminators (UAG, UGA, UAA). If the first cistron in the genomic viral RNA has a leaky termination codon (UAG or UGA) it might be suppressed by a host tRNA. Thus, some ribosomes can read through the codon into a downstream cistron, giving rise to a second longer functional polyprotein (reviewed by Bustamante & Hull, 1998). The read-through process requires at least two elements: (i) a suppressor tRNA; (ii) an appropriate nucleotide context surrounding the terminator codon (Skuzeski *et al.*, 1991; Valle *et al.*, 1992).

Translational frameshift

Normal translocation maintains a single reading frame that is fixed at the time of translocation initiation. Ribosomal frameshift is a strategy frequently employed by various organisms to produce more than one protein from overlapping reading frames (reviewed by Bustamante & Hull, 1998). It may occur in either direction, the ribosome may shift either -1 or +1 nucleotide or more, and hop to a distant location to change the reading frame. If this shift occurs near the end of the first ORF this can produce a fusion protein. A shift in the 3' direction (+1 frameshift) has also been described in the yeast retrotransposon Ty (Belcourt & Farabaugh, 1990); whereas a shift in the 5' direction (-1 frameshift) has been demonstrated in L-A virus of *Saccharomyces cerevisiae* (Wickner, 1993), retroviruses (Vickers & Ecker, 1992), and luteoviruses (Prufer *et al.*, 1992). The frameshift event requires a slippery site on the mRNA of the form XXXYYYZ (Wickner, 1993), which has the potential to adopt a pseudoknot folding pattern, that slows down the ribosome movement and allows it to change reading frames.

DsRNA viruses, (+) strand RNA viruses, and retroviruses all use their (+) strands for three functions: as mRNA, as the species packaged to make new virions, and as a template for replication (Wickner, 1993). If they alter these (+) strands for translation purposes, by splicing or editing, they will create mutant viruses removing a site necessary for packaging or replication. Presumably for this reason, dsRNA and (+) ssRNA viruses do not splice (excise internal regions after transcription) or edit their (+) strands. ‘Ribosomal frameshift’ and ‘read-through’ of termination codon are the two most common strategies deployed by viruses to produce different proteins using the same mRNA (Icho & Wickner, 1989).

1.8.5 Viral effects on fungi

The latent or symptomless relationship of many fungal viruses with their hosts suggests that fungi and their viruses have coevolved and co-adapted to a considerable degree (Ghabrial, 1980; Buck, 1986; Milgroom, 1999). Ghabrial (1980) suggested that the lack of extracellular infectivity of fungal viruses played a role as selective pressure upon virus pathogenicity. In order to make efficient use of host proteins many fungal viruses maintain only genes that are essential for their survival, such as RNA-dependant RNA polymerase and capsid genes. The host cell has evolved to support only a defined level of virus replication beyond which virus infection becomes pathogenic, leading to cell death. In the absence of an extracellular route of infection, host death means virus elimination; thus fungal viruses tend not to be extremely virulent (Ghabrial, 1998). Other authors have suggested a possible correlation between viral transmission and host virulence (Milgroom, 1999). Milgroom (1999) also suggested that the relative importance of vertical or horizontal transmission may provide clues to virus-host relationships. Viruses that depend primarily on vertical transmission are most likely to evolve towards reduced host, even to the point of being benign. In contrast, viruses that have the opportunity of horizontal transmission tend to be more deleterious to their hosts because they have more chances of survival. Mycovirus infection can be associated with various symptoms ranging from symptomless to severely debilitating or from hypovirulence to hypervirulence (Nuss & Koltin, 1990; Ghabrial, 1994).

1.8.5.1 Killer phenomenon

Some dsRNA mycoviruses offer advantages to their hosts. The “killer” phenotypes in yeasts (*Saccharomyces cerevisiae*) and in smuts (*Ustilago maydis*) are two such examples (Kandel, 1988; Koltin, 1988). Yeast and smut killer strains secrete a toxin to which they are immune, but that is lethal to sensitive strains. The toxin is synthesized as a preprotoxin, which is then processed by proteolysis and secreted as a mature toxin. It binds to cell-wall receptors after which the toxin makes proton leaks in the membrane of susceptible cells. Genetic and biochemical studies suggest that the toxin production is conferred by dsRNA satellites (M-dsRNA, H-dsRNA L-dsRNA), which are dependent on helper viruses (Hutchins & Bussey, 1983; Koltin, 1988). On the other hand, immunity is conferred by nuclear genes (Finkler *et al.*, 1992; Tao *et al.*, 1993; Ginzberg & Koltin, 1994). The helper viruses (*Saccharomyces cerevisiae totivirus L-A* and *Ustilago maydis totivirus HI*) are autonomously replicating and do not require dsRNA satellite for replication. They encode the RNA polymerase and coat protein needed either for their own replication and encapsidation or that of dsRNA satellites. Killer yeasts are commonly found in grapes and fermenting grape musts, and have been implicated as the cause of protracted fermentations (reviewed in Van Vuuren & Jacobs, 1992). In some wine producing areas nearly all the naturally occurring strains are killers, while in other areas the latter may be completely absent. Killer strains may have significant economic impact because they generally ferment slower and reduce the quality of the wine produced (Milgroom, 1999). There are no apparent adverse effects of these viruses on the growth, development or survival of infected yeasts. On the contrary, killer strains have been shown to have fitness advantages over non-killer strains because of their ability to inhibit toxic-sensitive strains (Milgroom, 1999).

1.8.5.2 Hypovirulence

Mycoviruses can also debilitate their hosts. When the host is a plant pathogenic fungus such as *Cryphonectria parasitica* (Van Alfen *et al.*, 1975), *Ophiostoma ulmi* (Brasier, 1991), *Helminthosporium victoriae* (Ghabrial & Mernaugh, 1983), dsRNAs can induce hypovirulence of the host and has been suggested as a potential biological control agents for the fungal disease. The best studied of these mycoviruses is *Cryphonectria parasitica hypovirus 1-EP713* (CHV1-EP713), which is associated with the

hypovirulence phenotype of the chestnut blight fungus, *Cryphonectria parasitica* (Shapira *et al.*, 1991b). *C. parasitica* infects chestnut trees through wounds in the bark and forms cankers that often kill the above-ground parts completely (Griffin, 1986; Anagnostakis & Kranz, 1987). Chestnut blight epidemics swept through eastern North America and Europe with devastating effects. Within 20 years the epidemic slowed considerably because of the natural appearance of the hypovirulent strains (reviewed by Heiniger & Rigling, 1994), which were infected with viruses and exhibited reduced virulence, causing superficial cankers only. For almost 20 years, hypovirulence was thought to be associated with dsRNAs in the cytoplasm of the fungus (Van Alfen *et al.*, 1975); however because it was not possible to infect fungi with purified viruses, Koch's postulates were only fulfilled following advances in molecular techniques (Choi & Nuss, 1992a).

CHV1-EP713 comprises three different dsRNAs associated with membrane vesicles: L-dsRNA is the main element, while the smaller dsRNAs (M and S) proved to be deletion products of L-dsRNA (Shapira *et al.*, 1991 a, b). L-dsRNA (+) strand (12,712 bp) contains two open reading frames, ORF A and B coding for 622 and 3165 amino acids, respectively. These ORFs are cleaved autocatalytically into different proteins. The processed ORF B contains helicase-like and polymerase-like domains with homology to those of plant *Potyvirus* (Shapira *et al.*, 1991). ORF A is the determinant of specific traits of the infected fungus, which include suppressed pigmentation, reduced conidiation, reduced expression of laccase and down-regulation of crypanin (Carpenter *et al.*, 1992; Choi *et al.*, 1992).

Using DNA-mediated transformation of *C. parasitica*, it has been possible to determine whether the phenotype traits exhibited by hypovirulent *C. parasitica* strains are the result of the presence of replicating dsRNA, or whether these traits are dependent on functions encoded by specific hypovirus genes. Transformation of dsRNA-free *C. parasitica* strains with a cDNA copy of ORF A was shown to confer some hypovirulence-associated traits such as reduced pigmentation, reduced laccase accumulation and suppressed conidiation (Choi & Nuss, 1992a). However, the complete hypovirulence phenotype was only obtained by transformation with a full-length cDNA

copy of CHV1-EP713 (Choi & Nuss, 1992a). Powell & Van Alfen (1987a, b) have demonstrated that specific host mRNA and polypeptides are down-regulated in dsRNA-containing strains. Hypovirus infection is believed to perturb the expression of specific developmentally regulated fungal host genes at mRNA level, such as those for laccase and crypanin. The extracellular laccase is a copper-containing phenol oxidase, known to occur in many plant and fungal species (Ghabrial, 1994). The biological function of this enzyme remains obscure, although laccase activity has been implicated in sporulation, pigmentation production, lignin degradation and pathogenesis (Ander & Eriksson, 1976; Law & Timberlake, 1980; Leatham & Stahmann, 1981; Anagnostakis, 1987; Bar-Nunn *et al.*, 1988). Another developmentally regulated gene that is affected by virus infection is crypanin (Carpenter *et al.*, 1992). It is a cell surface protein with lectin-like properties, particularly abundant in aerial hyphae and fruiting bodies.

1.8.6 Mushroom Viruses

Mycovirology (fungal virology) as a new branch of science was developed following the discovery of virus-like particles in diseased mushrooms in 1962. Hollings (1962) set a precedent for the previously unconsidered notion that fungi can be infected by viruses. Since then, mycoviruses have been reported in several other mushrooms. Magae & Hayashi (1999) reported two types of degenerative symptoms in pure white strain of *Flammulina velupes* mushrooms: one was a spontaneous brown colour change and another was malformation or reduced production of fruiting bodies. Two dsRNA elements (1.9 and 1.8 kbp) were detected in mycelium derived from brown-coloured mushrooms. These elements were located in the cytoplasm fraction and were associated with 50 nm diameter virus-like particles (Magae & Hayashi, 1999). Other virus-like particles have been reported in *Lentinus edodes* (Inoue, 1970) and the basidiomycete *Agrocybe aegerita* (Barroso & Labarere, 1990). Barroso & Labarere (1990) observed two types of viral complex in commercial *A. aegerita* mushrooms, a unencapsidated dsRNA complex (1900, 1800 and 1700 bp, respectively) associated with large vesicles or mitochondria, and another complex encompassing isometric mycoviruses. Although many viral particles and dsRNA elements were found in edible mushrooms (Schisler *et al.*, 1967; Inoue, 1970; Go *et al.*, 1992; Revill *et al.*, 1994; Park & Kim, 1996; Van der Lende *et al.*, 1996; Magae & Hayashi, 1999; Romaine & Goodin, 2002), very few have

been characterised. The best studied mycoviruses are the ones affecting *Agaricus bisporus* and *Pleurotus ostreatus*.

1.8.6.1 Mycoviruses infecting *Pleurotus ostreatus*

In *P. ostreatus* spherical and bacilliform viruses were isolated from malformed sporophores (Go *et al.*, 1992; Park & Kim, 1996). Van der Lende *et al.* (1995b) also demonstrated a possible correlation between the presence of dsRNA mycoviruses and slow growing mycelium. Yu *et al.* (2003) reported an epidemic disease in Korea affecting the oyster mushroom, ‘die-back disease’, which led to reduced yields in commercial farms. The epidemic was strongly associated with a ssRNA virus, oyster mushroom spherical virus (OMSV). OMSV is a 27 nm isometric virus with a 5.784 kbp ssRNA genome. Genomic structure and sequence analysis showed that OMSV shares closer similarity with tymoviruses than to other mycoviruses (Yu *et al.*, 2003). Another characterised virus infecting *P. ostreatus* is *Pleurotus ostreatus* virus 1 (PoV1), a partitivirus (Lim *et al.*, 2005). Unlike OMSV, PoV1 is not associated with diseased symptoms.

1.8.6.2 Mycoviruses infecting *Agaricus bisporus*

Viruses of the cultivated mushroom *A. bisporus* were the first viruses found in fungi (Hollings, 1962), probably because of their role in reducing mushroom production. Two main viral diseases affect *A. bisporus*: ‘La France’ disease and MVX disease.

1.8.6.2.1 ‘La France’ Disease

‘La France’ disease was first reported on the La France brothers’ farm in Pennsylvania, USA in 1948 (Sinden & Hauser, 1950). Similar symptoms were observed some years later, in UK, France, the Netherlands, Italy, Denmark, and Australia (Ghabrial, 1994). Several different designations (‘X-disease’, ‘watery stipe’, ‘brown disease’, and ‘die-back’) were used to describe La France disease (reviewed by van Zaayen, 1979). During the 1970’s, La France disease occurred at epidemic proportions in North America and Europe and was a major problem during commercial mushroom production (Romaine & Goodin, 2002). La France disease can affect all *A. bisporus* varieties, but does not infect

the alternative cultivated species *A. bitorquis* (Geels *et al.*, 1988), which is often used as ‘virus breaker’.

Symptoms of La France disease range from minor infections, resulting in an almost negligible yield loss, to severe infections, associated with malformed fruiting bodies (Romaine & Goodin, 2002). The first indication of La France disease is delayed emergence of fruiting bodies and bare-patches throughout the commercial beds where mycelium does not permeate the casing layer resulting in loss of yield. Fruiting bodies surrounding the bare areas typically assume a ‘drumstick’ phenotype, consisting of elongated stems and small mishapen caps. The caps sometimes turn dark brown and stems bend. There is also a tendency for the diseased fruiting bodies to mature prematurely and release infected spores (Romaine & Goodin, 2002). The infectious nature of La France disease was demonstrated before it was known to be caused by a virus (Schisler *et al.*, 1967). The La France virus can be transmitted by basidiospores, between 65-75% spores produced by infected mushrooms are virus-infected (Romaine *et al.*, 1993). The virus can also be transmitted horizontally from infected to healthy mycelium (Schisler *et al.*, 1967; Sonnenberg & Van Griesven, 1991).

Three types of virus particles have been identified in La France diseased mushrooms: two isometric particles with diameters of 34-36 nm and 25 nm respectively (predominant types) (Van Zaayan, 1979) and bacilliform particles measuring 50x19 nm (Revill *et al.*, 1994). Since all three viral particle types have also been observed in apparently healthy fruiting bodies (Van Zaayan, 1979), the major correlation occurs between the disease and the presence of nine dsRNA elements (Harmsen *et al.*, 1989; Koons *et al.*, 1989). The dsRNA pattern most frequently associated with La France diseased mushrooms consists of six major dsRNAs: L1 (3.8 kbp), L2 (3.1 kbp), L3 (3.0 kbp), L4 (2.8 kbp), L5 (2.6 kbp) and M2 (1.3 kbp), which are invariably present in infected mushrooms. Three minor dsRNA elements, M1 (1.7 kbp), S1 (0.9 kbp) and S2 (0.8 kbp) can be also observed but are often absent (Marino *et al.*, 1976; Wach *et al.*, 1987; Harmsen *et al.*, 1989; Romaine & Schlagnhauer, 1989). The 34-36 nm-virions can be co-purified with six dsRNA molecules (L1, L2, L3, L4, L5, and M2) and sometimes accompanied by minor dsRNAs (M1, S1, and S2) (Goodin *et al.*, 1992; Van

der Lende *et al.*, 1994). This virus was initially named La France isometric virus (LIV) (Goodin *et al.*, 1992; Romaine *et al.*, 1993). Subsequent authors preferred to rename it as *Agaricus bisporus* virus 1 (ABV1) since its role as the causal agent remained circumstantial (Van der Lende *et al.*, 1996).

The nine La France dsRNAs showed no cross hybridization under stringent conditions (Harmsen *et al.*, 1989), suggesting that each had a unique sequence and was not a defective (Tartaglia *et al.*, 1986; Shapira *et al.*, 1991a) or subgenomic RNA (Ni & Kemp, 1994). The most favoured interpretation is that the six major dsRNAs represent the ABV1 genome, whereas the minor elements (M1, S1 and S2), present in submolar amounts may be satellite RNAs (Romaine & Goodin, 2002). Cosegregation of dsRNA elements during sporogenesis of La France infected-mushrooms is consistent with the idea that they are interdependently replicating molecules, comprising the genome of a single virus (Romaine *et al.*, 1993). What is less clear is whether the dsRNAs are encapsidated individually or together in a single particle. Some authors have suggested a multiparticle system, similar to that found in many plant viruses (Goodin *et al.*, 1992; Romaine & Goodin, 2002). However, Van der Lende (1995a) suggested that the ABV1 genome could replicate as in *Reovirus*. X-ray diffraction studies of *reovirus* type 3 (Harvey *et al.*, 1981) indicate that dsRNAs are packaged in a well-ordered and semi-crystalline way. Their capsid core (25 nm in diameter) accommodates the entire genome of ten dsRNA molecules with a total length of about 23 kbp. Assuming that the observations made by Harvey *et al.* (1981) apply to ABV1, the nine dsRNAs (making a total length of 18.5 kbp) could be accommodated into a capsid of 20 nm (Van der Lende, 1995a). It is therefore physically possible for the ABV1 genome to be packaged in the observed 34-36 nm virion particle.

L1, L3, L5, M1 and M2 dsRNA elements have been completely sequenced (Harmsen *et al.*, 1991; Van der Lende *et al.*, 1996). Only the L1 dsRNA, exhibiting homology to proteins present in protein databases encodes a putative RNA-dependent RNA polymerase (Romaine & Goodin, 2002) whose best matching sequences occur in dsRNA viruses of *Saccharomyces cerevisiae*, *Ustilago maydis*, *Leishmania brasiliensis* subsp. *guyanensis* and *Giardia lamblia* (Van der Lende, 1996). The presence of an

RdRp in the L1 dsRNA sequence was consistent with the demonstration of RdRp activity associated with ABV1 (Goodin *et al.*, 1997). However, no other similarities were found between the other dsRNAs sequences and available protein sequences (Van der Lende, 1995). Given the minimal resemblance to dsRNA fungal viruses, ABV1 has not yet been assigned to a mycovirus family (Romaine & Goodin, 2002).

ABV1 is frequently found in co-infections with *mushroom bacilliform virus* (MBV) (Revill *et al.*, 1994; Romaine & Goodin, 2002; Section 1.8.2) in button mushrooms. Romaine *et al.* (1995) estimated that 60% of diseased mushrooms collected in North America during a 13-year period, were co-infected by ABV1 and MBV. Initially, MBV was thought to be a satellite virus, but single infection (Romaine *et al.*, 1995) and the presence of an RdRp gene in its genome (Revill *et al.*, 1994) dismissed this theory. Hybridization analyses also support the idea that MBV is a distinct virus from ABV1 (Romaine & Schlaghauser, 1991). No obvious symptoms were observed in MBV-singly infected mushrooms (Romaine & Goodin, 2002). The idea that ABV1 and MBV are independently replicating viruses does not exclude the possibility of a synergistic relationship between them. Indeed, the titre of MBV can be 12-fold higher in mushrooms infected with ABV1 than without (Romaine *et al.*, 1995).

1.8.6.2.2 MVX disease

In the mid 1990s a new, complex disorder of *A. bisporus* was observed on several British farms (Gaze, 1997). This disease was termed as ‘Mushroom Virus X’ or ‘MVX’ disease (Gaze *et al.*, 2000). Within a few years, MVX symptoms were also seen in the Netherlands and Ireland (Sonnenberg & Lavrijssen, 2004; Rao *et al.*, 2007). Crops affected by MVX disease develop bare patches due to arrested development of pins or delay in fruiting body formation. Other symptoms include premature veil opening, brown discoloration and malformed sporophores (Fig. 1.6). Estimates of UK crop losses due to MVX disease were approximately £50 m in 2000 and yield reductions of 15% were common (Grogan, *pers. comm.*). In Dutch farms symptoms were restricted to brown discoloured mushrooms and were reported to extend to some farms in Belgium and Germany linked to some Dutch companies (Sonnenberg & Lavrijssen, 2004). In Ireland the appearance of MVX symptoms appeared to be confined to the first flush of

mushrooms and often tended to decline or disappear in the subsequent flushes (Rao *et al.*, 2007).

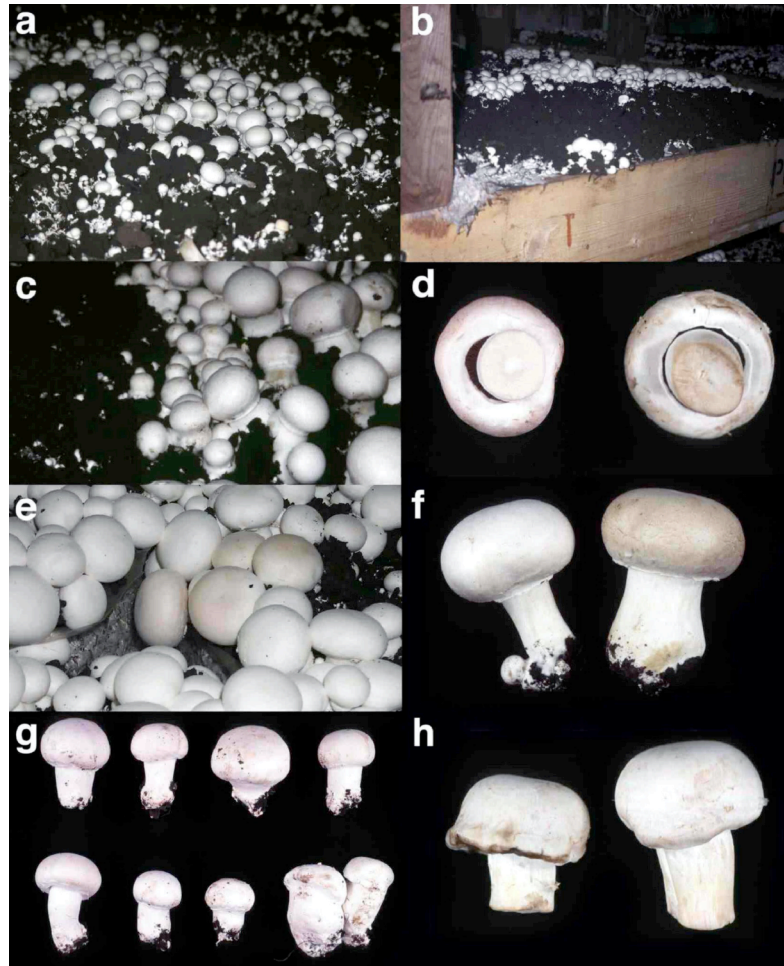


Fig. 1.6- MVX symptomology in *Agaricus bisporus* mushrooms. (a) pin suppression around a group of normal mushrooms; (b) bare cropping areas; (c) gradation in pin development from no pin on the left to fully developed mushrooms on the right; (d) premature veil opening; (e) ‘brown’ mushrooms; (f) an off-coloured mushroom (right) compared to a normal one (left); (g-h) malformed mushrooms (from Grogan *et al.*, 2003)

Studies of 389 MVX-infected mushroom samples carried out at Warwick HRI, UK allowed the identification of 26 dsRNA elements ranging in size from 640 bp to 20.2 kbp (Fig. 1.7; Grogan *et al.*, 2003). More recently, an additional dsRNA element of 3.5 kbp has been observed (Grogan *et al.*, 2004; Holcroft, *pers. comm.*). The researchers have extensively compared their results obtained from a wide range of sites and farms for dsRNA elements as expressed by banding patterns of MVX disease. Given the various sizes and numbers of dsRNAs together with the diverse range of symptoms

observed they concluded that MVX disease complex might comprise more than one virus.

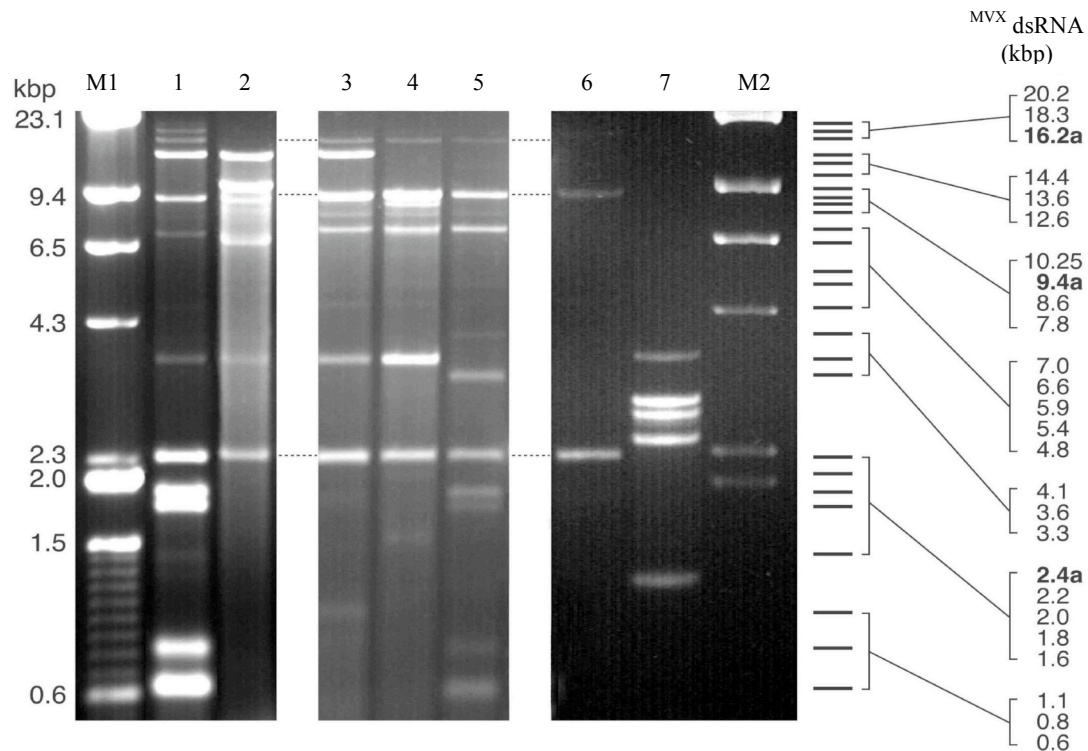


Fig. 1.7- dsRNA profiles from *Agaricus bisporus* mushrooms. M1 = molecular weight marker (λ DNA/HindIII ladder + 100 bp ladder, Invitrogen); M2 = molecular weight marker (λ DNA/HindIII ladder, Invitrogen); lanes 1-5 = different symptomatic MVX samples; lane 6 = asymptomatic mushroom; lane 7 = mushroom infected by La France disease. Molecular weights (kbp) are indicated for each dsRNA element. Asymptomatic dsRNAs are indicated by broken lines and highlighted in the schematic with the suffix “a” (from Grogan *et al.*, 2003)

Three dsRNA elements ($^{MVX}16.2_a$, $^{MVX}9.4_a$, $^{MVX}2.4_a$) were routinely found in mushrooms asymptomatic for MVX, whereas the remaining 24 dsRNAs were present exclusively in MVX-infected mushroom samples. The 4 low molecular weight dsRNAs ($^{MVX}2.0$, $^{MVX}1.8$, $^{MVX}0.8$, and $^{MVX}0.6$) were tentatively associated with brown symptoms Grogan *et al.*, 2003).

Other than the low weight dsRNAs, no clear relationship between the various MVX symptoms and the presence of specific dsRNA elements are present (Grogan *et al.*, 2003). DsRNA patterns may vary between samples collected in different sites and farms. The maximum number of dsRNAs found to occur in one sample is 17 (Grogan *et al.*, 2003).

Electron microscopy examinations have not revealed any morphological abnormalities or virus particles specifically associated with the disease (Sonnenberg & Lavrijssen, 2004; Rao *et al.*, 2007; Everard & Clay, unpubl.).

Hybridization analyses have indicated the presence of possible defective elements within the complex of viruses (Adie *et al.*, 2004). The ^{MVX}2.2 dsRNA hybridised with ^{MVX}18.3 and ^{MVX}16.2_a, while ^{MVX}9.4_a, 8.6, 7.8, and ^{MVX}7.0 formed a different hybridization group. Probes obtained from ^{MVX}14.4, 3.6, 1.8, 08, and ^{MVX}0.6 did not hybridize to any other ^{MVX}dsRNAs with the exception of the original element (Adie *et al.*, 2004). Sequence analyses of nine ^{MVX}dsRNA sequences obtained from random clones suggested similarity with various viral sequences (Adie *et al.*, 2004; Table 1.2).

Table 1.2- Summary of sequence analysis of ^{MVX}dsRNAs (from Adie *et al.*, 2004)

^{MVX} dsRNA (kbp)	Sequence generated (bp)	Homologies	Blast similarity	Conserved Motifs
14.4	558	<i>Vicia faba</i> dsRNA element causing male sterility (endornavirus); <i>Oryza sativa</i> dsRNA (endornavirus)	1.0e-07; 2.0e-04	NI
9.4a	925	Potexviruses: <i>fox mosaic virus</i> ; <i>clover yellow mosaic virus</i>	5.2e-08; 1.4e-07	NI
7.0	599	Novel sequence- no significant similarity	NA	Potential helicase DEAD box
1.8	657	Low similarity to <i>Picornaviridae</i>	0.55	RdRp GDD box motif
0.8	693	Mushroom bacilliform virus (1 clone)	6.0e-44	NI

NI: none identified; NA: not applicable

Work carried out at Warwick HRI suggested that ^{MVX}dsRNAs can be transmitted by spores and mycelia fragments (Gaze *et al.*, 2000; Grogan *et al.*, 2003; Adie *et al.*, 2004). Gaze *et al.* (2000) reported that MVX-infected spawn and mycelium used as inoculum for mushroom production in MVX-free areas could transmit MVX symptoms. In particular, when small amounts of MVX-infected spawn were mixed with a much larger

quantity of healthy spawn, sporophores subsequently produced were all infected. Infected mycelium was also reported as a possible source of infection (Gaze *et al.*, 2000), able to transmit ^{MVX}dsRNA elements through *in vitro* experiments (Adie *et al.*, 2004).

Whatever the dsRNA sequences and epidemiological works have revealed so far about the MVX disease, the specific viral aetiology still remains enigmatic. A number of issues for deciphering the molecular biology of this elusive and devastating disease remain to be explored.

1.9 Host Defence Mechanisms

Every organism suffers a constant battle with potential pathogens, but disease is often a relatively rare event. In crop science, plants have been used as a model to understand the reasons for pathogen failure. Either (i) the organism is unable to support the niche requirements of a potential pathogen and therefore is a non-host; or (ii) the organism possesses preformed structural barriers or toxic compounds that confine successful infections to specialized pathogens species; or (iii) upon recognition of the attacking pathogen induced-defence responses are elaborated and the invasion remains localized. All three types of interaction are said to be incompatible.

Successful pathogen invasion and disease (compatibility) ensue if the preformed organism barriers are inappropriate or the activated defence responses are ineffective (Hammond-Kosack & Jones, 1996). The most common mechanism associated with active defence is the hypersensitive response (HR); cells surrounding the primary infection site of the pathogen die due to a rapidly induced programmed cell death, resulting in a visible necrotic local lesion (reviewed by Golbach *et al.*, 2003). The induction of this response is preceded by a specific recognition between pathogen factors, produced from avirulence genes and infected organism gene products, produced from dominant resistance genes (Kenn, 1992; Staskawicz *et al.*, 1995). This type of resistance is limited to the inherited set of resistance genes (Laugé *et al.*, 1998) and thus cannot rapidly adjust to mutations of pathogens. Compared to fungal and bacterial

pathogens, viruses mutate relatively quickly because of the error prone RNA replicases (reviewed by Sijen & Kooter, 2000).

Certain organisms seem to have evolved a defence strategy that is activated by and directed against viral nucleic acids, rather than a strategy based on protein recognition. This mechanism, often referred to as Post-Transcriptional Gene Silencing (PTGS), provides a flexible surveillance system, which is able to cope efficiently with rapidly changing viruses (reviewed by Sijen & Kooter, 2000). These, in turn, produce proteins capable of suppressing host cell RNA silencing (reviewed by Silhavy & Burgyan, 2004). PTGS was first discovered in artificial systems where dsRNA was introduced by expression of transgenic constructs (van der Krol *et al.*, 1992) or by injection (Fire *et al.*, 1998). An early observation of PTGS was made in petunia (Napoli *et al.*, 1990). Trying to deepen the purple hue of petunias by boosting the activity of the chalcone synthase gene, the authors transferred additional copies of the gene into the plant hosts. Unexpectedly, many flowers became variegated or white instead of deep purple. Furthermore, they noted that the white colour could be passed to the next generation.

As plant researchers began to understand the significance of RNA silencing, Guo & Kemphues (1995), working on the nematode worm, *Caenorhabditis elegans* obtained surprising results with a technique involving ‘antisense’ RNA. In an effort to determine the function of the *par-1* gene, an antisense *par-1* RNA was injected into worms and this yielded the expected phenotype, embryonic lethality. However, injection of the control sense RNA, also created the same phenotype. Fire *et al.* (1998) reported that either sense- or antisense RNAs could inhibit gene expression and that dsRNA was ten fold more efficient than either strand alone.

In fungi, gene silencing was discovered during attempts to boost the production of an orange pigment in the mould *Neurospora crassa* (reviewed by Cogoni & Macino, 1997). The researchers introduced extra copies of a gene involved in making the carotenoid pigment, but found that some transformants bleached out rather than turning into deep orange. It soon became clear that PTGS was a widespread mechanism amongst different organisms and included the co-suppression observed in plants (Napoli

et al., 1990), quelling in fungi (Romano & Macino, 1992), and RNA interference in animals (Fire, 1999).

DsRNA is the key initiator molecule (Fire *et al.*, 1998; Wesley *et al.*, 2001) of PTGS, which is usually seen as a two step-reaction (Fig. 1.8, Buchon & Vaury, 2006).

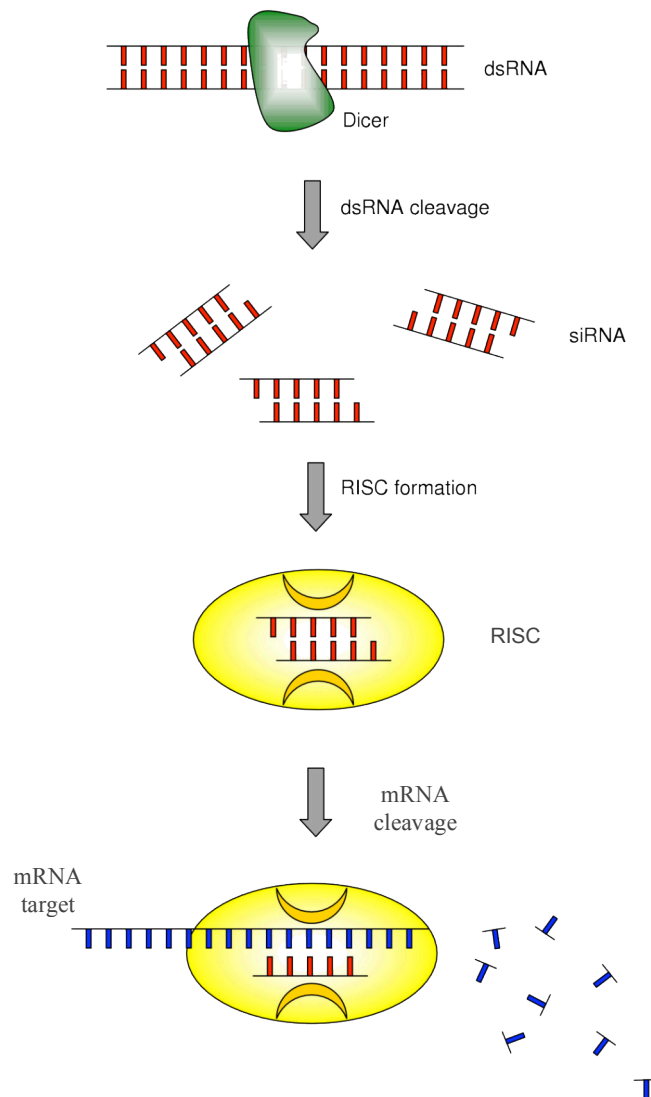


Fig. 1.8- Post-transcriptional Gene Silencing mechanism. dsRNAs are recognized by the RNA silencing pathway and cleaved by a Dicer-like protein to form siRNAs. These siRNAs serve as templates to guide the endonucleolytic cleavage of homologous mRNA

In the first step a dsRNA molecule (virus or transcribed transgene) is processed into short (21-25 nucleotides) small interference RNAs (siRNAs, Hannon, 2002). siRNAs are subsequently incorporated into a silencing complex, where they serve as templates

to guide the endonucleolytic cleavage of homologous mRNA (Hammond *et al.*, 2001). The first step is performed by a family of ribonucleases, the RNase III family, and more precisely by an enzyme called Dicer.

The latter is able to recognize and cleave dsRNA molecules to generate siRNAs. The second step of RNAi pathway is the formation of the RNA-Induced Silencing Complex (RISC), a large ribonucleoprotein complex considered to be a homology-dependent endonuclease, which seeks out and destroys homologous mRNA. The existence of such an RNA-based defence mechanism against invading elements could be particularly important for organisms such as plants and invertebrate animals, which lack protein-based adaptive immunity. Nevertheless, evidence has recently emerged that mammals also use PTGS to silence or destroy foreign genetic material detected in a cell (Svoboda *et al.*, 2004; Lecellier *et al.*, 2005).

Gene silencing against transposons and viruses can rely on another mechanism acting at a transcriptional level, Transcriptional Gene Silencing (TGS; reviewed by Buchon & Vaury, 2006). Until recently, both PTGS and TGS were considered separate pathways acting at the RNA and DNA level, respectively. Recently, a convergence of observations from diverse experimental systems suggested that a conserved mechanism might link both homology-dependent gene-silencing responses. TGS is often thought to involve a local chromatin modification whereby DNA and histones are chemically modified to recruit proteins inhibiting transcriptional activity and condensing chromatin. Hallmarks of TGS are DNA cytosine methylation and histone lysine methylation (reviewed by Buchon & Vaury, 2006). The link between altered chromatin structures and dsRNA induced gene silencing has emerged from plant and *Drosophila* systems (Hannon, 2002). In particular, alterations of either methyltransferases or chromatin remodelling complexes can affect both the degree and persistence of silencing in *Arabidopsis* (Jones *et al.*, 2001; Furner *et al.*, 1998). Mutations in genes required for PTGS decrease both co-suppression and transgene methylation (Fagard *et al.*, 2000). Hitherto, the mechanism linking TGS and PTGS pathways remains unclear (reviewed by Buchon & Vaury, 2006).

1.10 Aims and Objectives of this thesis

The research described in this thesis was principally aimed at studying one dsRNA element associated with the MVX disease of *A. bisporus*, ^{MVX}14.4.

^{MVX}14.4 was observed in *ca* 57% of MVX-infected mushrooms, showing a dsRNA pattern of 9 or more dsRNA elements (Adie *et al.*, 2004), and the element usually appeared as a very intense band in dsRNA profiles of MVX-infected mushrooms. Hybridization analyses (Adie *et al.*, 2004) indicated that ^{MVX}14.4 is most likely a unique element, with no defective interfering sub-elements.

To conduct molecular and epidemiological characterization of ^{MVX}14.4, the following specific objectives were identified:

- Generate full cDNA sequence for the ^{MVX}14.4 dsRNA using RT-PCR *fill in* strategy, cDNA cloning, sequencing, and sequence analyses.
- Investigate horizontal and vertical transmission of ^{MVX}14.4 using *in vitro* dual culture assay and single-spore progeny analysis, respectively.
- Characterize ^{MVX}14.4-like element observed in wild populations of *A. bisporus*.

A second aim was to investigate the occurrence of homology-dependent gene silencing phenomenon in *A. bisporus* as antiviral defence mechanism and its utility for functional genetic analysis of ^{MVX}14.4. To meet this second aim the following objectives were identified:

- Construct dsRNA forming-hairpin vectors using ^{MVX}14.4 sequences for transformation of *A. bisporus* and establish their utility in ‘silencing’ ^{MVX}14.4 dsRNA.
- Improve downstream transformation of *A. bisporus* investigating the use of phleomycin resistance gene as an alternative selectable marker.

- Additionally, evaluate the ability of different chemical compounds (hygromycin and cyclic AMP) to interfere with ^{MVX}14.4 replication by incorporation into laboratory culture media.

Chapter 2
GENERAL
MATERIALS AND METHODS

2. Summary

This chapter describes media, organisms, culture maintenance, solutions, and general experimental protocols used in this project. Details of more specific materials and methods are presented within relevant chapters.

2.1 Media

Unless otherwise stated, media were solidified, when applicable by the addition of 1.2% w/v Oxoid Technical Agar N° 3. All media and stock solutions were sterilised at 121° C (15 psi) for 15 min, unless otherwise stated. Heat sensitive solutions were filter sterilised using 0.2 µm Nalgene units (cat. No. 190-2520). Water used in all media and buffers was ultrapurified (18 MΩ) using an ELGA-Maxima Ultra Pure Water System.

2.1.1 Bacterial Media

Luria-Bertani (LB) medium (Little, 1987)

The composition of LB broth was as follows (g/L): 10 g bacto-tryptone, 5 g bacto-yeast extract, and 5 g NaCl.

SOC broth

The composition of LB broth was as follows: 2.0 g of bacto-tryptone, 0.5 g of bacto-yeast extract, 1 ml of 1M NaCl and 0.25 ml of 1M KCl were dissolved in 98 ml water, autoclaved, and cooled to room temperature. After autoclaving, 1 ml of 2M Mg²⁺ solution (203.3 g /L MgCl₂·6H₂O and 246.5 g/L MgSO₄·7H₂O) and 1 ml of 2M glucose were added.

Minimal Medium for *Agrobacterium* (MM, Hooykaas *et al.*, 1979)

The composition of MM was as follows (ml/L): 10 ml K-buffer (200 g/L K₂HPO₄, 145 g/L KH₂PO₄), 20 ml M-N buffer (30 g/L MgSO₄·7H₂O; 15 g/L NaCl), 10 ml 20% w/v glucose, 10 ml 0.01% w/v F₂SO₄, 2.5 ml 20% (NH₄)₂SO₄, and 1 ml 1% w/v CaCl₂·2H₂O.

***Agrobacterium* Induction Medium** (IM, Hooykaas *et al.*, 1979)

IM was composed as MM, but with the addition of 40 mM MES, 10 mM glucose and 0.5% v/v glycerol.

2.1.2 Mycological Media

Complete yeast extract medium (CYM, Raper *et al.*, 1972)

The composition of CYM was as follows (g/L): 20 g D-glucose; 2 g peptone; 2 g yeast extract; 0.5 g MgSO₄·7H₂O; 0.46 g KH₂PO₄; 1g K₂HPO₄.

Compost extract + Complete yeast extract medium (CE/CYM, Calvo-Bado *et al.*, 2000)

To prepare compost extract (CE), fresh compost (400 g phase II, Section 1.4) was oven dried in thin layers (3-4 h at 120° C) and ground to a fine powder using a Sample Mill (Cyclotec 1093). Dried compost was added to 1 litre of distilled water, simmered for 1 h, cooled and filtered through Miracloth (Calbiochem, cat. No. 475855). Solids were removed by centrifugation (7,000 xg, 20 min, 5° C, MSE-HS18). The remaining supernatant (*ca* 800 ml) was distributed into 400 ml aliquots and stored at - 20°C until required. For preparation of CE/CYM, 200 ml of CE and 20 ml of a stock solution 5x CYM were made up to 1 L with water and autoclaved for 20 min.

Malt, Peptone Agar (MPA, Challen, unpublished)

MPA was composed of 20 g/L malt extract and 5 g/L peptone.

2.1.3 Antibiotics, fungicides and other supplements

Antibiotics, anti-metabolites, and other supplements were prepared as stock solutions and filter sterilised through 0.2 µm Nalgene units (Section 2.1). Solutions were stored at -20° C with the exception of carboxin, which was stored at 4° C; and acetosyringone, which was made up as a fresh solution before use. Phleomycin working concentration was not determined. Table 2.1 shows the chemical stock and working concentrations. Compounds were routinely added to autoclaved media after cooling to 50° C.

Table 2.1- Chemical stock and working concentrations of antibiotics, fungicides and other supplements

CHEMICAL	TYPE	STOCK	SOLUTE	WORKING
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(abbreviation)		CONCⁿ		CONCⁿ
Acetosyringone (AS)	polyphenol	20 mg/ml	ethanol	40 µg/ml
Ampicillin (Amp)	antibiotic	100 mg/ml	water	100 µg/ml
Carbenicillin (Cb)	antibiotic	50 mg/ml	water	50 µg/ml
Bromo-cyclic AMP (Br-cAMP)	nucleotide	4.3 mg/ml	water	4.3 µg/ml
Carboxin (Cx)	fungicide	100 mg/ml	ethanol	15 µg/ml
Cefotaxime (Cf)	antibiotic	200 mg/ml	water	200 µg/ml
Cyclic AMP (cAMP)	nucleotide	369 mg/ml	water	369 µg/ml
Hygromycin B (Hyg)	antibiotic	100 mg/ml	water	25 µg/ml
Kanamycin (Kan)	antibiotic	30 mg/ml	water	30 µg/ml
Phelomycin (Phleo)	antibiotic	25 mg/ml	water	-
Rifampicin (Rf)	antibiotic	25 mg/ml	methanol	25 µg/ml
Tetracyclin (Tet)	antibiotic	12.5 mg/ml	ethanol	12.5 µg/ml

- = undetermined

2.1.4 Indicator Media

Media for colorimetric detection of *Escherichia coli* transformants were supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and isopropylthiogalactoside (IPTG). A 20 mg/ml stock solution of X-gal in N,N'-dimethyl-formamide was filter sterilised, and stored in the dark at -20° C in glass or polypropylene tubes. A 0.1 M stock solution of IPTG in water was filter sterilised and stored at -20° C. Using a sterile plastic spreader, 2 ml of X-gal and 2.5 ml of IPTG were added to 500 ml LB agar medium with the appropriate antibiotics.

2.2 Stock solutions and Buffers

All chemicals used in molecular procedures were of at least analytical grade. All glassware and water used for the preparation of solutions for RNA work were treated with dimethylpyrocarbonate (DMPC, Sigma cat. No. D-5520 [5% v/v ethanol; 0.1% v/v DMPC working solution]) and autoclaved prior to use.

Restriction endonucleases and other enzymes were obtained from commercial sources and used with the appropriate buffers according to the manufacturers instructions.

5x STE

A stock solution was prepared as follows (g/L): 30.25 g tris(hydroxymethyl)methylamine (TRIS); 1.65 g ethylenediaminetetracetic acid (EDTA); 29.6 g NaCl.

1x STE/ 15% v/v ethanol

The composition of 1xSTE/ 15% v/v ethanol was as follows (g/L): 200 ml 5x STE stock solution, 150 ml 100% v/v ethanol and 650 ml DMPC water. Ethanol was added to the solution under the fume-hood after autoclaving.

10% w/v SDS

50 g sodium dodecyl sulphate crystal (SDS) was dissolved in 500 ml water at 68° C.

Electrophoresis Buffers and Stock Solutions

Electrophoresis buffers and stock solutions were prepared according to Sambrook *et al.* (1989). The standard solutions were: 50x TAE (2M TRIS-base; 0.05M EDTA; pH 7.8 of the buffer was adjusted with glacial acetic acid); gel loading buffer type III (30% w/v sterile glycerol, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol FF); and ethidium bromide solution (10 mg/ml).

2.3 Maintenance of Fungal cultures

Fungal isolates and strains used are detailed within relevant chapters. They were maintained on CE/CYM agar plates at 4°C and sub-cultured every 3 months. Short/medium-term storage isolates were preserved in sterile water according to the Castellani's methodology (Cerezine & Kurozawa, 1992). Isolates were cultured on CYM or CE/CYM medium at 25°C for 2-3 weeks; 4-5 mycelial culture plugs were then removed from the culture and transferred to a 50 ml Falcon tube containing 20 ml of sterile water. Tubes were stored at room temperature for up to 2-3 years. Long-term cultures storage was achieved using liquid nitrogen (Challen & Elliott, 1986).

2.4 Bacterial strains

2.4.1 *Escherichia coli*

Two strains of *E. coli* competent cells were used for routine cloning: *E. coli* DH5 α , genotype F ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 *recA1 endA1 hsdR17*(rk⁻, mk⁺) *phoA supE44 thi-1 gyrA96 relA1* λ ⁻ (Invitrogen, cat No.18258-017); and *E. coli* strain XL1-Blu, genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacI*^qZ Δ M15 *Tn10* (*Tet*^r)] (Advantagen, cat No. Eco010).

Competent cells of *E. coli* SCS110, genotype *rpsL* (*Str*^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ (lac- *proAB*) [F' *tra36 proAB lacI*^qZ Δ M15] (Stratagene, cat No. 200247) were used for experiments described in Chapter 6. Strain SCS110 is deficient for two methylases (Dam and Dcm) found in most strains of *E. coli*.

E. coli strains were routinely cultured onto LB media with appropriate antibiotics at 37°C. Broth cultures were shaken at 225 rpm (New Brunswick Scientific- Edison NI USA).

2.4.2 *Agrobacterium tumefaciens*

A. tumefaciens strain AGL-1 (Lazo *et al.*, 1991) was used for *A. tumefaciens*-mediated transformation of *A. bisporus*. Strain AGL-1 was obtained from bacterial culture collections at Warwick HRI.

Strains of *Agrobacterium* are defined by their chromosomal background and resident Ti-plasmid. Strain AGL-1 carries a C58 chromosomal background and has proved useful for transformation of a range of *A. bisporus* strains (Leach, 2004). It contains a nopaline/L, L- succinamopine opine-type Ti-plasmid (pTiBo542) that is hypervirulent in the *virG* locus.

To prepare electro-competent cells *A. tumefaciens* was streaked onto LB agar supplemented with 25 µg/ml rifampicin and 50 µg/ml carbenicillin at 26° C, overnight. A single colony was inoculated to 50 ml LB broth with appropriate antibiotics. The culture was incubated at 26° C, 150 rpm, overnight. The cells were harvested by centrifugation at 3650 xg (Hermle Z382K), 10 min, 4° C and kept on ice to prevent further growth. *A. tumefaciens* cells were washed and centrifuged at 3650 xg three times in 50 ml of 10% glycerol. The final pellet was resuspended in 1 ml of ice-cold 10% v/v glycerol and 100 µl aliquots of electro-competent cells were stored at -80° C.

2.5 Nucleic acid extraction methods

Three different methods were used to extract nucleic acids from mushroom samples. Nucleic acids extraction used fungal mycelium or mushroom sporophores as starting material. All fungal material was freeze-dried (Edwards, Modulyo) prior to the extraction unless otherwise stated. Fungal mycelium (50 mg) was freeze-dried over 24 h. Sporophore tissue (3.5 g) was freeze-dried over *ca* 7 days.

2.5.1 dsRNA extraction (Valverde *et al.*, 1990b; Holcroft, *pers.comm.*)

This method combined a dsRNA phenol extraction with CF-11 cellulose column purification and was the first step for dsRNA profiling analysis.

Mushroom sporophores were ground to a fine powder in liquid nitrogen, using mortars and pestels. Powdered samples were transferred to 250 ml pots containing 16 ml of 1x

STE, 2 ml of 10% w/v SDS, 1 ml of 2% w/v bentonite aqueous solution and 18 ml of acid phenol (pH 4.3; Sigma, cat. No. P-4682). The mixture was emulsified by shaking for 30 min at 4° C and then centrifuged for 15 min at 9,000 xg (Europa 24 M- MSE). Supernatants were transferred to 50 ml tubes for further centrifugation (10 min, 9,000 xg, Hermle Z382K). Absolute ethanol was added to the purified aqueous phase to a final concentration of 15% v/v. For each sample two columns were prepared, comprising a sterile 20 ml plastic syringe plugged with glass wool, equilibrated with 25 ml of 1x STE/ 15% v/v ethanol and 1.5 g of CF-11 cellulose (Whatman, cat. No. 4021050). Samples were added to each column and washed with 40 ml of 1x STE/ 15% v/v ethanol. The dsRNA from the column was eluted by adding 10 ml of 1xSTE and collected in sterile tubes. Absolute ethanol was again added to each tube to a final concentration of 15% v/v and the wash process repeated, apart from a final elution with 6 ml of 1xSTE. Nucleic acids were precipitated at -20°C for 2h using 20 ml of absolute ethanol and 1 ml of 3M sodium acetate (pH 5.2). Samples were centrifuged at 9,000 xg for 25 min (Hermle Z382K) and the supernatants discarded. The pellets were resuspended in 100 µl DMPC water and further purified using Qiaquick PCR purification kit (Qiagen, cat. No. 28106), following the manufacturers protocol. Samples were finally eluted in 30 µl DMPC water.

2.5.2 TRI Reagent extraction (Chomczynski, 1993)

TRI Reagent was used to extract total RNA from mushroom mycelium. Samples were ground to a fine powder using small sterile pestles in the presence of 0.15 g of glass beads (106 µm, SIGMA, cat. No. G-4649). TRI reagent (1 ml, SIGMA cat. No. T 9424) was added to each sample and tubes were shaken vigorously for 15-30 s before centrifugation at 12,000 xg, 10 min, 4° C (Hawk 15/05- Sanyo MSE). Supernatants were transferred to clean microcentrifuge tubes and 0.2 ml chloroform was added. Samples were shaken gently for 15-30 s and centrifuged (12,000 xg, 15 min, 4° C). The upper aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube and 0.5 ml of isopropanol added. Samples were allowed to stand for at least 5 min at room temperature or longer at -20° C and then centrifuged. Supernatants were removed and the RNA pellets washed with 1 ml of 75% v/v ethanol, centrifuged (7,500 xg, 5 min, 4° C), air-dried for 5-10 min and then resuspended in 100 µl DMPC water. The RNA was

further purified using the QIAquick PCR purification kit (QIAGEN, cat. No. 28106) according to the manufacturer's protocol, prior to RT-PCR.

2.5.3 Chelex extraction

Crude DNA extraction for PCR screening was performed using a Chelex method (Challen *et al.*, 2003). Five plugs were cut from the periphery of actively growing mycelial agar cultures and transferred to a 1.5 ml microcentrifuge tubes. Glass beads (0.15 g) and 1 ml Chelex-Tris suspension (3% w/v Chelex, 1 mM Tris pH 8) were added to each tube and then vortexed for 1 min. Macerates were treated by three alternate cycles of freezing in liquid nitrogen and boiling for 1 min each and a final prolonged boiling of 5 min. Tubes were vortexed for an additional min, incubated at 55° C for 30-40 min and centrifuged (12,000 xg, 5 min; Hawk 15/05- Sanyo MSE). Finally 100 µl of supernatant was transferred to a clean 1.5 ml microcentrifuge tube. Samples were stored at -20° C or used directly for PCR.

2.6 Nucleic acids quantification

Quantity and purity of nucleic acids were determined using the spectrophotometer (Nanodrop®, Labtech) to read absorbance at 260 nm and 280 nm. A pure preparation of DNA and RNA had the ratio between the readings at 260 nm and 280 nm (OD₂₆₀: OD₂₈₀) of approximately 1.8 and 2.0, respectively.

2.7 Purification of dsRNA

DNA and ssRNA were removed from extracted dsRNA using DNase (1 U/µl, Promega, cat. No. M6101) and S1 nuclease (1 U/µl, Promega, cat. No. M5761). To remove DNA, 8 µl of RNA sample was added to 1 µl of RQ1 RNase free DNase and 1 µl of RQ1 RNase free DNase 10x reaction buffer. The mixture was incubated at 37° C for 15 min. The reaction was then stopped by addition of RQ1 DNase stop (1 µl) and incubation at 65° C for 10 min.

To remove ssRNA, the RNA preparation was treated with S1 nuclease. Each RNA sample was added to 1 μ l of S1 nuclease 10x reaction buffer and 1 μ l S1 nuclease. The mixture was incubated at 37° C for 1 h and 30 min. The dsRNA solution was then purified using the QIAquick PCR purification kit (QIAGEN, cat. No. 28106) according to the manufacturer's protocol.

2.8 Reverse Transcription-Polymerase Chain Reaction

2.8.1 Reverse Transcription (RT)

cDNA synthesis (reverse transcription) was performed using ThermoScript™ RT-PCR System (Invitrogen, cat. No. 11146-024), unless otherwise stated. For each reaction, 2 μ l of random hexamers (50 ng/ μ l), 1 μ g of purified RNA template and DMPC water up to 10 μ l were mixed in a 0.5 μ l microcentrifuge tube. Reactions were boiled for 5 min and then placed on ice. The second step-reaction was performed in 20 μ l volume and comprised the following: 1 μ l ThermoScript™ RT (15U/ μ l), 4 μ l of 5x cDNA synthesis buffer, 2 μ l dNTPs mix (10 mM), 1 μ l DTT (0.1M), 1 μ l Rnase OUT™ (40 U/ μ l), and 10 μ l template (from previous step). cDNA synthesis was progressed using a HYBAID MBS 0.5 G Thermal Cycler with the following parameters: 25° C for 10 min; 50° C for 45 min; and 85° C for 5 min (termination). To remove any remaining RNA template, each cDNA reaction was treated with 1 μ l *E. coli* RNase H (2U/ μ l) at 37° C for 20 min.

2.8.2 Polymerase chain reaction (PCR)

Sequence specific oligonucleotides primers for PCR were designed using the Clone Manager Professional Suite v 7 (Scientific & Educational software, Cary, NC). Specific primers used for each reaction are stated in relevant sections. PCR screenings were routinely carried out as follows unless otherwise stated. Reactions were prepared in 50 μ l volume using: 0.5 μ l Platinum *Taq* DNA polymerase (5 U/ μ l, Invitrogen), 5 μ l of 10x PCR buffer, 1.5 μ l MgCl₂ (50 mM), 1 μ l dNTPs (10 mM mix), 1 μ l forward primer (10 μ M), 1 μ l reverse primer (10 μ M), 20-60 ng DNA or 3 μ l cDNA obtained from cDNA synthesis (section 2.8.1). PCR reactions were progressed using a HYBAID MBS

0.2 G Thermal Cycler with the following parameters: initial denaturation at 96°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. Variations on PCR protocol are described in appropriate chapters/sections.

2.8.3 ^{MVX} dsRNA Test

An RT-PCR Test was previously developed at Warwick HRI to routinely screen four ^{MVX} dsRNA elements: ^{MVX}14.4, ^{MVX}3.6, ^{MVX}1.8 and ^{MVX}9.4a (Section 1.8.6.2.2). The cDNA synthesis and PCR reactions were carried out as described in Sections 2.8.1 and 2.8.2, respectively. Specific primers amplifying ^{MVX} dsRNA elements are listed in Table 2.2. To assess the quality of RNA templates, RT-PCR was performed using primers (AB18S_f1 and AB18S_r1) amplifying the *A. bisporus* ribosomal 18S RNA.

Table 2.2- Primers amplifying ^{MVX}14.4, ^{MVX}3.6, ^{MVX}1.8, ^{MVX}9.4a, and 18S RNA

Primer	Primer sequence Reference: Challen M.P. (<i>pers. comm.</i>)	Product Size (bp)	RNA Target
B3c198_f2 B3c198_r2	5'-TCTGGCGATGTGAAACCT-3' 5'-CTGCCAGAAGTAGTGATTAGTAG-3'	315	^{MVX} 14.4
B15c35_f1 B15c35_r1	5'-ACTAGGCAGGAGCAGATGAA-3' 5'-CCAACAATCGAGCGTCAGAA-3'	427	^{MVX} 3.6
B19b_f1 B19b_r1	5'-GAGGTTCACTGAGAGTTG-3' 5'-CTCGTATGTTTCATCGAC-3'	322	^{MVX} 1.8
BH2c102_f1 BH2c102_r1	5'-GAAGATGAGGAC-CGGCACAGT-3' 5'-CTCTCGCTTCTCCGAAGGTA-3'	241	^{MVX} 9.4a
AB18S_f1 AB18S_r1	5'-GGTAGGATAGAGGCCTACCA-3' 5'-TTCGCAGTAGTCGGTCTTGA-3'	617	18S

2.9 Electrophoretic Analysis of Nucleic acids

Nucleic acid samples were fractionated by electrophoresis through agarose gels prepared with agarose MP (Roche, cat. No. 1388991), 1xTAE buffer and gel loading buffer type III (Section 2.2). Ethidium bromide (0.5 mg/ml final concentration) was

added to agarose gel, which was then analysed under UV light using the UV transilluminator (UVP Biodoc-It™ System).

2.9.1 Electrophoresis of PCR products

PCR products were analysed on 1% w/v agarose gel after about 40 min run at 80 V. When PCR products were electrophoresed for DNA gel extraction, DNA bands were excised from a 0.6% w/v agarose gel, collected into 1.5 ml microcentrifuge tubes and gel purified using the QIAquick gel extraction kit (QIAGEN cat. No. 28704), according to the manufacturers protocol.

2.9.2 Electrophoresis for dsRNA Profile Analysis

Extracted dsRNA (Section 2.5.1) was fractionated on 0.8% w/v agarose gel after running overnight at 30V.

2.10 Cloning

2.10.1 pGEM®-T Easy Cloning

Routine cloning of amplified PCR products was carried out using pGEM®-T Easy vector System (Promega, cat. No. A1360). Following gel purification (Section 2.9.1), 700 ng DNA was ligated into the pGEM®-T Easy vector (Fig. 2.1 and Fig. 2.2.). Ligation reactions were set up in 10-12 µl final volumes using 1 µl pGEM®-T Easy vector (50 ng/µl), 5 µl of 2x rapid ligation buffer, 1 µl T4 ligase (3U/µl), and 3-5 µl PCR product and incubated overnight at 4° C. Following a brief centrifugation, 5-7 µl of each ligation reaction was transferred to a sterile 1.5 ml microcentrifuge tube containing 100 µl DH5α™ or XL-1 Blue competent cells for transformation (Section 2.4.1).

Competent cells were incubated on ice for 30 min, heat-shocked for 45s at 42° C and placed on ice for 2 min. Pre-warmed SOC broth (900 µl) was added to each tube and incubated in a shaking incubator (1 h at 37° C, 225 rpm, New Brunswick Scientific-Edison NI USA). Three aliquots (50 µl, 100 µl and 200 µl) of each transformation reaction were spread onto LB agar containing 100 µg/ml ampicillin and 80 µg/ml X-gal and incubated overnight at 37° C. Transformed colonies were isolated onto LB agar

with appropriate supplements, and also grown overnight in 15 ml LB broth (37° C, 225 rpm) supplemented with ampicillin (100 µg/ml). Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, cat. No. 27106), according to the manufacturers protocol. To confirm the integrity of recombinants, pGEM®-T Easy clones were restricted using *Not* I endonuclease (ROCHE, cat. No. 1014706), according to the manufacturers protocol.

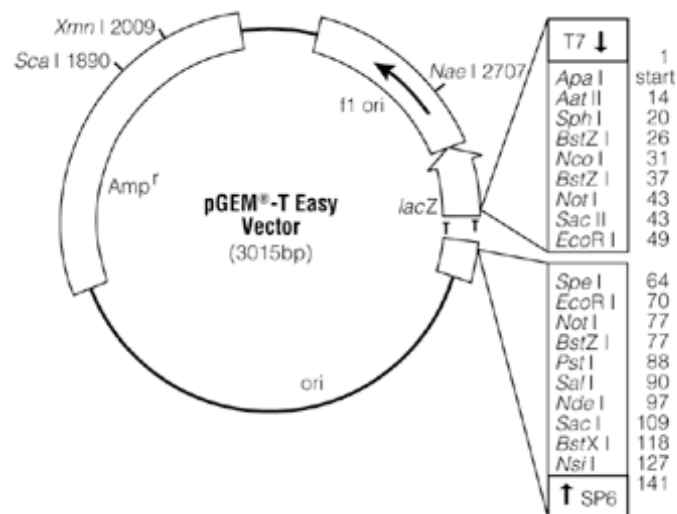


Fig. 2.1– The pGEM®-T Easy vector. The pGEM®-T Easy vector contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the coding region of the α -peptide enzyme β -galactosidase (*lacZ*). Insertional inactivation of the α -peptide allows recombinant clones to be directly identified by colour screening on indicator plates

2.10.2 pGREEN Cloning

Binary Ti vectors are the plasmid vectors of choice in *Agrobacterium*-mediated transformation protocols (Hellens *et al.*, 2000). The pGreen binary series are configured for easy cloning. This plasmid system allows any arrangement of selectable marker and reporter gene at the right and left T-DNA borders without compromising the choice of

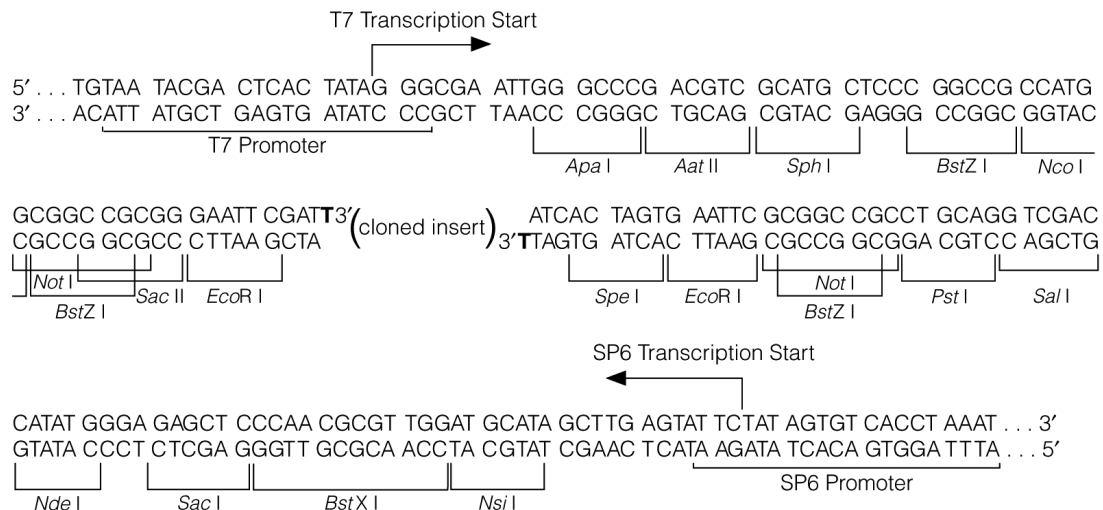


Fig. 2.2- The promoter and multiple cloning sequence of the pGEM[®]-T Easy vector. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase

restriction sites for cloning, since the pGreen cloning sites are based on the well-known pBluescript general vector plasmids. Its size and copy number in *E. coli* offers increased efficiencies in routine cloning protocols. pGreen can replicate in *Agrobacterium* only if another plasmid, pSoup (Fig 2.3) is co-transformed into the same host. pSoup provides replication functions *in trans* for pGreen. Removal of the replication gene (*RepA*) has enabled the size of pGreen vector to be kept to a minimum. pGREEN II (Fig. 2.4) is a modification of the original vector and was used in this study. Following the appropriate enzymatic digestion of the vector, ligation reactions and cloning of PCR products were carried out as described in Section 2.10.1. However, pGREEN clones were selected using kanamycin instead of ampicillin.

2.11 *Agrobacterium* Electroporation

A 100 μ l aliquot of *A. tumefaciens* electro-competent cells (Section 2.4.2) was thawed on ice and transferred to an electroporation cuvette (0.2 cm electrode gap, Invitrogen, cat. No. P450-50), containing 200 ng of pGREEN II and pSOUP (1:1 ratio). Plasmid DNA was previously dialysed on nitrocellulose membrane (0.025 μ M Millipore, cat. No. VSWPO4700) for 20 min before electroporation.

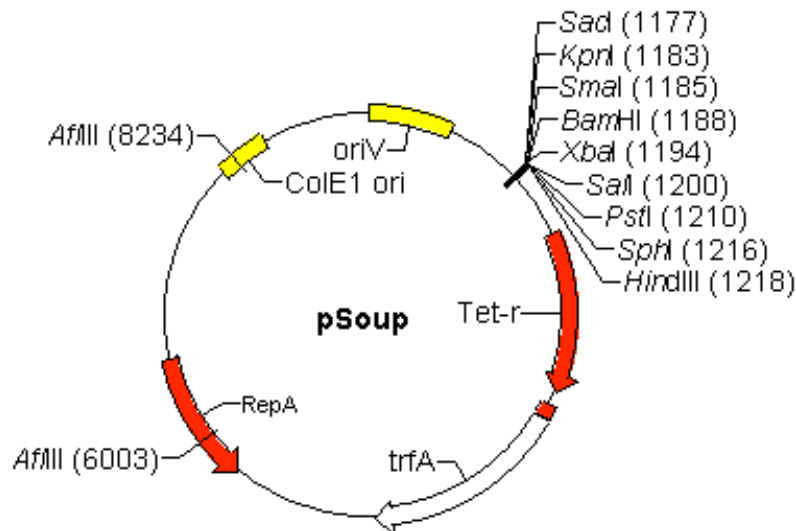


Fig. 2.3- pSOUP plasmid. pSOUP is co-transformed with pGREEN into *Agrobacterium* cells. ColE1 ori = plasmid origin for replication in *E. coli*; oriV = vegetative origin of plasmid replication; Tet-r = tetracycline resistance gene for selection of putative transformants; trfA = replication initiation gene; RepA = *Agrobacterium* replication gene

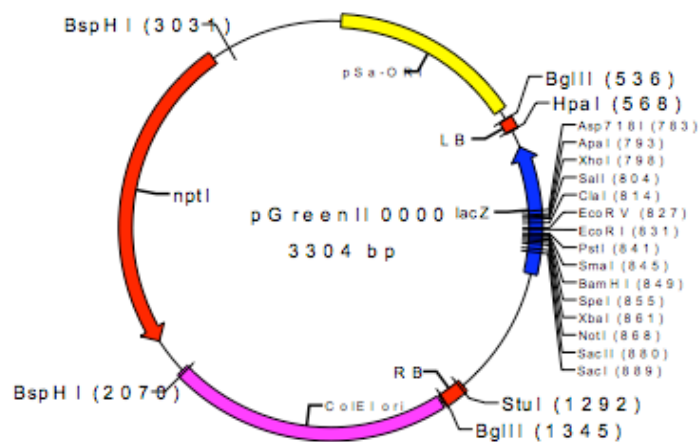


Fig. 2.4- pGREEN II binary vector. Npt I = kanamycin resistance gene enabling most bacterial selection in both *E. coli* and *Agrobacterium* transformation; pSa ORI = *Agrobacterium* replication origin; LB = T-DNA left border; RB = T-DNA right border; lacZ = β -galactosidase gene for blue/white bacterial colony screening; ColE1 ori = plasmid origin for replication in *E. coli*

One pulse of 12 ms (2.5 kV, 25 μ FD, 400 ohms) was applied and cells were immediately recovered in 1 ml of SOC medium followed by incubation for 6 h at 26 $^{\circ}$ C, 150 rpm (New Brunswick Scientific- Edison NJ USA). Serial dilutions (10 μ l, 50 μ l and

100 µl) of each transformant were plated to LB agar supplemented with kanamycin (30 µg/ml) and incubated at 26° C for 2-3 days. Individual colonies were then restreaked on the appropriate medium to confirm the transformation event prior to DNA extraction using QIAprep Spin Miniprep Kit (Qiagen, cat. N. 2710056). Plasmid integrity was determined by restriction with appropriate restriction enzymes.

2.12 DNA Sequencing

Sequencing reactions were performed from both ends of cloned fragments or PCR products using the ABI Prism® BigDye™ terminator cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase, Fs (Perkin-Elmer Applied Biosystems, cat. No. 403044).

2.12.1 Recombinant Clone Sequencing

The sequencing of inserts cloned in pGEM®-T Easy vector (Section 2.10.1) was performed in 10 µl volume reactions comprising: 2 µl ABI Prism® BigDye™ terminator cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase, 1 µl of 5xBuffer, 0.2 µl primer T7 or SP6 (10 pmol/µl), 800-1000 ng purified plasmid. Sequencing primers T7 (5'- TAATACGACTCACTATAGGG-3') and SP6 (5'- ATTTAGGTGACACTATAGAA-3') annealed flanking regions of the pGEM®-T Easy cloning site (Fig. 2.2). BigDye® terminator cycle sequence reactions were performed using GeneAmp 9600 Thermal Cycler according to the following parameters: 25 cycles of rapid thermal ramp (1° C/s) to 96° C, 96° C for 10s, rapid thermal ramp to 50° C, 50° C for 5 s, rapid thermal ramp to 60° C, 60° C for 4 min. Reaction products were sequenced in the Warwick-HRI Genomic Centre using an automated DNA capillary system (3130xl Genetic Analyzer, Applied Biosystems).

2.12.2 Direct Sequencing of PCR products

Amplified PCR products were processed for direct sequencing as follows: 2 µl ABI Prism® BigDye™ terminator cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase, 1 µl of 5xBuffer, 0.2 µl forward primer or reverse primer (10 pmol/µl), 60 ng of purified PCR product in a 10 µl total volume. Descriptions of

specific sequencing primers are stated in relevant sections of this thesis. BigDye® terminator cycle sequence reactions were progressed as described in Section 2.12.1.

2.13 Sequence analyses

Sequences were assembled and analysed using the DNASTar package (LaserGene version 5.07, Madison, Wisconsin). The Seqman module was used to view and assemble trace sequences into contigs, remove vector sequences. The Seqman program uses dual-end sequence data (forward and reverse sequence readings originating at opposite ends of the same fragments) to put contigs into groups. The Seqman program created new groups for each set of overlapping sequences where dual-end data implied physical linkage. If dual-end sequence data for a group of contigs was not available or inconsistent, contigs were assigned to the “unlocated contigs” group. Several parameters were set in the assembly of cDNA contigs, including minimum match percentage of overlapping bases (80%) and minimum length percentage of matching bases (100%).

Sequence similarity and database searches were performed using the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) and European Bioinformatics Institute (EMBL-EBI, <http://www.ebi.ac.uk/services/index.html>) facilities. Blast-N analysis compared nucleotide sequences against the nucleotide database. Blast-X analysis compared nucleotide sequences, translated in all six reading frames, against protein databases. Blast-P analysis compared predicted protein against protein databases. The best hits in the database list were used in pairwise comparisons using BL2seq (NCBI). Similarities were considered significant when the E-value was e^{-9} or lower (Altschul *et al.*, 1997).

Multiple sequence and protein alignments, construction of phylogenetic trees and bootstrapping analysis were performed using the MegAlign DNASTar module, which also enabled the alignment to be viewed as CLUSTAL W (Thompson *et al.*, 1994) phylogenetic tree. The GENEDOC program (version 2.6.0.2) was also used to view and facilitate presentation of multiple sequence alignments.

Predicted amino acid sequences and open reading frames (ORFs) were identified using the GeneQuest module (DNASTAR package) and the ORF Finder program (NCBI). The latter proved especially useful to identify conserved domains. Protein molecular weights were calculated using the SMS (Sequence Manipulation Suite, www.bioinformatics.vg/sms). Proteins often contain several modules or domains, each with a distinct evolutionary origin and function. NCBI's Conserved Domain database (CDD) is a collection of multiple sequence alignments of annotated functional domains and full-length proteins (Marchler-Bauer *et al.*, 2005) and was used to identify conserved domains present in a protein sequence. CDD contains domains imported from SMART, Pfam and COGs. The Pfam database (Finn *et al.*, 2006) enabled the identification of protein families for new putative domains.

2.14 Mushroom Transformation

Agrobacterium-mediated transformation was used to introduce transgenic sequences into *A. bisporus* mushrooms as previously described (de Groot *et al.*, 1998; Challen *et al.*, 2000; Chen *et al.*, 2000; Mikosch *et al.*, 2001; Burns *et al.*, 2006).

2.14.1 *Agrobacterium tumefaciens* Culture and Induction

All *A. tumefaciens* strains were cultured and transformed as described in Section 2.4.2 and 2.11, respectively. Starter cultures were established in 50 ml LB broth supplemented with 30 µg/ml kan and incubated at 26° C, 150 rpm (New Brunswick Scientific- Edison NJ USA), overnight. One aliquot (5 ml) of each LB culture was used to inoculate 50 ml MM broth, containing the appropriate antibiotics and incubated at 26° C, 150 rpm, overnight. An aliquot providing an OD₆₆₀ value of 0.15 (PV8720 UV/Vis Scanning Spectrophotometer, Philips) was transferred to 50 ml Falcon tubes in a volume of 20 ml IM broth. The culture was centrifuged for 8 min at 1000 xg (Mistral 1000). The pellet was resuspended in 50 ml IM broth supplemented with 30 µg/ml kan and 40 µg/ml of freshly prepared acetosyringone, incubated at 26° C, 150 rpm, for 6 h.

2.14.2 Gill Tissue Transformation

Chen *et al.* (2004) first reported *Agrobacterium*-mediated gill tissue transformation as an effective method to transform *A. bisporus*. Mushrooms used for gill tissue transformation were grown in the Warwick HRI (Wellesbourne) Mushroom Unit and harvested at developmental stages 3 and 4 (button and closed cup, Fig. 2.5), which had pink gill lamellae, spore maturation largely absent and veil not opened yet (Burns *et al.*, 2006).



Fig. 2.5- Button mushroom at developmental stage of closed cup. Mushrooms used for gill tissue transformation were grown in the Warwick HRI (Wellesbourne) Mushroom Unit and harvested at developmental stages 3 and 4 (button and closed cup). Stage 4 is differentiated by pink gill lamellae, spore maturation largely absent and veil stretched but intact

Gill tissue was aseptically excised in 2-5 mm pieces using a scalpel and suspended in 10 ml of induced *A. tumefaciens* cells and vacuum infiltrated (600 mmHg, Dry Seal Disseicator, General Electric) for 10 min. The gill tissue (20 pieces) was then transferred to IM agar covered with sterile cellophane membrane (A.A. Packaging Limited, cat. No. M/60756/VO), supplemented with 40 µg/ml of freshly prepared acetosyringone and incubated at 20° C for 2 days. *A. bisporus* tissue was transferred to MPA supplemented with 200 µg/ml cefotaxime and the appropriate antibiotic for the selection of putative transformants. Cultures were incubated at 25° C for up to 2 weeks and then transferred to MPA for another 3 weeks.

Chapter 3

MOLECULAR CHARACTERIZATION OF ^{MVX}14.4

3. Summary

This chapter describes the molecular techniques used to fully sequence and characterize ^{MVX}14.4 dsRNA. RT-PCR *fill in* and Single Primer Amplification techniques were carried out prior to cloning and sequencing. Sequence analyses showed that ^{MVX}14.4 is the first *Endornavirus* infecting edible mushrooms to be characterized.

3.1 Introduction

3.1.1 Double-stranded RNA elements

DsRNAs elements have a wide incidence in fungi: they have been reported in all major taxonomic groups (Buck, 1986; Nuss & Koltin, 1990; Section 1.8.2). They can be either encapsidated in mainly spherical particles of 25-30 nm or unencapsidated (Zhang *et al.*, 1994). When dsRNAs occur in the absence of detectable virus-like particles, dsRNA genomes appear associated with cytoplasmic structures, such as lipid-rich host vesicles (Hansen *et al.*, 1985; Lefebvre *et al.*, 1990; Nuss & Koltin, 1990), mitochondria or chloroplasts (Rogers *et al.*, 1987; Valverde *et al.*, 1990a; Ishihara *et al.*, 1993; Koga *et al.*, 2003). This cytoplasmic location of mycoviruses is a consequence of their limited means of transmission. No known vectors or evidence for their existence outside the host cytoplasm has been reported (Zhang *et al.*, 1994). Because of the high frequency of mating among fungi (Deacon, 1984), virus transmission through anastomosis is very efficient (Wickner, 1992) making the presence of a viral capsid unnecessary. For this reason, cytoplasmic genetic elements are the biological equivalent of conventional viruses for fungi (Wickner, 1992; Hong *et al.*, 1998). The intracellular life cycle of mycoviruses might also play an important role in pathogenicity. Fungi and mycoviruses have co-evolved and co-adapted in such a way that most mycoviruses are symptomless or even benign (Section 1.8.5).

In recent decades the scientific community has become increasingly aware of the presence of unencapsidated, linear dsRNA molecules in a wide variety of organisms, including a wide range of plants such as the common bean (Wakarchuk & Hamilton, 1985; Mackenzie *et al.*,

1988), cassava (Gabriel, 1987), alfalfa (Fairbanks *et al.*, 1988), pepper (Valverde *et al.*, 1990a; Valverde & Fontenot, 1991), cultivated rice (Moriyama *et al.*, 1995; Fukuhara *et al.*, 1995), wild rice (Moriyama *et al.*, 1995; Fukuhara *et al.*, 1995), broad bean (Pfeiffer, 1998); green algae (Ishihara *et al.*, 1992; Koga *et al.*, 1998; Koga *et al.*, 2003); insects (Miyazaki *et al.*, 1996) and protozoa (Wang & Wang, 1986, 1991; Stuart *et al.*, 1992). These elements have been termed endogenous dsRNAs (Fukuhara, 1999).

Although most plants and fungi possess endogenous dsRNA elements, fewer protozoa and animals appear to harbour such molecules (Miyazaki *et al.*, 1996). This observation led to the hypothesis that cell walls might serve as a barrier to the release of dsRNAs from cells (Brown & Finnegan, 1989). These dsRNA elements, ranging in size from 1.5 kbp to 20 kbp (Fukuhara, 1999) have some intriguing plasmid-like properties, which differ from those of conventional plant viruses (Moriyama *et al.*, 1999), but appear more similar to the mycovirus features: 1) they mostly have no obvious effects on their hosts; 2) their inheritance is mainly vertical; 3) they are present at a low constant concentration in their host cells (*ca* 100 copies/cell); 4) they are not associated with virus-like particles; 5) they are not transcripts of cellular DNAs (Moriyama *et al.*, 1995; Moriyama *et al.*, 1999).

Sequencing of some large endogenous RNAs longer than 10 kbp (Pfeiffer, 1998; Fukuhara, 1999, Coutts, 2005; Hacker *et al.*, 2005; Valverde & Gutierrez, 2005; Fukuhara *et al.*, 2006; Osaki *et al.*, 2006; Tuomivirta & Hantula, 2006) has revealed important information about a new viral group of virus, the *Endornavirus*, that infect plants, fungi and protists (Gibbs *et al.*, 2000; Fukuhara *et al.*, 2006; ICTVdB Management, 2006; Section 1.8.2).

3.1.2 A new virus genus: *Endornavirus*

3.1.2.1 *Endornavirus* and Symptoms

Endornaviruses have been mainly reported in plants, such as barley, bell pepper, broad bean, kidney bean, melon, bottle gourd, malabar, rice, spinach, seagrass (Pfeiffer, 1998; Gibbs *et al.*, 2000; Coutts, 2005; Fukuhara *et al.*, 2006; Valverde & Gutierrez, 2005).

However, some non-plant endornaviruses have been recently reported in protists such *Phytophthora* spp. isolate P441 (Hacker *et al.*, 2005) and fungi such as *Helicobasidium mompa* and *Gremmeniella abietina* type B (Osaki *et al.*, 2006; Tuomivirta & Hantula, 2006). Endornaviruses successfully fully sequenced have been described in cultivated rice (*Oryza sativa* spp. japonica; Moriyama *et al.*, 1995), wild rice (*Oryza rufipogon*; Moriyama *et al.*, 1999), broad bean (*Vicia faba* cv. 447; Pfeiffer, 1998), the ascomycete *G. abietina* (Tuomivirta & Hantula, 2006), and the basidiomycete *H. mompa* (Osaki *et al.*, 2006). Endornaviruses are similar to cryptoviruses (Boccardo *et al.*, 1987; Milne & Natsuaki, 1994; Section 1.8.2) in that they are efficiently transmitted through seed in the absence of vectors and are not associated with disease symptoms, with the exception of broad bean (Pfeiffer, 1993) and *H. mompa* (Osaki *et al.*, 2006). However, unlike the cryptoviruses, which produce particles containing a dsRNA bipartite genome about 2-3 kbp long, none of the endornaviruses have been reported to be associated with particles and their dsRNA genome is longer than 10 kbp (Gibbs *et al.*, 2000).

In broad bean line '447' a cytoplasmic male sterility (CMS) trait (Pfeiffer, 1998) deviates from the classical pattern of genetic male sterility described in several plants (Dewey *et al.*, 1986; Young & Hanson 1987; Lewings, 1993). The latter results from a mitochondrial dysfunction restricted to anther tissues leading to plants unable to shed viable pollen. At molecular level, CMS correlates with the expression of non-functional variant polypeptides produced as a consequence of mitochondrial rearrangements (Belliard *et al.*, 1979; Boeshore *et al.*, 1985). The '447' CMS line of *Vicia faba* is unusual in that CMS does not result from mitochondrial DNA rearrangements, but rather correlates with the presence of a new dsRNA virus, named *Vicia faba endornavirus* (VFV; Pfeiffer, 1998; ICTVdB Management, 2006). This virus is transmitted exclusively in a vertical mode and is permanently lost after restoration of male fertility by crossing with a restorer line, or as a consequence of spontaneous reversion to fertility (Scalla *et al.*, 1981). Early electron microscopy observations (Edwardson *et al.*, 1976) revealed that all tissues of male-sterile plants contain cytoplasmic membranous vesicles, which disappear after restoration of male fertility. These membranous structures do contain dsRNA molecules associated with RdRp

activity. They possibly represent a virus lacking capsid protein, maintained in the form of a replicative complex and contained in host-derived membranous vesicles rather than virions, very reminiscent of *Cryphonectria parasitica hypovirus* (Hansen *et al.*, 1985; Section 1.8.2 and Section 1.8.5.2). However, the involvement of VFV in the CMS trait remains circumstantial, since sequencing information to support this hypothesis has not been found and all attempts to convert fertile plants to male sterility by inoculation failed (Turpen *et al.*, 1988).

Helicobasidium mompa endornavirus 1-670 was identified as a hypovirulence factor in the basidiomycete *H. mompa*, causing violet root rot disease on more than 100 plant species (reviewed by Osaki *et al.*, 2006). Hypovirulence can be demonstrated by two experimental results: i) enhanced virulence after elimination of dsRNA virus and ii) reduction of virulence after reintroduction of the dsRNA virus (Ikeda *et al.*, 2003). Ikeda *et al.* (2003) demonstrated the relation between HmEV1-670 and hypovirulence in *H. mompa* removing the endornavirus by hyphal tip isolation technique and then reintroducing it by pairing the strain with an HmEV1-670 donor. Hypovirulence factor is increasingly proposed as a biocontrol agent (Section 1.8.5.2) for fungal pathogens such as *Rhizoctonia solani* (Castanho & Butler, 1978), *Ophiostoma ulmi* (Rogers & Buck, 1986), *Leucostoma persoonii* (Hammar *et al.*, 1989), *Cryphonectria parasitica* (Nuss, 1992), *Helminthosporium victoriae* (Huang & Ghabrial, 1996), *Sclerotinia homoeocarpa* (Zhou & Boland, 1997), and *Fusarium graminearum* (Chu *et al.*, 2002).

3.1.2.2 *Endornavirus* and Molecular Structure

The double-stranded nature of Endornaviruses has been confirmed by several authors through column chromatography on CF-11 cellulose and treatment with nucleases (Morris & Dodds, 1979; Schuster & Sisco, 1986; Lefebvre *et al.*, 1990). The dsRNAs are resistant to DNase I and RNase A in high-salt buffer, but sensitive to RNase A in low-salt buffer (Fukuhara, 1999). Although purification of virus-like particles was attempted by several procedures, no viral capsids have ever been detected by electron microscopy (Fukuhara, 1999). Differential centrifugation and sucrose density-gradient centrifugation were used to

determine subcellular localization of dsRNAs. Detailed examinations have revealed that endornaviruses are localized in cytoplasmic vesicles (Mackenzie *et al.*, 1988; Lefebvre *et al.*, 1990; Moriyama *et al.*, 1996) and are associated with their replicase (Lefebvre *et al.*, 1990). This enzyme is able to pursue *in vitro* RNA synthesis on preinitiated complexes, even after these have been released from the membranous vesicles by treatment with non-ionic detergent (Pfeiffer *et al.*, 1993). Labelled NTPs are incorporated exclusively into the dsRNA and resist RNase digestion at high salt concentrations. Newly synthesized RNA therefore remains associated with its template, indicating that RNA replication proceeds by a strand displacement mechanism according to a semi-conservative replication model (Pfeiffer *et al.*, 1993; Section 1.8.3).

3.1.2.3 *Endornavirus* and Genome Structure

Endornaviruses have a dsRNA genome longer than 10 kbp (Horiuchi & Fukuhara, 2004) encoding for a single open reading frame (ORF). Conserved motifs for RNA-dependent RNA polymerase (RdRp) and RNA helicase (Hel) have been found within all *Endornavirus* ORFs. An UDP glycosyltransferase (UGT) domain has also been found in *Oryza sativa* endornavirus (OSV), *Oryza rufipogon* endornavirus (ORV), *Phytophthora* endornavirus 1 (PEV1, Hacker *et al.*, 2005) and *Helicobasidium mompa* endornavirus 1-670 (Osaki *et al.*, 2006). The large putative proteins encoded by endornaviruses are assumed to be polyproteins that are processed by virus-encoded proteinases according to cleavage mechanisms reported for other viruses (Seipelt *et al.*, 1999; Adams *et al.*, 2005). However, proteinase motifs or proteolytic cleavage sites have not yet been reported for endornaviruses (Hacker *et al.*, 2005; Osaki *et al.*, 2006).

Previous laboratory work carried out at Warwick HRI produced 558 bp of cDNA sequence from the ^{MVX}14.4 dsRNA element (^{MVX}14.4) using random-primed cloning (Section 1.8.6.2.2). The chapter describes how ^{MVX}14.4 was fully sequenced and characterized revealing its *Endornavirus* features.

3.2 Materials and Methods

3.2.1 Isolations and Purifications of ^{MVX}14.4 dsRNA

^{MVX}14.4 infected-*A. bisporus* isolates were obtained from the epidemiological experiment, SSI experiment, carried out at Warwick HRI and described further in Chapter 4. The main source of ^{MVX}14.4 used was a single-spore isolate (SSI 61), which harboured only ^{MVX}14.4 as ^{MVX}dsRNA element. Thus, ^{MVX}14.4 was extracted from SSI 61 mushrooms using the dsRNA extraction method described in Section 2.5.1, digested with DNase and S1 nuclease (Section 2.7) to remove any remaining traces of DNA and ssRNA, and used as template for downstream RT-PCR *fill in* protocol.

3.2.2 RT-PCR *fill in*

Earlier work at Warwick HRI yielded 558 bp of ^{MVX}14.4 sequence (Adie *et al.*, 2004). ^{MVX}dsRNAs were extracted, purified and used as template for random-primed cDNA synthesis, cloning and sequencing. The identity of generated sequences was confirmed using Northern blotting.

In the present study, RT-PCR *fill in* using sequence-specific primers was used to expand the ^{MVX}14.4 sequence and bridge the gaps between non-overlapping clones. Given that the orientation and relative position of contigs were unknown, *ca* 80 primers targeting various contigs were designed and used as a pool (maximum 5 primers-pool) in RT-PCR *fill-in*. Primers were designed in such a way to span at least 100 bp of the known sequence.

cDNA synthesis reactions were prepared as follows: 0.5 µl of each 10 µM P_A primer (designed for a target contig) were mixed with 0.5 µl of each 10 µM P_B primer (designed for a different contig), 300 ng of purified RNA template and DMPC water up to 10 µl total volume. Reactions were boiled for 5 min and then placed on ice. The second step-reaction was performed in 20 µl volume and comprised the following: 1 µl Superscript™ II Reverse Transcriptase (200 U/µl, Invitrogen cat. No.18064-022), 4 µl of 5x cDNA synthesis buffer,

2 µl dNTPs mix (10 mM), 1 µl DTT (0.1M), 1 µl Rnase OUT™ (40 U/µl, Invitrogen cat. No.11146-024), and 10 µl template (from the previous step). cDNA synthesis was progressed using a HYBAID MBS 0.5 G Thermal Cycler with the following parameters: 50° C for 45 min (cDNA synthesis), 85° C for 5 min (termination). To remove any remaining RNA template, each cDNA reaction was treated with 1 µl *E. coli* RNase H (2 U/µl, Invitrogen) at 37° C for 20 min.

PCR amplification of cDNA products were performed in 50 µl volume reactions as follows: 0.5 µl Expand Long Template enzyme mix (Roche, cat. No. 1681834), 5 µl of 10x buffer I, 2 µl dNTPs (10 mM mix), 0.5 µl of 10 µM P_A primer (already used for cDNA synthesis), 0.5 µl of 10 µM P_B primer (already used for cDNA synthesis), 3 µl cDNA. PCR reactions were progressed using a HYBAID MBS 0.2 G Thermal Cycler according to the following parameters: initial denaturation at 94° C for 2 min, followed by 10 cycles of 94° C for 15 s, 50° C for 30 s, 68° C for 8 s, subsequently 20 cycles of 94° C for 15 s, 50° C for 30 s, 68° C for 8s with 5 s increment each cycle, and a final extension at 72° C for 5 min.

3.2.3 Single Primer Amplification Technique (SPAT)

To determine the ^{MVX}14.4 dsRNA termini, an adaptation of the SPAT technique published by Shapiro *et al.* (2005) and modified by A. Soares (Warwick HRI) was performed (Fig. 3.1 and Fig. 3.2). Extracted dsRNA (500 ng, Section 3.2.1) was ligated to an anchor, 5'-GACCTCTGAGGATTCTAAAC/iSp9/TCCAGTTTAGAATCC-3' (iSp9 is a carbon chain spacer) using 10 U T4 RNA ligase in a 10 µl reaction (New England Biolabs, cat. No M0204S). The ligation reaction was incubated at 10° C for 12 h and the product was precipitated at room temperature using Pellet Paint® NT Co-Precipitant (Novagen, cat. No. 70748) in 100 µl total volume as follows: 2 µl of Pellet Paint® were added to the ligation reaction, followed by 0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol. After gently mixing, the reaction was centrifuged at 12,000 xg for 5 min. The pellet was rinsed with 2 volumes of 75% v/v ethanol, centrifuged again and air-dried prior to resuspension in 8 µl of water to the pellet.

DETERMINATION of the 5' TERMINUS

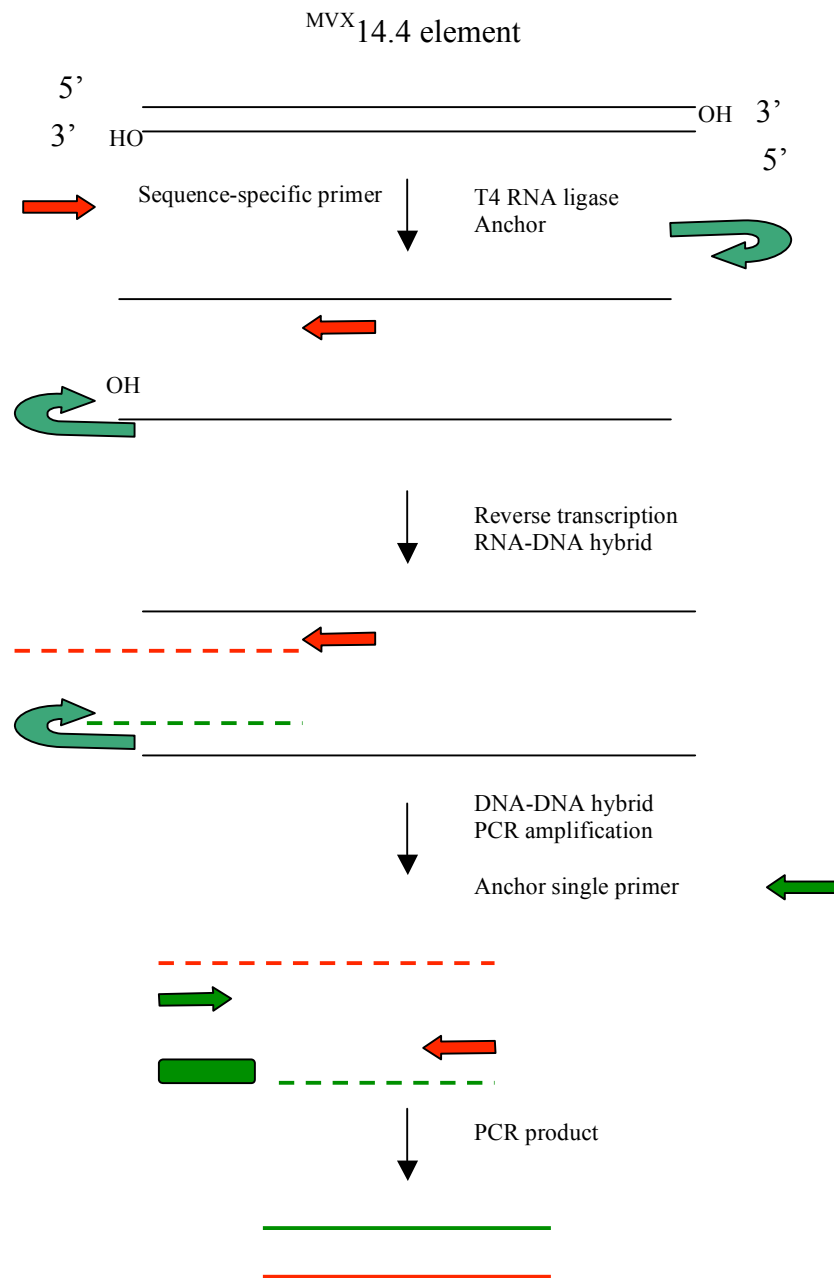


Fig. 3.1- Single Primer Amplification Technique to determine the 5' end. DsRNA was ligated to an anchor by T4 RNA ligase in order to carry out the cDNA synthesis using also a sequence-specific primer (complementary to the positive RNA strand). PCR was subsequently performed using the sequence-specific primer (used for the cDNA synthesis) and a single anchor primer (complementary to the anchor)

DETERMINATION of the 3' TERMINUS

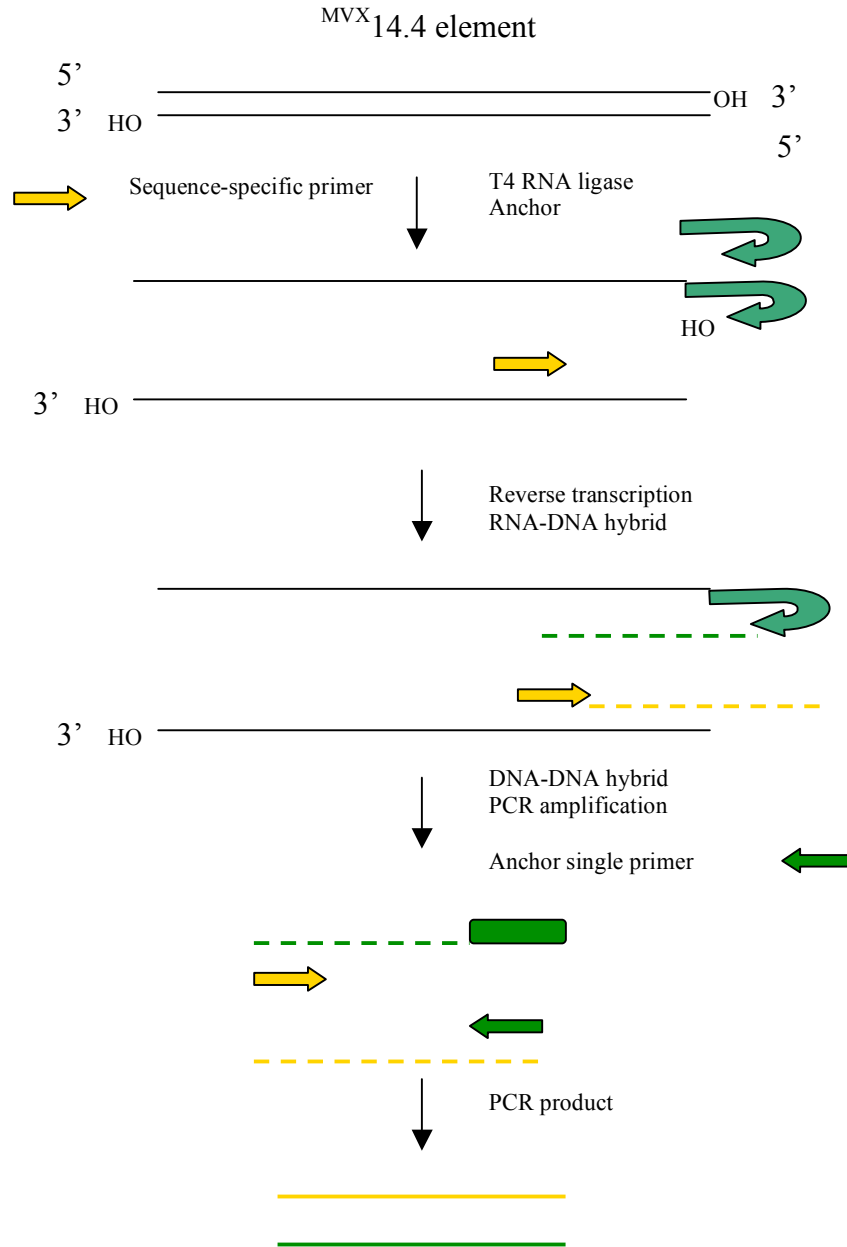


Fig. 3.2- Single Primer Amplification Technique to determine the 3' end. DsRNA was ligated to an anchor by T4 RNA ligase in order to carry out the cDNA synthesis using also a sequence-specific primer (complementary to the negative RNA strand). PCR was subsequently performed using the sequence-specific primer (used for the cDNA synthesis) and a single anchor primer (complementary to the anchor)

Following addition of 2 µl of specific primer (20 µM), the tube was boiled for 5 min and then kept on ice. ^{MVX}14.4 specific primers used in the successful RT-PCR protocol were as follows: c655R376 primer (5'- AAGCTGCCACGTCGTTGTAG-3') and Band3R268 (5'- GACTTCCGTGACTCCTGTTG-3') to determine the 5'-end, or c655F11909 (5'- TATGCTGTTGCTGGGTGATG-3') to determine the 3'-end.

The cDNA was synthesized using 1 µl Superscript™ II Reverse Transcriptase (200U/µl, Invitrogen cat. No.18064-022), 4 µl of 5x cDNA synthesis buffer, 2 µl dNTPs mix (10 mM), 1 µl DTT (0.1M), 1 µl Rnase OUT™ (40 U/µl, Invitrogen cat. No.11146-024), and 10 µl ligation reaction (from the previous step). cDNA synthesis was progressed using a HYBAID MBS 0.5 G Thermal Cycler with the following parameters: 42° C for 60 min (cDNA synthesis); 70° C for 15 min (termination). Finally, each cDNA reactions were treated with *E. coli* RNase H (Section 3.2.2).

Synthesized cDNA was purified using Pellet Paint® precipitation as above. PCR reactions were performed in 50 µl volumes using: 1 µl Expand Long Template enzyme mix (Roche, cat. No. 1681834), 5 µl of 10x buffer I, 2 µl dNTPs (10 mM mix), 2 µl of 20 µM anchor's single primer (5'-GAGGGATCCAGTTTAGAATCCTCAGAGGTC-3'), 2 µl of 20 µM specific primer (already used for cDNA synthesis), 26.5 µl cDNA. PCR reactions were progressed using a HYBAID MBS 0.2 G Thermal Cycler according to the following parameters: initial denaturation at 96° C for 2 min, followed by 35 cycles of 94° C for 30 s, 55° C for 30 s, 72° C for 3 min, and a final extension at 72° C for 10 min.

3.2.4 cDNA Cloning, Sequencing and Sequence Analysis

Amplified RT-PCR products were gel purified (Section 2.9.1) and ligated into pGEM®-T Easy vector (Section 2.10.1). Ligation reactions and transformation of DH5α™ or XL1-Blue *E. coli* competent cells were performed as described in Section 2.10.1. To confirm the presence of the clonal inserts, pGEM®-T Easy clones were restricted using *Not* I endonuclease (Roche, cat. No. 1014706), according to the manufacturer's protocol. Sequencing reactions were performed to generate sequences from both ends of clonal

inserts using ABI Prism® BigDye™ Terminator cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase, Fs (Perkin-Elmer Applied Biosystems, cat. No. 403044).

3.2.5 Searching for ^{MVX}14.4 sequence in *Agaricus bisporus* DNA

In order to determine specificity of ^{MVX}14.4 dsRNA sequences, PCR screening was performed on *A. bisporus* DNA using 6 pairs of primers designed for ^{MVX}14.4 RdRp and helicase regions (Table 3.1). *A. bisporus* DNA template was Chelex purified as described in Section 2.5.3. Primers designed were preliminary tested by RT-PCR using a control template (isolate SSI 61, further described in Chapter 4).

Table 3.1- Primers amplifying the ^{MVX}14.4 helicase and RdRp regions

Primer	Primer sequence Reference: this thesis	Product Size (bp)	DNA/RNA Target
B3f321 B3r634	5'-GGACAACTCAGAACTAATG-3' 5'-ATCGGAGAGCTAAAGAAATG-3'	314	^{MVX} 14.4 helicase
B3f114 B3r634	5'-AACGGTTTCAACAACAATCG-3' 5'-ATCGGAGAGCTAAAGAAATG-3'	521	^{MVX} 14.4 helicase
B3f92 B3r673	5'-CCTCGTTACCTGGTAACTG-3' 5'-GTATCATCGCTATGACTTCC-3'	582	^{MVX} 14.4 helicase
B3f2980 B3r3564	5'-TTTGGCGCAAGACACATGAG-3' 5'-GCTGCATCATTCGTCATACC-3'	585	^{MVX} 14.4 RdRp
B3f3038 B3r3319	5'-AAGTTGGATGCGTCTCACTG-3' 5'-GACATACTGCCGTCTTGTG-3'	282	^{MVX} 14.4 RdRp
B3f2823 B3r3332	5'-GAATTGGCAGCCAGAATGAG-3' 5'-GTCAGGACAGACAGACATAC-3'	510	^{MVX} 14.4 RdRp

Further assessment for sequence homology between ^{MVX}14.4 dsRNA and *A. bisporus* DNA led to sequence similarity analysis (BLAST search) of the ^{MVX}14.4 RdRp against cellular RdRps, present in public database (NCBI).

3.3 Results

3.3.1 Nucleotide sequence of ^{MVX}14.4 and deduced amino acid sequence

The completed *consensus* sequence of ^{MVX}14.4 dsRNA, assembled from more than 130 overlapping RT-PCR *fill in* sequences (Fig. 3.3 and Fig. 3.4) was 12750 bp (Annex 3.1). All regions were sequenced from more than two independently synthesized clones (Fig. 3.5). The ^{MVX}14.4 dsRNA termini were determined from at least three cDNA clones recovered from independent dsRNA extractions. For the 5' end, 7 sequences were generated, whereas 12 sequences were recovered for the 3' end. Integrity of the assembled *consensus* sequence was confirmed by RT-PCR screening using primers that span different regions of the entire sequence.

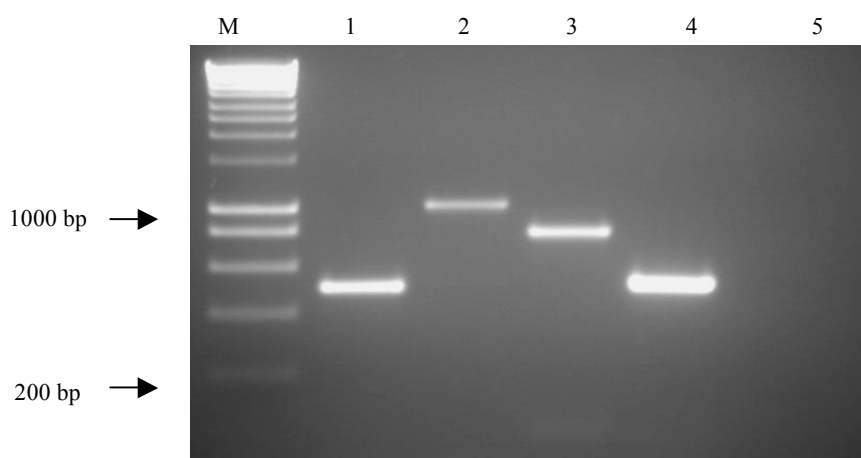


Fig. 3.3- RT-PCR *fill in* products. Examples of RT-PCR *fill in* products on 1% agarose gel. Lane 1-3 = RT-PCR *fill in* products; lane 4 = RT-PCR positive control (strain SSI 61); lane 5 = RT-PCR negative control (water); M = hyperladder I (Bioline)

A single open reading frame (ORF) was found in the plus strand (Annex 3.2), starting at nt 29 and ending at nt 12679, which encodes a putative protein of 4216 aa (369.13 kDa). This would imply a 5'-untranslated region (UTR) of 28 nt. The first methionine codon nt 29-31 is in a favourable context for translation initiation, with purine (A) residues at the -3 and +1 positions, according to Kozak's rules for ribosomal scanning (Kozak, 1986; Lütcke *et al.*, 1987).

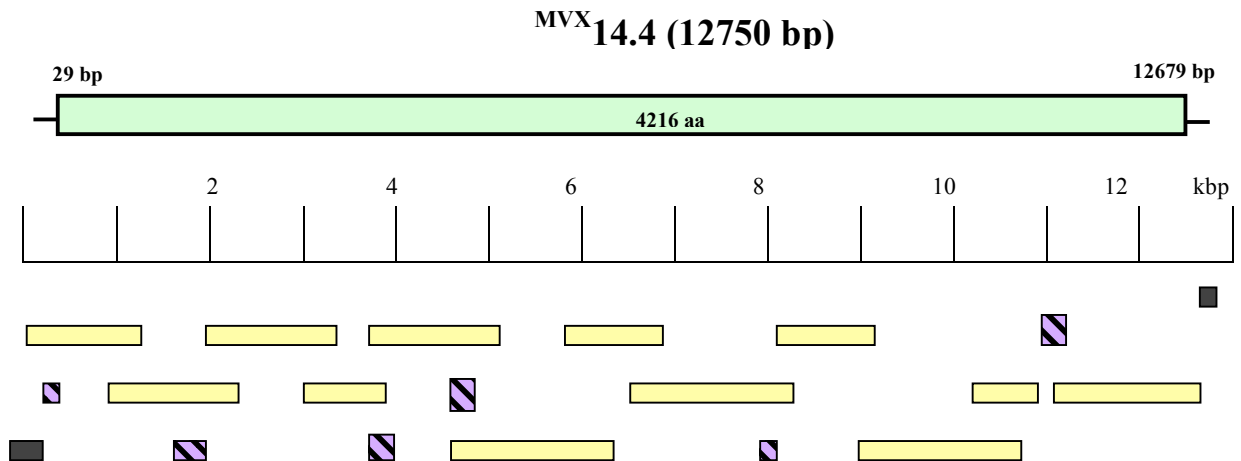


Fig. 3.4- Map of the major cDNA clones of ^{MVX}14.4 from *A. bisporus*. Specific-primed cDNA clones are represented by open boxes, random-primed cDNA clones are indicated by hatched shading boxes, and SPAT cDNA clones of the extremities by filled boxes

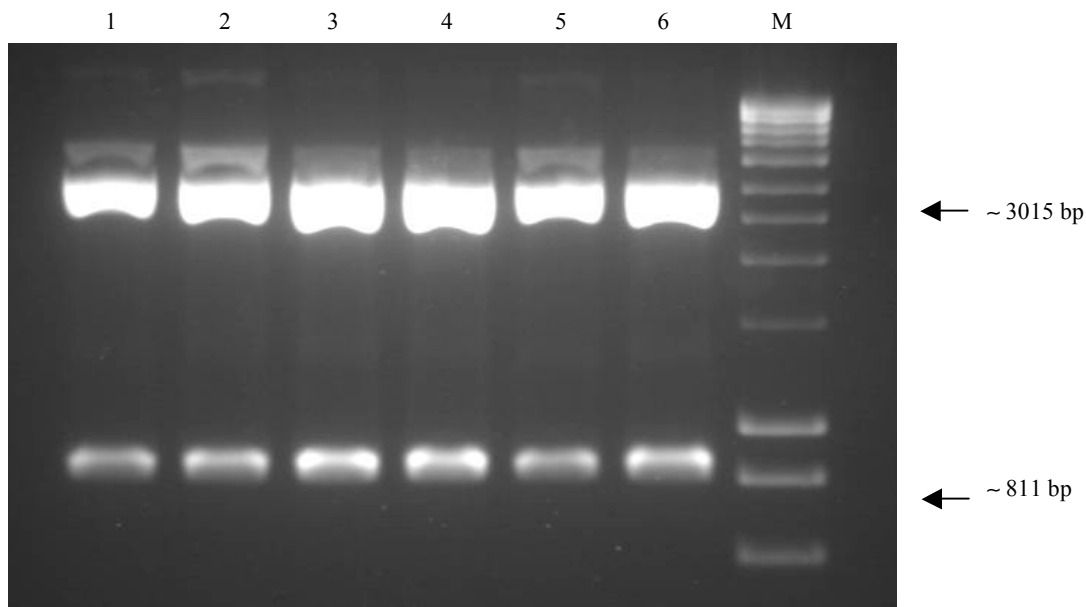


Fig. 3.5- Consistency of insert for the *Not* I digestion of RT-PCR *fill in* clones. *Not* I enzyme cut the insert (811 bp) out from pGEM[®]-T Easy vectors (3015 bp). Lane 1-6 = *Not* I-digested products of purified plasmids on 1.5% agarose gel; M = hyperladder I (Bioline)

The 3'-untranslated region (UTR) is 71 bp long, including a run of seven G residues at the 3' terminus. Other significant ORFs were not detected in alternative reading frames of the plus and minus strands of ^{MVX}14.4 dsRNA. A BLASTX search using the complete

sequence of the ^{MVX}14.4 dsRNA showed significant similarity to putative polyprotein sequences encoded by members of the novel virus genus *Endornavirus*, recently accepted by the ICTV (ICTVdB Management, 2006; Section 1.8.2 and 3.1.2). The best sequence similarity identified was with *Helicobasidium mompa* endornavirus 1-670 (HmEV1-670, composite E value = $11e^{-132}$), followed by *Vicia faba* endornavirus (VFV, composite E value = $5e^{-118}$), *Phytophthora* endornavirus 1 (PEV1, composite E value = $15e^{-115}$), *Oryza sativa* endornavirus (OSV, composite E value = $8e^{-111}$) (Annex 3.1-CD). Other than the *Endornavirus* genus, the next most similar alignments were with regions of RdRp of several viruses belonging to the *Closteroviridae* family, such as *Mint vein banding virus* (E value = $4e^{-11}$) and *Strawberry chlorotic fleck associated virus* (E value = $3e^{-10}$). The size of ^{MVX}14.4 (12750 bp) was most similar to PEV1 (13883 bp) (Fig. 3.6). The amino acid sequence of ^{MVX}14.4 showed a wide range of similarity with non-plant and plant endornaviruses (Table 3.2).

Table 3.2- Regions of significant similarity between ^{MVX}14.4 and PEV1, HmEV1-670, OSV, and VFV amino acid sequences identified by pairwise comparison using NCBI database

AMINO ACID RESIDUES		IDENTITY (%)	SIMILARITY (%)
^{MVX} 14.4	PEV1		
543-1580	543-1673	21%	38%
2737-2802	3106-3171	31%	53%
3716-4214	4117-4612	35%	53%
^{MVX} 14.4	HmEV1- 670		
488-2796	933-3577	20%	36%
2800-3700	-	No identity	No similarity
3723-4198	4883-5357	34%	54%
^{MVX} 14.4	GaEV		
1-3700	-	No identity	No similarity
3727-4158	2913-3343	26%	45%
^{MVX} 14.4	OSV		
495-1614	558-1785	21%	38%
1615-3600	-	No identity	No similarity
3604-4208	3984-4566	33%	51%
^{MVX} 14.4	VFV		
294-818	1593-2226	22%	40%
820-3700	-	No identity	No similarity
3722-4184	5239-5736	34%	52%

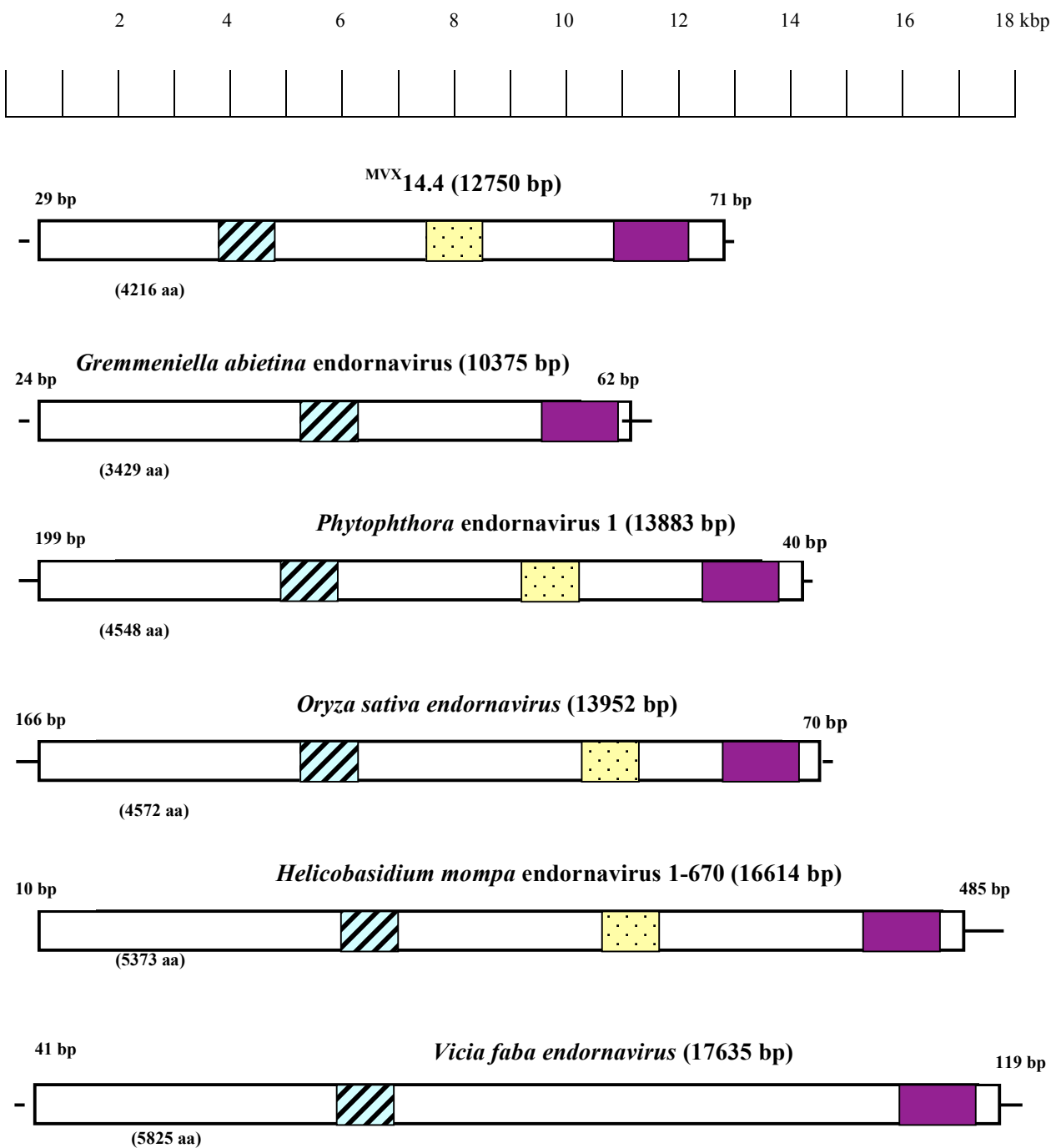


Fig. 3.6- Comparison of genome organization of ^{MVX}14.4, *Phytophthora* endornavirus 1, *Oryza sativa* endornavirus, *Helicobasidium mompa* endornavirus 1-670, and *Vicia faba* endornavirus. Small boxes indicate the positions of the helicase-like (diagonally hatched), UGT-like (dotted), and RdRp-like (filled) regions within the polyprotein. Horizontal lines on either side of the long rectangular boxes (ORFs) indicate the 5'- and 3'-UTRs. ORF lengths are indicated in brackets

Although the upstream region between aa 294 and aa 818 showed the highest similarity with VFV (identity = 22%; similarity = 40%); the other two-thirds of the polyprotein showed highest similarity with PEV1 and HmEV1-670.

3.3.2 RdRp-like, Helicase-like, and UGT-like regions

The region of ^{MVX}14.4 dsRNA polyprotein with the highest sequence similarity to *Endornavirus* was located near the C-terminus. Inspection of this sequence showed that conserved motifs characteristic of RNA-dependent RNA polymerase (RdRp) were present in ^{MVX}14.4 dsRNA. Motifs found (between *ca* aa 3700 and aa 4150) represented the signature of ssRNA virus superfamily III (Koonin & Dolja, 1993). Fig. 3.7 shows alignment of ^{MVX}14.4 with other 8 amino acid sequences within the conserved RdRp motifs III-VI (Koonin, 1991; Koonin & Dolja, 1993). ^{MVX}14.4 RdRp motifs showed high conservation with those of other endornaviruses. Neighbour-joining phylogenetic analysis of RdRp (motifs III-VI) of various endornaviruses and ssRNA viruses showed that ^{MVX}14.4 clustered within an *Endornavirus* clade (79% bootstrap support, Fig. 3.8). Preliminary phylogenetic analyses, using more diverse RdRps confirmed the highest similarity between endornaviruses and ssRNA viruses belonging to the alpha-like superfamily (data not shown). Pairwise identity analysis indicated a plant endornavirus (OSV) RdRp as the most similar to the ^{MVX}14.4 RdRp (59% similarity; Annex 3.3).

Another region of ^{MVX}14.4 polyprotein with high similarity to endornaviruses was located near the N-terminal part of the protein. Further inspection of this region (between *ca* aa 1300 and aa 1580) showed conserved motifs characteristic of RNA helicases of superfamily I (Koonin & Dolja, 1993, Fig. 3.9). When ^{MVX}14.4 sequence identified as helicase motif I-VI was used for BLASTP searching and pairwise identity analysis, the best match was with PEV1 (E value = $5e^{-15}$, 31% similarity; Annex 3.2-CD and Annex 3.3), which has previously been reported to contain helicase-like domains (Hacker *et al.*, 2005). The next most similar alignment was with helicase-like regions of *Phaseolus vulgaris* dsRNA element (E value = $3e^{-14}$) and VFV (E value = $2e^{-13}$).

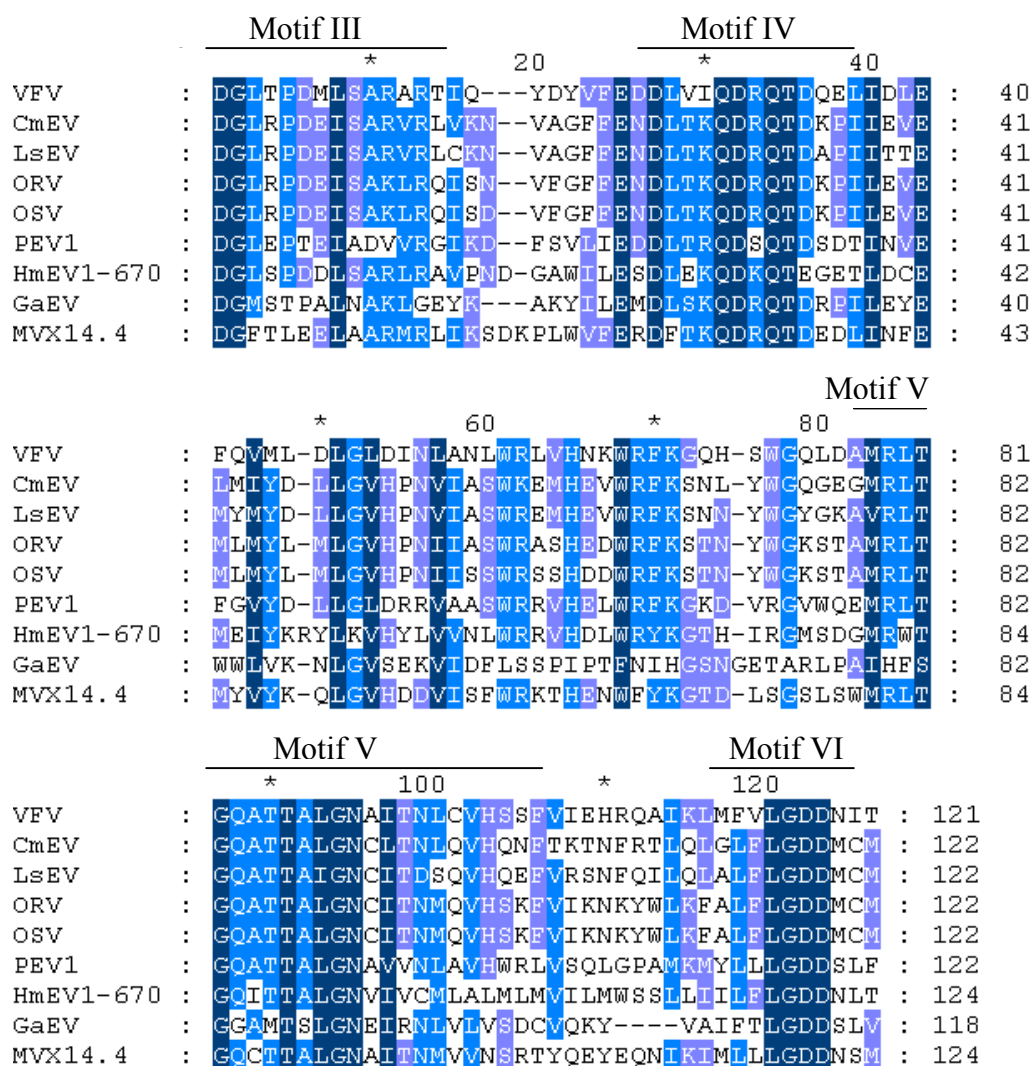


Fig. 3.7- ^{MVX}14.4 RdRp motifs. A multiple alignments of the amino acid sequences within the conserved RdRp motifs III-VI (Koonin, 1993) was constructed for 9 endornaviruses, including ^{MVX}14.4 using ClustalW algorithm. ^{MVX}14.4 RdRp motifs showed to be consistent with those of other endornaviruses. VFV: *Vicia faba endornavirus*; CmEV: *Cucumis melo endornavirus*; LsEV: *Lagenaria siceraria endornavirus*; ORV: *Oryza rufipogon endornavirus*; OSV: *Oryza sativa endornavirus*; PEV1: *Phytophthora endornavirus 1*; HmEV1-670: *Helicobasidium mompa endornavirus 1-670*; GaEV: *Gremmeniella abietina endornavirus*; MVX14.4: ^{MVX}14.4

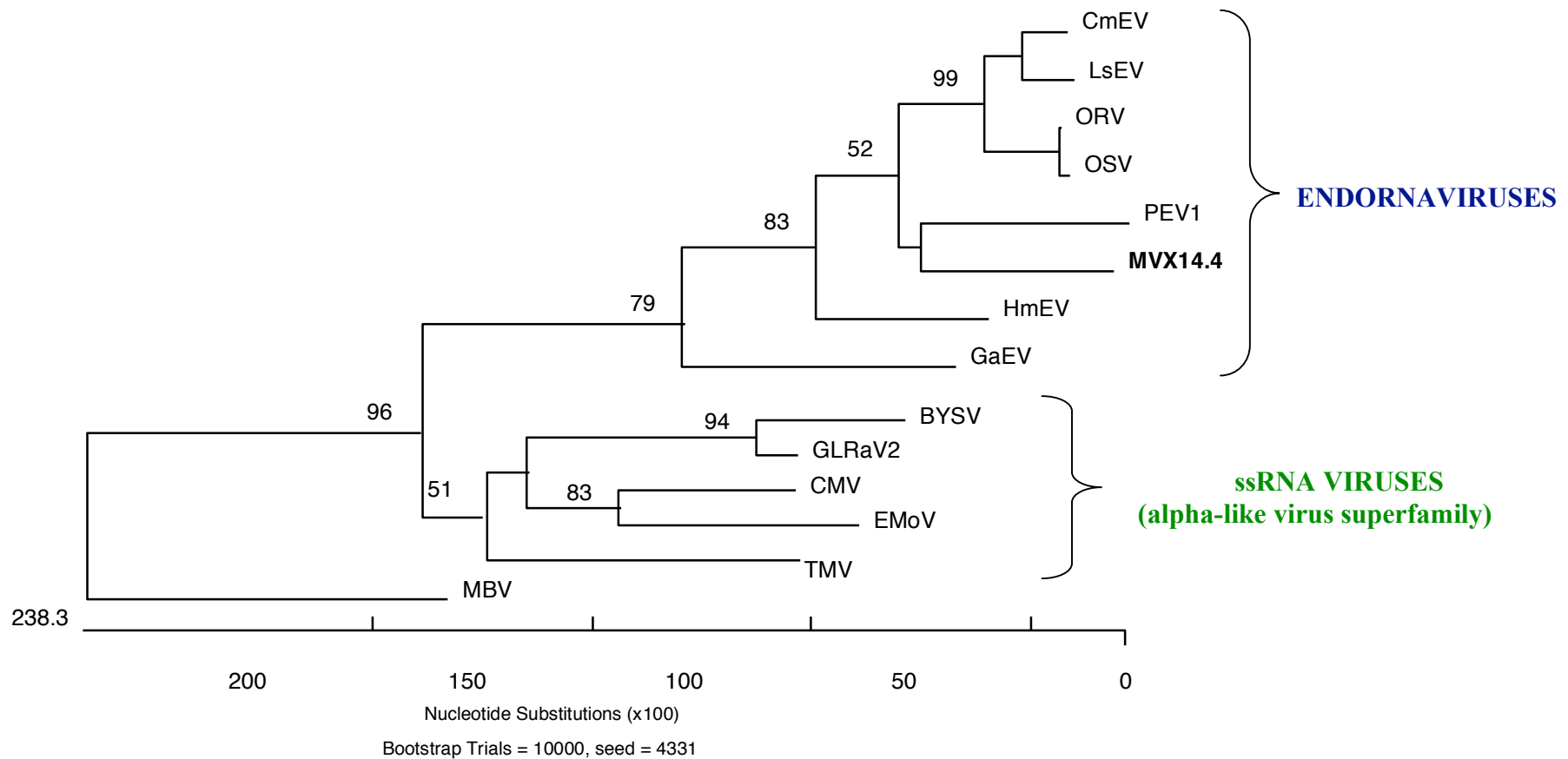


Fig. 3.8 – Phylogenetic tree of alpha-like ssRNA viruses and endornaviruses based on the RdRp domain. A Neighbour-joining phylogenetic tree of RdRp-like regions (motifs III-VI) of endornaviruses and ssRNA alpha-like viruses was constructed using the MegAlign package via ClustalW algorithm. Bootstrap support values (% of 10000 resamplings) over 50% are indicated on the branches. ^{MVX}14.4 clustered within the *Endornavirus* clade with 79% bootstrap support. CmEV: *Cucumis melo* endornavirus; LsEV: *Lagenaria siceraria* endornavirus; ORV: *Oryza rufipogon* endornavirus; OSV: *Oryza sativa* endornavirus;; PEV1: *Phytophthora endornavirus 1*; MVX14.4: ^{MVX}14.4; HmEV: *Helicobasidium mompa* endornavirus 1-670; GaEV: *Gremmeniella abietina* endornavirus; BYSV: *Beet yellow stunt virus (Closterovirus)*; GLRaV2: *Grape leafroll-associated virus 2 (Closterovirus)*; CMV: *Cucumber mosaic virus (Cucumovirus)*; EMOV: *Elm mottle virus (Ilarvirus)*; TMV: *Tobacco mosaic virus (Tobamovirus)*; MBV: *Mushroom bacilliform virus (Barnavirus)*

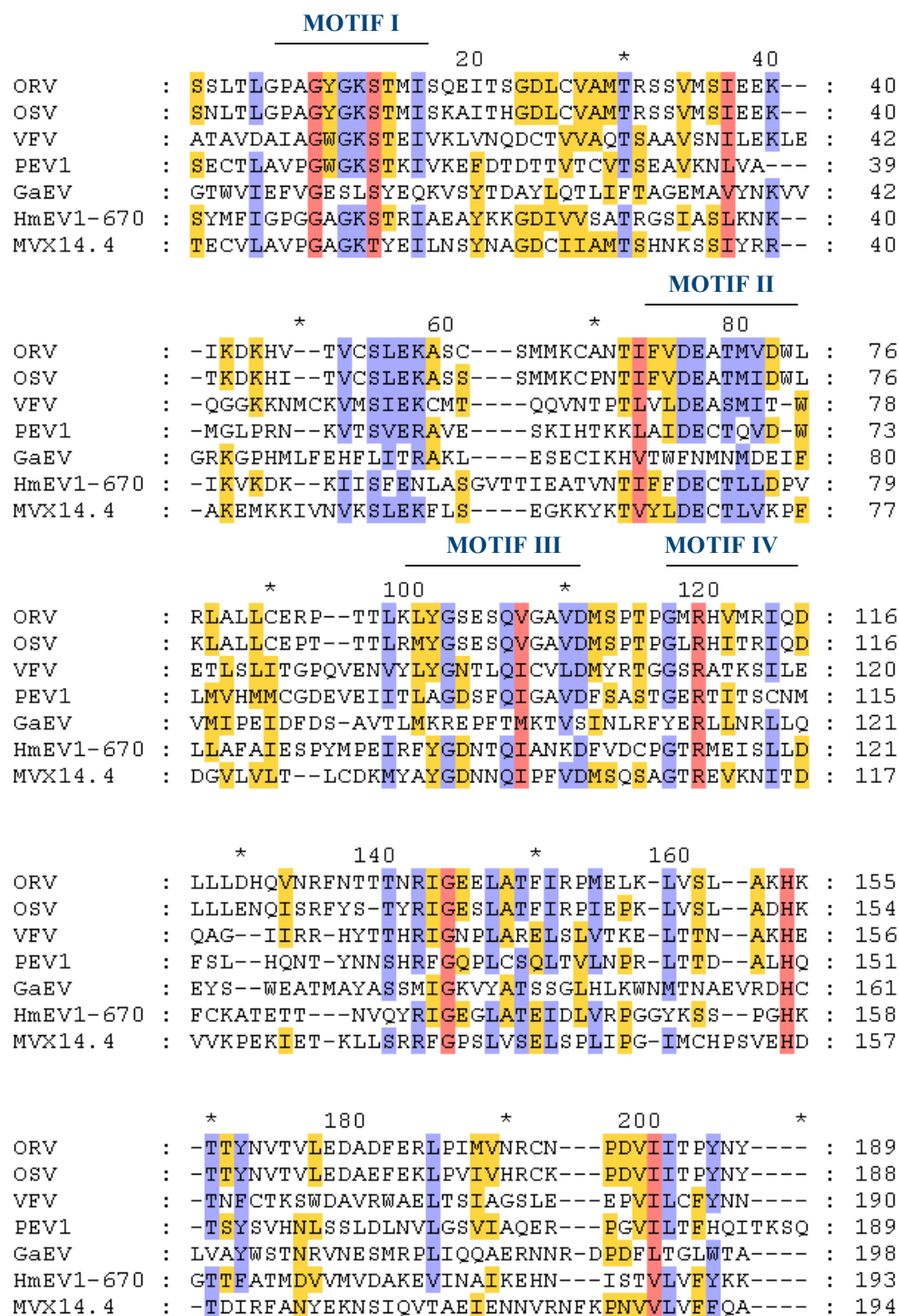


Fig. 3.9- ^{MVX}14.4 helicase motifs. A multiple alignment of the amino acid sequences within the conserved helicase motifs I-VI (Koonin, 1993) was constructed for 7 endornaviruses, including ^{MVX}14.4 using ClustalW algorithm. ORV: *Oryza rufipogon endornavirus*; OSV: *Oryza sativa endornavirus*; VFV: *Vicia faba endornavirus*; PEV1: *Phytophthora endornavirus 1*; GaEV: *Gremmeniella abietina endornavirus*; HmEV: *Helicobasidium mompa endornavirus 1-670*; MVX14.4: ^{MVX}14.4

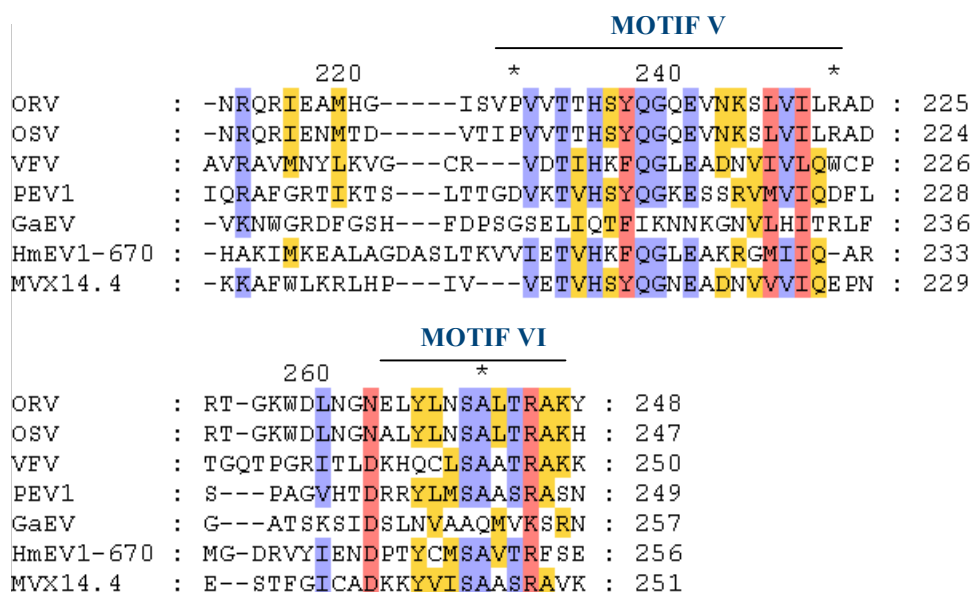


Fig. 3.9- (cont.)

A BLAST search also revealed that the region between the helicase and RdRp domains of ^{MVX}14.4 polyprotein (between *ca* aa 2450 and aa 2750) had significant homology with glycosyltransferases related to UDP-glucuronosyltransferases found in bacteria and fungi (Pfam COG1819). Fig. 3.10 shows the amino acid sequence alignment of the putative UDP-glycosyltransferases (UGTs) motif IV (Hacker *et al.*, 2005) found in ^{MVX}14.4 polyprotein with other viral, fungal and plant UGTs. The highest similarity for ^{MVX}14.4 UGT was found to be with PEV1 (41% identity), followed by the basidiomycete *Ustilago maydis* UGT (39% identity). Identity with the homobasidiomycete *Coprinus cinereus* was 26% (Annex 3.4).

Sequence similarity analysis outside the conserved RdRp, helicase, and UGT domains identified by BLASTP search confirmed similarity with HmEV1-670, except for the region between aa 2700-3700, which exhibited similarity with a retrotransposable element of the homobasidiomycete *Phanerochaete chrysosporium* (Table 3.3). The absence of capsid-like domain in the AbEV1 sequence was consistent with microscopy analyses as well as literature reported for other endornaviruses.

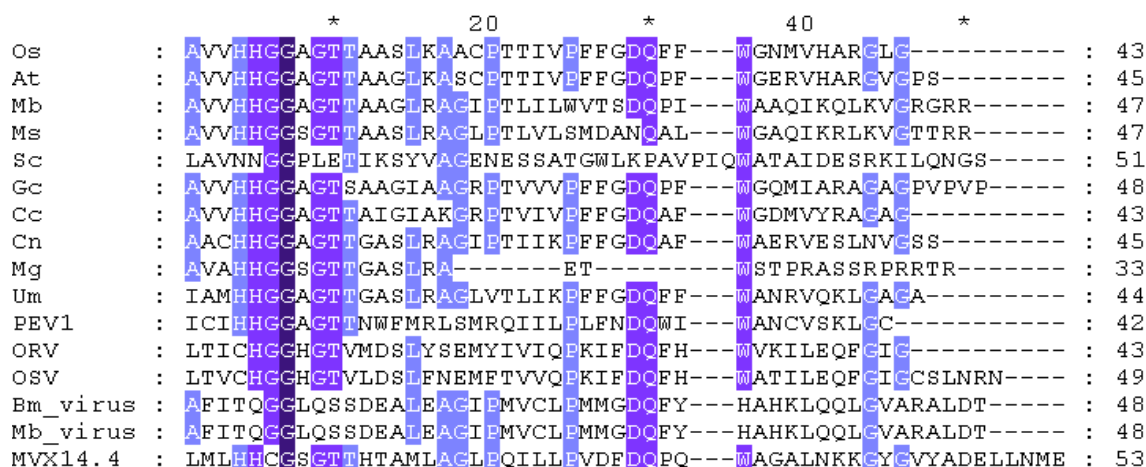


Fig. 3.10- ^{MVX}14.4 UGT motif IV. A multiple alignment of viral, fungal and plant amino acid sequences within the conserved UGT motifs IV (Hacker *et al.*, 2005) was constructed using ClustalW algorithm. Os: *Oryza sativa* (plant); At: *Arabidopsis thaliana* (plant); Mb: *Mycobacterium bovis* (bacterium); Ms: *Mycobacterium smegmatis* (bacterium); Sc: *Saccharomyces cerevisiae*; (Ascomycete); Gc: *Glomerella cingulata* (Ascomycete); Cc: *Coprinus cinereus* (Homobasidiomycete); Cn: *Cryptococcus neoformans* (Heterobasidiomycete); Mg: *Magnaphorte grisea* (Ascomycete); Um: *Ustilago maydis* (Heterobasidiomycete); PEV1: *Phytophthora endornavirus*; ORV: *Oryza rufipogon endornavirus*; OSV: *Oryza sativa endornavirus*; Bm_virus: *Bombix mori nuclear polyhedrosisvirus* (virus); Mb_virus: *Mamestra brassicae nucleopolyhedrovirus* (virus); MVX14.4: ^{MVX}14.4

Table 3.3- Similarities identified by BLASTP search (NCBI) between the ^{MVX}14.4 amino acid sequence and the database outside the conserved domains regions

AMINO ACID RESIDUES		IDENTITY (%)	SIMILARITY (%)
^{MVX} 14.4	DATABASE		
1- 1350	HmEV1-670	22%	38%
1600-2400	HmEV1-670	20%	38%
2700-3700	<i>Phanerochaete chrysosporium</i> RP-78 retrotransposable element	26%	43%

Pairwise comparison using BLASTP search (NCBI) was conducted in order to look for proteinase sequence similarity within ^{MVX}14.4 polyprotein. Analysis was carried out with chymotrypsin related cysteine, serine and papain-like cysteine proteases. No protease motifs, cysteine-rich regions nor cleavage sites were identified in ^{MVX}14.4 polyprotein.

Sequence similarity, phylogenetic and conserved motifs analyses, all supported inclusion of the ^{MVX}14.4 dsRNA element in the genus *Endornavirus*.

3.3.3 Searching for ^{MVX}14.4 sequence in *Agaricus bisporus* DNA

No ^{MVX}14.4 sequences were detected in *A. bisporus* DNA using PCR screening, whereas 18S-PCR product yielded the appropriate amplicon (data not shown). In order to test the reliability of the primers used, a preliminary RT-PCR was performed using ^{MVX}14.4-infected control material as template, giving positive results (data not shown).

No similarities were observed from BLAST searches between the ^{MVX}14.4 RdRp and eukaryotic RdRps (data not shown).

3.4 Discussion

3.4.1 *Agaricus bisporus* endornavirus 1

Sequence analysis, homology searches, phylogenetic analysis, and genomic organization, all support the conclusion that ^{MVX}14.4 is a new species of the genus *Endornavirus*. *Agaricus bisporus* endornavirus 1 (AbEV1) is proposed as an appropriate nomenclature for this virus.

Initial estimate of AbEV1 size using DNA molecular marker in agarose gel electrophoresis was *ca* 14.4 kbp (Gorgan *et al.*, 2003; Adie *et al.*, 2004). As a result of sequencing work carried out in this project, the correct size of AbEV1 was 12750 bp. This inconsistency was not unexpected, since RNA moves slower than DNA of the same molecular size in low density-gels (Livshits *et al.*, 1990).

Endornaviruses have been reported in plants (Wakarchuk & Hamilton, 1990; Fukuhara *et al.*, 1993; Pfeiffer, 1998), protists (Hacker *et al.*, 2005) and a few fungi, such as *Helicobasidium mompa* strain 670 (Osaki *et al.*, 2006) and *Gremmeniella abietina* type B

(Tuomivirta & Hantula, 2006). However, this is the first molecular characterization of an endornavirus that infects a homobasidiomycete and AbEV1 is the first dsRNA element from the MVX complex to be fully sequenced and characterized. Amongst fungal endornaviruses, AbEV1 has the second largest genome following HmEV1-670 (16614 bp). In full-length sequence comparison, AbEV1 sequence was most similar with HmEV1-670; however in Blast searches different regions of the genome showed various similarities with different endornaviruses. All regions containing functional domains (RdRp, helicase, and UGT) showed higher homology with non-plant endornaviruses, although the AbEV1 RdRp motifs sequence showed higher homology with plant endornaviruses in pairwise identity analysis. All regions of the AbEV1 polyprotein exhibited homology with endornaviruses, except for the non-coding region between aa 2700 and aa 3700, in which similarity with a homobasidiomycete retrotransposable element (*Phanerochaete chrysosporium* RP-78) was observed. These inconsistencies may be indicative of general genome heterogeneity in the *Endornavirus* genus, possibly due to RNA recombination occurring through horizontal transmission between parasitic and host organisms (Osaki *et al.*, 2006). Alternatively, it may be the result of limited *Endornavirus* sequences in the public databases as this is a virus genus only recently recognised.

AbEV1 showed a typical *Endornavirus* genomic organization. Its 5' UTR was consistently short (28 bp) as those of other endornaviruses. No significant sequence similarity was found between the AbEV1 5' UTR and 3' UTR, and those of other endornaviruses. A run of seven G residues was identified at the 3' UTR of AbEV1 sequence (TAG TGT GTG TGT GTG GGG GGG). The 3' ends of endornaviruses appeared to be rich in pyrimidines, e.g. OSV (GGG CAC CCC TCC CAA ACC CCG G) and VFV with three terminal G residues (TAC CAT CGG G).

The start codon at 29 bp was in a favourable context (AAC AAA ATG AAC), with purine (A) residues at -3 and +1 positions (Lutcke *et al.*, 1987). The presence of an A residue at position -3 appears to modulate the efficiency and rate of initiation for protein translation (Kozak, 1986). A similar context was found in *Gremmeniella abietina* endornavirus

(GaEV) where the *consensus* sequence for the translation initiation was the same as that most often observed in animals (TAC ACC ATG CAT; Lutcke *et al.*, 1987).

3.4.2 Phylogeny of *Agaricus bisporus* endornavirus 1

Members of the genus *Endornavirus* exhibit no significant homology with dsRNA viruses outside their genus, but rather their RdRp and helicase domains are most closely related to ssRNA viruses of alpha-like superfamily (Gibbs *et al.*, 2000; Fukuhara *et al.*, 2006; Hacker *et al.*, 2005). The latter includes various genera defined on the basis of several features (Koonin & Dolja, 1993; Goldbach & de Haan, 1994) such as: i) the presence of ssRNA genome with a 5'cap; ii) production of subgenomic RNA encoding a virion protein; iii) homologous RdRp and helicase amino acid sequences. Since some of these features are also shared by viruses not assigned to this supergroup, the best feature specifying the alpha-virus superfamily is the homology of the helicase and RdRp sequences (Gibbs *et al.*, 2000).

RNA viruses are rapid in evolution and this is reflected in enormous sequence divergence even among apparently closely related viruses (Holland *et al.*, 1982; Domingo *et al.*, 1985; Steinhauer & Holland, 1987). As a rule, only short amino acid sequence motifs thought to be involved in enzymatic functions are conserved (Koonin & Gorbalenya, 1989). An important element of the research strategy in comparative studies is 'gene context analysis' where both the sequence motifs in an individual protein, and the gene order and distance are conserved (Koonin & Dolja, 1993). Specific motifs within the RdRp domains of ssRNA viruses are highly conserved. Although overall sequence similarity among the ssRNA viral polymerases is quite low, three motifs (IV, V, and VI) defined as 'RdRp motif core' show unequivocal conservation throughout the whole class (Koonin & Dolja, 1993). Counterparts to the core RdRp motifs have been also detected in RNA dependent DNA polymerases, DNA-dependent DNA polymerases, and RdRps of negative-strand RNA viruses (Poch *et al.*, 1989; Delarue *et al.*, 1990; Xiong & Eickbusch, 1990). Clustering of the AbEV1 RdRp with other endornaviruses and alpha-like superfamily viruses is consistent with previous findings (Gibbs *et al.*, 2000; Hacker *et al.*, 2005; Fukuhara *et al.*, 2006; Osaki *et al.*, 2006). No significant similarities were observed between the AbEV1

RdRp and those of other dsRNA viruses such as cryptoviruses and hypoviruses previously suggested as related to endornaviruses for the mode of transmission and morphology, respectively (Pfeiffer, 1998; Fukuhara, 1999). The presence of RdRp motifs further confirms the relatedness between AbEV1 and other endornaviruses. The actual function of RdRp motifs is not known (Koonin & Dolja, 1993). However, Koonin (1991) suggested the direct involvement of motifs V and VI in substrate binding as demonstrated by site-directed mutagenesis (Inokuchi & Hirashima, 1987; Kroner *et al.*, 1989).

The identification of a typical helicase domain of superfamily I in AbEV1, upstream of the polymerase gene is also consistent with the endornaviruses (Osaki *et al.*, 2006) and closely related alpha-like viruses (Koonin & Dolja, 1993). All positive-stranded RNA viruses with genome size longer than 6 kbp encode for a putative RNA helicase thought to be involved in duplex unwinding during viral RNA replication, and maybe also translation (Gorbalenya and Koonin, 1989b). The presence of a helicase domain is a typical feature for dsRNA viruses such as endornaviruses, while the UGT domain is not always present (Hacker *et al.*, 2005). AbEV1 along with PEV1 (Hacker *et al.*, 2005), OSV (Hacker *et al.*, 2005) and HmEV1-670 (Osaki *et al.*, 2006) harbour a putative UDP-glycosyltransferases (UGT) domain within the polyprotein.

UGT genes have been identified in several families of DNA viruses (Markine-Goriaynoff *et al.*, 2004) and they have also been recently reported in RNA hypoviruses (Linder-Basso, 2002) as well as endornaviruses (Hacker *et al.*, 2005). Analysis of the AbEV1 UGT amino acid sequence revealed the presence of motif IV, but not the motif I reported in other endornaviruses (Hacker *et al.*, 2005). Sequence similarity analysis of UGT motif IV of AbEV1 showed the highest identity with PEV1, followed by a cellular UGT from *U. maydis*. Viral encoded UGT genes are considered to be have been acquired from their hosts during the evolutionary process (Markine-Goriaynoff *et al.*, 2004). Although Hacker *et al.* (2005) reported a fungal UGT from *U. maydis* as the most closely related to that of PEV1, in AbEV1 the best similarity was with another endornavirus (PEV1) suggesting that the UGT gene might not be a recent acquisition in AbEV1. UGTs have been suggested to be

beneficial to the virus interfering with host metabolism (O'Reilly, 1995). For example, baculovirus ecdysteroid glucosyltransferases inactivate ecdysteroid hormones by glucosylating them, thereby preventing moulting and pupation of infected larvae and increasing virus yield and spread (O'Reilly, 1995). It has also been suggested that UGTs maybe involved in pathogenicity mechanisms of plant pathogenic fungi (Hacker *et al.*, 2005). Since cellular sterol UGT genes are known to be required in plant pathogenic fungi (Sweigard *et al.*, 1998; Kim *et al.*, 2002), the expression of a viral UGT gene might modulate the pathogenicity of the host by gene silencing or other mechanisms (Hacker *et al.*, 2005; Osaki *et al.*, 2006). The role of the UGT domain in AbEV1 is not known, but it might interfere in some way with the mushroom development during MVX infection.

The long endornavirus polyproteins are believed to be processed by virus-encoded proteases via cleavage mechanisms (Seipelt *et al.*, 1999; Adams *et al.*, 2005). However, proteinase motifs, cysteine-rich regions and proteolytic cleavage sites were not identified in the AbEV1 polyprotein. Numerous RNA viruses produce functional proteins via proteolytic processing of polyprotein precursors (reviewed by Koonin & Dolja, 1993). The polyprotein expression strategy provides economy by encoding multiple proteins from a single open reading frame (Section 1.8.4). Since all proteins within a polyprotein are generated in equal amounts, differential proteolytic processing may provide a mechanism for regulating the availability of functional proteins required for early or late stages of infection (de Groot *et al.*, 1990). Virus-encoded proteases can be involved in the autoproteolytic cleavage of the viral polyprotein and can also interfere with the host metabolism preventing cellular protein synthesis initiation (Devaney *et al.*, 1988). Picornaviral proteinases are responsible for specific proteolysis of the eukaryotic initiation factor (eIF)4G, which is involved in the recruitment of capped cellular mRNA to the ribosome. The cleavage of eIF4G impairs this process and leads to the inability of the cell to initiate protein synthesis of its own mRNA (Seipelt *et al.*, 1999). In CHV1-EP713 the papain-like protease p29 located within N-terminal portion of ORF A, has been reported to be responsible for viral symptoms such as loss of pigmentation and reduction in asexual sporulation (Suzuki *et al.*, 1999). It appears that p29 alters host phenotype both directly through action of the protease on host factors

and indirectly by contributing to viral RNA accumulation. A large superfamily of virus-encoded proteases related to chymotrypsin-like cellular serine proteases has been described (Bazan & Fletterick, 1989; Gorbalenya *et al.*, 1989a). Some of these viral proteases have the substitution of Cys for the principal catalytic site, not found in cellular enzymes. The existence of classical cysteine proteases related to papain-like cellular proteases has been reported in several positive-stranded RNA viruses (Gorbalenya *et al.*, 1991) as well as some dsRNA viruses, e.g. hypoviruses (Suzuki *et al.*, 2003). The sequences of cellular and viral papain-like proteases are quite variable. The only reliable conserved region is a stretch of approximately ten amino acid residues centred at the catalytic Cys and no other conserved motifs could be detected (Gorbalenya *et al.*, 1991). Cysteine-rich regions have been described in PEV1 (Hacker *et al.*, 2005) and HmEV1-670 (Osaki *et al.*, 2006), but protease or proteolytic cleavage signatures have not yet been reported in endornavirus polyproteins. Due to their low degree of conservation, protease functions are difficult to be identified in viral sequences (Koonin *et al.*, 1992) and they are generally inferred from functional studies and site-directed mutagenesis that allow assignment of catalytic residue and target sequences.

Although endornaviruses have been reported in various plants, their presence in other organisms has not been extensively studied. Few reports have described endornaviruses in fungi. Results reported in this study support the hypothesis that ^{MVX}14.4 is the first ever endornavirus to be characterized in edible fungi.

Chapter 4

VERTICAL AND HORIZONTAL TRANSMISSION OF AbEV1

4. Summary

This chapter describes the epidemiological experiments set up to study the transmission via spores and anastomosis of ^{MVX}14.4, characterized and named as *Agaricus bisporus* endornavirus 1 (AbEV1). In order to demonstrate the vertical transmission of AbEV1, single spore isolates were cropped and analysed for the presence of ^{MVX}dsRNA elements. An *in vitro* dual-culture experiment was also set up to study AbEV1 horizontal transmission.

4.1 Introduction

4.1.1 DsRNAs Transmission via spores

Transmission of mycoviruses through spores is well known, but the efficiency of virus transmission differs between spore types (Ghabrial, 1998). In most cases transmission of dsRNAs through asexual spores (conidia) is very efficient (Buck, 1986, 1998). The vertical transmission rate of the most extensively studied mycovirus, *Cryphonectria parasitica hypovirus* (CHV), varies considerably among isolates showing a transmission of rate up to 100% in conidia (Enebak *et al.*, 1994; Melzer *et al.*, 1997; Russin & Shain, 1985), while there is no transmission of CHV via ascospores (Anagnostakis, 1988). *Ca* 10% of ascospore progenies from infected *Magnaporthe grisea* strain can contain dsRNA elements (Chun & Lee, 1997). Low transmission rates of dsRNAs into ascospores of *Aspergillus nidulans* have been reported, while conidia are always infected (Coenen *et al.*, 1997). Yeasts differ from most filamentous ascomycetes in that vertical transmission of dsRNA into ascospores is very efficient (Brewer & Faugman, 1980).

There is less information available about the transmission of mycoviruses in basidiomycetes. Many basidiomycetes do not produce conidia, but there are exceptions. For example, *Heterobasidion annosum* do produce conidia, which can transmit dsRNA elements with 3-55% efficiency (Ihrmark *et al.*, 2002). A high proportion of basidiospores was reported to contain dsRNAs in *Rhizoctonia solani* (Castanho & Butter, 1978) and *Ustilago maydis* (Day & Dodds, 1979). In *A. bisporus* basidiospores are considered the primary source of infection in the epidemiology of La France disease (Romaine *et al.*,

1993; Section 1.8.6.2.1); the spore infection rate range from 33% to 100% among the diseased basidiocarps, and diseased basidiospores show an increased rate of germination and viability (Schisler *et al.*, 1967; Dieleman van Zaayen, 1970; Romaine *et al.*, 1993). *Lentinus edodes* basidiospores can also transmit virus-like particles (Van Zaayen, 1979), whereas for the basidiomycete *Agrocybe aegerita*, the transmission of dsRNAs via basidiospores is very inefficient or non-existent (Barroso & Labarere, 2000).

4.1.2 DsRNA Transmission via fungal anastomosis

Mycoviruses can be transmitted horizontally through anastomosis (Osaki *et al.*, 2004). The ability of hyphae to anastomose (cell fuse) is well known in fungi and is especially developed in the ascomycetes and basidiomycetes (Deacon, 1984). During anastomosis there is complete fusion of hyphal walls and migration of cytoplasmatic particles in both directions through the hyphal bridges (Giovannetti *et al.*, 1999).

Anastomosis enables mycoviruses to exist intracellularly and is the only way for mycoviruses to be transmitted horizontally. No transmission vectors are known (Buck, 1986; Ghabrial, 1998). Plants, like fungi, have a cell wall, which acts as a barrier for virus penetration. Plant viruses circumvent the problem of transmission by exploiting vectors, e.g. insects, nematodes, chytrids, and protozoa (Brown *et al.*, 1995; Campbell, 1996; Perring *et al.*, 1999). The possibility that insects and nematodes play a similar role in fungi cannot be ruled out, but there are no reports so far. Fungal viruses have adopted a different strategy by not entering through the cell wall, but rather passing intracellularly (Wicker, 1992; Hong *et al.*, 1998). Numerous attempts to infect fungal mycelia with purified virus extracts have been made (Hollings, 1962; Hollings *et al.*, 1963, Dieleman-Van Zaayen & Temmink, 1968), but all have been either unsuccessful or have yielded spurious results due to possible infected spore contaminations (Ghabrial, 1980; Buck, 1986).

Removal of the cell wall may increase viral infection *in vitro* but not always, suggesting that the cell wall is not the only barrier viruses need to overcome during their infection process. Protoplasts of *Penicillium stoloniferum* could be infected at 10% rate with virus

extracts, according to Lhoas (1971). Pallett (1976) got similar results for *Penicillium chrysogenum*, *Marasmius androsaceus* and *Mucor hiemalis*. In other cases, protoplast infection with virus extracts gave very low frequency of infection as in *Cryphonectria parasitica* (Van Alfen *et al.*, 1984; *Pleurotus ostreatus* (van der Lende *et al.*, 1995b) and *Helminthosporium victoriae* (Ghabrial, 1986). It has also proved possible to infect protoplasts by fusing virus-infected with virus-free protoplasts as shown for *Pyricularia oryzae* (Lecoq *et al.*, 1979), *Gaeumannomyces graminis* (Stanway & Buck, 1984) and several *Aspergillus* spp. (Varga *et al.*, 1994; Coenen *et al.*, 1997; van Diepeningen *et al.*, 1998). El-Sherbeim & Bostian (1987) also reported infection of the yeast *Saccharomyces cerevisiae* during cell mating in a solution with virus particles. Although the infection rate was extremely low (6%), the experiment might suggest the intriguing possibility of a natural extracellular transmission route for mycoviruses during particular circumstances, such as mating, when the cell wall partially breaks down.

Anastomosis usually occurs between compatible strains (Osaki *et al.*, 2004). This involves the fusion of cell walls and mixing of cytoplasm between the paired isolates, which results in the formation of heterokaryotic filaments that contain a mixture of the nuclei of both parental strains in a common cytoplasm between the hyphal bridges (Begueret *et al.*, 1994). Such a reaction is typical of self-pairings. Perfect anastomosis is a rare event in non-self pairings because of incompatibility mechanisms (Hietala *et al.*, 2003).

4.1.3 Incompatibility Systems in Fungi

In fungi there are three different incompatibility systems. The first system, also referred to as heterogenic compatibility, makes it possible for unrelated individuals of the same species to anastomose, recombine and produce sexual spores, only if they have different alleles of mating-type genes (*mt* or *mat*; Begueret *et al.*, 1994). A second system is the interspecific incompatibility, which inhibits anastomosis between hyphae from different species (Paoletti *et al.*, 2006). A third system, the somatic or vegetative incompatibility system, is controlled by *vic* or *het* genes, allowing anastomosis between individuals with the same alleles in most cases (Begueret *et al.*, 1994).

In many species incompatibility between strains cultured on a medium can be detected by the presence of a barrage, an abnormal contact in the region where the incompatible mycelia fuse (Begueret *et al.*, 1994). Indeed, the hyphae of incompatible strains can fuse, but the heterokaryotic cells are rapidly destroyed by a lytic and degenerative reaction (Beisson-Schecroun, 1962; Boucherie *et al.*, 1981; Saupe *et al.*, 1995). Hyphal fusion between incompatible individuals results in a rapid compartmentation, organelle degeneration, shrinkage of the plasma membrane, septal plugging and death of the hyphal fusion cells and often surrounding cells (Glass & Kaneko, 2003). A similar lethal reaction has also been described in myxomycetes after plasmodia have fused (Carlile & Dee, 1967). In other cases, instability of the heterokaryon can lead to a post-fusion event, such as the specific loss of one of the two parental nuclei (Pittenger & Browner, 1961).

Genetic control of vegetative incompatibility has been well studied in several species of ascomycetes: eleven genes, including those at the mating-type locus, control somatic incompatibility in *Neurospora crassa* (Perkins, 1988), nine in *Podospora anserina* (Begueret *et al.*, 1994) and eight in *Aspergillus nidulans* (Croft & Dales, 1984). Detailed information concerning somatic incompatibility is lacking for most of Basidiomycetes, although several multiallelic loci have been suggested in *Heterobasidion annosum* (Hansen *et al.*, 1993), a single locus in *Phellinus gilvus* (Rizzo *et al.*, 1995), two loci in *Armillaria ostoyae* (Guillaumin, 1998) and three to four in *Collybia fusipes* (Marçais *et al.*, 2000). In contrast to somatic incompatibility reactions that occur between homokaryons in ascomycetes, those in basidiomycetes take place between secondary (heterokaryotic) mycelia (Hietala *et al.*, 2003). Mechanisms controlling somatic incompatibility have not been identified in *A. bisporus*. However, roughly 10-20% of pairings in this species might show behaviour consistent with some forms of somatic incompatibility (Hietala *et al.*, 2003).

The significance of vegetative incompatibility in the biology of fungal populations is not clear. Begueret *et al.* (1994) suggested a possible role for somatic incompatibility as a mechanism of genetic isolation and a barrier to cytoplasmic exchange; thus reducing the

spread of mycoviruses, mitochondria, plasmids and undesired cytoplasmic material within a fungal population. Vegetative incompatibility would contribute to population structure by limiting outbreeding and favouring evolution of isolated groups within a species (Esser & Blaich, 1973). The efficiency of transmission of mycoviruses between fungal isolates through anastomoses is dependent on the incompatibility (Buck, 1998). This has been extensively studied in *Cryphonectria parasitica*, where it has been shown that mycovirus transmission occurs most readily between isolates with the same *vic* genes, and decreases with increasing numbers of *vic* genes (Liu & Milgroom, 1996). Similar results have been presented for *Ophiostoma ulmi* (Brasier, 1986) and *Aspergillus nidulans* (Coenen *et al.*, 1997).

In *A. bisporus*, dsRNAs associated with La France disease have been shown to be readily transmissible between commercial strains (Sonnenberg & van Griensven, 1991), but the horizontal transmission from commercial to wild strains is restricted, probably due to vegetative incompatibility between strains (Sonnenberg *et al.*, 1995).

This chapter describes experiments carried out to examine the vertical and horizontal transmission of AbEV1 in *A. bisporus*. Preliminary analyses carried out at Warwick HRI involving crop infection has shown that dsRNAs associated with the MVX disease could be readily transmitted from MVX-infected to healthy compost irrespective of whether the infection occurs at spawning, at the end of the spawn-run or at casing (Grogan *et al.*, 2004). However, the expression of symptoms seems to depend both on the type of dsRNAs present in the infected mycelium and the time of infection. Further work suggested that ^{MVX}14.4 (AbEV1) could infect spores of *A. bisporus* very efficiently (Adie *et al.*, 2004). Thus, in order to investigate the vertical transmission and partitioning through spores of specific ^{MVX}dsRNA elements, single spore isolates (SSIs) of an MVX-infected strain (strain 1283, isolated from commercial mushrooms in the UK) were cropped. Harvested mushrooms were screened for the presence of ^{MVX}dsRNAs. Preliminary horizontal transmission experiments between commercial ^{MVX}dsRNAs-infected donors and genetically marked carboxin resistant mutants (Challen & Elliott, 1987) suggested that not all the acceptor

strains were equally receptive to the uptake of ^{MVX}dsRNAs, possibly because of vegetative incompatibility (Adie *et al.*, 2004). In this chapter details will be given about an *in vitro* transmission experiment set up in order to assess the transmission of AbEV1 from a donor to some acceptor strains of *A. bisporus*.

4.2 Materials and Methods

4.2.1 ^{MVX}dsRNAs Transmission via spores

MVX infection trials were performed in collaboration with Dr H.M. Grogan of Warwick HRI using single spore isolates that had previously been shown to contain ^{MVX}dsRNA elements (Adie *et al.*, 2004).

4.2.1.1 MVX ‘strain’ material

MVX-infected mushroom ‘strains’ (mushroom crops with different dsRNA patterns or dsRNA profiles) used for the experiments, are listed in Table 4.1 according to information provided by H.M. Grogan.

Table 4.1- MVX ‘strains’

MVX strain	Year isolated	Symptoms reported	^{MVX} dsRNA present
1283	2000	40-80% yield reduction; patchy crop with large bare areas and crop delay	AbEV1, ^{MVX} 9.4a, ^{MVX} 7.0, ^{MVX} 3.6, ^{MVX} 3.5, ^{MVX} 2.4a
A15	2002	none	^{MVX} 18.3, ^{MVX} 16.2a, ^{MVX} 2.4a

Strain 1283 was isolated in 2000 from UK mushroom farms experiencing MVX disease symptoms. Symptoms consisted mainly of yield loss and crop delay. Strain A15 was an isolate from the commercial Sylvan spawn, that contained only asymptomatic ^{MVX}dsRNAs (^{MVX}16.2a and ^{MVX} 2.4a) and ^{MVX}18.3 dsRNA, occasionally found in non-symptomatic mushrooms samples from sites with no history of MVX disease or recovered from this

disease (Grogan *et al.*, 2004). Preliminary hybridization analyses carried out at Warwick HRI are also consistent with the idea that ^{MVX}18.3 might be linked to an asymptomatic ^{MVX}dsRNA, ^{MVX}16.2a (Adie *et al.*, 2004).

4.2.1.2 SSIs Cropping

Single spore isolates (SSIs) were obtained from the MVX infected mushroom strain 1283 in earlier experiments at Warwick HRI (Adie *et al.*, 2004). Sixty single spore isolates were selected and grown on CE/CYM plates for 2-3 weeks at 25°C. Mycelia were previously tested for the presence of ^{MVX}14.4 (AbEV1) by RT-PCR and 47 spores (78%) proved positive.

In the present study, 10 SSIs were selected for a cropping experiment. The SSIs tested were: SSI 9, 27, 84, 127, 134 (AbEV1 negative), and SSI 1, 61, 121, 140, 160 (AbEV1 positive). The positive control for the presence of AbEV1 was 1283-2, an isolate of strain 1283. The negative control was A15-1, an isolate of the commercial strain A15.

Grain rye-spawn cultures (Elliott, 1985c) were prepared for compost inoculation. Agar plugs were cut from CE/CYM cultures and used to inoculate screw-capped glass jars, containing 150 g of sterilized, pre-cooked rye grain (Elliott, 1985c). Jars were incubated at 25°C for 2-4 weeks with weekly shaking to ensure a homogeneous colonization of all grains (Fig. 4.1). Colonised spawn (*ca* 20 g) was mixed with 3-3.5 kg phase II compost from the Warwick HRI Mushroom Unit in pots (*ca* 40 cm in diameter). For each treatment 3 replicates were prepared and 2 flushes harvested. A total of 36 pots were filled in and positioned in the growing chamber using a randomised plot statistical design (Greenland, 1990), developed in consultation with a Warwick HRI biometrician (Mead, *pers. comm.*).

Single Spore Isolate Experiment

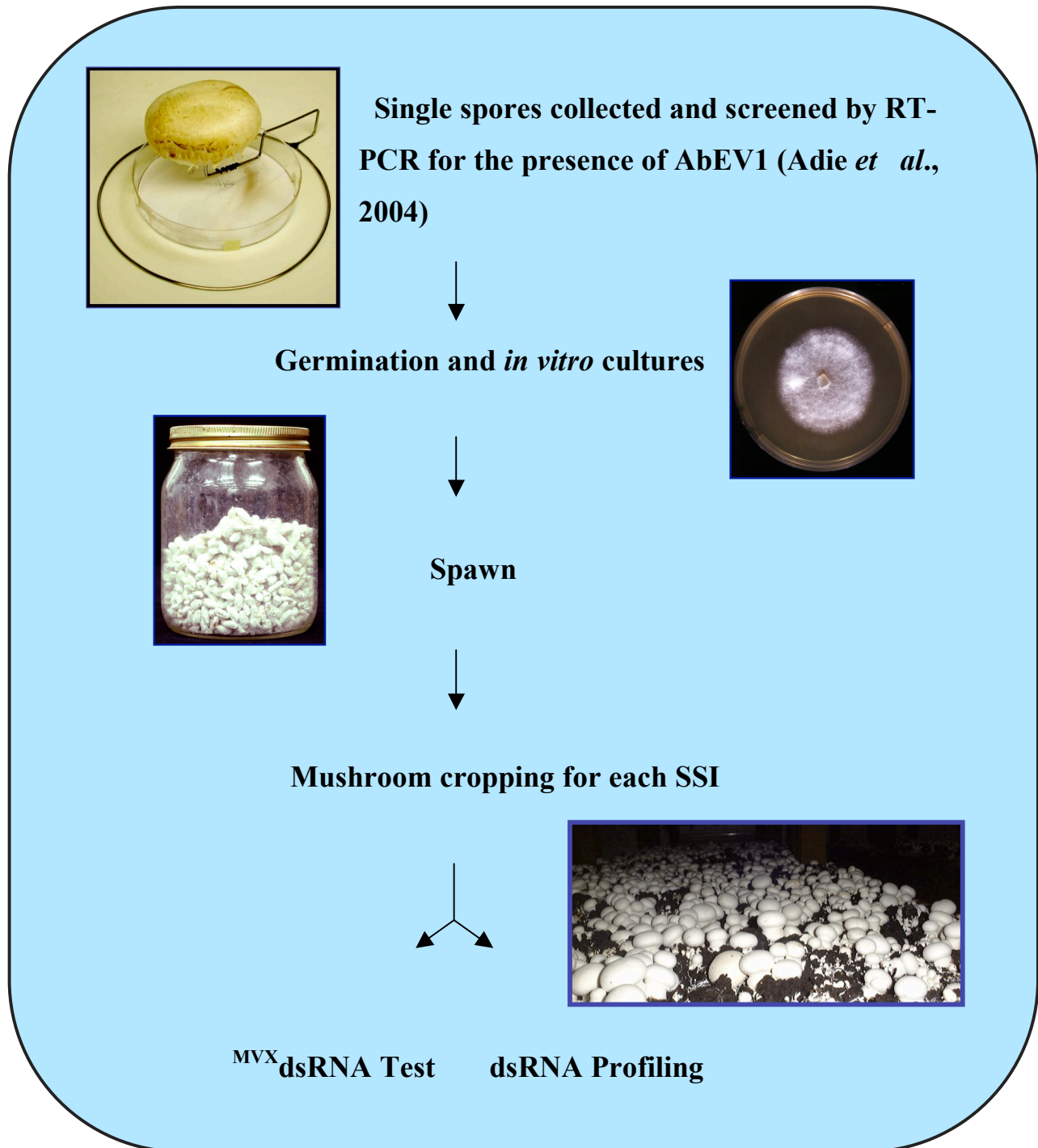


Fig. 4.1- Schematic diagram showing the SSI experiment

4.2.1.3 SSIs Molecular Analyses

Total RNA was extracted from harvested mushrooms by the TRI reagent method (Section 2.5.2) and used as template for the ^{MVX}dsRNA Test (Section 2.8.3). Additionally, the same mushroom samples were used for dsRNA Profile analysis (Section 2.9.2).

4.2.2 Horizontal Transmission of AbEV1

4.2.2.1 Strains

Dual-culture transmission experiment was conducted using the AbEV1-donor SSI 61 and two different AbEV1-free acceptor strains (A15-1 and C63-carb422). The MVX donor, SSI 61, was a single spore isolate obtained from the SSI experiment (Section 4.2.1.2), harbouring only AbEV1. The acceptor A15-1 was an isolate of a commercial strain (Section 4.2.1.2). The acceptor C63-carb422 was a mutant hybrid variety showing resistance to the fungicide carboxin (Challen & Elliott, 1987; Challen *et al.*, 1989) and was obtained from the fungal culture collections at Warwick HRI.

4.2.2.2 *In vitro* Transmission

Donors and acceptors were grown on CE/CYM agar (Calvo-Bado *et al.*, 2000) at 25° C for 8 weeks. The donor and acceptor plugs were positioned in the same plate *ca* 2 cm apart, so that advancing mycelia could anastomose. Two replicates for each dual culture assay were made. Sample isolations were made from the acceptor and donor colonies and let grow in CE/CYM broth for 2 weeks. Two sample isolations per plate (one from the donor and one from the acceptor mycelium, respectively) were taken at half way distance between the anastomosis zone and the periphery of the growing mycelium. Carboxin resistant acceptor isolates were grown on CE/CYM medium supplemented with carboxin (15 µg/ml) to confirm provenance of the mycelium.

The transmission of AbEV1 was assessed using RT-PCR (Section 2.8.3) with specific primers amplifying ^{MVX}14.4 dsRNA and 18S ribosomal RNA. RNA templates were prepared by the Tri Reagent extraction method (Section 2.5.2).

4.3 Results

4.3.1 ^{MVX}dsRNAs Transmission via spores

Mushrooms produced from the four MVX infected-single spore isolates (SSI 61, SSI 121, SSI 140, SSI 160), all proved positive for AbEV1 using the ^{MVX}dsRNA Test (Fig. 4.2) and the dsRNA Profile analysis (Fig. 4.3). Mushrooms produced from the five AbEV1-free SSIs, were not positive for AbEV1 as expected using the ^{MVX}dsRNA Test (Fig. 4.4) and the dsRNA Profile analysis (Fig. 4.5).

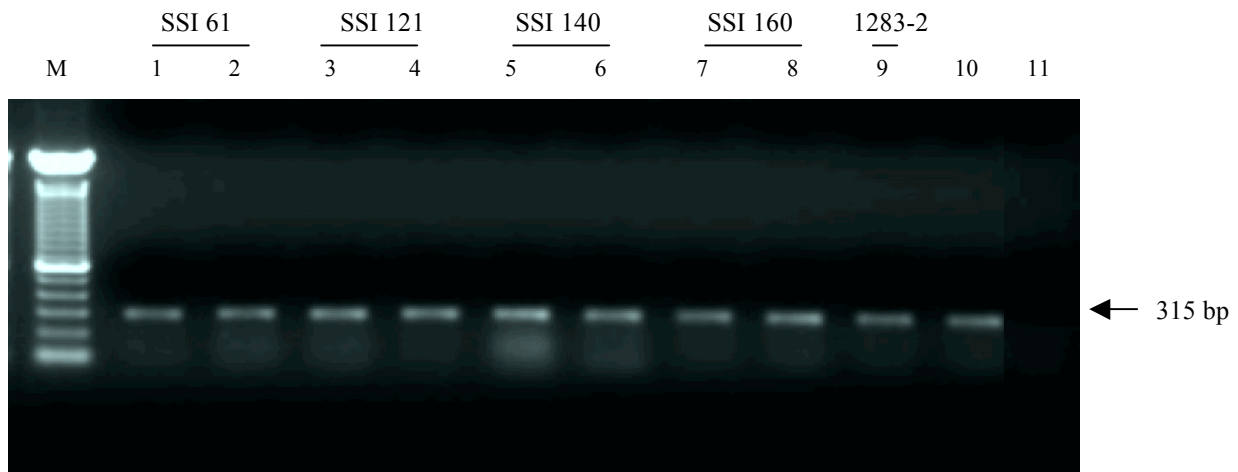
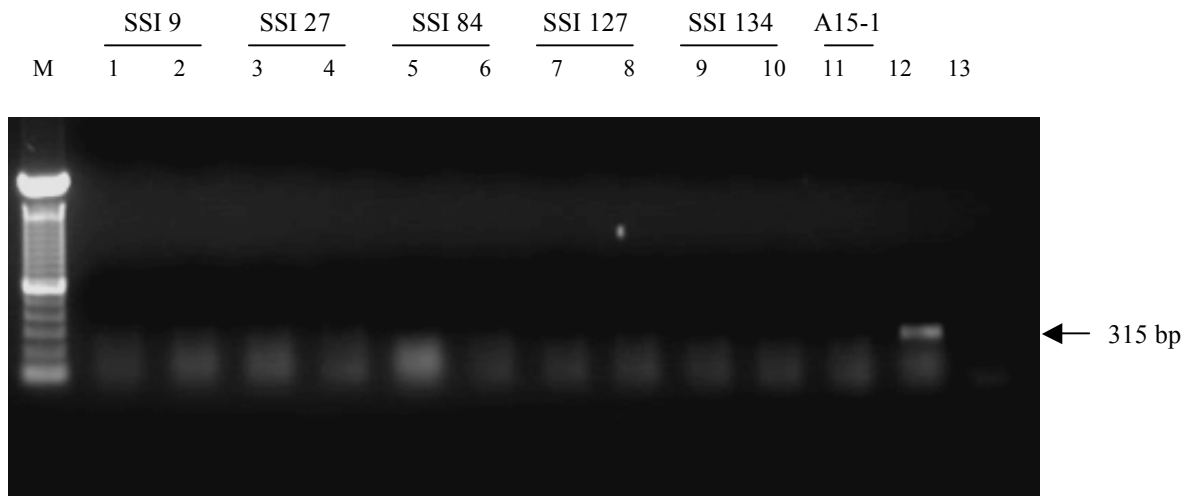
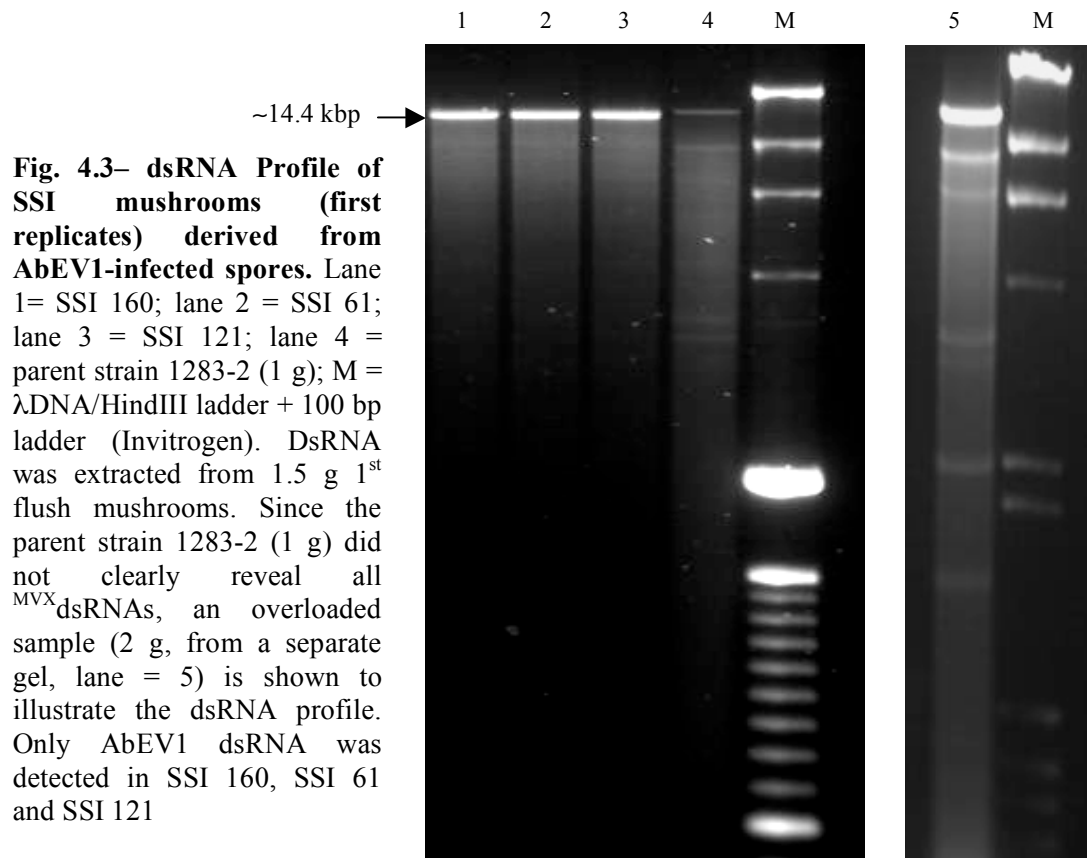


Fig. 4.2- RT-PCR screening in AbEV1 positive SSI mushrooms. First (lanes 1, 3, 5, and 7) and second replicates (lanes 2, 4, 6, and 8) of single spore isolate SSI 61, SSI 121, SSI 140, and SSI 160 were all positive for the presence of AbEV1. Lane 9 = parent strain 1283-2 (first replicate); lane 10 = RT-PCR positive control (strain 1283), lane 11 = RT-PCR negative control (water); M = 100 bp DNA ladder (Invitrogen)

Furthermore, all isolate mushrooms (SSI 9, SSI 27, SSI 84, SSI 127, SSI 134, SSI 61, SSI 121, SSI 140, and SSI 160) were free of asymptomatic ^{MVX}dsRNAs or other elements present in the 1283-2 parent strain (Table 4.2). One isolate, SSI 140, showed ^{MVX}3.5 in addition to AbEV1 (Fig 4.6). No differences were observed between 1st flush and 2nd flush mushroom samples.



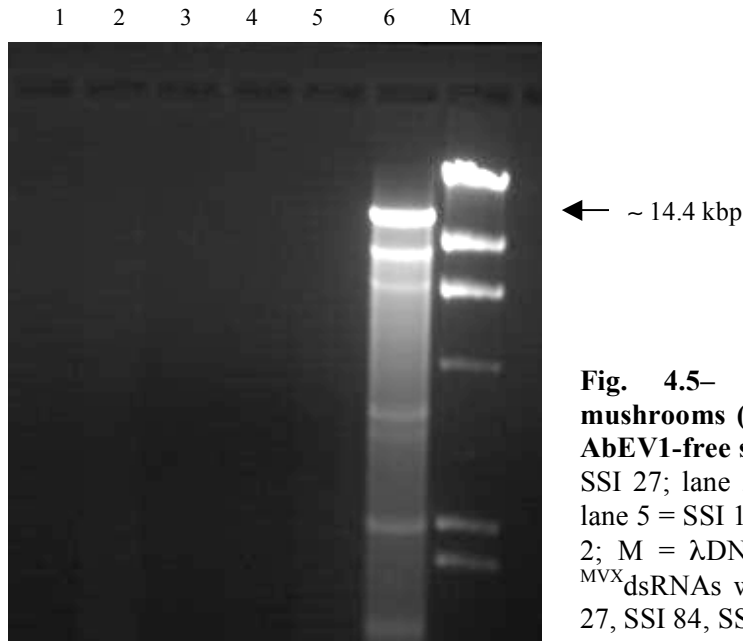


Fig. 4.5– dsRNA Profile for SSI mushrooms (first replicates) derived from AbEV1-free spores. Lane 1 = SSI 9; lane 2 = SSI 27; lane 3 = SSI 84; lane 4 = SSI 127; lane 5 = SSI 134; lane 6 = parent strain 1283-2; M = λ DNA/HindIII ladder (Invitrogen). ^{MVX}dsRNAs were not detected in SSI 9, SSI 27, SSI 84, SSI 127, and SSI 134

Table 4.2- Summary of RT-PCR analysis and dsRNA Profiles of SSI mushrooms. SSI 9, SSI 27, SSI 84, SSI 127, SSI 134 showed no presence of ^{MVX}dsRNAs. SSI 1 did not produce any mushrooms. SSI 61, SSI 121, SSI 160 harboured only AbEV1. SSI 140 exhibited both ^{MVX}3.5 and AbEV1. A15-1 was the negative control for the AbEV1 presence and contained only asymptomatic ^{MVX}dsRNAs (^{MVX} 18.3, 16.2a, 2.4a). 1283-2 was the MVX-infected parent strain

SSI CULTURE	^{MVX} dsRNAs (dsRNA Profile)	^{MVX} dsRNAs (RT-PCR)
SSI 9	-	-
SSI 27	-	-
SSI 84	-	-
SSI 127	-	-
SSI 134	-	-
SSI 1	no mush.	no mush.
SSI 61	AbEV1	AbEV1
SSI 121	AbEV1	AbEV1
SSI 140	AbEV1, ^{MVX} 3.5	AbEV1
SSI 160	AbEV1	AbEV1
A15-1 (negative control)	^{MVX} 18.3, 16.2a, 2.4a	-
1283-2 (parent strain)	AbEV1, ^{MVX} 7.0, 3.6, 3.5, 9.4a 2.4a	AbEV1, ^{MVX} 3.6, 9.4a

- = no detectable signal; No mush = no mushrooms produced

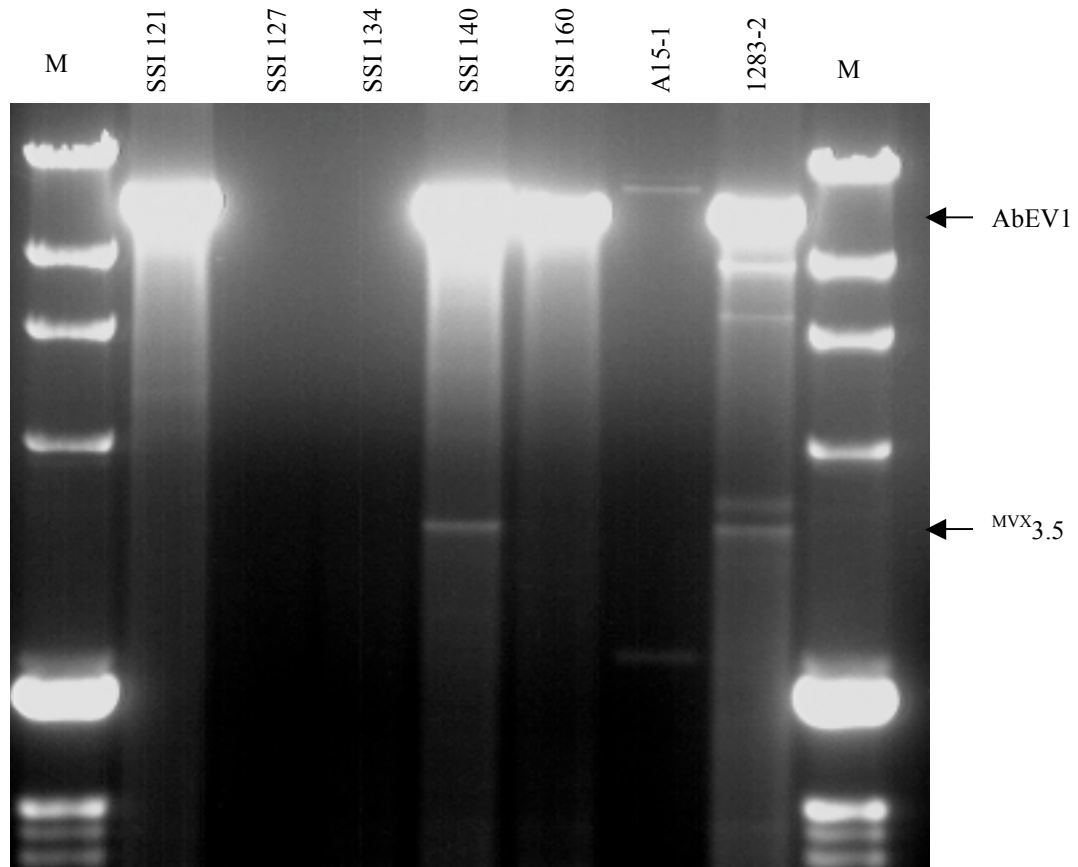


Fig. 4.6– dsRNA Profile for various SSI mushrooms. dsRNA was extracted from 3.5 g 1st flush mushrooms (second replicates). Isolates SSI 127 and SSI 134 showed no presence of ^{MVX}dsRNAs. Isolates SSI 121 and SSI 160 exhibited only AbEV1 dsRNA. SSI 140 exhibited both ^{MVX}3.5 and AbEV1. A15-1 was the negative control and contained only asymptomatic ^{MVX}dsRNAs (^{MVX} 18.3, 16.2a, 2.4a). 1283-2 was the MVX-infected parent strain. M = λ DNA/HindIII ladder + 100 bp ladder (Invitrogen)

SSI cultures on CE/CYM agar did not show any gross morphological abnormalities neither reduced growth unlike the parent strain (Fig. 4.7), with the exception of SSI 121 and SSI 140, which exhibited slower growth (data not shown).



Fig. 4.7- 1283-2 and SSI 61 agar cultures. Strains 1283-2 and SSI 61 were cultured on CE/CYM agar for 30 days. Growth of strain SSI 61 was not as slow as that of the parent strain 1283-2

4.3.2 AbEV1 Horizontal Transmission

Donor and acceptor agar plugs were positioned in the same plate *ca* 2 cm apart, so that advancing mycelia could anastomose (Fig. 4.8).

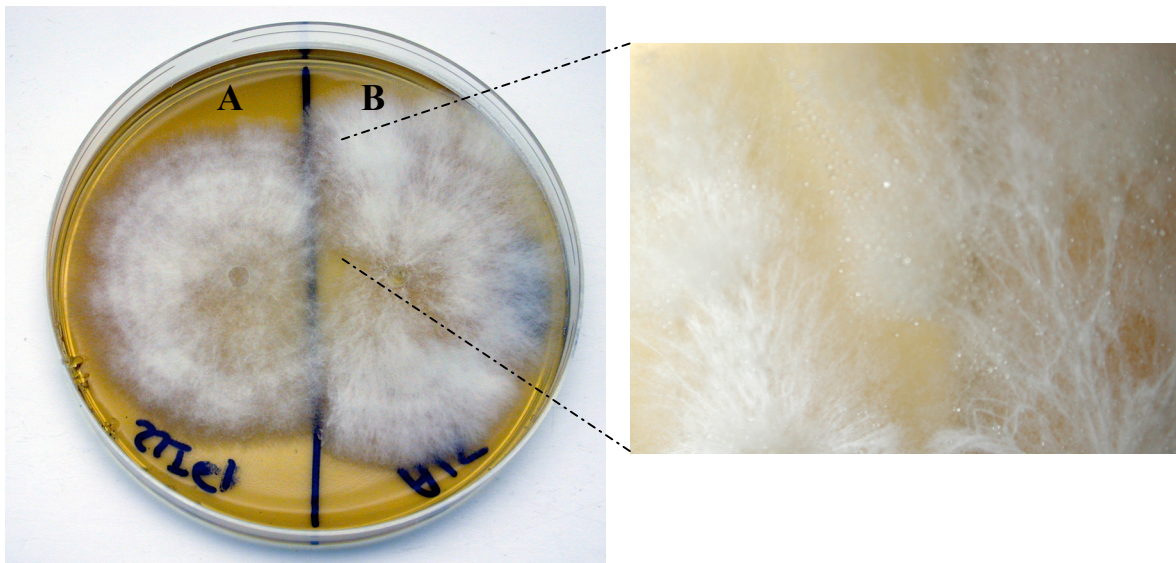


Fig. 4.8- *In vitro* dual culture assay for SSI 61 x A15-1. Donor (SSI 61; A) and acceptor (A15-1; B) cultures were allowed to anastomose on CE/CYM agar for 8 weeks. Contact zone between the two cultures has been magnified in the right hand sided photo

Sample isolations were made from the acceptor and donor colonies and let grow in CE/CYM broth for 2 weeks (Fig. 4.9). All carboxin resistant isolations were able to grow on CE/CYM medium supplemented with carboxin (15 µg/ml) confirming their provenance, whereas donor isolations from the same culture plate could not.



Fig. 4.9- A15-1 culture in CE/CYM broth. Sample isolations were made from the acceptor (A15-1) side of the dual culture plate and incubated in CE/CYM broth for 2 weeks

All samples tested yielded the appropriate 18S RT-PCR product and all donor isolations proved positive for AbEV1 by RT-PCR. Both A15-1 and C63-carb422 acceptor strains were infected by AbEV1 after anastomosis (Fig. 4.10).

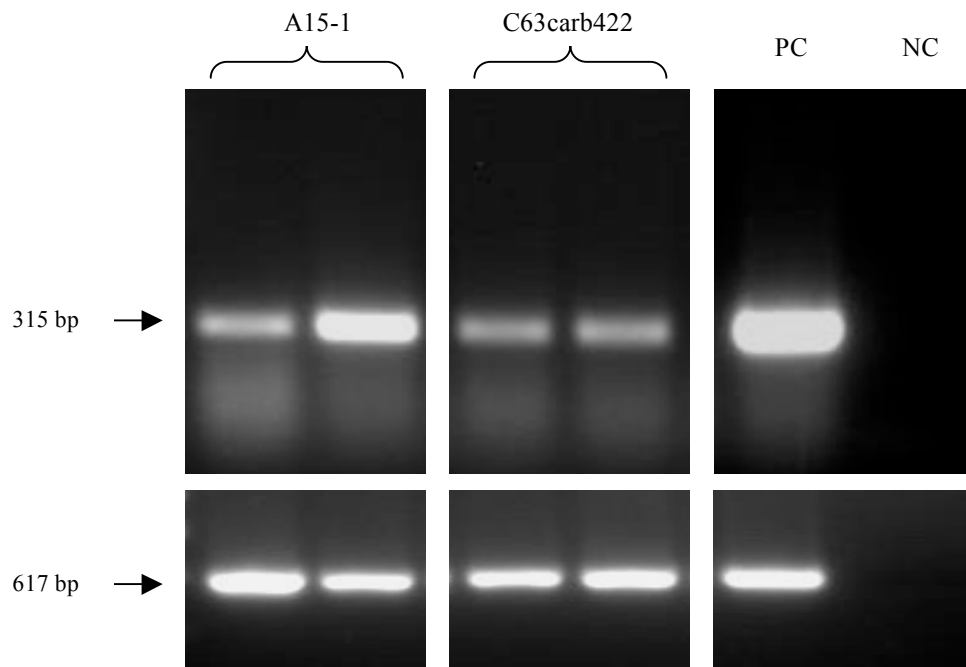


Fig. 4.10- RT-PCR screening for the AbEV1 presence in dual culture assay. Both A15-1 and C63-carb422 acceptor strains were infected by AbEV1 after 8 weeks-anastomosis. Indeed, A15-1 and C63- carb422 acceptors showed the appropriate 315 bp-amplicon in each replicate (photos in the first row) after anastomosis. All samples also yielded the appropriate 18S RT-PCR product (photos in the second row). Lane PC= RT-PCR positive control (strain SSI 61); lane NC= RT-PCR negative control (water)

4.4 Discussion

4.4.1 Vertical Transmission

Fruiting of various MVX infected-single spore isolates (SSIs) was used to demonstrate vertical transmission and stability of ^{MVX}dsRNA elements to *A. bisporus* offspring. Although MVX infected-SSI cultures were previously obtained (Adie *et al.*, 2004), stability of ^{MVX}dsRNAs throughout *A. bisporus* life cycle (spore-mycelium-mushroom) had not been previously demonstrated.

Mushrooms harbouring only AbEV1 were recovered from SSIs cropping (Table 4.2). The inability to detect AbEV1 in all SSI cultures (Adie *et al.*, 2004) and the loss of numerous

other MVX elements up to the fruiting stage suggested that partitioning of ^{MVX}dsRNAs may take place during basidiospore development. Segregation of viral elements during sexual sporogenesis has been reported in other fungi, e.g. *Ophiostoma ulmi* and *Gaeumannomyces graminis* (Brasier, 1983; McFadden, *et al.*, 1983; Rogers *et al.*, 1986). Segregation of dsRNA viruses during conidiogenesis has also been described in *Cryphonectria parasitica* (Romaine *et al.*, 1993), *Penicillium stoloniferum* (De Marini *et al.*, 1977), and *Saccharomyces cerevisiae* (Bruenn, 1986). Thus, vertical transmission of MVX elements could represent a stage where disease is transmitted, but also a moment of ‘recovery’ from MVX elements for some *A. bisporus* spores. Screening of single spore progeny can therefore be used to identify virus-free strains.

The transmission of AbEV1 as an independent element suggested its ability to replicate and survive as a unique virus. AbEV1 is often observed in high titre compared to other elements of the MVX complex and this might also play a role in the transmission efficiency as observed in *Heterobasidion annosum* (Ihrmark *et al.*, 2002). Moriyama *et al.* (1999) reported a copy number increase of more than 10-fold for *Oryza sativa endornavirus* in pollen grains compared to leaves, roots and seedlings. They suggested that this increase might be related to the high efficiency of dsRNA transmission via pollen.

Most of the infected SSI cultures (4/5) fruited regularly. However, one SSI culture (SSI 1) did not produce any mushrooms in fruiting trials. It is not known whether this was a consequence of viral infection. It is well known that some mycoviruses reduce fertility in the host (Anagnostakis, 1987; Brasier, 1986), while others seem to enhance their vertical transmission (Schisler, 1967). *Agaricus bisporus* mycelium infected with La France virus often produces basidiospores, which can germinate more frequently and more quickly than those from healthy mushrooms (Schisler, 1967). The mechanism of this response is not known, but it has been hypothesized that spores produced by diseased mushrooms germinate more quickly because they have less pigment and thinner walls (Schisler, 1967). Another effect is the production of taller mushrooms (elongated stems) that mature earlier and discharge spores ahead of healthy mushrooms (Schisler *et al.*, 1967; van Zaayen,

1979). In case of early infection of La France disease, fruiting can be suppressed over the entire bed or in well-defined areas (Schisler *et al.*, 1967). The inability of one MVX infected single spore isolate to fruit in the study described in this chapter could not be entirely correlated with the viral infection since it is well known that some *A. bisporus* spores are incapable of fruiting (Elliott, 1985b; Section 1.3.2).

In the present study AbEV1 was transmissible to the next progeny with a rate of 78% since 47 out of 60 spores harboured the virus (Adie *et al.*, 2004) and 100% (4/4) mushrooms produced from the infected single spore isolates exhibited the presence of AbEV1. This is consistent with the high transmission rate observed with other endornaviruses. Fukuhara (1999) found a transmission efficiency of 94% via pollen and 100% via eggs in rice infected with large endogenous dsRNAs. Transmission via spores/seeds is considered the main mode for endornaviruses. In plants, endornaviruses are transmitted to plant progeny only via seeds in a biparental (via pollen and ova) and non-Mendelian mode (Pfeiffer *et al.*, 1993; Moriyama *et al.*, 1995; Fukuhara, 1999). The observed inheritance of large-endogenous dsRNAs seems to be different from the so-called ‘uniparental’ or ‘maternal inheritance’ found for cytoplasmic components (e.g. chloroplasts and mitochondria), which are usually inherited only via eggs (Birky, 1995). The biparental transmission of endornaviruses also confirms the viral nature of these elements, in contrast with the early interpretation of large endogenous dsRNAs as plasmid-like elements (Turpen *et al.*, 1988; Fukuhara, 1999; Fukuhara *et al.*, 2005), localized in the cytoplasm (Lefevre *et al.*, 1990; Moriyama *et al.*, 1996; Koga *et al.*, 2003). Indeed viruses such as cryptoviruses (Section 1.8.2), which show some similarities with endornaviruses are only transmitted through seeds in a biparental mode; the transmission is generally found to be about 50% efficient when non-carrier plants are pollinated by carriers (Boccardo *et al.*, 1987).

All single spore isolate mushrooms harbouring AbEV1 routinely displayed reduced yields and 1-2 days crop delay (Grogan, *pers. comm.*), symptoms similar to the parent strain 1283 reported on farm. However, nothing is known about the molecular or physiological mechanisms causing these symptoms. Other studies have suggested that some viral

symptoms are caused indirectly by the rapid replication of the virus, which debilitates mushroom growth and fruiting rather than the direct activity of the virus (Wessels, 1994).

Virus replication might interfere with translation of highly expressed genes resulting in a diminished concentration of rare amino-acyl tRNAs. During fruiting and substrate colonisation proteins needed in high quantities such as hydrophobins might be affected by a viral disease. Hydrophobins have been identified in *A. bisporus* (Lugones *et al.*, 1996). ABH1p hydrophobin is particularly abundant at the surface of fruiting bodies; ABH3p hydrophobin is found in colonising mycelium of *A. bisporus*. These proteins, which occur uniquely in mycelial fungi, allow them to escape their aqueous environment, confer hydrophobicity to fungal surfaces in contact with air and mediate the attachment of hyphae to hydrophobic surfaces. Van der Lende (1995a) suggested that limitations of these and other hydrophobins could very well cause the decreased ability to colonise the substrate and failure to form abundant and normal-shaped fruiting bodies. Powel & Van Alfen (1987a, b) demonstrated the downregulation of specific host gene transcripts, such as the crypanin and laccase mRNAs in infected *Cryphonectria parasitica*. Virus regulated fungal genes have been identified in *Cryphonectria parasitica* (Fahima *et al.*, 1993), indicating the active role of viral genome at cellular level. In the case of the chestnut blight fungus hypovirus it has shown that pathogenicity traits result from a double effect between a general debilitation of the host due to the physical presence of the replicating virus and the differential regulation of specific viral coding domain (Choi & Nuss, 1992; Pfeiffer, 1998). Specifically, viral ORF A (Section 1.8.2) was identified as the determinant for altered fungal phenotype, such as reduced pigmentation, reduced laccase and crypanin accumulation and suppressed conidiation. The presence of a viral encoded protease (p29) in ORF A results in loss of pigmentation and reduction of sporulation as demonstrated through gene expression studies (Suzuki *et al.* 1999). This protein shares similarity with the N-terminal portion of the potyvirus-encoded helper component-protease (HC-Pro), a multifunctional protein implicated in aphid-mediated transmission, genome amplification, polyprotein processing, long-distance movement and suppression of PTGS.

However, symptoms shown by SSI mushrooms in this study were less severe than that shown by the parent strain 1283, and *in vitro* cultures did not generally show any reduced growth, apart from two SSI strains (Grogan, *pers. comm.*). This might suggest that other ^{MVX}dsRNAs may contribute more strongly to the yield reduction and crop delay symptoms observed on farms in MVX-infected 1283 mushrooms.

4.4.2 Horizontal transmission via mycelium

Anastomosis reactions lead to exchange of cellular material on a different scale according to the fungal species involved. Although bilateral nuclear migration is considered quite common in many basidiomycetes (Hintz *et al.*, 1988; Ihrmark *et al.*, 2002), nuclear migration does not occur in *A. bisporus* during anastomosis (Raper & Raper, 1972; Sonnenberg *et al.*, 1991). The two pairing heterokaryotic mycelia remain as two discrete entities and new heterokaryons are only formed in the junction zone (Challen, *pers. comm.*). The absence of nuclear migration in *A. bisporus* has been confirmed using carboxin resistant strains as MVX acceptor markers. The efficiency of mycovirus transmission between fungal isolates through anastomosis can be dependent on the vegetative compatibility of the isolates (Buck, 1998). However, nuclear and virus exchanges have also been reported between incompatible isolates of the basidiomycete *H. annosum* (Ihrmark *et al.*, 2002; Johannesson & Stenlid, 2004). Vegetative incompatibility may have a role in the transmission of MVX complex between different strains of *A. bisporus*. Not all strains seem equally receptive to the uptake of ^{MVX}dsRNA elements (Adie *et al.*, 2004; Challen, *pers. comm.*). This apparent ‘tolerance’ of some strains is an interesting observation, but it is not yet proven whether it is robust resistance or a delayed transmission.

In this study, AbEV1 was transmitted to both acceptor strains (A15-1 and C63-carb422) within 60 days. However, it is noteworthy that some early acceptor sub-culture samplings taken from the very periphery of the growing mycelium showed no presence of AbEV1, although isolates from the inner colony were positive. This result may be explained by the usual absence of virus in the hyphal tips of growing mycelium (Mori *et al.*, 1978; Nuss &

Koltin, 1990; Varga *et al.*, 1994; Ikeda *et al.*, 2003). Prolonged storage and subculturing might also result in increased virus concentration and in its presence in hyphal tip isolates (Varga *et al.*, 1994). Sonnenberg & van Griensven (1991) reported that dsRNAs concentration in samples depends on the time of contact and distance of the sampling point to the contact zone. Despite horizontal transmission of endornaviruses has not been demonstrated in plants (Wakarchuk & Hamilton, 1985; Valverde *et al.*, 1990; Zabalgogezcoa & Gildow, 1992; Moriyama *et al.*, 1996; Gibbs *et al.*, 2000), Ikeda *et al.* (2003) showed transmission via anastomosis for the fungal endornavirus *Helicobasidium mompa* endornavirus 1-670. These authors demonstrated that HmEV1-670 can be removed from infected strains by hyphal tip isolation and then reintroduced by anastomosis using an HmEV1-670 infected donor (Ikeda *et al.*, 2003; Osaki *et al.*, 2006).

The results of the current study represent the first report of horizontal transmission for AbEV1 to commercial mushroom strains, making the virus an ‘infectious threat’ for the mushroom industry. Commercial mushrooms can be more readily infected than wild mushrooms because of the favourable environment for virus spread, high host population density and low genetic variability. All these elements can be excellent selection factors for the evolution of viruses and can render viruses even more virulent (Milgroom, 1999).

Chapter 5

DETECTION OF MVX-like dsRNAs IN FEW MYCELIAL CULTURES OF WILD *AGARICUS* COLLECTIONS

5. Summary

This chapter describes the various techniques and experiments carried out to investigate the presence of MVX-like dsRNA elements in mycelial cultures derived from wild mushroom collections. Different *Agaricus* collections were screened for MVX dsRNA elements by RT-PCR (^{MVX}dsRNA Test) and an AbEV1-type element from collection ARP250 was further characterized by sequence analysis.

5.1 Introduction

Mycoviruses are widespread in fungal hosts, ranging from pathogenic fungi to edible mushrooms (Section 1.8.1). Their biological significance to their hosts is still poorly understood, since a large number of fungal viruses are associated with latent infections (Ghabrial, 1994). Fungal viruses do not have an extracellular phase, therefore they are transmitted intracellularly during cell division, sporogenesis and cell fusion. As a result of these modes of transmission the natural host range of mycoviruses is limited to individuals within the same or closely related vegetative compatibility groups (Anagnostakis, 1982). The presence of mycoviruses and unencapsidated dsRNAs has been reported for a large number of different plant fungal pathogens including *Ustilago maydis* (Wood & Bozarth, 1973), *Rhizoctonia solani* (Castanho *et al.*, 1978; Zanzinger *et al.*, 1984; Finkler *et al.*, 1985), *Gaeumannomyces graminis* (Stanway, 1985), *Pyricularia oryzae* (Hunst *et al.*, 1986), *Periconia circinata* (Matsumoto, 1987), *Phytophthora infestans* (Tooley *et al.*, 1989), *Ophiostoma ulmi* (Hong *et al.*, 1998), *Cryphonectria parasitica* (Choi & Nuss, 1992), *Sclerotinia sclerotiorum* (Li *et al.*, 1999), *Septoria nodorum* (Newton, 1987), *Helminthosporium victoriae* (Soldevila *et al.*, 2000), *Heterobasidion annosum* (Ihrmark *et al.*, 2001), *Discula destructiva* (Rong *et al.*, 2002), *Botrytis cinerea* (Castro *et al.*, 2003), *Coniothyrium minitans* (Cheng *et al.*, 2003), *Helicobasidium mompa* (Osaki *et al.*, 2004), *Phytophthora* spp (Hacker *et al.*, 2005), *Fusarium graminearum* (Chu *et al.*, 2004) and others.

Despite the broad number of mycoviruses reported in plant fungal pathogens, very little is known about the presence of dsRNAs in edible mushrooms. Van der Lende *et al.* (1995b) described dsRNAs and virus particles in slow growing cultivated *Pleurotus ostreatus*. Diseased oyster mushrooms were shown to harbour 7 dsRNA elements. One of these dsRNA, 2.4 kbp dsRNA, was associated with infective viral particles and could be horizontally transmitted to virus-free mycelium (van der Lende *et al.*, 1995b). In some cases, lack of an apparent correlation between the presence of dsRNAs and host phenotype has been reported. Lim *et al.* (2005) have described a new dsRNA virus with distinct similarities to partitivirus, named *P. ostreatus virus 1* (PoV1) infecting *P. ostreatus* latently. Despite most viruses infecting mushrooms have a dsRNA genome, Yu *et al.* (2003) reported a new virus with ssRNA genome of 5.784 kbp and a coat capsid of approximately 28.5 kDa. This virus, named *oyster mushroom spherical virus* (OMSV) was isolated from cultivated oyster mushrooms with severe epidemic oyster mushroom Die-back disease. Only three other ssRNA mycoviruses have been reported so far. The first, *mushroom bacilliform virus* (MBV) was found in *A. bisporus* (Tavantzis *et al.*, 1980). The second, *Sclerophthora macrospora virus B* (SmVB) was isolated in *S. macrospora*, the pathogenic fungus responsible for downy mildew in gramineous plants (Honkura *et al.*, 1983; Yokoi *et al.*, 1999). The third *Botrytis virus F* (BVF) infects *Botrytis cinerea*, an important fungus affecting a large number of economically important vegetables, flowers and fruit crops (Robyn *et al.*, 2001). Mycoviruses have been found also in *Lentinus edodes* (Inoue, 1970) and in another cultivated basidiomycete, *Agrocybe aegerita* (Barraso & Labarere, 1990). Virus-like particles have been found in some wild mushrooms, such as *Lentinus lepideus*, *Collybia peronata* and *Phaeolepiota aurea* (Mori *et al.*, 1978). Mycelial growth and malformations of fruiting bodies were observed.

Little is known about viruses in wild populations of *A. bisporus* (Milgroom, 1999). Sonnenberg *et al.* (1995) screened 133 wild isolates of *A. bisporus*, but they could not find any indications for the presence of viral dsRNAs associated with La France disease in the natural population. However, Adie *et al.* (2004) at Warwick HRI screened 40 commercial

varieties and 109 *Agaricus* isolates of various origin (mainly *A. bisporus*) for the presence of three ^{MVX}dsRNAs (AbEV1, ^{MVX}3.6 and ^{MVX}1.8) using RT-PCR, and within these collections only one isolate, ARP250 was positive for AbEV1.

5.2 Materials and Methods

5.2.1 Mushroom Isolates

The ARP250 isolate was kindly provided by R. Kerrigan (Sylvan Research, Pennsylvania, USA) and is a culture sample distributed through the ARP (*Agaricus* Resource Program) collection of wild strains. The wild ARP250 isolate was originally collected near Asilomar, California. A mycelium plug was grown on CE/CYM plates for 2-3 week at 25°C and the resulting mycelium gently collected in a 1.5 ml microcentrifuge tube.

Two other mushroom isolates were collected in Warwickshire (UK), P44 (harvested by R. Gaze near Warwick, Warwickshire) and W52-B (collected by R. Noble in Barford, Warwickshire, Fig. 5.1). Tissue cultures were prepared growing *ca* 1 cm-pieces of explant from pileus tissue of wild mushrooms on CE/CYM agar plates for 2-3 week at 25°C and the resulting mycelia were collected in a 1.5 ml microcentrifuge tube.

5.2.2 ^{ARP250}dsRNAs Screening

5.2.2.1 Cropping Experiment

A cropping experiment was set up with the ARP250 isolate. Grain rye-spawn culture (Elliott, 1985c) was prepared for compost inoculation. Agar plugs were cut from ARP250 agar cultures and used to inoculate screw-capped glass jars, containing 150 g of sterilized, pre-cooked rye grain (Elliott, 1985c). Jars were incubated at 25°C for 2-4 weeks with weekly shaking to ensure a homogeneous colonization of all grains. Colonised spawn (*ca* 20 g) was mixed with 3-3.5 kg phase II compost from the Warwick HRI Mushroom Unit in pots (*ca* 40 cm in diameter). A total of 3 replicates were prepared and the first flush

harvested. DsRNA was extracted from harvested mushrooms as described in Section 2.5.1, and analysed through electrophoresis in order to obtain a dsRNA Profile.



Fig. 5.1- *Agaricus bisporus* isolate (W52-B) collected in Warwickshire. *Agaricus bisporus* mushrooms (W52-B) collected in Barford, Warwickshire, UK. Mushrooms presented scaly and brown-coloured cap (photo kindly provided by M. Challen)

5.2.2.2 Molecular Characterization of an AbEV1-like element in ARP250 isolate

The ARP250 isolate was cultured on CE/CYM agar medium and the resulting mycelium collected after 2-3 weeks to obtain the starting material for total RNA extraction (TRI-Reagent, Section 2.5.2). Recovered total RNA was then used as a template for RT-PCR analysis (^{MVX}dsRNA Test, Section 2.8.3).

An AbEV1-like element (^{ARP250}14.4) was found in ARP250 isolate. In order to characterize it sequencing work was carried out. Total RNA extracted by Tri-Reagent from ARP250 culture was used as a template for the RT-PCR assay (Section 2.8.1 and 2.8.2). Primers used (Table 5.1) were designed on specific AbEV1 regions sequences (helicase, polymerase and glycosyltransferase domains, Section 3.3.2).

Table 5.1- Primers amplifying helicase, RdRp, and GT regions in both AbeV1 and ^{ARP250}14.4

Primer	Primer sequence Reference: this study	Product Size (bp)	RNA Target
B3f92 B3r673	5'-CCTCGTTACCTTGGTAACT-3' 5'-GTATCATCGCTATGACTTCC-3'	ca 582	^{ARP250} 14.4 helicase
RDRP11027 RDRP11933	5'-CGAAGTTCAGGTAGGATTG-3' 5'-ATCATCACCCAGCAACAG-3'	ca 907	^{ARP250} 14.4 RdRp
B3-F7654 B3-R7934	5'-TGACGGCATAACCGAAGAG-3' 5'-GTTCTGCAAACCGCAATGG-3'	ca 281	^{ARP250} 14.4 glycosyltransferase

Obtained RT-PCR products were electrophoresed, gel purified and sequenced directly as previously described (Section 2.12.2) using primers listed in Table 5.1. Additional primers (RDRPr190, 5'-TCTGGCTGCCAATTCTTCA-3'; RDRPf57, 5'-TCGTGCACCAAACAA TGC-3') were used to complete the sequencing of the long RdRp amplicon (~ 900 bp). All regions were obtained from more than four independently sequencing reactions. Sequences obtained were analysed as described in Section 2.13.

5.2.3 DsRNA Screening in other Isolates

Total RNA was extracted from cultures of P44 and W52-B isolates by TRI reagent extraction (Section 2.5.2) and used as a template for the RT-PCR assay (^{MVX}dsRNA Test, Section 2.8.3).

5.2.4 Isolates Characterization

Nucleic acids from isolates described in Section 5.2.1 were extracted using the Chelex method (Section 2.5.3) and used as a template for the PCR assay (Section 2.8.2) amplifying the Internal Transcribed Spacer (ITS) regions of *Agaricus* ribosomal DNA (rDNA). Primer its1extB (5'-AACAAGGTTTCCGTAGGTGAACCTGC-3') and its4extA (5'-TTCTTTTCCTCCGCTTATTGATATGC-3') amplified a product of ca 700 bp for the rDNA region. Obtained PCR products were electrophoresed, gel purified and sequenced directly as previously described (Section 2.12.2). Direct sequencing reactions were

performed in order to generate sequences for the region between the small ribosomal subunit and 5.8S subunit using primer its1 (5'-TCCGTAGGTGAACCTGCGG-3') and its2 (5'-GCTGCGTTCTTCATCGATGC-3'), and the region between the large ribosomal subunit and 5.8S subunit using primer its3 (5'-GCATCGATGAAGAACGCAGC-3') and its4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Phylogenetic analysis based on ITS sequences, obtained from 4 independent sequencing reactions per sample, were analysed and compared to ITS sequences from other well defined *Agaricus* isolates (Challen *et al.*, 2003). A neighbour-joining phylogenetic tree of ITS sequences was constructed using the MegAlign package via ClustalW algorithm (Thompson *et al.*, 1994).

5.3 Results

5.3.1 ARP250 Isolate

ARP250 mushrooms cropped at Warwick HRI had brown-coloured, slightly scaly caps (Fig. 5.2, Fig. 5.3, and Fig. 5.4).

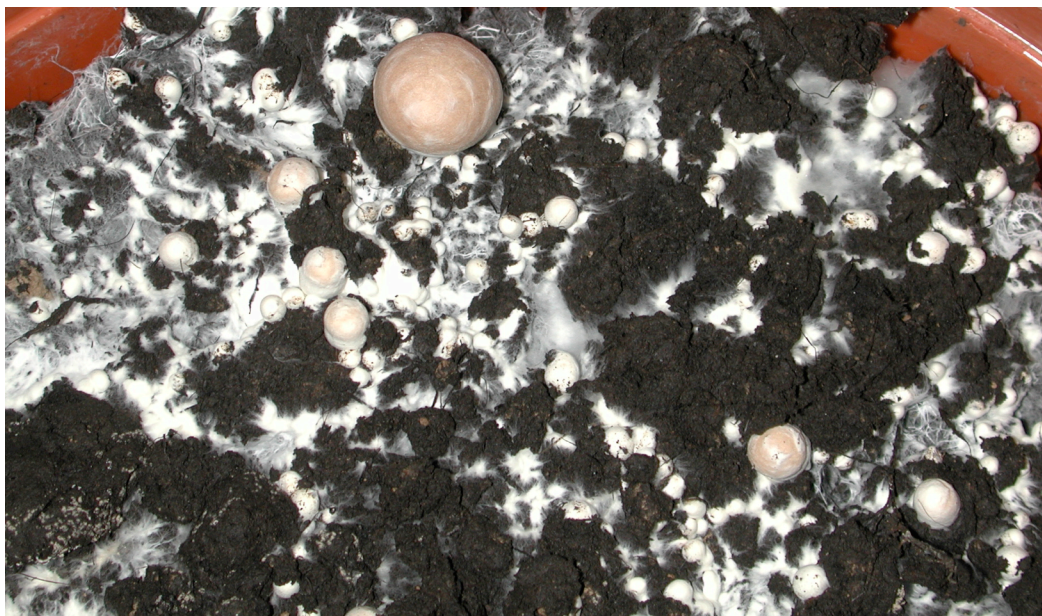


Fig. 5.2- ARP250 isolate fruiting in compost culture. Brown-coloured pins of ARP250 isolate emerging from colonised casing



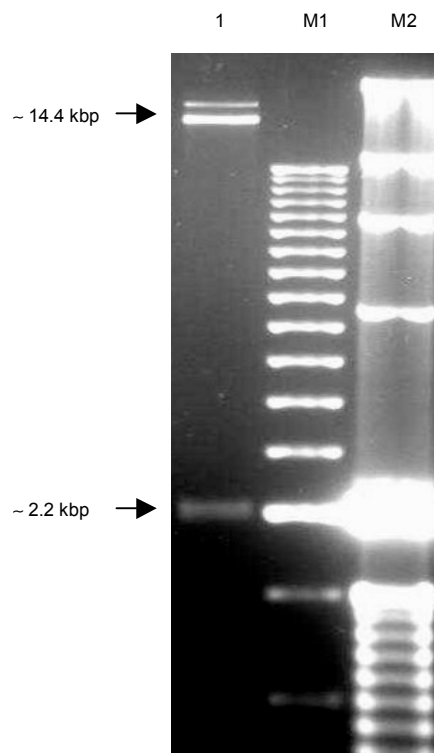
Fig. 5.3 - ARP250 mushrooms. Two ARP250 mushrooms harvested from Warwick HRI Mushroom Unit and showing brown and scaly caps



Fig. 5.4- ARP250 mushroom close-up. ARP250 cap with visible brown-coloured scales

Extracted dsRNA from harvested mushrooms was used to obtain a dsRNA Profile, which revealed the presence of a *ca* 14.4 kbp-band and two further RNA elements, one at *ca* 2.2 kbp (^{ARP250}2.2) and a second at *ca* 16.2 kbp (^{ARP250}16.2) (Fig. 5.5). These appeared equivalent in size to MVX elements found in MVX-infected cultivated mushrooms, ^{MVX}16.2a, ^{MVX}14.4 (AbEV1), and ^{MVX}2.2.

Fig. 5.5- dsRNA Profile for the ARP250 isolate. The ARP250 isolate showed the presence of some ^{MVX}dsRNA-like elements similar to ^{MVX}16.2a, ^{MVX}14.4 (AbEV1), and ^{MVX}2.2 found in MVX-infected cultivated mushrooms. These elements have been named ^{ARP250}16.2, ^{ARP250}14.4, and ^{ARP250}2.2, respectively. None of the other ^{MVX}dsRNAs-like were found. Lane 1 = ARP250 mushroom sample; M1 = molecular weight marker (500 bp ladder, Invitrogen); M2 = molecular weight marker (λ DNA/HindIII ladder + 100 bp ladder, Invitrogen).



RT-PCR analysis using the ^{MVX}dsRNA Test confirmed the presence of a possible AbEV1-like element, yielding an amplicon of 315 bp. The isolate yielded the appropriate 18S RT-PCR product, but amplicons for the ^{MVX}3.6, ^{MVX}1.8 and ^{MVX}9.4a were not recovered (Fig. 5.6).

RT-PCR amplicons of *ca* 582 bp, 907 bp, and 281 bp were recovered for the ^{ARP250}14.4 helicase, RdRp and glycosyltransferase regions, respectively. RT-PCR products were gel purified and sequenced directly. A ‘consensus’ sequence of 482 bp from the sequencing work was produced for the ^{ARP250}14.4 helicase region. The sequence showed 98% similarity

with AbEV1 at the DNA level (Annex 5.1). A mutation from C to T in nt position 404 (aa 135) results in a mutation in the second position of the translated codon turning the amino acid threonine found in AbEV1 into isoleucine in ^{ARP250}14.4 (Fig. 5.7)

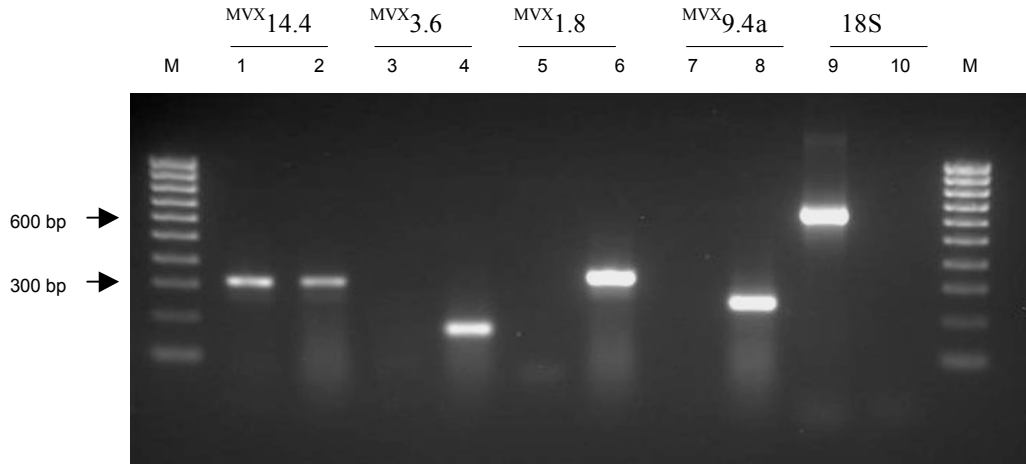


Fig. 5.6- RT-PCR screening using the ^{MVX}dsRNA Test for ARP250 isolate. The ARP250 isolate was found positive only using primers for ^{MVX}14.4 (lane 1). Amplicons for ^{MVX}3.6 (lane 3), ^{MVX}1.8 (lane 5), and ^{MVX}9.4a (lane 7) were not recovered. The isolate yielded the appropriate 18S RT-PCR product (lane 9). A positive control (strain 1283) was used for each pair of primers (lanes 2, 4, 6, and 8). Lane 10 = RT-PCR negative control (water); M = molecular weight marker (Hyperladder IV, Bioline)

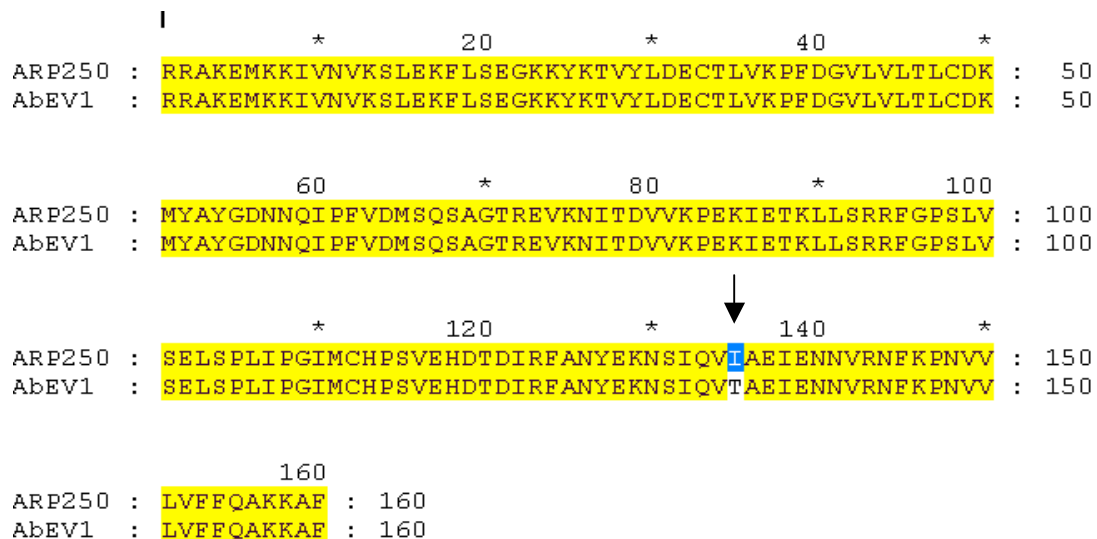


Fig. 5.7 -Pairwise alignment of ^{ARP250}14.4 and AbEV1 amino acid sequence in the helicase region. The alignment was constructed using the MegAlign package and displayed via GENEDOC program. The two sequences share 99% identity. Identities are highlighted in yellow, while dissimilarities are highlighted in blue (arrow). ARP250: ^{ARP250}14.4

This threonine → isoleucine change does not occur in the helicase conserved motifs (Section 3.3.2) (Fig. 5.8). Other polymorphisms detected in the DNA sequence would not result in any translational change in amino acid residues.

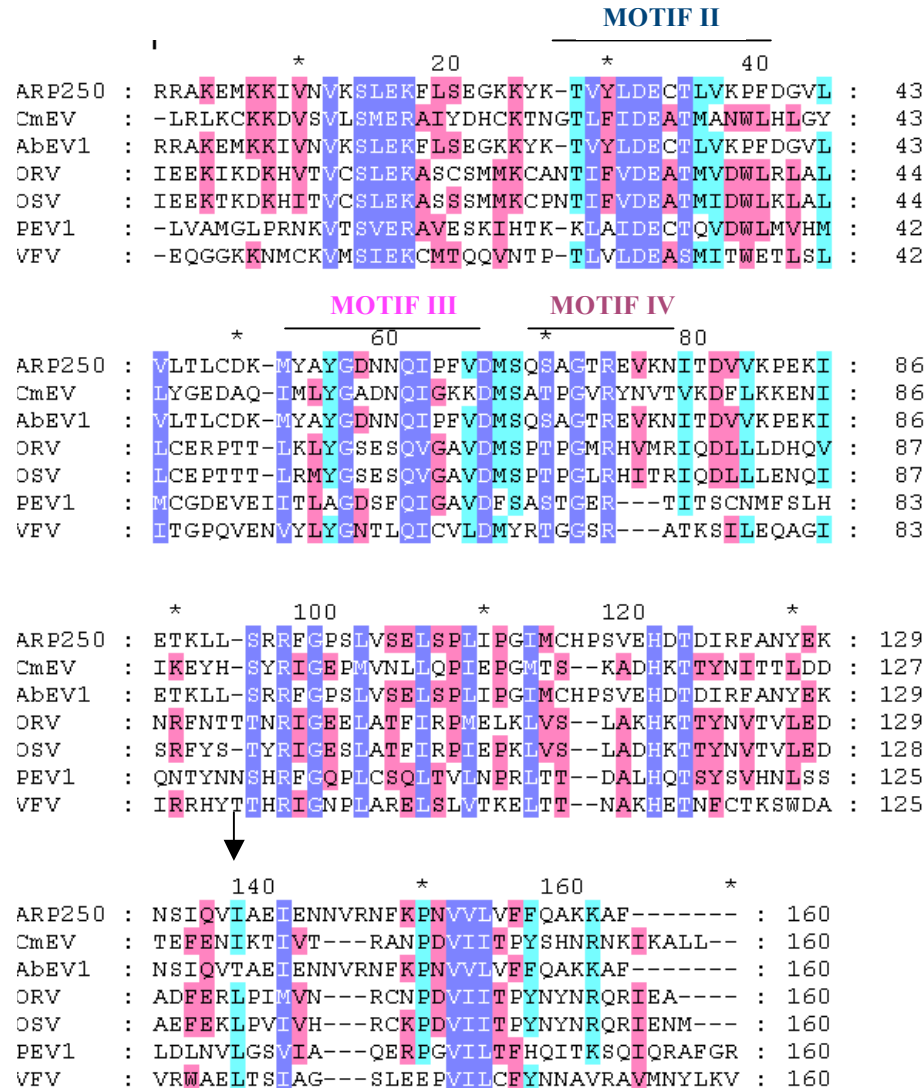


Fig. 5.8 - ^{ARP250}14.4 helicase motifs. Multiple alignment of amino acid sequences within the conserved helicase motifs II, III, IV (Koonin, 1993) was constructed for 7 endornaviruses, including ^{ARP250}14.4 using ClustalW algorithm. ARP250: ^{ARP250}14.4; CmEV: *Cucumis melo* endornavirus; AbEV1: *Agaricus bisporus* endornavirus 1; ORV: *Oryza rufipogon* endornavirus; OSV: *Oryza sativa* endornavirus; PEV1: *Phytophthora endornavirus 1*; VFV: *Vicia faba* endornavirus. ARP250 sequence showed an isoleucine amino acid (I) in position 138 which did not match with the AbEV1 threonine in the same position (arrow)

Sequence similarity was also found for the ^{ARP250}14.4 polymerase domain. The region amplified (895 bp) through sequencing showed 97% similarity with AbEV1 at the DNA level (Annex 5.2). Mutations were homogenously distributed throughout the DNA sequence and occurred in the third position of the translated codons resulting in no differences (100% similarity) with AbEV1 RdRp amino acid sequence (Fig. 5.9).

```

                *           20           *           40
ARP250 : MVKYPEMSKPVITKGVYQTFNAVSSRLHSVVVKYRKKHKMDVVSEF : 44
AbEV1  : MVKYPEMSKPVITKGVYQTFNAVSSRLHSVVVKYRKKHKMDVVSEF : 44
OSV    : MTKYPIKSRPVLTKICFEEGRSITGRIFSVMNLRITVTPDPEKIL : 44

                *           60           *           80
ARP250 : DRCANCYFVVPNYQHVLDEFKNDPITYNVNNIVDWMNRRKNSSVV : 88
AbEV1  : DRCANCYFVVPNYQHVLDEFKNDPITYNVNNIVDWMNRRKNSSVV : 88
OSV    : WDVCNAYEKEEGWEHNI PHEKNDLLIITPEDVKNWIEENKDCFGV : 88

                *           100          *           120          *
ARP250 : AAELIELMQTGWFNHPINSVKVHSKLESLLKEDEIGYQYGQQKI : 132
AbEV1  : AAELIELMQTGWFNHPINSVKVHSKLESLLKEDEIGYQYGQQKI : 132
OSV    : EKELNDLLAGELLIKPLNDVNVHLKLESLLKDKHISIMKEQQ-A : 131

                140           *           160           *
ARP250 : RIIVHQTYAICAI FADIFLQAKERFKQLLGEKCVYADGFTLEEL : 176
AbEV1  : RIIVHQTYAICAI FADIFLQAKERFKQLLGEKCVYADGFTLEEL : 176
OSV    : RIIVWQRKAVCSLFAKLEVRCKDRLKTLLVDHILYVDGLRPDEI : 175

                180           *           200           *           220
ARP250 : AARMRLIKSDKPLWVFERDFTKQDRQTD EDLINFEMYVYKQLGV : 220
AbEV1  : AARMRLIKSDKPLWVFERDFTKQDRQTD EDLINFEMYVYKQLGV : 220
OSV    : SAKLRQIS--DVFGEFENDLTKQDRQTDKPILEVEMLMYLM LGV : 217

                *           240           *           260
ARP250 : HDDVVSFWRKTHENWFYKGTDLSGSLSWMRLTGQCTTALGNAIT : 264
AbEV1  : HDDVVSFWRKTHENWFYKGTDLSGSLSWMRLTGQCTTALGNAIT : 264
OSV    : HPNIISSWRSSHDDWRFKSTNYW GKSTAMRLTGQATTALGNCTIT : 261

                *           280
ARP250 : NMVVNSRITYQEYEQNIKIMLLLGDD : 289
AbEV1  : NMVVNSRITYQEYEQNIKIMLLLGDD : 289
OSV    : NMQVHSKFVIKNKYW LKFAFLGDD : 286

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Fig. 5.9- Multiple alignment of ^{ARP250}14.4, AbEV1 and OSV amino acid sequence in the RdRp region. The alignment was constructed using the MegAlign package and displayed via GENEDOC program. The ^{ARP250}14.4 and AbEV1 sequences share 100% identity, while the identity is 54% between ^{ARP250}14.4 and a different endornavirus such as OSV within the RdRp region

The amino acid ^{ARP250}14.4 deduced sequence exhibited the usual RdRp conserved motifs III, IV, V, VI (data not shown, Section 3.3.2).

A ‘*consensus*’ sequence of 256 bp was produced for the ^{ARP250}14.4 glycosyltransferase region. The sequence showed 98% similarity with AbEV1 at the DNA level (Annex 5.3) and 100% similarity for the deduced amino acid sequence (Annex 5.4).

ARP250 sequences from the ITS region were also obtained. Phylogenetic analyses based on ITS sequences of ARP250 isolate and other various wild *Agaricus* isolates allowed the identification of ARP250 isolate as *A. bisporus*. Isolate ARP250 clustered within the *A. bisporus* clade with 90 % bootstrap support (Fig. 5.10) and showed 100% identity with isolate BISP_RWK 1885 (data not shown).

5.3.2 Mushrooms from the Warwickshire

The two isolates collected in Warwickshire (P44 and W52-B) did not yield any MVX-like elements in the RT-PCR screening (data not shown). ITS sequences were obtained for P44 and W52-B isolates and phylogenetic analyses revealed that P44 was an *A. vaporariorum* collection (with 100% bootstrap support) and that W52-B was an *A. bisporus* collection (with 90 % bootstrap support; Fig. 5.10).

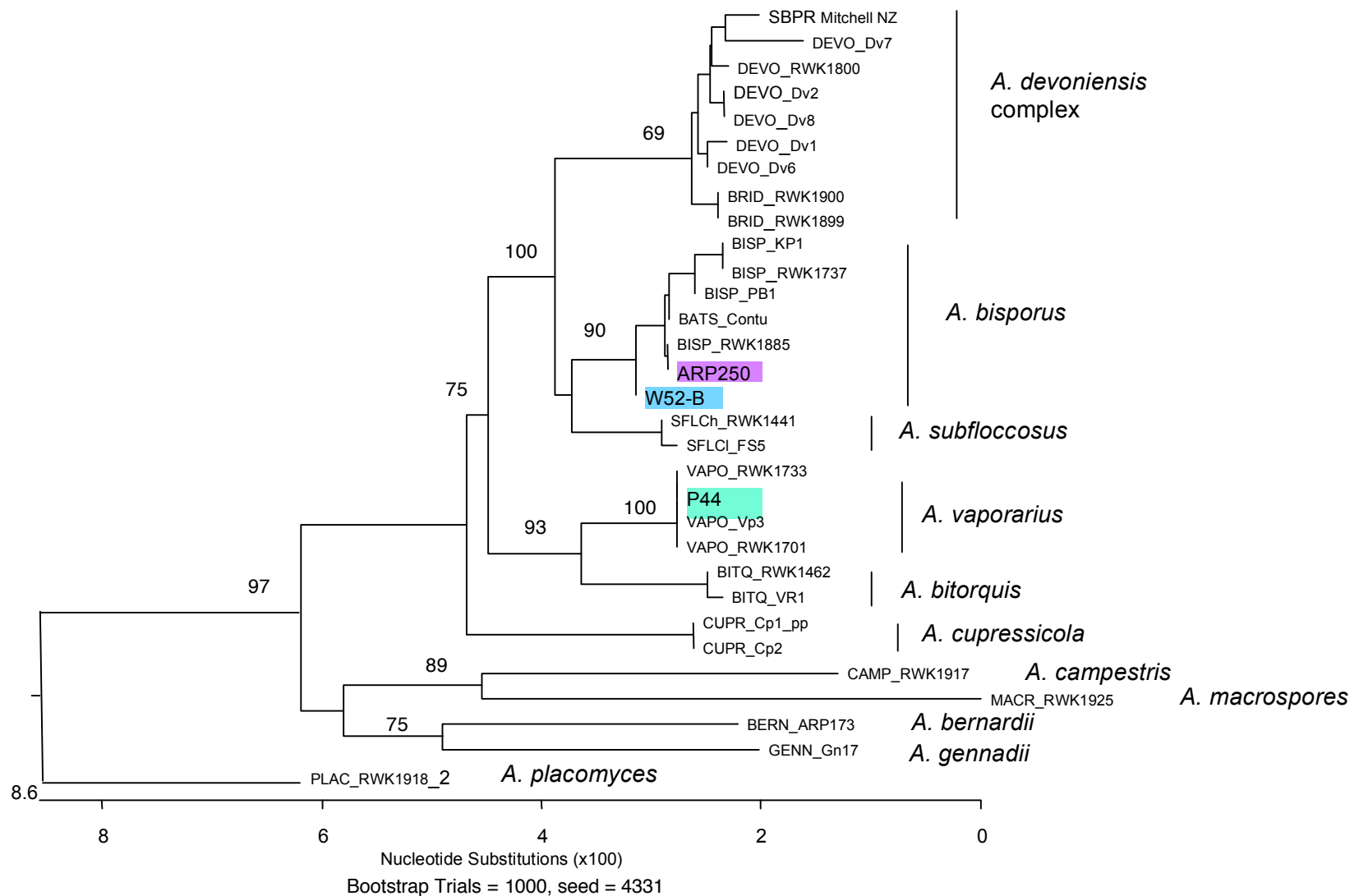


Fig. 5.10– Phylogenetic tree of *Agaricus* isolates inferred from ITS sequences. A neighbour-joining phylogenetic tree of ITS sequences was constructed using the MegAlign package via ClustalW algorithm. Bootstrap values (% of 1000 resamplings) over 65% are indicated on the branches. ITS sequences of isolates ARP250, W52-B, and P44 are compared with established *Agaricus* ITS sequences (Challen *et al.*, 2003). Isolates ARP250 (highlighted in violet) and W52-B (highlighted in torquoise) clustered within the *A. bisporus* clade with 90 % bootstrap support, whereas the isolate P44 (highlighted in green) clustered within *A. vaporarius* clade with 100% bootstrap support

5.4 Discussion

Agaricus bisporus has been cultivated for over a century, first in Europe (primarily France), then in eastern North America and most recently in western North America (Petersen & Hughes, 1999).

In the present study three different *Agaricus* collections (isolates P44 and W52-B from the UK and isolate ARP250 from California) were identified using phylogeny analysis based on ITS sequences. ITS sequences are ribosomal repeat units encoding for ribosomal RNA (rRNA). Nuclear RNA genes in fungi are arranged as tandem repeats with several hundred copies per genome. In filamentous fungi, each repeat ranges from 7.7 to 12 kbp in size (White *et al.*, 1990). Each rRNA repeat unit contains three rRNA genes: the small nuclear (18S-like) rRNA, the 5.8S rRNA, and the large nuclear (28S-like) rRNA. Within each repeat unit, 5.8S region is flanked by bipartite internal transcribed spacers (ITS), ITS1 and ITS2, which separate the 5.8S rRNA gene from the 18S and 28S gene, respectively. Since ITS regions tend to be highly polymorphic, they have been used extensively in fungal taxonomic and phylogenetic studies (Calvo-Bado *et al.*, 2000). Phylogeny analysis revealed that isolates ARP250 and W52-B were *A. bisporus* collections, whereas isolate P44 was an *A. vaporariorum* collection.

RT-PCR screening of the three *Agaricus* isolates for MVX-like elements revealed the presence of AbEV1-like virus only in isolate ARP250. The presence of this element was further confirmed through dsRNA profile analysis, showing dsRNA molecules with very similar molecular size to those present in cultivated MVX-infected isolates (16.2 kbp, 14.4 kbp and 2.2 kbp). Sequence similarity analyses of the 14.4 kbp element (^{ARP250}14.4) found in ARP250 isolate showed moderate differences at DNA level (97-100% identity) with AbEV1 sequence for conserved domains (helicase, RdRp and glycosyltransferase).

It is generally accepted that viruses, especially RNA viruses mutate quite easily since RdRp replicate their genome with lower fidelity than DNA polymerase (Wang *et al.*, 2005). It has been reported that RNA enzymes are less accurate than DNA enzymes because they lack proofreading activities resulting in an error rate of replication several order of magnitude higher than that affecting DNA replication (Drake & Holland, 1999). The average mutation rate of RNA viruses ranks in the order of 10^{-4} to 10^{-5} misincorporation for nucleotide acid per round of copying. This means that each daughter genome will contain on average one or two mutations when compared to the parental sequence (Batschelet *et al.*, 1976). However, due to the degeneracy of the genetic code, mutations of nucleotide bases do not always correspond to mutations at amino acid level. ^{ARP250}14.4 sequence was highly conserved at amino acid level (100% identity) in the ^{ARP250}14.4 polymerase and glycosyltransferase domains, despite the mutations in DNA sequence. However, one mutation appeared in the helicase region showing a change from threonine to isoleucine. These two amino acids have quite different properties; threonine is a polar uncharged amino acid, isoleucine is a hydrophobic amino acid. The amino acid change did not occur in the helicase conserved motifs and would not be expected to play a critical role in enzyme function.

Virus isolates, whose genomes differ by 3-4 % have been reported as belonging to the same virus species (Schwemmler *et al.*, 1999). Gessain *et al.* (1993) reported an amino acid sequence variability ranging between 3 and 25% for different strains of human T-cell leukaemia retrovirus type I. Wild rice endornavirus (ORV) polymerase amino acid sequence exhibits 92% identity with the cultivated rice endornavirus (OSV) polymerase sequence (this study). The minor difference between AbEV1 and ^{ARP250}14.4 suggests that these viruses are closely related and that ^{ARP250}14.4 may represent a wild accession of AbEV1.

The 'wild' ARP250 isolate, obtained through the activities of the Agaricus Resource Program (Kerrigan, 1991, 1996), was collected near Asilomar, California. In wild isolates

from California, Kerrigan *et al.* (1995) differentiated two strains based on RFLP patterns. One strain, usually found fruiting in lawns, was similar to the commercial strains originally domesticated in Europe, whereas the other strain, fruiting at the margin of native woods, was unique and probably endemic to California. Other populations of *A. bisporus* with European RFLP patterns have probably escaped from commercial production sources and from home compost piles. Very little is known about European and UK wild mushroom diversity.

Although it is possible that the presence of ^{ARP250}14.4 could have arisen through contamination of laboratory cultures, a number of different observations suggest that this is not the most likely explanation. The observation that an AbEV1-type RT PCR product could be amplified from ARP250 tissue culture derivatives was first made independently in an USA laboratory. The USA mushroom industry has not experienced outbreaks of the MVX disease and the laboratory concerned did not handle large numbers of MVX infected strains (Challen, *pers. comm.*). Two other wild collections from independent locations in Greece have also been found to yield RT PCR amplicons using primers for ^{MVX}3.6. Several different laboratories have observed that total dsRNA extractions from many different wild mushroom collections often reveal very complex dsRNA profiles (Kerrigan, 2004). Collectively these observations suggest that it is quite likely that ^{MVX}dsRNA elements can occur in wild mushroom collections, but screening of nucleic acid preparations isolated directly from wild mushroom collections would be required to eliminate chance of contamination.

Recently, the ^{ARP250}14.4 was transmitted from ARP250 isolate to commercial *A. bisporus* strains through dual culture assay, demonstrating the potential of wild isolates as dsRNA donor (Holcroft, *pers. comm.*). Conversely, no horizontal transmission has been demonstrated yet from commercial to wild *Agaricus* strains (Challen, *pers. comm.*).

Future studies aiming at the screening of further wild *Agaricus* collections might reveal the geographic and genetic diversity of AbEV1-like strains, and provide further insight into the origin of MVX infection. Moreover, it would be interesting to extend the screening for ^{MVX}dsRNAs to other genera since virus transmission through anastomosis has been reported between incompatible *Heterobasidion annosum* isolates (Ihrmark *et al.*, 2002).

Chapter 6

AbEV1 SILENCING

6. Summary

This chapter describes experiments carried out to examine homology-dependent gene silencing (HDGS) pathways in *A. bisporus*. ‘Hairpin vectors’ with self-complementary AbEV1 sequences were introduced into *A. bisporus* mushrooms using *Agrobacterium*-mediated transformation technique and recovered transformants screened by PCR and RT-PCR analyses. Progress in the development of alternative selectable marker (resistance to phleomycin) for mushroom transformation is also described. Furthermore, the effect of different compounds (hygromycin and cyclic adenosine monophosphate) on AbEV1 replication has been evaluated.

6.1 Introduction

6.1.1 Homology-Dependent Gene Silencing in fungi

During the past decade it has become evident that different organisms can react to the introduction of foreign nucleic acids by inducing gene silencing mechanisms that are based on the recognition of nucleic acids sequence homology (Meyer & Saedler, 1996; Cogoni & Macino, 2000). Gene silencing is achieved via diverse strategies: homologous sequences can be inactive at the transcriptional level involving DNA methylation (Vaucheret *et al.*, 1998) or at post-transcriptional level involving sequence-specific RNA degradation/translation repression (Olsen & Ambros, 1999; Heneghan *et al.*, 2007). Fungi appear particularly well equipped to fight against parasitic sequences (Cogoni & Macino, 1999). In *Neurospora crassa* at least two gene silencing mechanisms exist, quelling and repeat-induced point mutation, which act during the vegetative and reproductive cycles respectively.

6.1.1.1 Post-Transcriptional Gene Silencing in fungi

Quelling is the post-transcriptional gene silencing (PTGS) phenomenon first described in *N. crassa* (Romano & Macino, 1992; Section 1.9). Wild-type (orange) strains of *N. crassa* were transformed with *albino* (*al-1*, *al-2*, *al-3*) transgenes, required for carotenoid biosynthesis. Transformed strains frequently showed white (albino) phenotypes, indicating that both the endogenous *albino* gene and the *albino* transgene were inactivated. Since then, quelling has been observed for several different genes (reviewed by Cogoni & Macino, 1997). Analysis of quelled *Neurospora albino-1* (*al-1*)

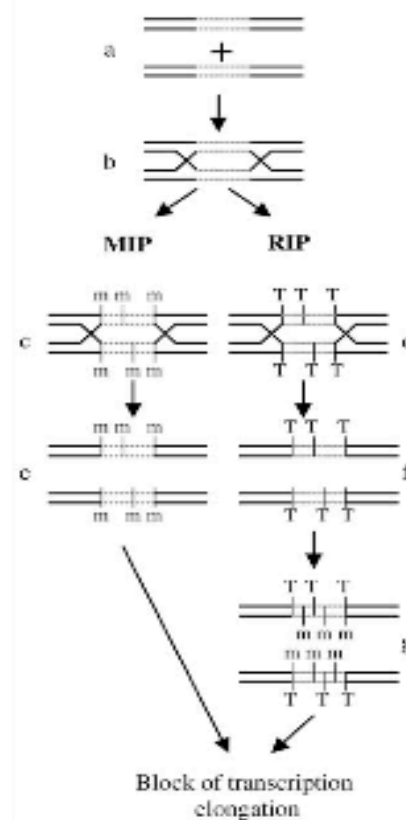
transformants revealed drastic gene-specific reductions in levels of *al-1* mRNA. Using nuclear run-off assays it was found that quelled strains produce the same amount of *al-1* primary transcript as the wild type strain in the nucleus indicating that quelling does not affect transcription, but acts at a post-transcriptional level (Cogoni *et al.*, 1996). Quelling is a dominant phenomenon in heterokaryons with nuclei from quelled and wild-type strains, suggesting the presence of mobile signal spreading across cells (Cogoni *et al.*, 1996).

Studies on quelling-deficient (*qde*) mutants of *N. crassa* along with studies on other eukaryotes have paved the way for the identification of genes required for PTGS in different organisms (Cogoni & Macino, 2000). Homology between the *N. crassa qde-1* gene (Cogoni & Macino, 1999), a tomato gene (Schiebel *et al.*, 1998), the *Arabidopsis* SDE1/SG2 gene (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000), and the *C. elegans ego-1* gene (Smardon *et al.*, 2000) provided the first experimental evidence that PTGS phenomena are mechanically related across kingdoms (reviewed by Cogoni & Macino, 2000). Similarly, the protein product encoded by the second *qde* gene, *qde-2*, was shown to be a piwi-PAZ domain (PPD or Argonaute) protein (Catalanotto *et al.*, 2000), an essential and conserved component of the RNA silencing pathway in a variety of eukaryotic organisms. The *qde-3* gene encodes a putative RecQ-type DNA helicase (Cogoni & Macino, 1999), which has been recently shown to play a role in recombination repair (Pickford *et al.*, 2003; Kato *et al.*, 2004). PTGS has also been reported in other fungi including fungi from the *Ascomycota*, *Basidiomycota*, and *Zycomycota* (Liu *et al.*, 2002; Kadotani *et al.*, 2003; Fitzgerald *et al.*, 2004; Mouyna *et al.*, 2004; Rappleye *et al.*, 2004; Hammond *et al.*, 2005) as well as the *Oomycota* (Latijnhouwers *et al.*, 2004). RNA silencing pathways have been reported in fungi including *Aspergillus nidulans* (Hammond & Keller, 2005), *Candida albicans* (De Backer *et al.*, 2001), *Coprinus cinereus* (Heneghan *et al.*, 2007), *Magnaporthe oryzae* (Kadotani *et al.*, 2003), *Neurospora crassa* (Romano & Macino, 1992), and *Schizophyllum commune* (de Jong *et al.*, 2006).

6.1.1.2 Transcriptional Gene Silencing in fungi

Repeat-Induced Point Mutation (RIP), a form of transcriptional gene silencing (TGS) was characterised in *N. crassa* (Cambareri *et al.*, 1989; Cambareri *et al.*, 1991). RIP is highly regulated, occurring in the premeiotic phase (Selker, 1990) where the two nuclei of opposite mating type share a common cytoplasm in dikaryotic cells before karyogamy. RIP can inactivate the expression of unpaired DNA sequences in each nucleus (reviewed by Nakayashiki, 2005). RIP takes place as mutagenesis of unpaired sequences by G: C to A: T transition and methylation of the remaining cytosines (Fig. 6.1, Selker *et al.*, 1993). A similar phenomenon was discovered in *Ascobolus immersus*, methylation-induced premeiotically (MIP, Rhouhim *et al.*, 1992; Rossignol & Faugeron, 1995). MIP also occurs in the sexual phase but involves only DNA methylation (Barry *et al.*, 1993), which like RIP inhibits transcription elongation (Fig. 6.1).

Fig. 6.1- RIP and MIP gene silencing mechanisms. Paired DNA hybrids (b) between partially homologous sequences are the substrate for specific *de novo* DNA methyltransferases in *Ascobolus* during the methylation induced premeiotically process (c) or are hypermutagenized by repeat induced premeiotically process in *Neurospora* via C to T transitions (d). DNA methylation is maintained even in the absence of continuous DNA-DNA pairing (e). In *Neurospora* mutagenized sequences (f) constitute substrate for methyltransferases (g). In both *Neurospora* and *Ascobolus*, DNA methylation has been demonstrated to interfere with transcription elongation probably by inducing chromatin condensation



6.1.2 Gene Silencing as a Virus Resistance Mechanism

There are a growing number of indications that support the idea that various homology-dependent gene silencing (HDGS) phenomena correspond to host-defence responses against parasitic nucleic acids such as transposons, RNA, or DNA viruses and viroids (Baulcombe, 1999; Matzke *et al.*, 2000). Several lines of research indicate that gene silencing at PTGS is a general antiviral defence mechanism in plants (reviewed by Vance & Vaucheret, 2001) and is considered an RNA-based gene silencing phenomenon. The strongest evidence in support of this comes from the silencing of RNA viruses performing their life cycle exclusively in the cytoplasm of the host cell (Lindbo & Dougherty, 1992). Further support resides in the observations that dsRNA molecules are inducers of PTGS in several organisms (Fire *et al.*, 1998; Kennerdell & Carthew, 1998; Montgomery *et al.*, 1998; Ngô *et al.*, 1998; Misquitta & Paterson, 1999; Sanchez-Alvodoro & Newmark, 1999). Although dsRNA does not naturally occur in a cell and is not a product of normal gene expression, it can be produced as a replication intermediate form by RNA viruses (reviewed by Fire, 1999).

In the last few decades PTGS has been strongly linked to the historically known pathogen-derived resistance (PDR, reviewed by Rovere *et al.*, 2002; Goldbach *et al.*, 2003; Zadeh & Foster, 2004), which confers protection to plants against viral infections by transferring of virus-derived transgene into the plant host. Some of these PDR events can be explained at a molecular level by the PTGS phenomenon resulting in the degradation of both viral RNA and virus-derived transgene, and resistance of the plant to the virus (Baulcombe, 1999). Early in infections, transgene expression is unaffected by the virus and the normal viral symptoms are produced. As infection progresses upper leaves are free of virus, resistant to secondary viral infection, and contain lower concentration of transgene transcript. The plant exhibiting this response is called 'recovered' (Lindbo *et al.*, 1993).

Although PTGS was first described and understood in artificial systems, this phenomenon is considered a natural defence mechanism against viral infection and transposons mobilization (reviewed by Chicas & Macino, 2001). This notion is supported by several observations. Firstly, plants can recover from natural viral

infections via a process similar to PTGS (Matthews, 1992; Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al Kaff *et al.*, 1998; Ratcliff *et al.*, 1999). Secondly, the finding that many viruses encode proteins that suppress RNA silencing (Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Baulcombe, 1999) supports indirectly the idea that RNA silencing is a natural defence mechanism, which viruses have to counteract (Anandalakshmi *et al.*, 2000). Further evidence has come from plant mutants defective in PTGS showing hypersusceptibility to virus infection (Mourrain *et al.*, 2000).

No virus system is available for fungi and our understanding of RNA silencing in fungi comes primarily from gene expression studies (Cogoni, 2001; Catalanotto *et al.*, 2004; Heneghan *et al.*, 2007). Although RNA silencing has not been shown to serve directly as an antiviral defence mechanism in fungi, Segers *et al.* (2006) demonstrated that the fungal virus, hypovirus CHV1-EP713 (Section 1.8.5.2) encodes a protein suppressing the RNA silencing pathway in *Cryphonectria parasitica* and in a heterologous plant system, providing additional evidence for cross-kingdom conservation of RNA silencing mechanisms.

6.1.3 PTGS Viral Suppressors

The hypothesis that PTGS can form an important part of the innate response was first supported by the observation that many plant viruses encode proteins that inhibit PTGS (Voinnet *et al.*, 1999). *Tomato bushy tomodusvirus* and *Turnip mosaic virus* have both been shown to encode suppressors of RNA silencing, which reduce cellular antiviral effects allowing the respective viruses to accumulate to high titres (Vance & Vaucheret, 2001; Zamore, 2004; Dunoyer & Voinnet, 2005). Silencing suppressors have also been identified for a large number of other plant viruses and a growing number of animal viruses, including (+) and (-) ssRNA, and ssDNA viruses (Silhavy & Burgyan, 2004). Recently a gene silencing viral suppressor was characterised in the fungal virus, *Cryphonectria hypovirus* 1-EP713 (Segers *et al.*, 2006).

Theoretically, viruses could inhibit RNA silencing by: i) inhibiting viral siRNAs generation; ii) interfering with silencing effector complexes; iii) interfering with the synthesis of movement silencing signal (Silhavy & Burgyan, 2004). A striking feature

of PTGS suppressors is the huge diversity in sequence and structure and their presence in virtually any type of virus (reviewed by Moissiard & Voinnet, 2004). Such diversity is an example of evolutionary convergence and can be explained by the fact that silencing suppressors evolved as an additional feature of unrelated proteins that already had diverse functions (reviewed by Moissiard & Voinnet, 2004). Given the diversity in sequence of silencing viral suppressors, currently such proteins are best identified through functional assays. Because of its simplicity the reversal assay is largely used (reviewed by Moissiard & Voinnet, 2004). The virus carrying the putative suppressor protein is inoculated into the organism showing silencing for a stably integrated reporter gene, e.g. green fluorescent protein (GFP, Fig. 6.2). In the absence of silencing suppressor, the silenced GFP phenotype is unaffected. In the presence of a suppressor it reverts and GFP is expressed (Voinnet *et al.*, 2000; Dunoyer *et al.*, 2002; Hamilton *et al.*, 2002; Bucher *et al.*, 2003;). The reversal assay provides evidence that silencing viral suppressors are not sequence specific and interfere with PTGS pathways already triggered (Brigneti *et al.*, 1998).

Anandalakshmi *et al.* (2000) suggested the involvement of calcium in regulating PTGS suppression. They identified a cellular PTGS suppressor, which is a plant calmodulin-related protein functionally similar to the helper component-proteinase (Hc-pro) of potyviruses, suppressing PTGS in plants. Since calmodulin and related proteins normally act by binding calcium, this finding points to a role for calcium in PTGS pathway.

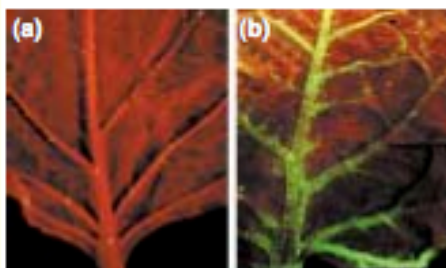
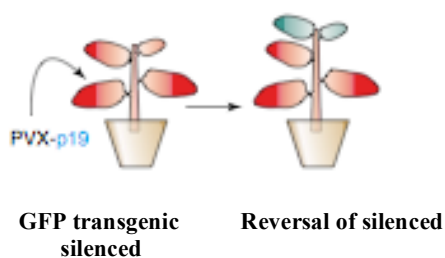


Fig. 6.2- Reversal GFP assay in plant system. In the reversal assay (above panel), the *Tombusvirus* silencing suppressor, p19 is expressed from a recombinant PVX vector that is inoculated into a silenced green-fluorescent protein (GFP) transgenic plant. The protein p19 leads to the reversal of the silencing phenomenon shown in the drawing by green fluorescence in the upper leaves. (Below panel): (a) leaf of a transgenic plant where GFP is uniformly silenced, therefore red under UV illumination; (b) initial reversal of GFP silencing induced in a new emerging leaf by infection of PVX expressing the p19 protein (from Voinnet, 2001)

6.1.4 RNA Silencing Technology

Double-stranded RNAs are potent activators of RNA silencing and can be used to trigger sequence-specific degradation of target RNAs (Tenllado *et al.*, 2004). Different methods can be used to induce RNA silencing depending on the organism to transform: i) injection of dsRNA molecules into the organism (Fire *et al.*, 1998; Wang *et al.*, 2005); ii) soaking the organism in a solution containing the dsRNAs (Tabara *et al.*, 1998); iii) transformation with transgenes expressing self-complementary RNA molecules (Waterhouse *et al.*, 1998; Hamilton *et al.*, 1998). Although RNA silencing is still a relatively novel technique (Weld *et al.*, 2006), it has been recently used to down-regulate genes in filamentous fungi (Nokayashiki, 2005; Heneghan *et al.*, 2007; Section 6.1.1). One approach is to use ‘hairpin’ vectors, which integrate ectopically. They contain regions of the target gene transcribed as self-complementary hairpin structure, which comprises a double-stranded loop region and a base-paired stem. This structure mimics a dsRNA molecule, which can trigger a RNA silencing mechanism. Hairpin constructs have been used to silence *gfp* gene in various fungi, e.g. *Magnaporthe oryzae* (Kadotani *et al.*, 2003), *Venturia inaequalis* (Fitzgerald *et al.*, 2004), *Coprinus cinereus* (Heneghan *et al.*, 2007). Several fungal endogenous genes have also been silenced, including genes involved in fungal virulence (Latijnhouwers *et al.*, 2004; Mouyna *et al.*, 2004; Rappleye *et al.*, 2004).

In the past decades one of the major technical limitations for this technology has been the transformation of fungi. Transformation is a powerful technology whereby genes can be transferred within or between different species (Challen *et al.*, 2000). It may reduce the need for traditional breeding, enabling direct modification of the genome. It can be used to add new genes, delete or modify the expression of existing genes. Successful transformation of fungi has been achieved by: i) protoplasts electroporation (Ward *et al.*, 1989; Ozaki *et al.*, 1994; Ruiz-Diez and Martinez-Suarez, 1999; Kuo *et al.*, 2004; Amey *et al.*, 2002), ii) particle bombardment (Lorito *et al.*, 1993; Parker *et al.*, 1995; Davidson *et al.*, 2000; Hazell *et al.*, 2000), iii) and *A. tumefaciens*-mediated transformation (Amey *et al.*, 2002; Michielse *et al.*, 2005; Burns *et al.*, 2006).

Application of biotechnology to the basidiomycete *A. bisporus* was hampered until recently by the lack of an efficient transformation system. Although protoplast-based transformation was reported for this fungus (van de Rhee *et al.*, 1996a, b), this proved relatively ineffective (Challen *et al.*, 2000). It was not until *A. tumefaciens* mediated-transformation was developed that an efficient and reproducible method became available (de Groot *et al.*, 1998; Chen *et al.*, 2000; Mikosch *et al.*, 2001; Burns *et al.*, 2005). *Agrobacterium*-mediated transformation has been shown to produce a significantly higher frequency of transformation and more stable transformants in fungi (Meyer *et al.*, 2003; Idnurm *et al.*, 2004). Under appropriate conditions, *A. tumefaciens* is able to transfer DNA (T-DNA) to a wide range of fungi and fungal tissues. *Agrobacterium tumefaciens* is a plant pathogen soil-borne bacterium causing the crown gall tumor in a wide range of plants (Mikosch *et al.*, 2000). The bacterium transfers a part, the T-DNA, of its tumor inducing (Ti) plasmid to plant cells. The T-DNA then integrates into the plant nuclear genome. The induction of T-DNA transfer depends on a set of virulence (*vir*) genes, which are located on the Ti plasmid. The *vir* genes are induced by compounds secreted from wounded plant cells, such as acetosyngone (Kado *et al.*, 1991). This natural transformation system has been used in plant research for more than 25 years and has recently been used to transform filamentous fungi (de Groot *et al.*, 1998; Dunn-Coleman and Wang, 1998). Fungi that were recalcitrant to transformation by other systems have been successfully transformed by co-cultivation with *Agrobacterium* (Challen *et al.*, 2000; Foster *et al.*, 2004; Mills *et al.*, in press; reviewed by Weld *et al.*, 2006).

To date the only successful selective marker for *Agrobacterium*-mediated transformation of *A. bisporus* is the *E. coli* hygromycin resistance gene, *hph* (Burns *et al.*, 2006). To progress the development of alternative selective marker for *A. bisporus*, the phleomycin resistance gene (*ble*, Drocourt *et al.*, 1990) was tested in this project. Phleomycin is a glycopeptide antibiotic of the bleomycin family (Gatignol *et al.*, 1988), isolated from a mutant strain of *Streptomyces verticillus*. It binds and intercalates DNA, thus destroying the integrity of the double helix. The phleomycin resistance gene was initially isolated from the bacterium *Streptoalloteichus hindustatus* (Drocourt *et al.*, 1990) and encodes a small protein with strong affinity for the phleomycin antibiotics

family (Gatignol *et al.*, 1988). When these antibiotics are bound by the *Sh ble* proteins, phleomycins can no longer be activated by ferrous ions and oxygen to break down DNA (Gotignol *et al.*, 1988).

6.2 Materials and Methods

6.2.1 Silencing of *Agaricus bisporus* endornavirus 1

A homologous-dependent gene silencing study was carried out using ‘hairpin constructs’ with specific sequences from AbEV1. Constructs with AbEV1 self-complementary sequences were introduced through *Agrobacterium*-mediated transformation into two types of *A. bisporus* strains: i) AbEV1-infected strains and ii) AbEV1-free strains. AbEV1-free transformants were subsequently challenged in an *in vitro* dual-culture assay using an AbEV1 donor. Positive transformants from both sets of mushroom strains were screened for the presence of the virus in order to detect possible silencing.

6.2.1.1 Mushroom strains

Gill tissue of two *A. bisporus* strains was used for fungal transformation. Strain SSI 61 was obtained from the epidemiological experiment (SSI Experiment) described in Section 4.2.1 and was used to produce AbEV1-infected transformants. The commercial strain A15 (Section 4.2.1.1) and a carboxin resistant mutant C63-carb422 (Section 4.2.2.1) were used to produce AbEV1-free transformants.

6.2.1.2 Construction of Silencing Vectors

Silencing vectors (hairpin vectors) were constructed using the hairpin expression vector pRNAiDE001 (Eastwood *et al.*, unpublished; Fig. 6.3), which contained the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase II (Ab *gpdII*) promoter (350 bp), *Aspergillus nidulans* tryptophan synthetase (An *trpC*) terminator (766 bp), *Escherichia coli uidA* (β -glucuronidase, GUS) intron (325 bp), and an ampicillin resistance gene as selectable marker for bacterial cloning. The hairpin cassette was constructed inserting twice in opposite orientations (sense and antisense) the same RT-PCR product in order to obtain inverted repeats.

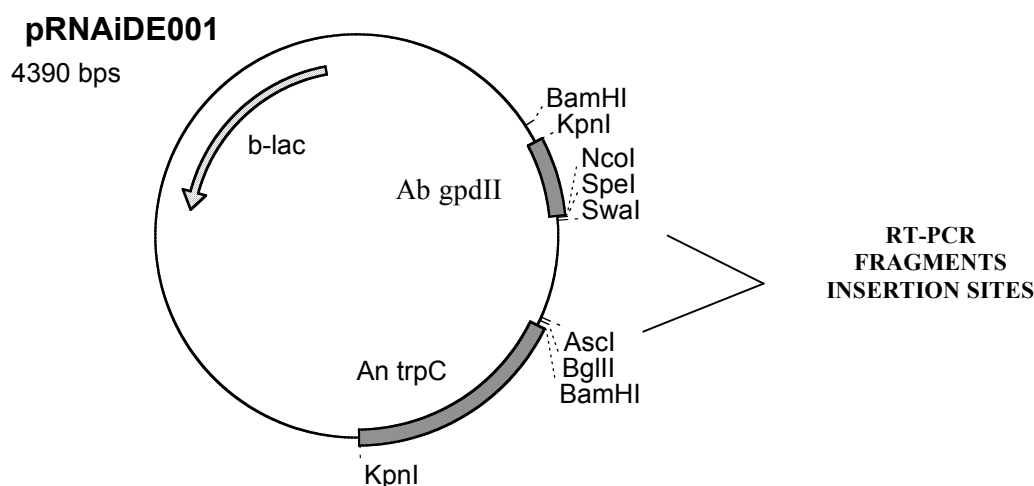


Fig. 6.3- pRNAiDE001. This plasmid is 4390 bp long and contains an ampicillin resistance gene (b-lac) as selectable marker for bacterial cloning. Ab *gpdII* = *Agaricus bisporus gpdII* promoter, An *trpC* = *Aspergillus nidulans trpC* terminator

Three different AbEV1 regions of the RNA-dependent RNA polymerase (RdRp) and helicase domains (Section 3.3.2) were recovered using RT-PCR amplification and primers (Table 6.1) to introduce appropriate *SpeI*-*BglII* and *SmaI*-*AscI* restriction sites.

Table 6.1- Primers amplifying the AbEV1 helicase and RdRp regions

Primer	Primer sequence Reference: this thesis	Restriction enzymes	Product Size (bp)	RNA Target	Frag
B3f89ext B3r516ext	5'- <u>GACAAGATCTACTAGT</u> -CGGCCTCGTTACCTTGGTAACTG -3' 5'-AGAGGCGCGCCATTTAAAT-TGACGGAGTGTTGGTTC -3'	<i>BglII/SpeI</i> <i>AscI/SmaI</i>	463	AbEV1 helicase	F1
B3f89ext B3r311ext	5'- <u>GACAAGATCTACTAGT</u> -CGGCCTCGTTACCTTGGTAACTG -3' 5'- AGAGGCGCGCCATTTAAAT-CCCGAATTATGTGTCATCC-3'	<i>BglII/SpeI</i> <i>AscI/SmaI</i>	258	AbEV1 helicase	F2
B3f90ext B3r522ext	5'- <u>GACAAGATCTACTAGT</u> -GGCCTCGTTACCTTGGTAAATG -3' 5'- AGAGGCGCGCCATTTAAAT-ACCATTGACGGAGTGTTG -3'	<i>BglII/SpeI</i> <i>AscI/SmaI</i>	468	AbEV1 helicase	F3
B32793ext B3r3317ext	5'- <u>GACAAGATCTACTAGT</u> -TGC GTGTATGCCGACGGCTTC-3' 5'- AGAGGCGCGCCATTTAAAT-CATACTGCCGTCTTGTGTTGG	<i>BglII/SpeI</i> <i>AscI/SmaI</i>	560	AbEV1 RdRp	F4
B3f2985ext B3r3333ext	5'- <u>GACAAGATCTACTAGT</u> -CGCAAGACACATGAGAATTGG -3' 5'- AGAGGCGCGCCATTTAAAT-AGTCAGGACAGACAGACATAC-3'	<i>BglII/SpeI</i> <i>AscI/SmaI</i>	384	AbEV1 RdRp	F5
B3f2793ext B3r3061ext	5'- <u>GACAAGATCTACTAGT</u> -TGC GTGTATGCCGACGGCTTC -3' 5'- AGAGGCGCGCCATTTAAAT-TGTCCAGTGAGACGCATCCAAC-3'	<i>BglII/SpeI</i> <i>AscI/SmaI</i>	304	AbEV1 RdRp	F6

Frag = AbEV1 RT-PCR amplicon; RdRp = RNA-dependent RNA polymerase. Restriction sites are underlined in each oligonucleotide

Amplified AbEV1 fragments were ligated into pRNAiDE001 plasmid using *SpeI-SwaI* and *BglII-AscI* restriction digests and cloned into *E. coli* competent cells (Section 2.10.1). The overall organization for the hairpin cassette was: Ab *gpdII* promoter, sense AbEV1 insert, spacer (GUS intron), antisense AbEV1 insert, and An *trpC* terminator (see Results, Fig. 6.5). Hairpin expression cassettes were excised by enzymatic digestion with *KpnI* (Roche, cat. No. 899186) and inserted into pGREENhph01 vectors (Eastwood *et al.*, unpublished).

The pGREENhph01 vector (Fig. 6.4) comprised a klenow modified 3984 bp *BglII-HindIII* *AngpdA*-hph-AntrpC expression cassette encoding the hygromycin B resistance gene, excised from pAN7-1 (Punt *et al.*, 1987), cloned into *EcoRV* site of pGREENII (Hellens *et al.*, 2000). The pGREENhph01 plasmid was linearized and dephosphorilated by enzymatic digestion with *KpnI* and shrimp alkaline phosphatase (Roche, cat. No. 1758250) respectively. The resulting hairpin constructs were cloned into *E. coli* competent cells (Section 2.10) and purified plasmids were restricted using *BglII* (Roche, cat. No. 348767) to confirm the presence and orientation of inserts.

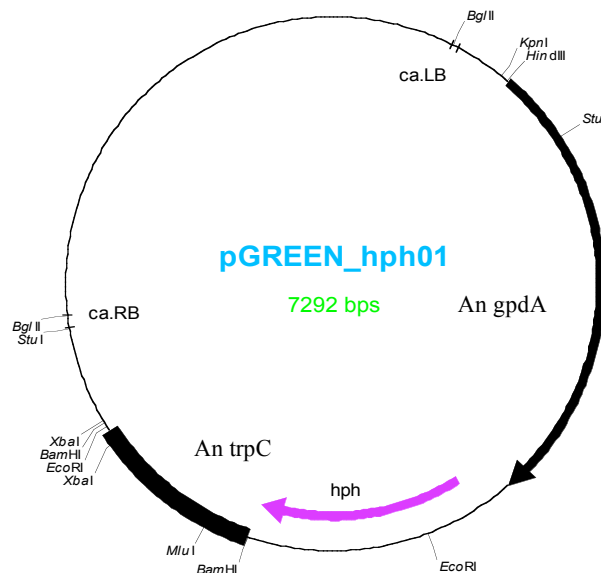


Fig. 6.4- The pGREENhph01. It comprises a klenow modified 3984 bp *BglII-HindIII* *AngpdA*-hph-AntrpC expression cassette encoding the hygromycin B resistance gene

6.2.1.3 *Agrobacterium*-mediated Transformation of *Agaricus bisporus*

Hairpin vectors and the pSOUP helper plasmid (Section 2.11) were co-transformed into *A. tumefaciens* by electroporation (Shen & Forde, 1989; Section 2.11). pGREENhph01 only was also electroporated into *A. tumefaciens* to obtain a control strain, NC. Purified plasmids (QIAprep Spin Miniprep kit, Qiagen, cat. No. 27106) were digested with *Bgl*III to assess their integrity and screened by PCR (Section 2.8.2) with appropriate primers (Table 6.2).

Table 6.2- Primers amplifying sense and antisense fragments in *Agrobacterium* transformants. Primers amplified either the sense insert by annealing with the *gpdII* promoter and the GUS spacer region, or the antisense insert by annealing with the *trpC* terminator and the GUS spacer region

Primer	Primer sequence Reference: this thesis	Product Size (bp)	DNA Target
GPDGUS-2210 GPDGUS-1420	5'-TTCCGGTCACATCCACCATC-3' 5'-CCGGTTCGTTGGCAATACTC-3'	724	F1 sense insert
GUSTRPC-769 GUSTRPC-1433	5'-TGCCAACGAACCGGATACCC-3' 5'-GCGGTCGGCATCAGATCTAC-3'	598	F1 antisense insert
GPDGUS-2210 GPDGUS-1420	5'-TTCCGGTCACATCCACCATC-3' 5'-CCGGTTCGTTGGCAATACTC-3'	519	F2 sense insert
GUSTRPC-769 GUSTRPC-1433	5'-TGCCAACGAACCGGATACCC-3' 5'-GCGGTCGGCATCAGATCTAC-3'	393	F2 antisense insert
GPDGUS-2210 GPDGUS-1420	5'-TTCCGGTCACATCCACCATC-3' 5'-CCGGTTCGTTGGCAATACTC-3'	645	F5 sense insert
GUSTRPC-769 GUSTRPC-1433	5'-TGCCAACGAACCGGATACCC-3' 5'-GCGGTCGGCATCAGATCTAC-3'	519	F5 antisense insert
GPDGUS-2210 GPDGUS-1420	5'-TTCCGGTCACATCCACCATC-3' 5'-CCGGTTCGTTGGCAATACTC-3'	565	F6 sense insert
GUSTRPC-769 GUSTRPC-1433	5'-TGCCAACGAACCGGATACCC-3' 5'-GCGGTCGGCATCAGATCTAC-3'	439	F6 antisense insert

Primers amplified either the sense fragment by annealing with the *gpdII* promoter and the GUS spacer region, or the antisense fragment by annealing with the *trpC* terminator and the GUS spacer (see Results, Fig. 6.5). Primers altHYG1 (5'-CTCTCGGAGGGCGAAGAATC-3') and altHYG2 (5'-GGGCGTCGGTTTCCACTA TC-3') amplified the hygromycin resistance cassette giving an amplicon of 987 bp. Positive *Agrobacterium* transformants were cultured and induced for virulence as

described in Section 2.14.1. Induced *Agrobacterium* transformants were used for gill tissue transformation of AbEV1-infected and AbEV1-free mushroom strains as described in Section 2.14.2. *Agaricus* gill tissue was cultured on MPA supplemented with 200 µg/ml cefotaxime and 25 µg/ml hygromycin for 2 weeks at 25° C and then MPA supplemented with 25 µg/ml hygromycin for another 3 weeks.

6.2.1.4 Analysis of Putative *Agaricus* Transformants

Putative *Agaricus* transformants were recovered and assessed by PCR for presence of transgene using the appropriate primers already used for *Agrobacterium* transformants screening (Section 6.2.1.3) after DNA extraction (Chelex method, Section 2.5.3). PCR screening was performed according to the protocol reported in Section 2.8.2.

6.2.1.5 Virus Inoculation

In vitro dual-culture anastomoses were set up in duplicates between AbEV1-free transformants and an AbEV1 donor strain (SSI 61) on CE/CYM medium for 60 days as described in Section 4.2.2.2. Transformants were also challenged with the AbEV1-free donor, A15 as negative control.

The dual-culture challenge was replicated twice for all transformants. Subculture isolations from the carboxin resistance acceptors (C63-carb422) were also tested on CE/CYM medium supplemented with carboxin (15 µg/ml) to confirm the integrity of acceptor strain.

6.2.1.6 Analysis of Hairpin Expression and Viral RNA

Transcript analyses were conducted on *Agaricus* transformants. Total RNA was extracted from samples by TRI-Reagent method (Section 2.5.2) and then subjected to DNase treatment (Section 2.7) to remove any traces of contaminating DNA. Transformants were screened by RT-PCR (Section 2.8.1 and 2.8.2) for expression of the hairpin construct using the appropriate primers (Table 6.3), which spanned the spacer and the specific AbEV1 insert. Given the difficulties to detect the hairpin transcript a second pair of primers (PTGS-1118, 5'-GTTGGATGCGTCTCACTG -3'; PTGS-1393c, 5'-TACTGCCGTCTTGTGTTGG-3') targeting only the viral insert of

fragment 5 (F5) was designed to yield a RT-PCR product of 276 bp. Primers altHYG1 (5'-CTCTCGGAGGGCGAAGAATC-3') and altHYG2 (5'-GGGCGTCGGTTTCCAC TATC-3') amplified the hygromycin resistance cassette giving an amplicon of 987 bp.

Table 6.3- Primers amplifying the hairpin cassette in *Agaricus* Transformants. Primers annealing with the GUS spacer region and AbEV1 inserts

Primer	Primer sequence Reference: this thesis	Product Size (bp)	RNA Target
PTGS-2792c GPDGUS-1420	5'-CCTCGTTACCTTGGTAACTG -3' 5'-CCGGTTCGTTGGCAATACTC-3'	653	F1 sense insert
GUSTRPC-769 PTGS-1583	5'-TGCCAACGAACCGGATACCC-3' 5'-CCTCGTTACCTTGGTAACTG -3'	571	F1 antisense insert
PTGS-2792c GPDGUS-1420	5'-CCTCGTTACCTTGGTAACTG -3' 5'-CCGGTTCGTTGGCAATACTC-3'	448	F2 sense insert
GUSTRPC-769 PTGS-1583	5'-TGCCAACGAACCGGATACCC-3' 5'-CCTCGTTACCTTGGTAACTG -3'	366	F2 antisense insert
PTGS-1118 GPDGUS-1420	5'-GTTGGATGCGTCTCACTG -3' 5'-CCGGTTCGTTGGCAATACTC-3'	522	F5 sense insert
GUSTRPC-769 PTGS-2066	5'-TGCCAACGAACCGGATACCC-3' 5'-AGTTGGATGCGTCTCACTG -3'	441	F5 antisense insert
PTGS-1118 GPDGUS-1420	5'-GTTGGATGCGTCTCACTG -3' 5'-CCGGTTCGTTGGCAATACTC-3'	250	F6 sense insert
GUSTRPC-769 PTGS-2066	5'-TGCCAACGAACCGGATACCC-3' 5'-AGTTGGATGCGTCTCACTG -3'	169	F6 antisense insert

AbEV1-free transformants were screened for the expression of hairpin construct before and after the *in vitro* dual-culture challenge. Absence of contaminating DNA was confirmed using primers for the *A. bisporus* succinate dehydrogenase gene (*sdh*), SDHF1 (5'-AGTGCATTCTCTGCGCTTGT-2') and SDHR1 (5'-TTGCGTTGAGCAC CATGAGT-3'), designed around the intron *sdh* gene to differentiate 194 bp genomic DNA products from 137 bp cDNA amplicons. In some cases results were confirmed using RT-PCR analysis with 100 µM primer concentration when 10 µM primers concentration did not show any detectable amplicon.

To determine whether AbEV1 was silenced in *Agaricus* transformants total RNA was extracted using the Tri-Reagent method (Section 2.5.2). RT-PCR screening was

performed according to the protocol reported in Section 2.8.1 and 2.8.2, using primers B3198f2 and B3198r2 (Section 2.8.3), which enabled to discriminate the AbEV1 sequence from the hairpin construct. Absence of contaminating DNA was assessed by screening for *A. bisporus sdh* gene as described above. In some cases it was necessary to use 100 μM of primer concentration during the RT-PCR analysis rather than the usual 10 μM primer concentration.

6.2.1.7 Quantitative Evaluation of AbEV1 Silencing and Hairpin Expression

Quantification of the viral RNA was assessed by Reverse Transcription and Quantitative PCR (RT-qPCR) in AbEV1-free transformants after the challenge with AbEV1 donor. Quantification of hairpin transcripts was assessed by RT-qPCR in AbEV1-free transformants before and after the challenge with AbEV1 donor. Total RNA from all transformants was obtained as described in Section 2.5.2 and cDNA synthesised (Section 2.8.1) using Superscript™ II Reverse Transcriptase (200 U/ μl , Invitrogen cat. No.18064-022) instead of Thermoscript™ RT.

qPCR reactions were performed using the SYBR Green I technology. To detect the AbEV1 RNA, qPCR primers (Table 6.4) amplifying a region distinct from the hairpin sequence were designed. These primers spanned the AbEV1 helicase region. qPCR detection of the hairpin was also performed using primers annealing with the ‘antisense’ transcript in the region between the AbEV1 insert and the *trpC* terminator.

Table 6.4- Primers used for the qPCR screening

Primer	Primer sequence Reference: this thesis	Product Size (bp)	RNA Target
Hel-f469 Hel-r578	5'-ACCGTTCACAGTTACCAAGGT-3' 5'-GCGGCTGAGATCACATACTTT-3'	110	AbEV1 helicase RNA
F1-f107 F1-r184	5'-GTAACGAGGCCGACTAGTAGATCTG-3' CGTCAAGCTGTTTGATGATTTTCAG-3'	78	F1 antisense transcript
F5-f84 F5-r160	5'-CGTCAAGCTGTTTGATGATTTCA-3' 5'-TGTGTCTTGCGACTAGTAGATCTGATG-3'	77	F5 antisense transcript
F18S R18S	5'-ACGAACGAGACCTTAACCTGC-3' 5'-GACGCTGACAGTCCCTCTAAGAA-3'	78	18S rRNA

Primers were designed using the Primer Express package (Applied Biosystem PRISM, version 2.0). Quantification was performed against an external standard curve (Ingham *et al.*, 2001), made with a serial dilution (1, 1/4, 1/16, 1/64) of either a control transformant (non-silenced) or a non-transformed sample for detection of the AbEV1 silencing, or a plasmid containing the hairpin construct for detection of hairpin cassettes. Expression of target genes were normalized against the endogenous *A. bisporus* 18S rRNA (F18S and R18S primers, Table 6.4). Reactions to amplify AbEV1 helicase RNA and 18S rRNA were performed simultaneously.

qPCR reactions were prepared in 15 µl reaction volumes: 2x qPCR Mastermix (Eurogentec, cat. No. RT-SN2x-03WOU+), 0.75 µl of each primer (10 µM), and 3 µl diluted cDNA (1:3). Two replicates of each sample were aliquoted in 384-well reaction PCR microplates (Axygen Scientific, cat. No. 3212905) and the plate sealed with optically clear heat seal film (Applied Biosystems, cat. No. 4314320).

qPCR reactions were performed using the ABI PRISM® 7900HT Sequence Detection System (Perkin Elmer-Applied Biosystem) according to the following parameters: initial denaturation at 50° C for 2 min; 95° C for 10 min; followed by 40 cycles of 95° C for 15s, 60° C for 1 min; and a final dissociation stage of 95° C for 15s, 60° C for 15s and 95° C for 15s. Negative controls (water and non-transformed *A. bisporus*) were included in each experiment. Two independent RNA extractions were performed for each transformant tested. For the AbEV1 silencing assay RNA was extracted from two independent dual-culture anastomosis challenges.

6.2.2 Antiviral compounds

Two hygromycin resistant transformants, SSI61_NC_10 and SSI61_NC_1 (control transformants of AbEV1-infected *Agaricus* strain, cultured on hygromycin selective medium for 30 days, Section 6.2.1.4) were used to determine whether hygromycin had an effect on virus replication. Transformant SSI61_NC_10 was positive for AbEV1, whereas SSI61_NC_1 was negative for AbEV1 using RT-PCR screening after 30 days culture on MPA supplemented with 25 µg/ml hygromycin (Results, Section 6.3.1.2). Both transformants were cultured for further 60 days in duplicate on MPA and

CE/CYM media, with and without 25 µg/ml hygromycin. Total RNA was then extracted by Tri-Reagent (Section 2.5.2), and subjected to RT-PCR (Section 2.8.1 and 2.8.2) using primers B3c198_f2 and B3c198_r2, and primers AB18S_f1 and AB18S_r1 to screen for the presence of AbEV1 RNA and 18S rRNA, respectively (Section 2.8.3).

Similarly, to determine if 3': 5'-cyclic adenosine monophosphate (cAMP, Sigma, cat. No. 6885) induced any MVX defence response, *A. bisporus* MVX-infected isolates (SSI 61 and 1283 strains, Section 4.2.1.2) were cultured on CE/CYM medium with and without cAMP (1mM) for 3 months in triplicate. Isolations of the two cultures were then transferred onto CE/CYM with and without 8-bromoadenosine 3': 5'-cyclic monophosphate (Br-cAMP, 10 µM, Sigma-Aldrich, cat. No. B77880) for a further 3 months. Total RNA was extracted by Tri-Reagent (Section 2.5.2) and subjected to RT-PCR (Section 2.8.1 and 2.8.2) periodically (2, 4, and 6 months) to screen for the presence of AbEV1 RNA, 3.6 kbp dsRNA, and 18S rRNA using the following primer combinations: B3c198_f2 and B3c198_r2; B15c35_f1 and B15c35_r1; and AB18S_f1 and AB18S_r1 (Section 2.8.3).

6.2.3 Phleomycin Resistance Marker

6.2.3.1 Phleomycin Sensitivity Test

To determine the sensitivity of *A. bisporus* to phleomycin, sensitivity tests were performed. Mycelium and gill tissue of strain A15 (Section 4.2.1.1) were cultured on MPA and CE/CYM supplemented with phleomycin at different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, and 250 µg/ml) for 30 days. Gill tissue was also cultured on MM and MPA medium (also supplemented with cefotaxime, 200 µg/ml), both amended with phleomycin at the different concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, and 250 µg/ml).

6.2.3.2 Construction of Phleomycin Resistance Vector

The phleomycin resistance (phleo^R) cassette was excised from the plasmid FHJS3 (Shuren *et al.*, unpublished) and introduced into pGREENII (Section 2.10.2). To accomplish the cloning of the phleomycin resistance cassette some vector sequencing

was required. Primers (Table 6.5) were designed for the *Sh ble* gene (EMBL X52869) and used for direct sequencing (Section 2.12.2) of pFHJS3. Sequence similarity analysis revealed the organization of the phleomycin resistance cassette for pFHJS3. The phleomycin resistance cassette was excised using *KpnI* and *NsiI* (Promega, cat. No. R6531), and cloned into *KpnI-PstI*-restricted pGREENII vector, purified from *E. coli* SCS110 competent cells (Section 2.4.1) The resulting construct, pGREENphleo, was cloned into DH5 α or XL1-blue *E. coli* competent cells. Clonal inserts were confirmed using PCR (Section 2.8.2) performed at 58° C (annealing temperature), using primers (FHJS3-1014, 5'-AAGTTGACCAGTGCCGTTCC-3'; FHJS3-1305 5'-CCACGAAGTGCACGCAGTTG-3') that amplified the phleomycin resistance cassette and yielded a 352 bp PCR product.

Table 6.5- Primers used to generate sequences of the phleomycin resistance cassette from pFHJS3

Primer	Primer sequence Reference: this thesis
Phlc24	5'-AACGGCACTGGTCAACTTGG-3'
Phl347	5'-TGCACTTCGTGGCCGAGGAG-3'
Phl336	5'-CGGCAACTGCGTGCACTTCG-3'
Phl523	5'-TGCTTCCGGCTCGTATGTTG-3'
Phl421	5'-GGATCTCCGAGTCCAATGTC-3'

6.2.3.3 *Agrobacterium*-mediated Transformation for the phleo^R construct

pGREENphleo and pSOUP were co-transformed into *A. tumefaciens* by electroporation (Section 2.11). Plasmid integrity was assessed by PCR screening using primers FHJS3-1014 and FHJS3-1305 (Section 6.2.3.2). Positive *Agrobacterium* transformants were cultured and induced for virulence as described in Section 2.14.1. Induced *Agrobacteria* containing the phleomycin resistance constructs were used for gill tissue transformation (Section 2.14.2) of mushroom strain A15 (Section 4.2.1.1). *Agaricus* gill tissue was transferred onto MPA supplemented with 200 μ g/ml cefotaxime and phleomycin at various concentrations (50 μ g/ml, 100 μ g/ml, 150 μ g/ml, and 200 μ g/ml).

6.3 Results

6.3.1 Silencing of AbEV1

A total of 10 hairpin constructs were obtained by cloning in opposite orientations, sequences from the AbEV1 helicase or RNA-dependent RNA polymerase (RdRp) domains (Table 6.6). The hairpin expression cassette driven by the *A. bisporus gpdII* promoter could be in a unidirectional or divergent orientation with respect to the *hph* gene (Fig. 6.5). A total of 6 hairpin constructs (RNAi_F1U, RNAi_F1D, RNAi_F2U, RNAi_F5U, RNAi_F5D, and RNAi_F6U) were introduced into *A. tumefaciens* via electroporation. An empty construct (RNAi_NC), containing only the hygromycin resistance (hyg^R) cassette was also transformed into *Agrobacterium* as negative control.

Table 6.6- Hairpin constructs. Hairpin constructs were obtained inserting twice in opposite orientations sequences from the AbEV1 helicase or RdRp domains

CONSTRUCT	VIRAL INSERT	HAIRPIN ORIENTATION
RNAi_F1U* ¹	AbEV1 helicase	unidirectional
RNAi_F1D*	AbEV1 helicase	divergent
RNAi_F2U*	AbEV1 helicase	unidirectional
RNAi_F2D	AbEV1 helicase	divergent
RNAi_F3U	AbEV1 helicase	unidirectional
RNAi_F4U	AbEV1 RdRp	unidirectional
RNAi_F4D	AbEV1 RdRp	divergent
RNAi_F5U*	AbEV1 RdRp	unidirectional
RNAi_F5D* ¹	AbEV1 RdRp	divergent
RNAi_F6U*	AbEV1 RdRp	unidirectional
RNAi_NC* ¹	empty	-

* = construct introduced into *A. tumefaciens*; *¹ = construct introduced into *A. tumefaciens* and used for *A. bisporus* gill tissue transformation; RdRp = RNA-dependent RNA polymerase; - = absence

6.3.1.1 Hairpin constructs into AbEV1-free strains

Two *Agaricus* AbEV1-free strains (C63-carb422 and A15) were infiltrated with cultures of *A. tumefaciens* transformants carrying one of the following hairpin constructs: RNAi_F1U, RNAi_F5D, or RNAi_NC. Agroinfiltrated gill tissue showed selective mycelial growth according to the transformation efficiency rate (Table 6.7).

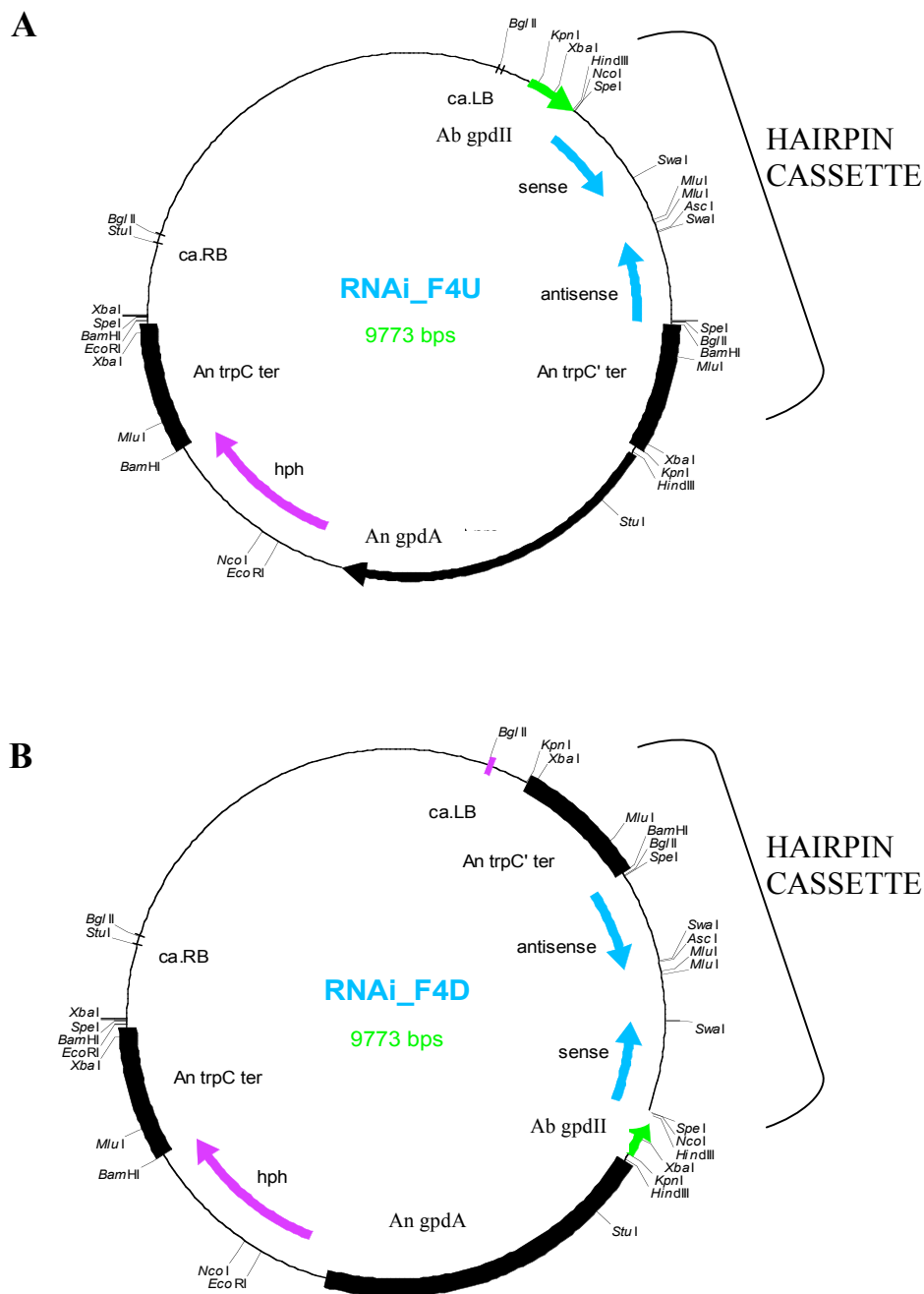


Fig. 6.5- Unidirectional or divergent hairpin construct. The hairpin expression cassette driven by *A. bisporus gpdII* promoter could be in a unidirectional (A) or divergent orientation (B) with respect to the *hph* gene

Strain C63-carb422 showed an initial general growth on hygromycin selective medium (Fig. 6.6), followed by further growth of a limited number of putative transformants (Table 6.7).

Table 6.7- Recovery of hygromycin resistant (hyg^{R}) colonies from AbEV1-free strain

Transformant Name (transformed strain_construct)	Putative Transformant (hyg^{R} colonies /gill tissue pieces)	Transformation Efficiency
c63carb_F1U	0/100	0%
c63carb_F5D	9/99	9%
c63carb_NC	7/100	7%
A15_F1U	5/101	5%
A15_F5D	4/99	4%
A15_NC	5/100	5%



Fig. 6.6- Hygromycin resistant colonies. Agroi infiltrated gill tissue of *Agaricus* strain C63-carb422 showed an initial general growth after 4 weeks on hygromycin selective medium (right) compared to the non-transformed control (left), followed by a further growth only of a limited number of putative transformants

Putative hygromycin resistant (hyg^{R}) transformants were tested by PCR for presence of the hairpin cassette and/or hygromycin resistance cassette. PCR screening confirmed the presence of hyg^{R} cassette for all hyg^{R} colonies (30 hyg^{R} transformants out of 599 agroi infiltrated gill tissue pieces). From 18 hyg^{R} colonies tested, 4 (2 from C63-carb422 *Agaricus* strain and 2 from the A15 *Agaricus* strain) contained the AbEV1 RdRp-hairpin cassette, whereas 4 (from A15 transformed strain only) contained the AbEV1 helicase-hairpin cassette (Table 6.8). A total of 12 control transformants contained only the hyg^{R} cassette as expected.

Table 6.8- Putative AbEV1-free transformants screened by PCR for the presence of hairpin and hygromycin resistance (hyg^R) cassettes

Transformant Name (transformed strain_construct)	Positive Transformant (hairpin construct / hyg^R tested)	Hyg^R cassette (hyg^R tested/ hyg^R colonies)
c63carb_F5D	2/9	9/9
c63carb_NC	-	7/7
A15_F1U	4/5	5/5
A15_F5D	2/4	4/4
A15_NC	-	5/5

- = undetectable

RT-PCR screening of total RNA extracted from *Agaricus* transformants confirmed the expression of hyg^R cassette for all transformants (Fig. 6.7), but did not reveal expression of the hairpin constructs using primers spanning the hairpin cassette or primers targeting only the viral insert (Fig. 6.8).

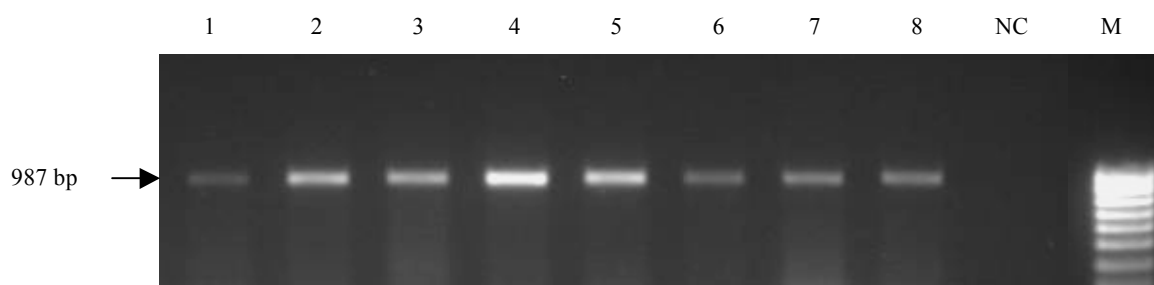


Fig. 6.7- RT-PCR screening for the expression of the hyg^R cassette. M = hyperladder IV (Bioline), lane 1-8 = putative transformants selected on hygromycin selective medium, NC = RT-PCR negative control (water). Transcripts of the hygromycin resistant cassette were detectable in all transformants

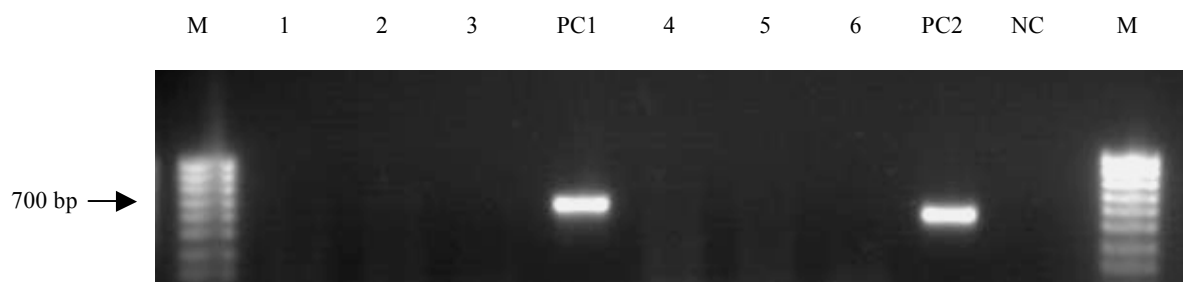


Fig. 6.8- RT-PCR screening for expression of the hairpin cassette. M = hyperladder IV (Bioline), lane 1-3 = hyg^R transformants F5D screened for the sense insert, lane 4-6 = hyg^R transformants F5D screened for the anti-sense insert, PC1 = RNAi_F5D plasmid used as PCR positive control, PC2 = RNAi_F5D plasmid used as PCR positive control, NC = RT-PCR negative control (water). Transcripts of the hairpin cassette were not detectable in any transformants

There was also no detectable expression of the hairpin construct using RT qPCR (Fig. 6.9; Annex 6.1).

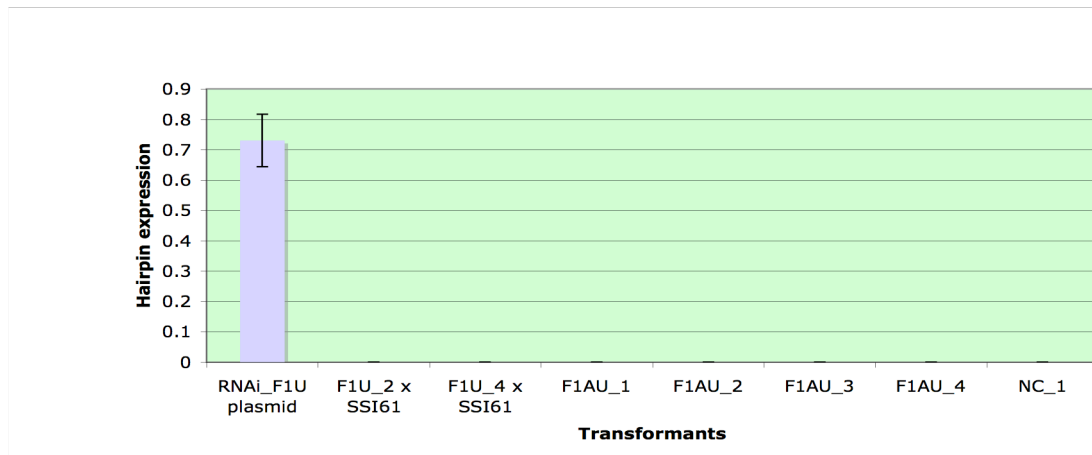


Fig. 6.9- RT-qPCR detection of the AbEV1 helicase-hairpin cassette. Relative quantification was obtained normalising the targeted insert (F1) with 18S rRNA. RNAi_F1U plasmid was used as reference for the relative quantification of the hairpin cassette, whereas the control (non-silenced) transformant, NC_1 (containing only the hyg^R cassette) was used as negative control. Some transformants (F1U_1, F1U_2, F1U_3, and F1U_4) were screened before the dual-culture challenge with AbEV1 and others (F1U_2 x SSI61 and F1U_4 x SSI61) after. All transformants showed no detectable transcript of the hairpin cassette compared to the reference (RNAi_F1U plasmid)

Following infection with AbEV1 through *in vitro* dual-culture anastomosis, transformants were screened for the presence/absence of the virus by RT-PCR. Although all control transformants were positive for AbEV1 by RT-PCR, a total of 63% (5/8) transformants carrying the viral construct did not show detectable AbEV1 RNA (Table 6.9) after dual-culture challenge with AbEV1 donor. From 4 transformants harbouring AbEV1 RdRp-hairpin construct, 2 (50%) had no detectable virus. From 4 transformants harbouring AbEV1 helicase-hairpin construct, 3 (75%) had no detectable virus (Fig 6.10). All templates produced a 137 bp-RT PCR product for the *sdh* gene confirming RNA presence (Fig. 6.11).

When AbEV1-free transformants were challenged through *in vitro* dual-culture anastomoses with *Agaricus* strain A15 (control), none exhibited AbEV1 RT-PCR products as expected (data not shown). Using RT-qPCR analysis reduction of AbEV1 RNA was observed for transformants where AbEV1 was not detectable by RT-PCR.

Table 6.9- Summary of *Agaricus* Transformant Screening. After challenging AbEV1-free transformants with a virus donor, RT-PCR screening was carried out to detect the presence of AbEV1. RNA quality was assessed using primers discriminating between the endogenous succinate dehydrogenase (*sdh*) gene and the *sdh* transcript. Although control transformants (c63carb_NC and A15_NC transformants) were all positive for AbEV1 RNA, 5 (1 from C63-carb422 *Agaricus* strain and 4 from the A15 *Agaricus* strain) out of 8 transformants carrying the hairpin construct had no detectable AbEV1 (bold)

Transformant Name (transformed strain_construct_n.)	AbEV1 RNA	<i>sdh</i> transcript
c63carb_F5D_4	+	+
c63carb_F5D_9	-	+
c63carb_NC_2	+	+
c63carb_NC_3	+	+
c63carb_NC_4	+	+
c63carb_NC_5	+	+
c63carb_NC_6	+	+
c63carb_NC_7	+	+
c63carb_NC_8	+	+
A15_FIU_1	-	+
A15_FIU_2	-	+
A15_FIU_3	+	+
A15_FIU_4	-	+
A15_F5D_1	-	+
A15_F5D-2	+	+
A15_NC_1	+	+
A15_NC_2	+	+
A15_NC_3	+	+
A15_NC_A	+	+
A15_NC_M	+	+

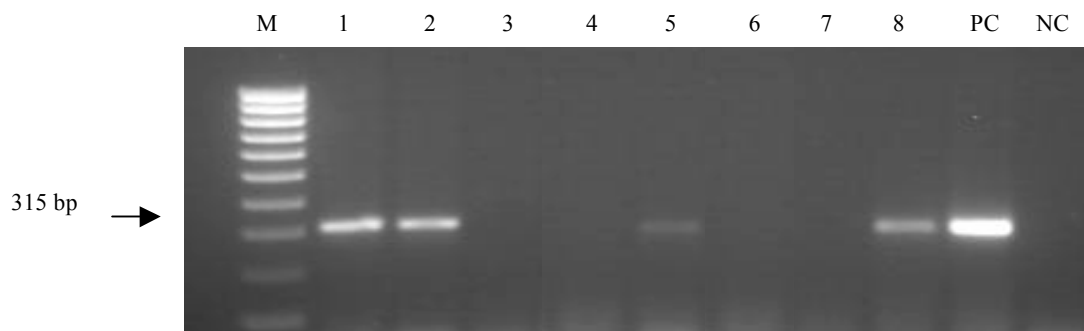


Fig. 6.10-RT-PCR screening of AbEV1 sequence in transformants (strain A15) following dual-culture anastomosis. From 4 transformants strain A15 (lane 3-6) harbouring the AbEV1 helicase-hairpin construct, 3 (lane 3, 4, and 6) had no detectable virus. One transformant (lane 5) showed an extremely faint detectable signal. From 2 transformants strain A15 (lane 7 and 8) harbouring AbEV1 RdRp-hairpin construct, 1 (lane 7) had no detectable virus. Control transformants (lane 1 and 2) showed a strong signal for AbEV1 sequence. M = hyperladder IV

(Bioline), lane 1 = control transformant 1, lane 2 = control transformant 2, lane 3 = A15_F1U_1 transformant, lane 4 = A15_F1U_2 transformant, lane 5 = A15_F1U_3 transformant, lane 6 = A15_F1U_4 transformant, lane 7 = A15_F5D_1 transformant, lane 8 = A15_F5D_2 transformant, lane PC = RT-PCR positive control (strain SSI 61), lane NC = RT-PCR negative control (water)

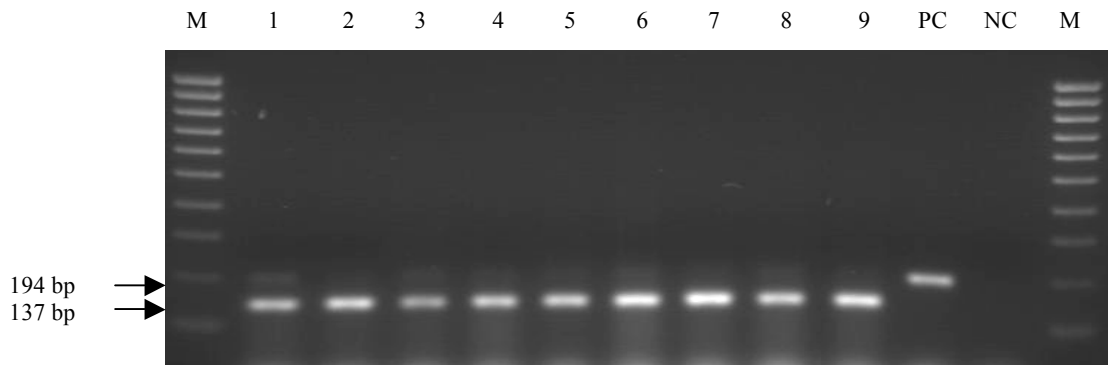


Fig. 6.11-RT-PCR screening of *sdh* gene in transformants (strain A15). All templates contained RNA in higher amount (137 bp band) compared to DNA (194 bp band). M = hyperladder IV (Bioline), lane 1 = control transformant 1, lane 2 = control transformant 2, lane 3 = A15_F1U_1 transformant, lane 4 = A15_F1U_2 transformant, lane 5 = A15_F1U_3 transformant, lane 6 = A15_F1U_4 transformant, lane 7 = A15_F5D_1 transformant, lane 8 = A15_F5D_2 transformant, lane 9 = RT-PCR positive control (strain SSI 61), lane PC = *A. bisporus* strain A15 DNA, lane NC = RT-PCR negative control (water)

A relative reduction of 100% was observed for two transformants (A15_F1U_1 and A15_F5D_1). In the other two transformants (A15_F1U_2 and A15_F1U_4) there was *ca.* 81-84% reduction of AbEV1 amplicon (Annex 6.2) compared to the control transformant (with no hairpin construct; Fig. 6.12). The non-transformed strain A15 did not show any detectable AbEV1 signal in the RT-qPCR screening as expected.

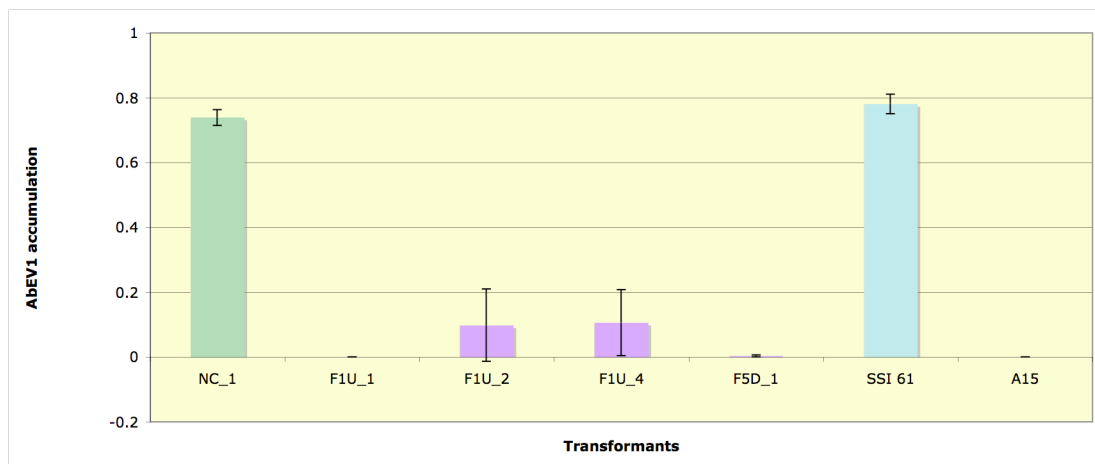


Fig. 6.12- Relative quantification of AbEV1 in *Agaricus* transformants. Relative quantification was obtained by normalising the AbEV1 RNA with 18S rRNA in *Agaricus*

transformants strain A15. The control transformant NC_1 was used as reference for the virus quantification in transformants (F1U_1, F1U_2, F1U_4, and F5D_1) after the *in vitro* dual-culture challenge with AbEV1 donor. AbEV1 RNA was also quantified in the AbEV1 donor (SSI 61) and in the non-transformed strain A15 (negative control).

6.3.1.2 Hairpin constructs into AbEV1-infected strains

To test whether hairpin construct could interfere with AbEV1 replication once the viral infection is already established, the AbEV1-infected strain SSI 61 was also transformed with the AbEV1 helicase-construct. The empty pGREEN-hph01 vector was used as control transformant (NC). The recovery of transformants using this *Agaricus* strain proved relatively efficient (Table 6.10). A total of 20 putative hyg^R colonies transformed with SSI61_F1U construct were selected to be PCR screened for the presence of hairpin and hyg^R cassettes. Of these, 11 showed the presence of the entire hairpin cassette (sense and anti-sense fragments).

Table 6.10- Recovery of hygromycin resistant (hyg^R) colonies from AbEV1-infected transformants

Transformant Name (transformed strain_construct)	Putative Transformant (hyg^R colonies /gill tissue pieces)	Transformation Efficiency
SSI61_F1U	65/99	66%
SSI61_NC	40/103	40%

The control transformants (SSI 61_NC) showed only the presence of the hyg^R cassette as expected. AbEV1 was not detectable by RT-PCR in 6 of these 11 transformants (Table 6.11). Several control transformants (6/20) also did not yield the expected viral amplicon (Fig. 6.13) suggesting instability of AbEV1 through the transformation procedure.

Table 6.11- Putative AbEV1-infected transformants screened by PCR for the presence of hairpin and hyg^R cassettes. A total of 11 out of 20 hyg^R transformants showed the presence of the entire hairpin cassette (sense and anti-sense fragments) by PCR. The control transformants showed only the presence of hyg^R cassette

Transformant Name (transformed strain_construct)	Hairpin cassette (hairpin construct / hyg^R tested)	Hygromycin cassette (hyg^R tested/ hyg^R colonies)
SSI61_F1U	11/20	20/20
SSI61_NC	-	20/20

- = undetectable

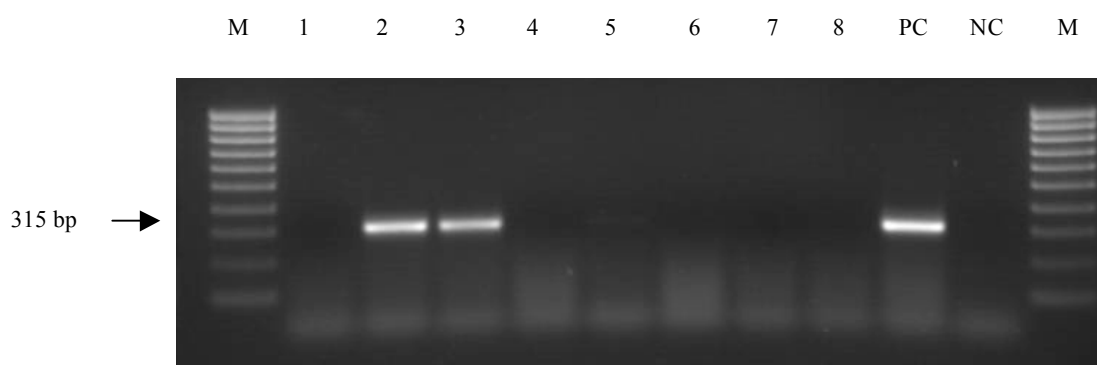


Fig. 6.13- RT-PCR screening of AbEV1 in AbEV1 infected-transformants (experiment I). Unexpectedly, not all control transformants (SSI61_NC_1, SSI61_NC_4, SSI61_NC_5, SSI61_NC_6, SSI61_NC_7, and SSI61_NC_8) showed the presence of AbEV1. M = hyperladder IV (Bioline), lane 1-8 = control transformants from SSI61_NC_1 to SSI61_NC_8, lane PC = RT-PCR positive control (strain SSI 61), lane NC = RT-PCR negative control (water)

Control transformants were cultured for a further one month on selective medium and re-screened for AbEV1. Surprisingly, AbEV1 was not detectable in two additional control transformants after the second screening (Fig. 6.14), although total RNA was present in all samples (Fig. 6.15). Although hyg^R transcript was detectable in all transformants, no expression of the hairpin cassette was detected (data not shown).



Fig. 6.14-RT-PCR screening of AbEV1 in AbEV1 infected-transformants (experiment II). Unexpectedly, all control transformants (SSI61_NC) previously screened (see Fig. 6.13) were negative to the presence of AbEV1 when screened a month later. M = hyperladder IV (Bioline), lane 1-8 = control transformants from SSI61_NC_1 to SSI61_NC_8, lane PC = RT-PCR positive control (strain SSI 61), lane NC = RT-PCR negative control (water)

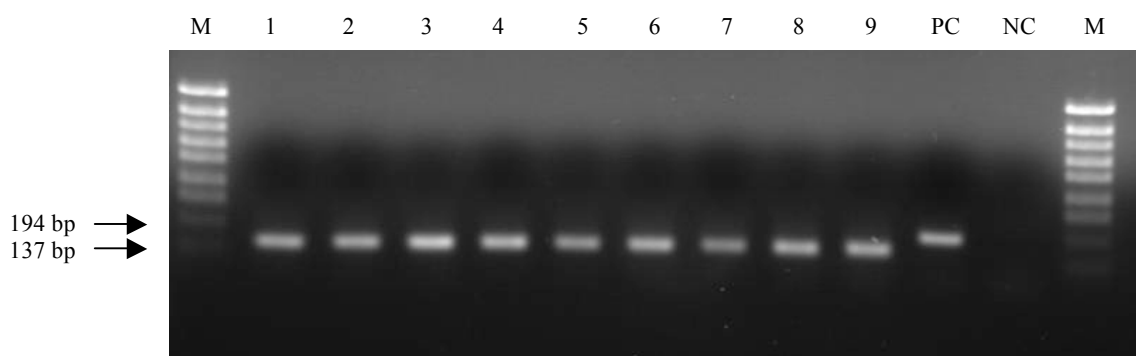


Fig. 6.15- RT-PCR of the *sdh* gene in AbEV1 infected-transformants (experiment II). All samples contained RNA in higher amount (137 bp band) compared to DNA (194 bp band). M = hyperladder IV (Bioline), lane 1-8 = control transformants from SSI61_NC_1 to SSI61_NC_8, lane 9 = RT-PCR positive control (strain SSI 61), lane PC = *A. bisporus* strain A15 DNA, lane NC = RT-PCR negative control (water)

6.3.2 Effect of hygromycin and cAMP on AbEV1 replication

To investigate whether the hygromycin was interfering with AbEV1 replication two hygromycin resistant (hyg^R) transformants (SSI61_NC_10 and SSI61_NC_1) were cultured for 60 days on medium with and without hygromycin. Hygromycin transformants showed undetectable virus when cultured on medium supplemented with hygromycin, whereas showed detectable virus in antibiotic absence. Intriguingly, the transformant SSI61_NC_1, negative for AbEV1 (Section 6.2.2) appeared to regain the virus when cultured in the absence of antibiotic (Fig. 6.16).

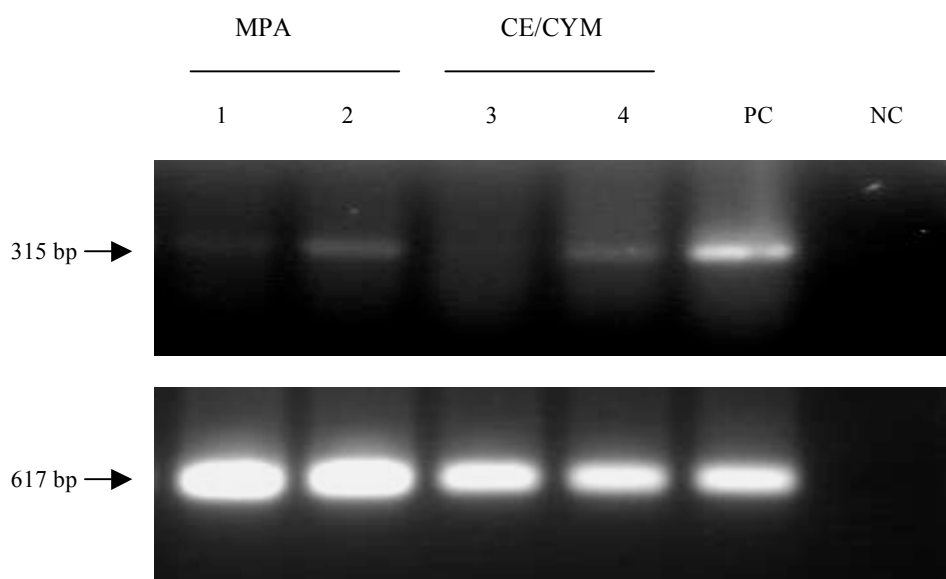


Fig. 6.16-RT-PCR screening of AbEV1 for the Hygromycin Test. The hyg^R transformant SSI61_NC_1 (negative for AbEV1 after 30 days-culture on MPA supplemented with hygromycin) showed poor or undetectable signal of AbEV1 (above photo) when cultured for 60

days on MPA medium (lane 1) and CE/CYM medium (lane 3) supplemented with hygromycin, whereas the virus appeared again on MPA (lane 2) and CE/CYM (lane 4) in antibiotic absence. All samples yielded the appropriate 18S RT-PCR products (below photo). Lane PC = RT-PCR positive control (strain SSI 61); lane NC= RT-PCR negative control (water)

It has been reported that cyclic adenosine monophosphate (cAMP) might be involved in the hypersensitive response of higher plants to fungal elicitors (Bent *et al.*, 1994; Zhao *et al.*, 2003). In order to test whether cAMP is involved in the defence mechanism of *A. bisporus* against AbEV1 two isolates, one of which infected with only AbEV1 and the other infected with multiple dsRNA components, were cultured for 6 months on CE/CYM medium in the presence or absence of cAMP. Cultured isolates screened for AbEV1 (Fig. 6.17) and 3.6 kbp dsRNA (data not shown) did not show any reduction in viral components in the presence of cAMP. All samples were normalised with the *sdh* cDNA.

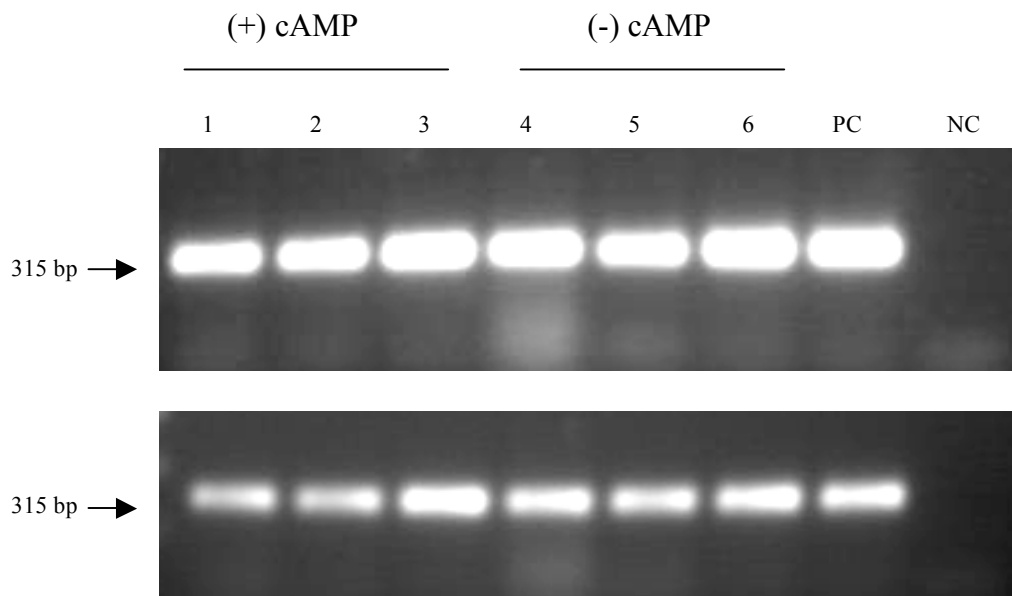


Fig. 6.17- RT-PCR screening of AbEV1 for the cAMP Test. In order to test whether cAMP is involved in the defence mechanism of *A. bisporus* against AbEV1 the isolate SSI61 was cultured for 6 months in triplicates on CE/CYM medium in the presence (lanes 1-3) or absence of cAMP (lanes 4-6). No difference in the virus presence could be found after 1 month (above photo) and 6 months of cAMP treatment (below photo). Lane PC = RT-PCR positive control (strain SSI61); lane NC = RT-PCR negative control (water)

6.3.3 Phleomycin Resistance Marker

6.3.3.1 Phleomycin Sensitivity Test

To determine the working concentrations of phleomycin for *A. bisporus*, mycelium and gill tissue were plated on media containing different concentrations and grown for 3-4 weeks. Differences were observed in the growth and response of different tissue types (Table 6.12).

Table 6.12-Summary of *A. bisporus* growth responses to different phleomycin concentrations. *A. bisporus* mycelium and gill tissue were plated on media at different phleomycin concentrations and grown for 3-4 weeks

[phleo] ($\mu\text{g/ml}$)	Mycelium (MPA)	Mycelium (CE/CYM)	Gill tissue (MPA)	Gill tissue (MM)
25	no growth	growth	-	-
50	no growth	growth	growth	growth
100	no growth	no growth	growth	growth
150	no growth	no growth	growth	growth
200	-	-	poor growth	poor growth
250	-	-	poor growth	poor growth

[phleo] = phleomycin concentration; - = not tested; MPA = malt peptone agar medium; CE/CYM = compost extract + complete yeast extract medium; MM = minimal medium

Agaricus mycelium appeared more sensitive to phleomycin than gill tissue. On MPA medium the mycelium made no growth, at any concentration. On the richer medium, CE/CYM, the mycelium grew at lower phleomycin concentrations (25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$).

Gill tissue showed significant regeneration into mycelium (more than 50%) on both MPA and MM media at concentrations ranging between 50-150 $\mu\text{g/ml}$, but poor growth (less than 20%) at phleomycin concentrations of 200 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ (Fig. 6.18).

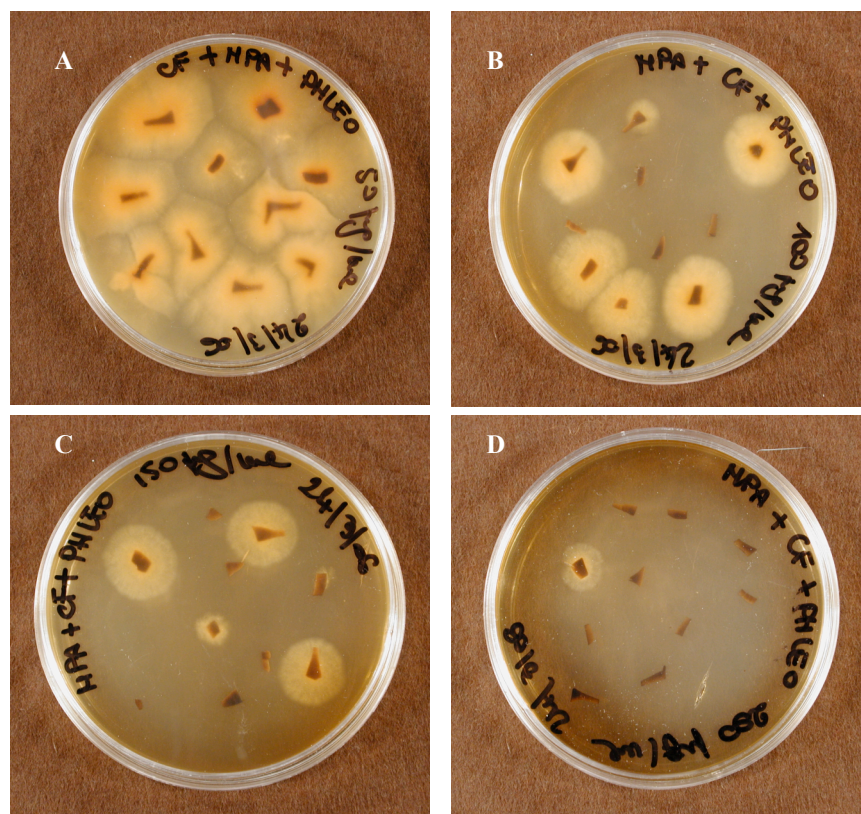


Fig. 6.18- Phleomycin Sensitivity Test on MPA medium supplemented with phleomycin and cefotaxime. *Agaricus bisporus* gill tissue showed a significant growth (more than 50%) at phleomycin concentration ranging between 50 µg/ml and 150 µg/ml (A-C), but poor growth (less than 20%) at phleomycin concentration of 200 µg/ml (D)

6.3.3.2 Construction of Phleomycin Resistant Vector

The phleomycin resistance cassette ($phleo^R$) sequence was identified in the plasmid FHJS3 by direct sequencing. Sequence similarity analyses of the 2060 bp sequence generated yielded the following construct organization: *A. bisporus gpdII* (211 bp) promoter, phleomycin resistance gene (*Sh ble*, 374 bp), and *A. bisporus gpdII* terminator (283 bp, Fig. 6.19).

The $phleo^R$ cassette was excised from pFHJS3 and inserted into the pGREENII binary resulting in the 4176 bp pGREEN_phleo construct (Fig. 6.20). This was cloned using DH5 α *E. coli* competent cells and recombinants identified by a PCR screening. Purified plasmid was introduced into *A. tumefaciens* with pSOUP.

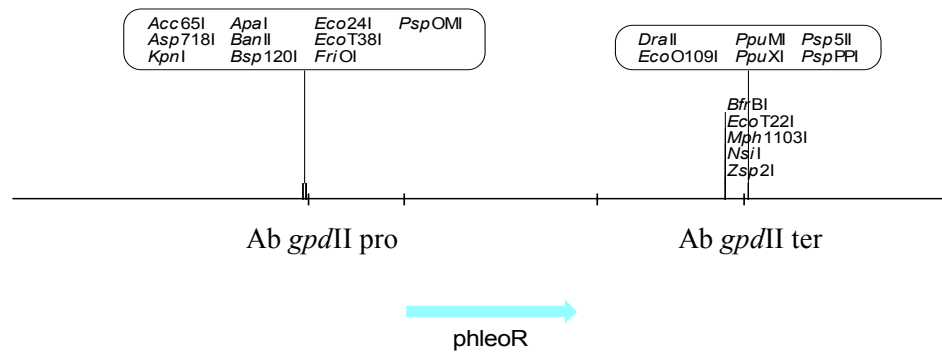


Fig. 6.19- The *phleo*^R expression cassette from pFHJS3. Sequence similarity analyses of the 2060 bp indicated the following construct organization: *A. bisporus gpdII* promoter (Ab *gpdII* pro), phleomycin resistance gene (*phleoR*), and *A. bisporus gpdII* terminator (Ab *gpdII* ter)

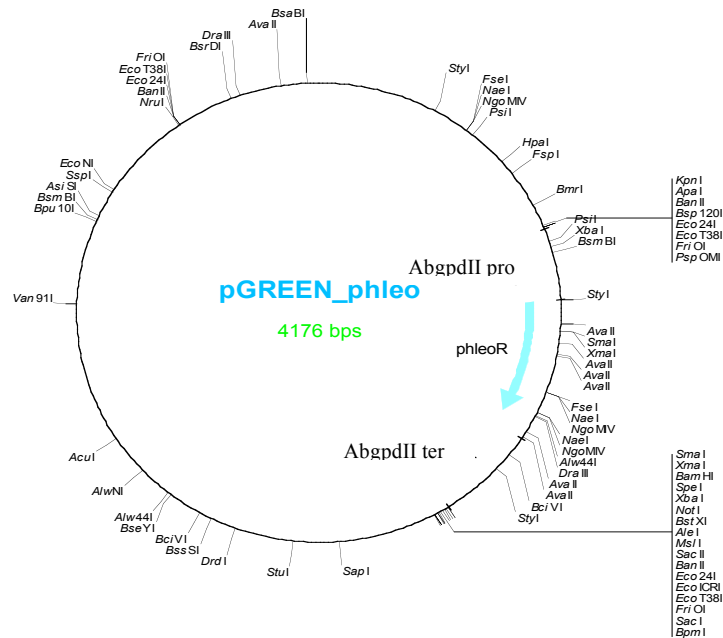


Fig. 6.20- The pGREEN_{phleo} construct. The *phleo*^R cassette was excised as a *KpnI-NsiI* fragment and cloned into *KpnI-PstI*-restricted pGREENII

In *Agrobacterium*-infiltration experiments, *A. bisporus* gill tissue showed signs of re-growth on phleomycin amended-medium after 3-4 weeks (Fig. 6.21). This re-growth was equal for transformed and non-transformed gill tissue suggesting that the selection was not reliable.



Fig. 6.21- Recovery of phleomycin resistant colonies from gill tissue transformation. Non-transformed colonies (left) showed signs of growth as well as agroinfiltrated *Agaricus* gill tissue (right) after 3-4 weeks on MPA medium supplemented with phleomycin (200 µg/ml)

6.4 DISCUSSION

6.4.1 Interference of AbEV1 replication

Homology-dependent gene silencing (HDGS) phenomena are often considered mechanisms evolved as defence responses to invading viruses (Matzke & Matzke, 1998). HDGS can be exploited as a molecular tool to investigate the function of viral genes, especially in viruses with large and complex genomes containing many putative proteins with unknown function (Matzke & Matzke, 1998).

In this project an HDGS approach was initiated to investigate functional domains of the AbEV1 virus. *A. bisporus* mushrooms were transformed using an *Agrobacterium*-infiltration technique. Although it is possible to efficiently transform many homobasidiomycetes most progress has been made with model species such as *Coprinus cinereus* (Binninger *et al.*, 1987; Heneghan *et al.*, 2007) and *Schizophyllum commune* (Muñoz-Rivas *et al.*, 1986). Considerable effort has been directed at transformation of the recalcitrant *A. bisporus* but early work was not successful (Challen *et al.*, 1991; Royer & Horgen, 1991; Li & Horgen, 1993; Challen & Elliott, 1994). This early lack of success was attributed to several factors; e.g. low level of integration of transforming DNA, poor expression of foreign sequences, DNA modification after integration or low competence of certain strains. The first report of *Agrobacterium tumefaciens* as tool to transfer T-DNA to *A. bisporus* used germinating

basidiospores (De Groot *et al.*, 1998). Later the use of gill tissue has proved more efficient (Chen *et al.*, 2000; Leach *et al.*, 2004). Burns *et al.* (2006) reported that gill tissue obtained from mushrooms immediately prior to veil-break (stage 4) might be more amenable to integrative transformation possibly because cells are preparing for karyogamy and meiosis (DNA recombination). Vacuum infiltration (Bechtold *et al.*, 1993) plays an important role in the transformation efficiency of fungi (Burns *et al.*, 2006). The vacuum physically creates a negative atmosphere pressure that causes air spaces between cells to decrease. Increasing the pressure afterwards allows the infiltration medium and therefore the bacterium, to relocate into the host tissue.

It is well known that transformation efficiency is also affected by several parameters including *Agrobacterium* strain, host tissue physiology (Godwin *et al.*, 1992), host metabolic stage, and transformation vector. The *A. tumefaciens* L,L-succinamopine-type used in this study for *Agaricus* transformation was previously reported (Chen *et al.*, 2000; Leach *et al.*, 2004; Burns *et al.*, 2006) as a successful strain for gill tissue transformation of button mushrooms. Virus-infected strains showed to be more transformable (40%-66% transformation efficiency, Table 6.10) than virus-free strains (0%-9% transformation efficiency, Table 6.7). The reason for this is unclear. There are evidences supporting the notion that cell permeability changes upon viral infection (Kohn, 1979; Carrasco & Smith, 1980; Pasternak & Micklem, 1981). Consistent with this idea, some observations point out that class of semipermeable compounds, which do not normally pass through the cell membrane, become easily permeable in virus infected cells (Macintyre *et al.*, 1991). It is appealing to speculate that virus infected cells might be also more permeable to DNA uptake than non-infected cells as shown in the present study, but further work would be needed to test this hypothesis.

Agaricus gill tissue was transformed with constructs containing viral sequences arranged in hairpin expression cassettes. These manipulations appeared to interfere with AbEV1 replication. This ‘interference’ occurred using both RdRp and helicase sequences. Silencing did not appear affected by hairpin cassette orientation since it worked with both unidirectional and divergent constructs. Similar observations have been made in plant system (Ruiz *et al.*, 1998). Control transformants did not exhibit

AbEV1 silencing and consistently proved able to be infected by AbEV1 following anastomosis.

In this study it was not possible to detect hairpin transcripts, either by RT-PCR or RT-qPCR. Similar observations have been made in other hairpin transformation experiments in *A. bisporus* using a range of endogenous genes (Seargent & Challen, unpublished). Although RT-qPCR can provide evidence of down-regulation of target genes, transcripts for the hairpin construct can be often not detectable (Challen, *pers. comm.*). The reason for this is not clear.

Gene silencing mechanisms in fungi can be complex compared to other organisms (Section 6.1.1, Cogoni & Macino, 1999) and despite extensive studies the mechanism behind PTGS is still poorly understood. Nevertheless, sufficient correlative evidence has accumulated for it to be suggested that TGS and PTGS may be fundamentally linked (Matzke and Matzke, 1995; Bingham, 1997; Wassener & Pélissier, 1998). The processes could have in common modifications to chromatin structure and/or DNA methylation, provoked by specialized RNA products, such as aberrant RNAs (Jones *et al.*, 1998). However, this area has not been fully explored and the evidence is often conflicting.

Transgene methylation has also been implicated in PTGS induced by viruses. In previous studies, virus-induced gene silencing initiated by an RNA virus, *pea seed-borne mosaic virus* (PsbMV), was associated with *de novo* methylation of homologous nuclear DNA sequences (Jones *et al.*, 1998). Similarly, viroid RNA also has the ability to direct methylation (Wassener *et al.*, 1994; Jones *et al.*, 1999; Pélissier *et al.*, 1999). The finding that promoterless constructs can induce PTGS (van Blokland *et al.*, 1994; Stam *et al.*, 1997) has led to the hypothesis of nuclear step and RNA-DNA interaction during PTGS pathways (Wassener & Pélissier, 1998; reviewed by Chicas & Macino, 2001). According to English *et al.* (1996) transgene methylation could lead to the formation of aberrant RNA molecules (aRNAs) possibly due to transcript termination within the methylated region, that affect the transcription of the transgene. These aRNAs would constitute the silencing signal for homologous dependent gene silencing.

It may be desirable in future studies to search for any possible methylation of the hairpin construct or any production of small interference RNAs/aRNAs in order to better understand the underlying silencing mechanism of AbEV1 in *A. bisporus*.

6.4.2 Antiviral Compounds

In transformation experiments using AbEV1-infected *Agaricus* strain, the hygromycin resistant transformants showed loss of AbEV1 by RT-PCR analysis over a 2-3 months culture on hygromycin-supplemented medium. Moreover, removal of hygromycin from the selective medium led to reappearance of AbEV1 after a further one month-culture. It has been reported by several authors, that antibiotics can affect virus replication (Carrasco, 1978; Contreras & Carrasco, 1979; Lacal *et al.*, 1980; Fulbright, 1984; Elias & Cotty, 1996). Hygromycin B is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* (Pittenger *et al.*, 1953), which inhibits protein synthesis in both prokaryotes and eukaryotes through interference with ribosomal translocation as well as with aminoacyl-tRNA recognition (Cabanas *et al.*, 1978; Gonzales *et al.*, 1978; Singh *et al.*, 1979; Eustice & Wilhelm, 1984). Hygromycin B has been reported to block several viruses such as vesicular stomatitis virus, herpes simplex virus type 1, Sendai virus, and Leishmania RNA virus 1-4 (Benedetto *et al.*, 1980; Ro *et al.*, 1997). Despite the potential antiviral properties of hygromycin, the mechanism of action remains unknown. Macintyre *et al.* (1991) suggested a possible block in translation of the viral RNA polymerase, which is likely one of the first products of gene expression in virus replication cycle. Given that the host cell protein synthesis is not inhibited by the antibiotic in transformants carrying the hygromycin resistance gene (*hph*), the inhibition must be selective. Hygromycin B binds to cellular rRNA in wild-type cells, and it is possible that it also binds to viral RNA, inhibiting its transcription and/or translation. This study represents the first known report of hygromycin B interfering with replication of a fungal virus.

The potential of 3': 5'- cyclic adenosine monophosphate (cAMP) to inhibit AbEV1 was also tested. There is an increasing number of reports indicating that cAMP is a critical signalling molecule involved in mediating defence responses in plants and fungi (Lee *et al.*, 2003; Jiang *et al.*, 2005, Yu, *pers. comm.*). Jiang *et al.* (2005) reported a transient

increase in endogenous cAMP amount in *Arabidopsis* in response to *Verticillium* toxins, while exogenous application of cAMP resulted in an improved host resistance to the elicitor. In fungi, the cAMP signalling cascades are remarkably conserved (Lee *et al.*, 2003) and are involved in fungal pathogenicity, filament growth, germ tube formation, and sexual development (reviewed by Kronstad, 1997; Adachi & Hamer, 1998; Kinane *et al.*, 2000; Thines *et al.*, 2000). The involvement of cAMP in viral infection was demonstrated in *cryphonectria parasitica* hypovirus (CHV) by Choi *et al.* (1995). The authors speculated that the viral attenuation caused by CHV might be the result of interference with signalling transduction pathway. To test this hypothesis they isolated the *cpg-1* gene encoding G α subunits (Fig. 6.22), expression of which is reduced in the hypovirulent chestnut blight fungus strain (Lee *et al.*, 2003).

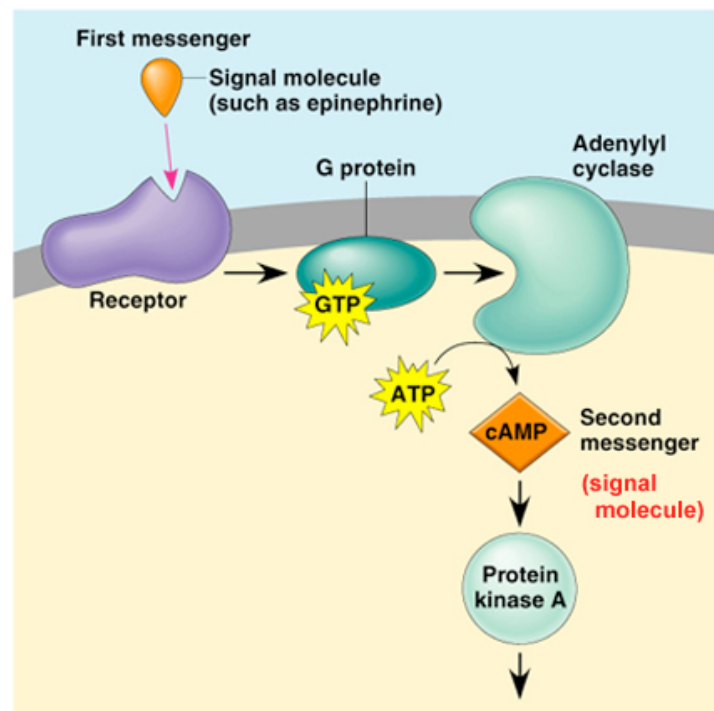


Fig. 6.22- Cyclic AMP signalling cascade. Cyclic AMP is an important signalling molecule in prokaryotes and eukaryotes. Two key enzymes, adenylyl cyclase, located in the plasma membrane, and phosphodiesterase, located in the cytoplasm, are presumed to be responsible for biosynthesis and biodegradation of cAMP in living organisms. Cyclic AMP is involved in perception of extracellular biotic and abiotic stimuli and subsequent transduction of the signal. In a classical cAMP pathway, a transmembrane cell surface receptor senses a specific extracellular signal that is transmitted into cells via heterotrimeric G-proteins. The G-proteins transmit the signal to adenylyl cyclase, which synthesizes the second messenger cAMP (Lee *et al.*, 2003)

This type of subunit inhibits adenylyl cyclase (cAMP biosynthesis enzyme) and interference with *cpg-1* gene expression would result in high cAMP level (reviewed by Kronstad, 1997). PTGS (RNAi) experiments with *cpg-1* in virus-free strains of *Cryphonectria parasitica* resulted in transformants with high level of cAMP and viral symptoms, such as slow growth, uneven colony margins, and attenuated virulence. These observations provide compelling evidence that viral infection influences cAMP pathway in the fungus *Cryphonectria parasitica*. Although there are reports suggesting the involvement of endogenous cAMP in defence signalling cascade, the detailed mechanism is still unclear (Jiang *et al.*, 2005). Characterization of knockout mutants of cyclic nucleotide-gated cation channel genes has provided molecular evidence for cAMP involvement in ion flux as already demonstrated in plant defence responses (Clough *et al.*, 2000; Belagué *et al.*, 2003). Cyclic AMP might regulate calcium-permeable ion channel, such as cyclic nucleotide-gated channels, increasing cytosolic Ca^{2+} concentration in the cell (Jiang *et al.*, 2005).

In this project, applications of exogenous cAMP to *A. bisporus* MVX-infected cultures did not result in any detectable loss of viral RNA elements.

It might be appropriate in future studies to apply cAMP to viral infected *Agaricus* cultures in a wider range of concentrations and examine more closely AbEV1 replication using quantitative approaches.

6.4.3 Phleomycin selective marker

To date the most selective marker used for *Agrobacterium*-mediated transformation of *A. bisporus* is the *E. coli* hygromycin resistance gene, *hph* (Leach *et al.*, 2004, Burns *et al.*, 2006). To progress the development of alternative selective marker for *A. bisporus*, the phleomycin resistance *ble* gene from *Streptoalloteichus hindustatus* (*Sh*) was tested. The *Sh ble* gene has been reported as an efficient selective marker for plants and fungi (Perez *et al.*, 1989; Schuren & Wessels, 1994, Rogers, *pers. comm.*). Most notably, the *Sh ble* gene was used to transform the homobasidiomycete *Schizophyllum commune* (Schuren & Wessels, 1994).

Although there have been some limited success using *ble* gene in *A. bisporus* (Moore *et al.*, 1995), in this project the anti-metabolite proved an unreliable selection system. *Agaricus* gill tissue had a particular tolerance to the antibiotic and could regenerate at elevated concentrations (up to 250 µg/ml); although mycelial growth was very poor. A novel phleomycin resistance binary vector was prepared using homologous regulatory sequences and was tested in *A. bisporus* using *Agrobacterium*-mediated transformation. In practice, the high background growth of spurious transformants made the system unworkable even using different media.

Since mushroom transformation via protoplasts proved to be successful using direct selection with *Sh ble* (Shuren & Wessels, 1994; Rogers, *pers. comm.*), modifications of *Agaricus* transformation protocol might give better results.

Chapter 7

GENERAL CONCLUSION

7. General Conclusion

The cultivated mushroom *Agaricus bisporus* is one of the most economically important horticultural product and the highest value protected crop cultivated in the United Kingdom, with an annual production of approx 5 million tons (Scrase & Elliott, 1998). *Agaricus bisporus* is also of importance for a number of other reasons. Mushrooms are cultivated from compost produced by animal faeces and waste straw; thus the industry is able to recycle considerable amount of such waste products. In recent years, various mushrooms have been appreciated for their medicinal properties and it is claimed that *A. bisporus* produces a number of compounds of potential biomedical/neutriceutical importance (Chen *et al.*, 2004). There is considerable interest in exploiting *A. bisporus* for bio-manufacturing of heterologous proteins primarily for reduced manufacturing costs but also because the glycosilation machinery of fungi may be more similar to mammalian cells (Velco *et al.*, 2004; Zhang *et al.*, 2004).

Despite the economic and biotechnological importance, diseases affecting *A. bisporus* mushrooms are relatively poorly studied. Viral diseases for fungi were not considered until Hollings (1962) suggested a viral etiology for 'La France' disease. Double-stranded RNAs (dsRNAs), presumably of multiple viral origins are causal agents for diseases often consistently associated with complex dsRNA profiles (Rao *et al.*, 2007). The emergence of a new disease termed Mushroom Virus X (MVX) disease, exhibited a wide range of symptoms (e.g. barren patches, arrested pins, premature veil opening, brown discoloration, and distortions). A variable compendium of 26 novel dsRNA elements, ranging in size between 20.2 kbp to 0.64 kbp, have shown to occur in diseased fruiting bodies (Grogan *et al.*, 2003).

In this project, an individual dsRNA element (Chapter 3) associated with the MVX disease was cloned, sequence characterised and named *Agaricus bisporus* endornavirus 1 (AbEV1). The virus was first isolated from samples collected from British mushroom farms. However, Sonnenberg & Lavrijssen (2004) reported a dsRNA element (dsRNA VXL1) with similar features to AbEV1 such as high molecular weight (> 12 kbp), high titre compared to other fungal dsRNAs, and presence in 50% of samples.

AbEV1 was originally estimated to be *ca.* 14.4 kbp by agarose gel analysis, but proved to be 12,750 bp from sequencing. Three major putative functional domains, characteristic of the *Endornavirus* genus were identified. The Endornaviruses have monopartite dsRNA genomes ranging between 10 to 18 kbp. No virus-like encapsidation structures are found, but genomes are often associated with cytoplasmic vesicles (Sections 1.8.2 and 3.1.2.2). Endornaviruses have been reported for a wide range of organisms, from plants to protists, and fungi. The non-kingdom specificity of infection is quite unusual for viruses. However, RNA viruses can readily adapt and expand their host range through their ability to mutate. It is believed that almost 50 RNA viruses, including human immunodeficiency virus, *Ebola virus*, *SARS coronavirus* have shifted from nonhuman hosts to humans since the World War II (Morse, 1993; Brault *et al.*, 2004). Other examples derive from animal and plant viruses transmitted via arthropods (Gray & Banerjee, 1999). Over 500 animal viruses are classified as arboviruses, able also to be transmitted and replicate in arthropods (Nuttall *et al.*, 1994). Additionally, there are many hundreds of plant viruses (Blunt *et al.*, 1996), most of which are dependent upon a transmission vector between inoculations into plant hosts. Plant-infecting viruses have evolved many interesting and biologically complex associations with their vectors, which include arthropods, nematodes, and fungi (Gray & Banerjee, 1999). These findings are evidence of virus ability to infect and adapt to various hosts, even across kingdom under selective pressure.

Several authors failed to recognise endornaviruses as conventional viruses, originally referring to them as plasmid-like elements (Moriyama *et al.*, 1999). The absence of capsids and often obvious symptoms, coupled with mainly vertical inheritance are uncommon features in animal and plant virology, although less unusual within the mycovirology (Sections 1.8.1, 1.8.2, and 3.1.1). For example, hypoviruses are mycoviruses lacking conventional capsids and packaging their dsRNA genome in host-encoded vesicles, whereas narnavirus members protect their RNA genome in the form of ribonucleoprotein complexes (Section 1.8.2). A group of plant virus, showing similarities with endornaviruses, the cryptoviruses, are normally present at low concentration, produce no or very slight disease symptoms and transmit vertically

(Boccardo *et al.*, 1987). Moreover, like cryptoviruses, endornaviruses can transmit biparentally (Fukuhara, 1999).

Since viruses are obligate parasites, they have evolved and co-adapted with their host in such a way that in some cases it is difficult to distinguish them from the host. For instance, pararetroviruses can integrate into the host genome entering in a latent phase and causing infection only in response to stress (Harper *et al.*, 2002). Tobacco vein clearing virus (TVCV) is a distinct member of the family *Caulimoviridae*, differing from typical caulimoviruses in both genome organization and biological properties. TVCV has not been transmitted experimentally from *N. edwardonii* to any other *Nicotiana* spp. or other plant species by mechanical, aphid, or graft inoculation, but is transmitted through seed to 100% of progeny plants (Lockhart *et al.*, 2000). However, its symptoms and virions appear only under certain conditions, which are not clearly defined (Harper *et al.*, 2002). Although increases in symptoms expression and virions are correlated with seasonal day length and temperature changes, the mechanism underlying this correlation remains to be elucidated (Harper *et al.*, 2002).

Although the *Vicia faba endornavirus* (VFV) sequence showed homology with broad bean nuclear genome of both sterile and fertile lines (Turpen *et al.*, 1988), endornavirus sequences were not found in rice DNA (Moriyama *et al.*, 1995) and similarly AbEV1 sequences have not been observed in *Agaricus bisporus* DNA (Section 3.3.3). Moreover, endornavirus genome organization does not support the notion of endornaviruses as retroviruses or retrotransposons. The absence of integrase and reverse transcriptase genes (Zaki, 2003), and the presence of helicase and RNA-dependent RNA polymerase (RdRp) genes suggest that these RNA elements are capable of autonomous replication (Moriyama *et al.*, 1995).

Phylogenetic analyses based on these two AbEV1 enzymes sequences (helicase and RdRp) also confirmed the hypothesis that the endornavirus shares a common ancestor with ssRNA viruses as already reported in literature for other endornaviruses (Gibbs *et al.*, 2000). It has been speculated that endornaviruses originated from a ssRNA virus, which converted into the more stable dsRNA form after losing its virion protein gene.

Several lines of evidence support the notion that loss of the virion protein gene affects the relative balance of concentration between (+) and (-) strand RNA synthesis of ssRNA viruses (Nassuth *et al.*, 1983; van der Kuyl *et al.*, 1991). Particularly informative were the experiments conducted on alfalfa mosaic virus, a plant virus belonging to the *Bromoviridae* family having a tripartite RNA genome. The coat protein of this virus is encoded by the RNA3 molecule. Nassuth & Bol (1983) demonstrated that there is an altered balance of (+) and (-) strand RNA synthesis induced by RNA1 and RNA2 of alfalfa mosaic virus in the absence of RNA3. Van der Kuyl *et al.* (1991) demonstrated that a frameshift early in the coat protein resulted in a 100-fold reduction in (+) strand accumulation and a 3- to 10-fold increase in (-) strand accumulation of alfalfa mosaic virus. It is conceivable that expression of genomic RNA1 and RNA2 results in the formation of a replicase activity generating equal amount of viral (+) and (-) strand RNAs, and an RNA3-encoded product, possibly the coat protein, is responsible for a switch to an asymmetric production of viral (+) strand RNA (Nassuth & Bol, 1983).

Support for the functional importance of the two domains found in AbEV1 came from gene silencing studies using hairpin vectors (Section 6.2.1). ‘Silencing’ of AbEV1 was obtained by targeting the AbEV1 helicase and RdRp sequences through homology-dependent gene silencing strategy. Transformants carrying AbEV1 sequences under expression of the AbGPD promoter were resistant to the uptake of AbEV1 dsRNA. This is an exciting observation and is the first time that homology-dependent gene silencing approaches have been used in homobasidiomycete mushrooms against viral sequences. Both helicase and RdRp sequences were able to initiate silencing in 50% or more of transformants, indicating that both sequences have a functional role in AbEV1 replication. Evidence of homology-dependent gene silencing in fungi as antiviral defence mechanism have been indirectly provided by demonstrating the presence of an RNA silencing suppressor in the mycovirus *Cryphonectria hypovirus 1* (Segers *et al.*, 2006). In this project, silencing of AbEV1 could not be defined as post-transcriptional gene silencing phenomenon since transcription of the hairpin silencing-construct could not be detected by RT-PCR. It would be interesting to characterise the nature of the gene silencing pathway in AbEV1-silenced transformants. Northern blotting might

reveal hairpin expression and/or provide evidence of siRNAs indicative of DICER activity. It may be also advisable to further investigate the involvement of different mechanisms other than PTGS in this study. Various gene silencing pathways (TGS and PTGS; Section 6.1.1) have been described for fungi, which might be inter-related in some way and play an important role in AbEV1-silenced transformants.

Agaricus bisporus endornavirus 1 (AbEV1) harboured also a putative glycosyltransferase domain, which has been identified in other endornaviruses (Hacker *et al.*, 2005; Osaki *et al.*, 2006). Glycosyltransferase enzymes are extremely interesting and highlight the crucial roles of glycans in numerous important processes, including viral infection. Over millions of years, viruses have acquired mechanisms to mimic, hijack or sabotage host processes that favour their replication. Viruses reach that goal either by regulating expression of host glycosyltransferases or by expressing their own glycosyltransferases (Markine-Goriaynoff *et al.*, 2004). Through glycosylation some viruses have the ability to avoid host anti-viral mechanisms. For example, some bacteriophages express α - and β -glucosyltransferases which glycosylate their DNA to make it resistant to host restriction endonucleases (Gram & Ruger, 1986). Other viruses actively alter host metabolism. Most baculoviruses encode an ecdysteroid glucosyltransferase, which glycosylates insect moulting hormones. Expression of this enzyme allows the virus to block moulting and pupation of infected insect larvae (O'Reilly & Miller, 1989). Although there is no evidence for function of the AbEV1 glycosyltransferase, interesting information of the effect of AbEV1 on host metabolism might be gained from further study of this enzyme in MVX infections.

In some endornaviruses studies, the presence of a discontinuity (break) in the coding strand upstream of the helicase domain has been shown to result in more than one endornavirus mRNA transcript (Fukuhara *et al.*, 1995; Pfeiffer, 1998; Hacker *et al.*, 2005). Similarly the fungal dsRNA *Cryphonectria parasitica hypovirus* (CHV) contains two open reading frames (ORFs) translated in a 'stop/restart' mode (Choi *et al.*, 1991). The discontinuity identified in endornaviruses is unusual because no stop codon has been reported for the subgenomic mRNA and the long ORF continues through the nick. Although the biological implications of this nick in the (+) strand of RNA sequence

remain unknown (Moriyama *et al.*, 1999), the discontinuity has been reported in endornaviruses infecting rice (OSV), wild rice (ORV), and broad bean (VFV). The discontinuity can be identified by polyadenylating the 3' end of the dsRNA molecule. There are up to three places where the poly(A) tail can ligate, the 3' terminus from the (+) strand, the 3' terminus from the (-) strand, and the 3' terminus from any internal break in the dsRNA molecule. *In vitro* polyadenylated dsRNA was therefore used in rice and broad bean as a template to prime reverse transcription with oligo(dT) and specific primers were subsequently used to extend the clones obtained towards the 5' end of the RNA (Pfeiffer, 1998). It may be appropriate in future studies to investigate for any possible discontinuity in AbEV1 in order to gain a better understanding of endornavirus genome expression.

As part of a wider effort to investigate the presence of PTGS suppressors (Section 6.1.3) in AbEV1, a construct carrying the phleomycin resistance gene was produced. An alternative selectable marker for transformation is required to permit use of a GFP suppression assay (Moissard & Voinnet, 2004) of hygromycin resistant, GFP transformants of *A. bisporus* (Section 6.1.3). In such experiment, AbEV1 would be inoculated into *A. bisporus* mushrooms where GFP was down-regulated. In the absence of silencing suppressor in AbEV1 genome, the silenced GFP phenotype would be unaffected, whereas in the presence of a suppressor the fluorescence phenotype would be reverted. Hygromycin resistant transformants of *A. bisporus* expressing GFP have been produced in a joint project between Warwick HRI and Bristol University (Burns *et al.*, 2005) and GFP silencing has also been demonstrated in homobasidiomycete mushrooms such as *Coprinus cinereus* (Heneghan *et al.*, 2007). The production of a silencing construct carrying an alternative selective marker to hygromycin (e.g. phleomycin) is a prerequisite to testing such a GFP suppression assay. However in this project, phleomycin resistance (phleo^R) could not be developed into a workable system for selection of *A. bisporus* transformants; the mushroom appeared to have a high tolerance to the antibiotic. Further studies are required to develop alternative transformation markers for *A. bisporus*.

The AbEV1 dsRNA was found to be present in about 60% (Adie *et al.*, 2004) of MVX infected commercial mushrooms. In culture samples derived from wild *Agaricus* collections an AbEV1-type virus (^{ARP250}14.4) has only been detected in a single strain (ARP250) when screening more than 100 isolates. This is perhaps not surprising since it is well known that enclosed environments such as mushroom houses can favour the spread of pests and diseases because of high host population density, low host genetic diversity, high concentration of spores, etc. (Milgroom, 1999). Moreover, the observation that AbEV1-like dsRNA may occur in the wild is interesting, and gives some credence to the hypothesis that MVX infections may have arisen from wild sources. Molecular characterization of the dsRNA element in the ARP250 isolate revealed high homology between the ‘wild’ viral sequence and the commercial mushroom strains. Although it is possible that the presence of ^{ARP250}14.4 could have arisen through contamination of laboratory culture, a number of different observations suggest that this is not the most likely explanation (Section 5.4). How the virus might have spread from wild to cultivated mushroom strains is unclear and merits further study. AbEV1 from commercial mushrooms has been demonstrated to transmit through spores and anastomosis (Chapter 4). Interestingly, ^{ARP250}14.4 has also been transmitted to various MVX-free strains through *in vitro* anastomosis (Holcroft *et al.*, unpublished). It is conceivable therefore, that MVX infected wild strains have anastomosed with commercial lines.

Notably, contaminated spores have been indicated (Gaze *et al.*, 2000) and demonstrated as such in this study as a source of AbEV1 transmission. It has also been demonstrated that some virus-infected mushrooms such as La France-infected mushrooms, produce spores earlier and with higher frequency than healthy mushrooms (Schisler *et al.*, 1967; Dieleman-van Zaayen, 1970). The reason for this is not known, but it has been hypothesized that diseased mushroom spores germinate more quickly as they have less pigment and thinner walls (Schisler, 1967). Future studies might focus on determining spore production and germination of AbEV1/MVX infected-mushrooms compared to healthy mushrooms.

It is intriguing to consider whether the mode of virus transmission might affect virulence towards the fungal host in the evolution of virus-fungal interaction. Viruses that depend primarily on vertical transmission are more likely to evolve reduced virulence towards their fungal hosts, even to the point of being benign (Milgroom, 1999). Endornaviruses infecting fungi, including AbEV1 can also transmit horizontally (Ikeda *et al.*, 2003; this project). This factor along with low genetic diversity in cultivated mushroom population might provide the selective pressure to yield more virulent strains (Milgroom, 1999).

An interesting observation during the homology-dependent gene silencing experiments was the loss of AbEV1 dsRNA during the transformation protocol. Hygromycin has previously been reported to inhibit the replication of several viruses, but the mechanism of action remains incompletely understood (Section 6.4.2). The antimetabolite may block translation of the viral RNA polymerase, which is likely one of the first products of gene expression in virus replication cycle (Macintyre *et al.*, 1991). Several techniques have been used so far to ‘cure’ fungi from viruses and dsRNA elements, including hyphal tip isolations (Ikeda *et al.*, 2003), heat treatment (Nair, 1973), and antibiotics such as cycloheximide (Elias & Cotty, 1996). It appears from these studies that hygromycin B also has potential as antiviral agent against fungal viruses. Although AbEV1 reappeared after removal of hygromycin selective pressure (Section 6.3.2), higher hygromycin concentration or longer incubation lapse might completely cure MVX infected mushrooms.

Collectively, the studies reported in this thesis represent first significant advances in the molecular characterization of the MVX disease. AbEV1 is the first endornavirus to be characterised in homobasidiomycete mushrooms and is the first MVX dsRNA element to be fully sequenced. Approaches used to clone and sequence AbEV1 can now be applied to other dsRNA elements associated with MVX disease. Homology-dependent gene silencing experiments allowed functional dissection of AbEV1 and provided a powerful tool for fungal virology to investigate the interaction between virus and mushroom.

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ANNEX 3.1

ENTIRE NUCLEOTIDE SEQUENCE OF ^{MVX}14.4

Entire nucleotide sequence (12750 bp) generated by recombinant clone sequencing

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|ACT AAC TAA CCG TTA GCC CTT AAA CAA AAT GAA CTC ATT AGC TAA AGT AGA AAC      54
ATA TCA CAC ACA CAC ACG AGG GGG TGG TGT CCC CTC TAA AAT ACG TTG TTA CAC      108
CAG TTT CAA AAA AAC GCT AAC AGC CAT ACA ACG TAA CAA ACA CTG TCT TGG ACT      162
TCA AAT CGA TGA AGA TTT TCC AAA GAC AGT AAA AAT GTC CGA TCC TGT TGA TGA      216
ATA TGC CGA CAG AAT TAA ATT AAT AGT TGG CGC AAC AGG AGT CAC GGA AGT CAA      270
ACA TAA ATA CTC TCA CAT GGT TGG AGG GGC TGT TTA CTT CAA GGA AGC CAT GAA      324
AAT TAG TGG TCT TAC CAG AGA AGA GTT ACT CTC AGA AAT CCT CCA AAC GGT GAG      378
ATC GAA ATT CGT TTG TCT ATT TGA ATG TCA CAA ACA AGA TGA TCC CAT TAA CAT      432
GGT GAT GGA ACT CGG CAA TGA AAT TTT AAA CAA TTT GCC AGA AGA TGA TGA TGA      486
AAA TAT TGA TTC GTT ATG TGA AAC AAT TTC AGA CTT ATA TCT AGG TGA ATA CAA      540
TAG TTA CGA TTA TGA GGA AGA AGA ACA TGA ACA TTG TGA GGA TGA AAT GGA GTT      594
GTT AGA TGA TAT CTA CAA CGA CGT GGC AGC TTA CTT TGA AGT GTC AGA TAT GTA      648
TGA TAC ACC ATT TAC ACC CGA ACA ATG TTT AGA ACT CCT AAA ACA ATT CGA TGA      702
ACC AAT CAC TTC AAG TGT TGA CAT TTT AAA ATT GCA TCT AAA TGT TAA GCA CTT      756
ACC AAA ACT TGT TAA CGT GGC TTA TTG TGC CAA TTG TGA AAT GGA AGC AAT CCT      810
AGG TCA TAA CCA CAA AAA TGT CCT CAC TAA TTG CAT TTG TTT AAA TTG TTA TAA      864
AGA GTT GAC AAT TAA ACC ATT AGG TAA AAT CGG ATC CAC GGT GCC TGA TCC AAT      918
GAG TCT GAC AGA ATA TTT AGA CAA TGG TAA TAG CAT TAG TGG TGC CTT TGA GAA      972
TAT TGC TCA AGA GCA CGA TTT GAG AGT GGT TGT GCA AGC ATT ATC TAG GCT CAA      1026
CGC CAA ATT AAT AAC TTT ACA CGA ACC GAT AGA TAA CTT GAC CAA ATC ATA CCT      1080
TAA GAA ACT AGC ACC AGA ACT AGT GTT TGC ACG AGG TAA AGG TAG TCT TGT TGG      1134
TAA CAC ATT ACT AGA CAA TCT AAC AAT TGG TTT AAC AGC AAA TCT ACA CAA CTC      1188
TGG TGA AAC CAC GTT ACT CAT CAG TGA ACA CCC TCC ACC ATC ACC AGC ATA TAT      1242
TAA TTG GAA CAG ATG GCC CGA CAG CAA TCA AAA TTA CGG ATT TAG AAA CAC TGT      1296
GGT GTC AAT TGA CCG TAT AGT AGC TGG TTT CAA ACT ACT TAG TCA ACT AGA AGT      1350
CGA CTG TTC TGT CGT GTT TTG TTT GCC GAC TGG CAT AGC TGG TGT CAC AGA TTA      1404
TGA TAC AAC CAC TAA CAT ACG TTT TGA TAG GTC AAA CAA TTC TTG TTT AAT GAC      1458
ACA TGA TAT GAG AAC AGA AGT ACA CTA TAT TAA TTC CAC ACA TAA AAT GTT GTT      1512
ATT CAA TGC TAA CAT AAT GAG CAT TAA TGA TAA ATT GTT TGT CAT TAG TAG ACA      1566
AAC TTA TGA TAG TAT ATG TGT CGT CAC GGT ACG TAG AGT TGT GAC TTT CAA AGA      1620
TTG GAC AAT TTA CAA TCC AGA TTT GCG AAA CAC GTT TAC TAT CCC TAC GAT TAC      1674
CTA CAA CAG CGC TCT TGG TTT AGT CGG AGC ACC GAT CGT CGT GAT CAA AAC TGT      1728
CAA ATT ACA CAA AGA ATT GTT GAC TAC TTT AAT TAC ACG AAA CTT GAC CGG CAA      1782
TGT GAG TGC CAA AGA ACT CGC GGA GTA CGG ATT GGG CTT CGC TCA TTC AAA GTA      1836
CAA CTT ATT CGA CAA ATC ATT CAG TCA TTA TGA CGT GAG TGC TCA AAT GAT CAT      1890
GGA TCA CGT TTT CAT ATC CGT ACT ATT GTG CAC AAG AGC TAC CAA CAA AAG ACG      1944
CGC GTT ACT GAA TTC TGC CAC ACC TGA TTT AAC AGG TAT ATT GAA CTT AAT TGG      1998
TTA CAA TTC ACT CGG CTA CCT CGC AGC CAC TAT TAA AAA GAT GTT AGA CAA AAA      2052
TTC TAC TAT AAC TGC ACT CAA CGA CTT TTC ACA AAC AAT AAC AGT TTT AAC AGA      2106
ATA TGC TAA TGA TCC AGT TTG GGA CGA AAT TGA GAG TTG GAC ACA CAC TGC TAC      2160
TAA CGA AAA CAT TCA ACT AAT CAC GCA TTA CGA AAC GCC CCT TGG TTG CTG CCA      2214
CAC TGA TAT ACA CAA CGA CAG TTG TGG TTG TTG TGG TTT GGG AAA TTC CAC TTT      2268
TAG GTG CAC CCA CTG CGC TAA TAC ACC AGC ACA ATT AAT TGA GAC TCT GAA TAT      2322
AGT AGT TGA AGA AAA AAT AGA ACC GTC AGA GCA AGT GAA GAC ACG TAA TGA AAA      2376
AAT ATT TGA TTA CAA TGG CAT ACC AGC AAG GAA GGC AGT GTT GCC TTG CGT CGA      2430
ACT ATC AGA CGG TTC ACA TGA ACA CAC TTG TGT GGT TTG CAA CAA TCG TTA TAA      2484
ACA CAC ACA CAA TCA TGA GAG TAG TAT GCA TCC ACT TTT CGT TGG CGA TTG TCC      2538
ATG GTG TGA ACC AGA ATT AAC AAC AAA AAA CAA AGG AGA AAA AAT TGA TTA TTT      2592
AGA CTC AAA CAA TAA CAT TAA CAA ACC AGT TAA AAC CGA CGC AAA TGT TCC TAA      2646
CAA GCC TAT AAC ACC TGG ACC CGC CAA ACT ACT CAA TGA CGT TCC AGA AAA AAC      2700
AGA GCG TAC ACC AGA ATC AAC AGT ACC TGA AAC ACC GTG GGG AAA CTC AAA TAG      2754
ACT AAG TGT TTT ATA CTC ACA ATT AAG TCA AGC AAC CAG TGT ACA TTT CGC TGA      2808
GCT GAT TTA CAG CAA AGG AGC ACG GTT CGT GAA CCT TTA CAC AAT CAC TAA TGA      2862
AAA ACA AAG TTT AAT ATT GTA CTG CCC ATT TGG GAC TCA CGT TGT GAC TCC ACA      2916
AAG TTT CGT TGT CAA AGA CAC AAT GCC CGT AGC AAA TTC AGG TTT AGA TAC TTG      2970
TGC CTA TGA CGC ATT AAA TTT CTT TTA TCA AAA TAG ATC AAC TAT TGA AGA TTT      3024
TAA AGC TGT AAG CAA AAC TGA TAA CTT TTG GAG TAA CGA TGA AAT TAT CTC TGT      3078
TGC TGA ACT TAA GAA ATT GAA CGT TGT GGT TTT GTC AAA GCA AAG TGG AAT AAT      3132
CCA CAA AGC AAC TAA TAG TGA CGA ATT TGC AGT TAT TGT TCA CAA TGA TTT CTT      3186
AGA TAA ACC AGG CGC ACA CTG GAC ACC AGC CAA AGT GTC ACA AAT TTC AAA TTG      3240
TAC TAT AAC GCC AGT TTG GAC CTT GAA TGC AGA CTT GGA ACA GTA TAA CAA GAT      3294
GTC AAA CAT GAA ATA TAA GAA ACA TTA CGA TTT AGT GAA ACT AGA TAT TGA ACA      3348
ATT GCT AAC CGT AAA CCT AGA AAT AGT TAA AAT TTC AAC AAA CAC TTT AGA ACT      3402
ACT TCG TCA CGC AAA ACC TAA CAT TGT TGT TGA TGT TAA GCA TGG TCA CGA ATT      3456
CCT TCA AAC AAC AAG TTT GAG TCA AGA TAG TAT GGA ATT AGA AAC AGT TGA AAT      3510
ACC AGT CAA ATT TTC AGC TTT GAT GAA TTT GTT GAA CGA TTC GAG TAA GAC TGT      3564

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TCT	TGA	ACA	TGA	TAA	ATT	AAC	AAG	ACA	TTA	CAA	TGT	CGC	ATT	AAC	TAA	TAC	TAT	3618
CGA	CGA	ATT	GTA	CGA	GGC	CGA	GAT	CAC	TGA	AGC	TAT	CAG	AAT	ATT	CTT	AAA	ATG	3672
CAA	ATT	CGG	TGC	CAA	TAT	TAC	AAA	TTA	TCA	TAA	AAT	GAG	GGT	AAT	TAA	AAG	ATT	3726
TGA	TGA	TTT	GAT	AGA	ATT	AGA	AAA	TTT	AGA	TTT	ACC	AAA	ACT	TAA	AGT	GGG	AGA	3780
CAT	CAT	CCA	TTT	AAG	TCA	CCA	GTC	GAA	CAT	GAT	AGC	GGT	ACC	GAT	TTT	AAA	AAC	3834
TCG	TCA	CAA	TAC	ATT	AGT	AGT	AAA	AAT	AAC	TGG	TGT	CGC	AGT	TGG	TGT	TCA	ACT	3888
TTT	GTT	TGG	AAT	TAC	AAA	GGG	ATC	CGC	TGG	AAG	TAT	GAT	CAG	CGC	AAT	TTT	CGG	3942
TTT	GTT	AAG	GGT	TCC	CAA	ATT	AAG	CAA	CAA	ACT	TTT	TGA	ACA	ATT	AAG	TAA	TAC	3996
AGA	ATG	TGT	CCT	AGC	TGT	TCC	GGG	AGC	AGG	GAA	AAC	ATA	CGA	AAT	ATT	AAA	TAG	4050
TTA	TAA	CGC	AGG	AGA	TTG	TAT	CAT	CGC	TAT	GAC	TTC	CCA	CAA	TAA	ATC	AAG	CAT	4104
TTA	CCG	GAG	AGC	TAA	AGA	AAT	GAA	GAA	AAT	TGT	AAA	CGT	TAA	ATC	TTT	AGA	GAA	4158
ATT	CTT	GAG	TGA	GGG	GAA	GAA	ATA	CAA	GAC	TGT	GTA	CTT	GGA	TGA	GTG	TAC	TTT	4212
AGT	TAA	ACC	ATT	TGA	CGG	AGT	GTT	GGT	TCT	CAC	ATT	GTG	CGA	CAA	AAT	GTA	TGC	4266
GTA	TGG	TGA	CAA	CAA	TCA	AAT	TCC	ATT	CGT	CGA	TAT	GTC	GCA	GTC	AGC	TGG	GAC	4320
TAG	GGA	AGT	TAA	AAA	TAT	TAC	TGA	CGT	TGT	CAA	ACC	CGA	AAA	GAT	TGA	GAC	CAA	4374
ATT	ATT	ATC	TAG	AAG	ATT	TGG	ACC	TTC	ATT	AGT	TTC	TGA	GTT	GTC	CCC	GTT	AAT	4428
CCC	CGG	AAT	TAT	GTG	TCA	TCC	ATC	TGT	TGA	ACA	TGA	CAC	TGA	CAT	TAG	ATT	TGC	4482
AAA	CTA	TGA	GAA	AAA	TTC	TAT	ACA	AGT	GAC	TGC	AGA	AAT	AGA	AAA	TAA	CGT	CAG	4536
GAA	CTT	TAA	ACC	TAA	TGT	AGT	GTT	AGT	ATT	TTT	CCA	AGC	GAA	GAA	AGC	ATT	TTG	4590
GTT	GAA	GAG	GTT	ACA	TCC	GAT	TGT	TGT	TGA	AAC	CGT	TCA	CAG	TTA	CCA	AGG	TAA	4644
CGA	GGC	CGA	TAA	TGT	TGT	GGT	TAT	TCA	AGA	ACC	AAA	TGA	AAG	CAC	ATT	CGG	CAT	4698
TTG	TGC	AGA	TAA	AAA	GTA	TGT	GAT	CTC	AGC	CGC	ATC	CAG	AGC	GGT	TAA	ACA	CTT	4752
GTT	TTG	GAT	ATC	GAT	CGG	GAT	GTA	CCA	ACA	CAA	CAA	AGG	ATT	AAG	TTC	AAG	AGT	4806
CTA	CAC	ATT	ATC	CCC	ACA	GCG	TGG	TGG	TGG	TAT	ACA	TAA	AAC	ATT	CAA	TGC	CAT	4860
ATT	AGA	CAA	CAT	AAC	AGG	CGT	AGA	AAT	GGT	GTC	CAA	TAC	AGG	AAC	AAT	GAT	TGA	4914
TGC	TGA	CAA	ATT	TAA	TGC	ATC	TAA	ATC	AGC	GTA	TGA	AGA	TAA	ATA	CGG	TGT	AAA	4968
AGT	TTC	TTT	AGC	AAA	TAA	CTC	TGA	TGG	TCA	ATC	CCT	AGA	AGT	TCG	TAA	AGT	TGG	5022
GAT	ACT	AAT	GGT	GAG	ATT	TGA	CAT	AGT	AAA	TGG	TGT	GGC	CAC	CAC	TTC	ATA	TGA	5076
CAG	ATT	GAA	TAT	GGT	CAC	GTC	AAA	AGA	TCT	GTC	TGA	TCT	AAA	CGA	ACT	GTT	GAA	5130
ACC	CGC	GGT	CAT	TCA	ATG	CGA	TAA	ATT	CGA	CAC	GAA	ATT	TAA	CAC	CAC	TAT	TGA	5184
TTC	ACA	ACA	AGC	CGA	TAT	AAT	AAC	ATT	CAA	TAT	GCA	ACT	TAG	TGC	CTT	GCA	TTC	5238
GGC	TTG	TGC	AAG	CAC	ACA	AGT	TAC	ATT	TTA	TAA	CGA	TTC	TAC	TGA	GGT	AAG	CAT	5292
CAT	TTG	TAG	GAC	GAC	TCA	TAA	CAA	ATT	TAG	AAT	TTC	ATT	ATC	TTT	AAA	TGA	CGA	5346
TTA	TTG	CCT	TAA	GGT	ACA	ACC	CAT	GAT	TAT	TGG	AAG	AAA	TTT	TGA	AAC	TCA	TGT	5400
GCA	TAA	TGT	TCT	CAC	TAC	TCT	TGG	TTA	TCA	AGA	TGG	TAG	TGA	AGA	ATA	TCC	GTT	5454
TAG	CCC	TGC	GGG	ACC	AAT	TAA	AAC	TAC	GGT	AAT	TAA	TCA	CAC	TTT	TAA	TCA	AAT	5508
CAT	GAA	TTA	TTT	AAA	AAC	TGT	AGT	GCC	GGG	CAT	AAT	TGA	ACA	AAA	AAT	GAG	TGA	5562
CAG	CAA	AAA	ATT	TTT	AAA	TAA	GAT	TTG	TGA	ACT	AAC	AAA	AGA	GAA	TGA	AAT	GGT	5616
ACT	GCT	TCA	TAA	TCA	CGA	AAC	AGG	TTG	TTA	TGT	AGT	CAA	GAC	TCC	AAT	AAA	TTA	5670
TAA	GTG	TGT	GAC	TCC	CAA	GTG	TAC	ATA	CAA	CAC	AAA	TGC	ATA	CAA	TGC	GTT	AAT	5724
GAT	TTT	CTT	GAA	CGC	CAC	TAG	TTT	TAA	GAC	TAG	TGC	AAT	GAC	TAT	TTT	CGC	TTT	5778
ACT	TAC	GAA	GTC	ACT	CAT	TGG	ATC	TCG	TAG	TGA	TAA	TGG	AAT	AGA	AGG	ACT	GAT	5832
GCT	TCC	ATG	GCA	CTA	CCA	CAA	GAC	GCA	CGC	AAC	TAA	AGC	TTT	CAA	ACA	TTA	CAT	5886
CGA	CAA	GAG	TGC	TTT	ATT	ATA	TAT	GAC	TAG	TAA	AAT	TGG	TGA	AAA	AAG	AAC	AAT	5940
CTA	CGT	GAC	AGA	ACA	AAA	CAG	AGA	CAA	AAT	CAT	GAA	TAT	GGT	ACG	AGA	TGC	AGG	5994
TAT	AGA	TGT	TAA	TGT	GAA	CAT	ACA	TCG	TTT	CTG	CCC	ATC	GTT	GCC	CTT	GGG	TGT	6048
TGA	AAT	TGT	TGG	TGT	TTT	AAT	GGT	CTT	AAG	TCT	AGA	TAA	GTC	TAA	GCA	AGA	CAG	6102
AAA	AAT	CAA	AAT	CTA	CGG	ACC	ACA	CGC	AGG	TGT	TCT	AGC	CGC	AAA	TGG	GTT	CTT	6156
AAA	TTT	ACA	AAA	AGT	TAG	AGA	TTA	TCA	TGA	ATT	ATC	TAC	TTA	TGA	GTT	GCA	TGC	6210
CGT	CGA	TGA	TCG	AAT	GTT	GAG	TTC	AGT	AAA	AAA	CAG	TGT	TAG	CGA	GGC	ATT	AAA	6264
GAA	AAA	GCA	GTT	TGA	TAA	CAC	GAA	CAC	TGA	AAA	AGA	TGA	CGC	TTT	TAT	CAA	AGC	6318
GTT	GGG	AAC	ACC	TAA	TGA	GAT	TAA	TTA	TGC	CGC	ACT	TTA	TGG	TTA	TGA	TTA	CAA	6372
TCT	GAT	AAC	GAA	TTC	TAA	TTT	GTT	AAC	AGA	CTT	ATC	AGA	GTT	TGA	AAC	CGT	CAA	6426
GTT	AAT	AAC	AGA	TTT	AGA	TTC	GAT	TGA	GCA	GTG	TAC	TGA	AGG	CTC	ACC	GAC	CTT	6480
CGT	TGA	CAA	TCC	AAT	GTG	GCA	ATT	TAC	TTT	CAA	AAT	ACC	TAA	AAT	TTT	GAA	TGC	6534
CCT	AAT	ACA	AAC	AGG	CGC	TGA	CGA	AAA	CAA	ACT	ACT	CAG	TTA	TGA	TTC	CAC	GTT	6588
TGT	GGG	GTT	GGG	TGC	TGT	AGT	GGG	TGA	CAT	TGA	ACA	AGG	TGT	TAG	TAA	ACA	ACT	6642
ATA	CAG	TCA	ACT	GGC	AGG	ATT	TGT	GCT	ACG	TGA	TCA	AAT	AAC	ACT	AAC	AGT	ACC	6696
ACG	CTT	GAA	TAG	TAG	TAT	GAT	TGA	TGG	GGG	AGT	AAA	CGT	TAC	CAG	CCA	AGA	GAT	6750
CAC	TGT	GTC	ACG	TAA	ATT	GTT	CCG	CGA	TTG	CGG	GTT	GAG	ATT	GTT	AAG	AGA	AAA	6804
TCA	CGA	TAT	CGA	CAG	TTT	ACT	AAA	ACA	CGT	TCG	CAC	AAA	ACA	ACA	TGC	TAC	TTA	6858
TTA	CTC	AGA	TAG	TGG	TAG	TTA	CAG	ACA	ATA	TAA	AGA	GAC	ACC	CGG	AGC	AGC	TAT	6912
GGG	TTT	GTG	TCT	AGC	TGT	TTA	CCT	GAG	ATA	TAA	CAA	TAA	ATT	TAC	TGA	GCT	GTT	6966
CGA	TAC	AAT	TAA	ATG	GCT	ACA	ACC	TGA	AAC	AGT	CAA	ACA	AGA	AGG	TAT	ATT	TGA	7020
GTT	GTT	GTT	AGA	TAA	CTT	AGG	TAG	TAG	CGC	TAA	AGA	AAT	GGT	GTT	GAA	CGT	TGT	7074
TAG	CAA	ACT	CTC	AGG	TAT	TGC	TGG	TAT	GAA	TTT	ACC	ACA	AAA	AGA	CTT	TAT	TAA	7128

TGA AAC GAT GAG TGT GAT GAA TGT AGA AGA GAG AAT ATC TAC TAG ACG CCT ATT	7236
AAT GCG GGG AAC CAA GCA GAT TCT AAT GAC TAG TGG GGC GGC GAG CAT TGA TGA	7290
AGG GGC AAA TAA AGT GAC CTT ACA ATC CAA GTA TCT CCA TAA ATT GTT CAG CAA	7344
TAT GTC AAC GCT GAT GCA ATC CGT TAG TGC AAG ACA TGA CCC TGT CAC TTT AAT	7398
GAA AGA CTT CAC CAA ACC AAA GAG TAT ATG CTG TAT TTG CAT TGG TTC TTT TGG	7452
TGA CAT AAC CAA CCA TCT CCA AGT AGC AAA CAT GGC TGT CCA TGA AGG TGT TAA	7506
AGT TTC ATT AAT CTT TCC AGA CAG CTA TTT GGA TCA GGT GAG GAG TAT TGC AGA	7560
CTC GAA AGT TAC CCT AGT GCC ACA AAG ATT CAG TGT AGA CAA TCA CAT CAA ACT	7614
ATT GAA TGA GTT AAG GAC TGA CAA CCT TGA GGT TTT AAT GAC GGC ATA CAC CGA	7668
AGA GAA AGG AGC ATT ACT TTA TAA TGA TTT TGA TTA CAA GAT TTT GAA ACC AGA	7722
CTT GGT TGT TGG AAC TTG TTT GAC TGT ACA GGG ACC AAT AAT CGC CAA AAA GTT	7776
CAA TGC AAA CTA CCT TGA ATT GAA TGC ATT ACC TTG GAA TTT AGT AAG AGC CGA	7830
AAA TAA AAG ATT CGA CTC ACT TAA AAA TGT ATT ATA TCG GGG TTA CAG AGC AAT	7884
GCA ACT AGA AAC CAT CAT AAA GCA ATA CGC CAT TGC CGG TTT GCA GAA CCC CAC	7938
ACC TCA AGA CAT AAT AAG TGA AAA TTA CAC AAG TGT AAA TGT AAT AGA TCC AAT	7992
CTT TGG TTT AGT GGC TGA GAA AGA AAA CCA CTC GGT AGT TGG GTT TTT ACC ATT	8046
TAA ATA CAC AAA TTC AAA GAC AAT TTT AAC AAC CAA AAC ATG TGT ATT AAC ATT	8100
AGG TTC TAT GAA AAC CAA ACA GCA TGC AAA CAA ACT AAT ATC AGT AGC TAA AAA	8154
ACT GTT GAG TTT TGG TGA ACA TGT TAC GAT TGT TGA AGG AGG TGT CAA ATT TAA	8208
CAA AGA ATT TAT AGA CCG GTC AGA TAA ATT ACA AAT TGT AAG CAA TGT GGA TTA	8262
TAC CTC ACA ATT AGT TCA CTG TAA ATT AAT GTT ACA TCA CTG CGG CTC AGG AAC	8316
AAC ACA CAC CGC CAT GTT AGC AGG ATT ACC ACA AAT TTT GTT GCC AGT TGA TTT	8370
TGA TCA ACC TCA ATG GGC GGG AGC GCT TAA TAA GAA AGG GTA CGG AGT GTA TGC	8424
TGA TGA ACT GTT AAA CAT GGA ATT AGA TGA TTT CAC CCA TTG GTT CAA CGT CAC	8478
GTT AGA GAA AAC CAA GAA TGA CTC AAA TAA AAT GAT TCG GCA TAC CGC TAC AAA	8532
CAT AGC ACA TGC CTT GAA ATT AAC AGA TAA TGC AAT CAC TGA TAG TGT TAC ATT	8586
GAC AAC AGA CAT TAC AAT TGA GGA AGA AGC AAT AAT TAG AGA CAA CAT ACA TAA	8640
ATG TAA CCT CCA TAA ATG TAA ACC TTT AGA GAC GAT GAG TGT TTT GAT TAA TAC	8694
ATG TTG TGG TGA ACG TAA AAG ACA CTG TGT CAT TGG TGA GAC AGA AGA GTG TTA	8748
CAT AAT AGT GAA TTC AAC ACA TTA CCC TGA TCA CAG AAT TGG GTG GAA CAG AAA	8802
ACA ACA TAT TGC TAT ACA ACC CAT CAT GCC AAT TCC AGT ACT GCG TGG TCA TTA	8856
CGA AAT AGC CAC TTT GGG TTA TCA TGC TCA TGG AAT GTG TGA AGC AAA TAG TGA	8910
ATC ACC TCC TAA CTT TTA TAT AAA ACC TAA TCA AGT ATC TGA ATT TAA CAA ATC	8964
GAT AGA TTG GAT TGA TTT AGT TAG ATT ACA CAA TGG GAG ATG GTC AAA CCG AGG	9018
AGT GTT CGG AAT CGA ACC AAG GAC AGG TAT TTG CAA TTG TTG TCA CAG ATC AAT	9072
GTG GTT ATT AAG ATC ACA AAA TGA AAA GTT GTG CAC ATC CTG TCA TAT ACA AAT	9126
CAA TTG GCC TCA ATT CAC TTT CAC AGA CTC CGT ATC CAC CAC CAA AAC CGA TAA	9180
TTT TAA AGC CAA ATC TAG AGC AGG ACA CAT TAT TTT TGA GCT TTC GAA GTT ACA	9234
AGA ATC TCA CGT GTT ATT AGA TCC TAA AGG GAA TAG CAA TTG CGT TCC CAA CTG	9288
TTT ACG TGC TGT TGC AAA GTA TAG TAC AAA GGT GTC CAG CAC GAT CAA TAA TGC	9342
ATT TAT TGA TTT TGA AAT TGG TGA GTT CAC CAG TAT AGA GAA AAT TAT TCA AAT	9396
TTG TCG ATT CAT GAG AGT GGA CAT ACA AGT TGA CCA TCC AAA TGT AAA AAC TGG	9450
GGG CAG ATT GTT GCA CAA TTT AGA GTT TGA TAA GAC TCC AAA ACT GAG GCT GTT	9504
AAT AAC GGG TGA AAG AGA ACA GCA CTG CAC ATT AAC CGC CGC TAT CAA AAT ACC	9558
TGC TGC ACA GAA ATC AAT AAC TTT AAT AGA AAC CGA AAA CCC ATG CGA TGA CAT	9612
GAA TTC ATT GAT AAC TAC TGA CTT GAT TGA CAA TGC AGA CAT GGA TTT CAA TCA	9666
AAT TTT AAA AAC AAA CCT TAA AAC CTT CAG GTC AAC AAA GTA CAC AGA CGT TAT	9720
AGC AAA AAC AAC AGA TGG TAA ATT GAA CAT TTA TGG TAG AAG GAA TTT GTT GGT	9774
GGA AGC AAA TAT TGT ATT CAG AAA GTT CAA ATC TCA TGA AAT GTA CTT TGG TGA	9828
ATT ACC GGA GTC ATT TAA CAG ATT TGA GGA CAG AAT ACT TTG TGT CCC GAC TAG	9882
AAC CGG TAA TGT TAT TGG GTT AGT CAC AAG ATT AAA CAA CAC GGA AAA ACA ACT	9936
AAT TGT TTT GAC TGG ACA AAG ACC TTT ACC TGT GGT AAA TTA TGT GAT CGA CGC	9990
AAG TGT ATC AAT TAC CAA GGT CAA AAA ACA CAA AAA ACT TAT TAA AAA TGA TAC	10044
TGT TAC AGT GGG CGC GTT CAA TGA AAA AAG TAG ACG CGA ATT GTC TAC CAT TTA	10098
CCC AGG GAT CAG TGT GAA TAG AAA CTG CCA GAA AGT AGT GAT TAG TAG TCT AGA	10152
CAC TAG AAA TCA TCA CTT GTA TAG TGA GTG GAA TGA AAT GCA CAC TTT GTT AAA	10206
TAG AAA CAC AAT GGG TGA AAC ACT CAT TGA ATT GTA CGG CAA ACT GGA TGG TGC	10260
AAC AGT GCC AAC ACT GGT CGA TTC AAA CTT AAC ACA AAG TAT ATG TGA ACA ATT	10314
TTC TAG AAC GAC AAG GGA CCA AAT TTT CAG ATT TGG TGG GGA CCA AAA ACC TAC	10368
TGT TGA AAC TTG GAC CCG TGA TAA ACC AAC TGG CTT GTT CCT TGA AGA GCA AGG	10422
TTT CAC ATC GCC AGA AAA CGA GAG GTA TGT ATT AGA TTA CGA GCA GTT TAA AGA	10476
TAT AAG TGA GTT GAA TGA TAC AGA AAC CAT GAA CAA GTT AAT AAG TTG CGA TTT	10530
GAT CGG ATA CAT TTC ACT CAA GAA TTG TGT GAA ATA TTT CGA AAT CAT TAA ATC	10584
GAC TGA GGA TAT TGT CGC ATT TAC GGG TGT GGA TAA AAA TGA ATT AAA TCG TGA	10638
ATT GAT TGA TTA TGA TGA GGC TAG CTT AAC AGT GAC TTG TGG GCC GAT CTG GAA	10692

AGG	CGG	ATT	GCC	CGA	GAT	AAC	GAC	GAT	GGA	TTC	TCT	TAA	CAG	GAC	TGT	CTT	TGT	10746
GAG	ACC	AGA	AGA	TAT	TAA	GGA	GTC	GAA	CGG	TGA	ATT	GAC	GCT	AGT	TTG	GTT	CAG	10800
GTC	TAA	TTG	TTT	AAT	TTT	GAC	AAC	CTT	TGT	ATG	GAA	AAA	GAA	AGT	AAG	AGG	TGG	10854
TTC	TTA	TGG	TCA	AAA	ACA	GGA	TCT	CGA	GGA	TAG	AAA	ACA	AAA	TAT	TGA	CAG	TGA	10908
CAA	CAA	CAA	ACT	TTA	TTG	GTT	TAA	TGA	GGA	GAT	TGA	AGA	AAG	ATT	AGA	ACA	CCG	10962
CGG	TGC	CGA	ATC	CTG	GAT	AGA	CAA	AGG	ATC	AAA	ACC	AAA	AAA	CAC	TCA	ATT	ACC	11016
AGC	TTT	AGC	TGC	CAA	TGA	AGT	TGA	ACT	CGA	TGC	CTT	ATG	TAG	TGT	TTT	CAA	TGA	11070
TGG	AAT	GAG	GGG	TTA	CGA	CGG	GAA	AAC	GTT	TGT	TGC	TTC	TGA	ACC	TGC	TAA	TAA	11124
AAA	GTT	TTT	AAT	GTT	TAC	TGA	ACC	AGA	CAC	GCC	AAA	CTT	TGT	TTG	CCA	TGA	AAC	11178
GGG	AGA	AGG	ACC	AGA	AAT	AGG	AAC	GGA	AGT	GAT	GGA	TAT	AAT	TGA	CGA	TGT	TGA	11232
CTT	GGC	CGA	TCA	CGT	CAC	AAG	ACT	AGC	TCC	GAA	AAC	TGG	AAT	AAT	TAA	GTC	ACA	11286
CGA	AGT	TCC	AGG	TAG	GAT	TGT	CAC	TAA	CAC	TAA	GAC	AAC	TAT	GGT	TAA	ATA	TCC	11340
AGA	GAT	GTC	TAA	ACC	TGT	TAT	AAC	GAA	AGG	GGT	CTA	CCA	AAC	ATT	CAA	TGC	TGT	11394
TTC	ATC	AAG	ACT	GCA	TAG	TGT	CGT	GAA	ATA	TCG	CAA	ACA	TAA	AAT	GGA	TGT	TGT	11448
GTC	TGA	GTT	TGA	CAG	ATG	TGC	TAA	TTG	TTA	TTT	TGT	GCC	TAA	TTA	TCA	ACA	TGT	11502
ATT	GGA	TGA	ATT	TAA	GAA	TGA	TCC	CAT	AAC	CTA	CAA	TGT	TAA	CAA	CAT	AGT	AGA	11556
TTG	GGT	GAA	TAG	GAG	AAA	AAA	CAG	TTC	TGT	CGT	AGC	TGC	CGA	ATT	GAT	TGA	ACT	11610
GAT	GCA	AAC	TGG	TTG	GTT	TAA	TCA	TCC	AAT	CAA	CAG	CGT	GAA	AGT	GCA	CAG	TAA	11664
ACT	TGA	ATC	ATT	GTT	GAA	AGA	AGA	TGA	AAT	TGG	TTA	TCA	ATA	TGG	ACA	ACA	AAA	11718
AAT	ACG	CAT	TAT	CGT	GCA	CCA	AAC	ATA	TGC	GAT	TTG	TGC	GAT	ATT	TGC	AGA	TAT	11772
ATT	TTT	GCA	AGC	AAA	AGA	AAG	ATT	TAA	ACA	ACT	CCT	GGG	TGA	AAA	ATG	CGT	GTA	11826
TGC	CGA	CGG	CTT	CAC	ACT	TGA	AGA	ATT	GGC	AGC	CAG	AAT	GAG	ATT	AAT	CAA	ATC	11880
AGA	CAA	ACC	ATT	ATG	GGT	ATT	TGA	GCG	TGA	CTT	CAC	AAA	ACA	AGA	CAG	ACA	AAC	11934
AGA	CGA	AGA	TTT	AAT	AAA	TTT	TGA	AAT	GTA	CGT	TTA	CAA	ACA	ACT	TGG	AGT	TCA	11988
TGA	CGA	CGT	AAT	AAG	TTT	TTG	GCG	CAA	GAC	ACA	TGA	GAA	TTG	GTT	CTA	CAA	AGG	12042
TAC	TGA	CTT	GAG	TGG	GTC	GTT	AAG	TTG	GAT	GCG	TCT	CAC	TGG	ACA	ATG	TAC	AAC	12096
GGC	ATT	AGG	AAA	TGC	TAT	AAC	TAA	TAT	GGT	TGT	GAA	TAG	CAG	AAC	TTA	TCA	AGA	12150
ATA	TGA	ACA	AAA	CAT	TAA	AAT	TAT	GCT	GTT	GCT	GGG	TGA	TGA	TAA	TTC	AAT	GAT	12204
CAG	TGA	CTG	TGA	AAT	AAA	TGT	TCA	CAA	TAG	TAG	AAA	AAT	TAG	TAG	GGA	TTA	TTA	12258
CAA	CAT	GGA	GTC	AAA	AGA	TTT	TTG	TAA	AAA	ACA	CAC	TGG	CAT	ATT	TTT	GAG	AAT	12312
GAT	ATT	ACA	CCA	ACA	ACA	AGA	CGG	CAG	TAT	GTC	TGT	CTG	TCC	TGA	CTT	TTT	ACG	12366
TTT	ACG	AAG	AAG	ATA	TGA	AGT	TAC	TAA	CGG	AGT	GCA	CAA	AAT	GGG	TGA	CGA	AGA	12420
ACT	TAA	ACT	ACG	TGG	TTT	AAG	TTA	TTT	GTA	CAT	GTT	GGG	TCG	TAA	CAA	AGT	TAC	12474
TGA	AGA	AGC	AGT	GAC	ATC	TTT	GGG	TTA	TAA	CGA	CGA	ATT	ATT	GCC	GAC	GTA	TTA	12528
CAA	TTT	TGA	AGA	AGC	AGT	TAT	GGC	TGT	GGC	AAC	TAA	CAA	TGG	TAT	GAC	GAA	TGA	12582
TGC	AGC	AAG	AAA	TGA	GTT	TAA	TCT	ATT	GAT	TGA	TAT	GAT	CAA	AGC	AAA	CAA	GTC	12636
TTA	CGA	TGT	AGA	CTA	TGT	GGT	TAC	CAG	TAG	TCA	AAT	TCG	TTA	AAC	TGA	TTA	TTG	12690
CGT	ATT	GAG	TGA	AAA	CGT	TTG	TTT	CAC	TCA	ACG	CGC	AAT	TAG	TGT	GTG	TGT	GTG	12744
GGG	GGG																	12750

ANNEX 3.2

^{MX}14.4 OPEN READING FRAME

A single open reading frame (ORF) was found in the ^{MX}14.4 plus strand sequence, starting at nt 29 and ending at nt 12679, which encodes a putative protein of 4216 aa

Frame	from	to	Length	Length
+2	29 bp	12679 bp	12651bp	4216 aa

```

29 atgaaactcattagctaaagtagaaacatatcacacacacacacga
   M N S L A K V E T Y H T H T R
74 ggggggtggtgtcccctctaaaatacgttgttacaccagtttcaaa
   G G G V P S K I R C Y T S F K
119 aaaacgctaacagccatacaacgtaacaaacactgtccttggaactt
   K T L T A I Q R N K H C L G L
164 caaatcgatgaagattttccaaagacagtaaaaaatgtccgatcct
   Q I D E D F P K T V K M S D P
209 gttgatgaatatgccgacagaattaataatagttggcgcaaca
   V D E Y A D R I K L I V G A T
254 ggagtcacggaagtcaaacataaatactctcacatgggttgagggg
   G V T E V K H K Y S H M V G G
299 gctgtttacttcaaggaagccatgaaaattagtggtcctaccaga
   A V Y F K E A M K I S G L T R
344 gaagagttactctcagaaatcctccaaacggtgagatcgaaattc
   E E L L S E I L Q T V R S K F
389 gtttgtctatttgaatgtcacaacaagatgatcccattaacatg
   V C L F E C H K Q D D P I N M
434 gtgatgaaactcggcaatgaaattttaacaatttgccagaagat
   V M E L G N E I L N N L P E D
479 gatgatgaaaatattgattcgttatgtgaaacaatttcagactta
   D D E N I D S L C E T I S D L
524 tatctaggtgaatacaaatagttacgattatgaggaagaagaacat
   Y L G E Y N S Y D Y E E E E H
569 gaacattgtgaggatgaaatggagttgtagatgatatctacaac
   E H C E D E M E L L D D I Y N
614 gacgtggcagcttactttgaagtgtcagatatgtatgatacacca
   D V A A Y F E V S D M Y D T P
659 ttacacccgaacaatgtttagaactcctaaaacaattcgatgaa
   F T P E Q C L E L L K Q F D E
704 ccaatcacttcaagtgttgacattttaaaattgcatctaaatgtt
   P I T S S V D I L K L H L N V
749 aagcacttaccaaaacttgtaacgtggcttattgtgccaattgt
   K H L P K L V N V A Y C A N C
794 gaaatggaagcaatcctaggtcataaccacaaaaatgtcctcact
   E M E A I L G H N H K N V L T

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839 aattgcatttggtttaaattggttataaagagttgacaattaaacca
 N C I C L N C Y K E L T I K P
 884 ttaggtaaaatcggatccacgggtgcctgatccaatgagtcctgaca
 L G K I G S T V P D P M S L T
 929 gaatatttagacaatggtaatagcattagtggtgcctttgagaat
 E Y L D N G N S I S G A F E N
 974 attgctcaagagcacgatttgagagtggttgtgcaagcattatct
 I A Q E H D L R V V V Q A L S
 1019 aggctcaacgccaattaataaactttacacgaaccgatagataac
 R L N A K L I T L H E P I D N
 1064 ttgaccaaatacacaacttaagaaactagcaccagaactagtggtt
 L T K S Y L K K L A P E L V F
 1109 gcacgaggtaaaggtagtcttgggtgtaaacacattactagacaat
 A R G K G S L V G N T L L D N
 1154 ctaacaattggtttaaagcaaatctacacaactctgggtgaaacc
 L T I G L T A N L H N S G E T
 1199 acgttactcatcagtgaaacaccctccaccatcaccagcatatatt
 T L L I S E H P P P S P A Y I
 1244 aattggaacagatggcccgcacagcaatcaaaattacggatttaga
 N W N R W P D S N Q N Y G F R
 1289 aacactgtggtgtcaattgaccgtatagtagctgggttcaacta
 N T V V S I D R I V A G F K L
 1334 cttagtcaactagaagtcgactggttctgctggttttggttgccc
 L S Q L E V D C S V V F C L P
 1379 actggcatagctgggtgtcacagattatgatacaaccactaacata
 T G I A G V T D Y D T T T N I
 1424 cgttttgataggtcaaacattcttggtttaatgacacatgatatg
 R F D R S N N S C L M T H D M
 1469 agaacagaagtacactatattaattccacacataaaaatggttgtta
 R T E V H Y I N S T H K M L L
 1514 ttcaatgctaacataatgagcattaatgataaattggttgcatt
 F N A N I M S I N D K L F V I
 1559 agtagacaaacttatgatagtatatgtgtcgtcacgggtacgtaga
 S R Q T Y D S I C V V T V R R
 1604 gttgtgactttcaagattggacaatttacaatccagatttgcca
 V V T F K D W T I Y N P D L R
 1649 aacacgtttactatccctacgattacctacaacagcgtctctgggt
 N T F T I P T I T Y N S A L G
 1694 ttagtcgggagcaccgatcgtcgtgatcaaaactgtcaaattacac
 L V G A P I V V I K T V K L H
 1739 aaagaattggtgactactttaattacacgaaacttgaccgggcaat
 K E L L T T L I T R N L T G N
 1784 gtgagtgccaagaactcgcggagtagcggattgggtctcgtcat
 V S A K E L A E Y G L G F A H

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1829 tcaaagtacaacttatttcgacaaatcattcagtcattatgacgtg
S K Y N L F D K S F S H Y D V
1874 agtgctcaaatgatcatgggatcacggttttccatattccgtactattg
S A Q M I M D H V F I S V L L
1919 tgcacaagagctaccaacaaaagacgcgcggttactgaattctgcc
C T R A T N K R R A L L N S A
1964 acacctgatttaacaggtatattgaacttaattgggttacaattca
T P D L T G I L N L I G Y N S
2009 ctcggctacctcgcagccactatta5aaaaagatgttagacaaaaat
L G Y L A A T I K K M L D K N
2054 tctactataactgcactcaacgacttttcacaaacaataacagtt
S T I T A L N D F S Q T I T V
2099 ttaacagaatatgctaataatgatccagtttgggacgaaattgagagt
L T E Y A N D P V W D E I E S
2144 tggacacacactgctactaacgaaaacattcaactaatcacgcat
W T H T A T N E N I Q L I T H
2189 tacgaaacgccccttgggttgctgccacactgatatacacaacgac
Y E T P L G C C H T D I H N D
2234 agttgtgggttggttggttgggaaattccacttttaggtgcacc
S C G C C G L G N S T F R C T
2279 cactgcgctaatacaccagcacaattaattgagactctgaatata
H C A N T P A Q L I E T L N I
2324 gtagtgaagaaaaaatagaaccgtcagagcaagtgaagacacgt
V V E E K I E P S E Q V K T R
2369 aatgaaaaaatatttgattacaatggcataccagcaaggaaggca
N E K I F D Y N G I P A R K A
2414 gtgttgcccttgcgctcgaactatcagacggttcacatgaacacact
V L P C V E L S D G S H E H T
2459 tgtgtggtttgcaacaatcgttataaacacacacacaatcatgag
C V V C N N R Y K H T H N H E
2504 agtagtatgcatccacttttcggtggcgattgtccatgggtgtgaa
S S M H P L F V G D C P W C E
2549 ccagaattaacaacaaaaaaacaaaggagaaaaaattgattattta
P E L T T K N K G E K I D Y L
2594 gactcaacaataacattaacaaaccagttaaaaccgacgcaaat
D S N N N I N K P V K T D A N
2639 gttcctaacaagcctataacacctggaccgccaactactcaat
V P N K P I T P G P A K L L N
2684 gacgttccagaaaaaacagagcgtacaccagaatcaacagtacct
D V P E K T E R T P E S T V P
2729 gaaacaccggtggggaaactcaaatagactaagtgttttataactca
E T P W G N S N R L S V L Y S
2774 caattaagtcaagcaaccagtgtagatttcgctgagctgatttac
Q L S Q A T S V H F A E L I Y
2819 agcaaaggagcaggttcgtagaacctttacacaatcactaatgaa
S K G A R F V N L Y T I T N E
2864 aaacaaagt5ttaattgtactgcccatttgggactcacggtgtg
K Q S L I L Y C P F G T H V V

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2909 actccacaaagtttcggttggtcaagacacaatgcccgtagcaaat
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 2954 tcaggtttagatacttgtgcctatgacgcattaaatttcttttat
 S G L D T C A Y D A L N F F Y
 2999 caaaatagatcaactattgaagattttaagctgtaagcaaaact
 Q N R S T I E D F K A V S K T
 3044 gataacttttgagtaacgatgaaattatctctgttgctgaactt
 D N F W S N D E I I S V A E L
 3089 aagaaattgaacggttggttttggtcaagcaaagtggataatc
 K K L N V V V L S K Q S G I I
 3134 cacaaagcaactaatagtgacgaatttgcagttattggtcacaat
 H K A T N S D E F A V I V H N
 3179 gatttcttagataaaaccaggcgcacactggacaccagccaaagt
 D F L D K P G A H W T P A K V
 3224 tcacaaatttcaaattgtactataacgccagtttggaccttgaat
 S Q I S N C T I T P V W T L N
 3269 gcagacttggaacagtataacaagatgtcaaacatgaaatataag
 A D L E Q Y N K M S N M K Y K
 3314 aacattacgatttagtgaaactagatattgaacaattgctaacc
 K H Y D L V K L D I E Q L L T
 3359 gtaaacctagaatagttaaaatttcaacaaacactttagaacta
 V N L E I V K I S T N T L E L
 3404 cttcgtcacgcaaaaacctaacattggttggtgatgtaagcatggt
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 3449 cacgaattccttcaacaacaagtttgagtcaagatagtatggaa
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 3494 ttagaaacagttgaaataccagtcacaaatttccagctttagaat
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 3539 ttggtgaacgattcagagtaagactggtccttgaacatgataaatta
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 3584 acaagacattacaatgtcgcattaactaatactatcgacgaattg
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 3629 tacgaggccgagatcactgaagctatcagaatattcttaaaatgc
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 3674 aaattcgggtccaatattacaaaattatcataaaatgagggttaatt
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 3719 aaaagatttgatgatttgatagaattagaaaatttagatttacca
 K R F D D L I E L E N L D L P
 3764 aaacttaaagtgaggagacatccatttaagtcaccagtcgaac
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 3809 atgatagcgggtaccgattttaaactcgtcacatacattagta
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 3854 gtaaaaataactgggtgtgcgagttggtggttcaacttttgtttgga
 V K I T G V R V G V Q L L F G
 3899 attacaaaggaatccgctggaagtatgatcaggcgaattttcgggt
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 3944 ttggttaagggttcccaaattaagcaacaaactttttgaacaatta
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3989 agtaatacagaatgtgtcctagctggtccgggagcagggaaaaca
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 4034 tacgaaatattaatatagttataacgcaggagattgtatcatcgct
 Y E I L N S Y N A G D C I I A
 4079 atgacttcccacaataaatcaagcatttaccggagagctaaagaa
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 4124 atgaagaaaattgtaaacgttaaatctttagagaaattcttgagt
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 4169 gaggggaagaaatacaagactgtgtacttggatgagtgtacttta
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 4214 gttaaacatttgacggagtggtggttctcacattgtgcgacaaa
 V K P F D G V L V L T L C D K
 4259 atgtatgcgtatggtgacaacaatcaaattccattcgtcgatatg
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 4304 tcgcagtcagctgggactaggggaagttaaaaatattactgacgtt
 S Q S A G T R E V K N I T D V
 4349 gtcaaaccgaaaagattgagaccaaatattatctagaagattt
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 4394 ggaccttcattagtttctgagttgtccccgttaatccccggaatt
 G P S L V S E L S P L I P G I
 4439 atgtgtcatccatctgttgaacatgacactgacattagatttgca
 M C H P S V E H D T D I R F A
 4484 aactatgagaaaaattctatacaagtgactgcagaaatagaaaat
 N Y E K N S I Q V T A E I E N
 4529 aacgtcaggaactttaaacctaattgtagtggttagtatttttccaa
 N V R N F K P N V V L V F F Q
 4574 gcgaagaaagcatttttggttgaagaggttacatccgattgttgtt
 A K K A F W L K R L H P I V V
 4619 gaaaccgttcacagttaccaaggtaacgaggccgataatggtgtg
 E T V H S Y Q G N E A D N V V
 4664 gttattcaagaaccaaataaaaagcacattcggcatttgtgcagat
 V I Q E P N E S T F G I C A D
 4709 aaaaagtatgtgatctcagccgcatccagagcgggttaaacacttg
 K K Y V I S A A S R A V K H L
 4754 ttttgatcgcgatcgggatgaccaacacaacaaggattaagt
 F W I S I G M Y Q H N K G L S
 4799 tcaagagtctacacattatccccacagcgtggtggtggtatacat
 S R V Y T L S P Q R G G G I H
 4844 aaaacattcaatgccatattagacaacataacaggcgtagaaatg
 K T F N A I L D N I T G V E M
 4889 gtgtccaatacaggaacaatgattgatgctgacaaatttaatgca
 V S N T G T M I D A D K F N A
 4934 tctaatacagcgtatgaagataaatacgggtgtaaaagtttcttta
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 5024 atactaatggtgagatttgacatagtaaatggtgtggccaccact
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5069 tcatatgacagattgaat**atgg**tcacgtcaaaagatctgtctgat
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 D T K F N T T I D S Q Q A D I
 5204 ataacattcaat**atg**caacttagtgccttgacattcggcttggtgca
 I T F N **M** Q L S A L H S A C A
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 5294 atttgtaggacgactcataacaaatttagaatttcattatcttta
 I C R T T H N K F R I S L S L
 5339 aatgacgattattgccttaaggtacaaccc**atg**attattggaaga
 N D D Y C L K V Q P **M** I I G R
 5384 aattttgaaactcatgtgcataatggttctcactactcttggttat
 N F E T H V H N V L T T L G Y
 5429 caagatggttagtgaagaatatccggttagccctgcggaaccaatt
 Q D G S E E Y P F S P A E P I
 5474 aaaactacggtaattaatcacacttttaatacaaat**atga**attat
 K T T V I N H T F N Q I **M** N Y
 5519 ttaaaaaactgtagtgccggacataattgaacaaaa**atg**agtgac
 L K T V V P D I I E Q K **M** S D
 5564 agcaaaaaatttttaataagatttgtgaactaacaaaagagaat
 S K K F L N K I C E L T K E N
 5609 gaa**atg**gtactgcttcataatcacgaaacaggttggtatgtagtc
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 5699 aacacaaatgcatacaatgcggtta**atg**attttcttgaacgccact
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 5744 agttttaagactagtgca**atg**actattttgcgtttacttacgaag
 S F K T S A **M** T I L R L L T K
 5789 tcaactcattggatctcgtactgataatggaatagaaggactg**atg**
 S L I G S R T D N G I E G L **M**
 5834 cttccatggcactaccacaagacgcacgcaactaaagctttcaaa
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 5879 cattacatcgacaagagtgctttattatat**atg**actagtaaaatt
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 G E K R T I Y V T E Q N R D K
 5969 atc**atga**at**atgg**tacgagatgcaggtatagatgttaatgtgaac
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 6014 atacatcggtttctgcccacggttgccttggggtggtgaaattggt
 I H R F C P S L P L G V E I V
 6059 ggtgtttta**atg**gtcttaagtctagataagtctaagcaagacaga
 G V L **M** V L S L D K S K Q D R
 6104 aaaatcaaatctacggaccacacgcaggtgttctagccgcaaat
 K I K I Y G P H A G V L A A N

6149 gggttcttaaatttacaaaaagtttagagattatcatgaattatct
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 6194 acttatgagttgcatgccgtcgatgatcgaa**atg**ttgagttcagta
 T Y E L H A V D D R **M** L S S V
 6239 aaaaacagtgttagcggagcattaaagaaaaagcagtttgataac
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 6284 acgaacactgaaaaagatgacgcttttatcaaagcgttggaaca
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 6329 cctaatagagattaattatgccgcactttatggttatgattacaat
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 6374 ctgataacgaattctaatttgtaaacagacttatcagagtttgaa
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 6419 accgtcaagttaataacagatttagattcgattgagcagtgact
 T V K L I T D L D S I E Q C T
 6464 gaaggctcaccgaccttcgttgacaatcca**atg**tggaatttact
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 6554 gacgaaaacaaactactcagttatgattccacgtttggtggggttg
 D E N K L L S Y D S T F V G L
 6599 ggtgctgtagtggggtgacattgaacaaggtgtagtaacaacta
 G A V V G D I E Q G V S K Q L
 6644 tacagtcaactggcaggatttggtgctacgtgatcaataacacta
 Y S Q L A G F V L R D Q I T L
 6689 acagtaccacgcttgaatagtagt**atg**attgatggggaagtaaac
 T V P R L N S S **M** I D G E V N
 6734 gttaccagccaagagatcactgtgtcacgtaaattggtcccgcat
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 6779 tgcggggtgagattggttaagagaaaatcacgatatcgacagttta
 C G L R L L R E N H D I D S L
 6824 ctaaaacacgcttcgcacaaaacaacatgctacttattactcagat
 L K H V R T K Q H A T Y Y S D
 6869 agtggttagttacagacaatataaagagacacccggagcagct**atg**
 S G S Y R Q Y K E T P G A A **M**
 6914 gatttggtgtctagctgtttacctgagatataacaataaatttact
 D L C L A V Y L R Y N N K F T
 6959 gagctgttcgatacaattaaatggctacaacctgaaacagtcaaa
 E L F D T I K W L Q P E T V K
 7004 caagaaggtatatttgagttggtgtagataacttaggtagtagc
 Q E G I F E L L L D N L G S S
 7049 gctaaagaa**atg**gtggtgaacggtgtagcaactctcaggtatt
 A K E **M** V L N V V S K L S G I
 7094 gctggt**atg**aatttaccacaaaaagactttattaaaacgatatgg
 A G **M** N L P Q K D F I K T I W
 7139 aaagttgtgagaaat**atg**gctttccatcaggaagac**atg**ttcact
 K V V R N **M** A F H Q E D **M** F T
 7184 gaaacg**atg**agtggt**atg**aatgtagaagagagaatatctactaga
 E T **M** S V **M** N V E E R I S T R


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7229 cgcctattaatgCGGGGAACCAAGCAGATTCTAATGACTAGTGGG
    R L L M R G T K Q I L M T S G
7274 gCGGCGAGCATTGATGAAGGGGCAAATAAAGTGACCTTACAATCC
    A A S I D E G A N K V T L Q S
7319 aagtatctccataaattgttcagcaatatgtcaacgctgatgcaa
    K Y L H K L F S N M S T L M Q
7364 tCGTtagtgcaagacatgaccctgtcactttaatgaaagacttc
    S V S A R H D P V T L M K D F
7409 accaaaccaagagtatatgctgtatttgcattgggtcttttgggt
    T K P K S I C C I C I G S F G
7454 gacataaccaaccatctccaagtagcaaacatggctgtccatgaa
    D I T N H L Q V A N M A V H E
7499 ggtgttaaagtttcattaatctttccagacagctatttggatcag
    G V K V S L I F P D S Y L D Q
7544 gtgaggagtattgcagactcgaaagttaccctagtgccacaaaga
    V R S I A D S K V T L V P Q R
7589 ttcagtgtagacaatcacatcaaactattgaatgagtttaaggact
    F S V D N H I K L L N E L R T
7634 gacaaccttgaggttttaatgacggcatacaccgaagagaaagga
    D N L E V L M T A Y T E E K G
7679 gcattactttataatgattttgattacaagattttgaaaccagac
    A L L Y N D F D Y K I L K P D
7724 ttgggtgttggaacttgtttgactgtacagggaccaataatcgcc
    L V V G T C L T V Q G P I I A
7769 aaaaagttcaatgcaaactaccttgaattgaatgcattaccttgg
    K K F N A N Y L E L N A L P W
7814 aatttagtaagagccgaaaataaaaagattcgactcacttaaaaat
    N L V R A E N K R F D S L K N
7859 gtattatatCGGGGTACAGAGCAATGCAACTAGAAACCATCATA
    V L Y R G Y R A M Q L E T I I
7904 aagcaatacGCCATTGCCGGTTTGCAGAACCCACACCTCAAGAC
    K Q Y A I A G L Q N P T P Q D
7949 ataataagtgaaaattacacaagtgtaaatgtaatagatccaatc
    I I S E N Y T S V N V I D P I
7994 tttggtttagtggctgagaaagaaaaccactcggttagttgggttt
    F G L V A E K E N H S V V G F
8039 ttaccatttaaatacacaaattcaaagacaattttaacaaccaa
    L P F K Y T N S K T I L T T K
8084 acatgtgtattaacattaggttctatgaaaaccaaacagcatgca
    T C V L T L G S M K T K Q H A
8129 acaaaactaatatcagtagctaaaaaactgttgagttttggtgaa
    N K L I S V A K K L L S F G E
8174 catgttacgattggttgaaggaggtgtcaaatttaacaagaattt
    H V T I V E G G V K F N K E F
8219 atagaccggtcagataaattacaaattgtaagcaatgtggattat
    I D R S D K L Q I V S N V D Y
8264 acctcacaattagttcactgtaaattaatgttacatcactgcggc
    T S Q L V H C K L M L H H C G

```

8309 tcaggaacaacacacacaccgccatggttagcaggattaccacaaatt
 S G T T H T A M L A G L P Q I
 8354 ttggttgccagttgattttgatcaacctcaatgggcgaggagcgctt
 L L P V D F D Q P Q W A G A L
 8399 aataagaaaggggtacggagtgatgctgatgaactgttaaacatg
 N K K G Y G V Y A D E L L N M
 8444 gaattagatgatttcacccattgggttcaacgtcacggttagagaaa
 E L D D F T H W F N V T L E K
 8489 accaagaatgactcaaataaaaatgattcggcataccgctacaaac
 T K N D S N K M I R H T A T N
 8534 atagcacatgccttgaaattaacagataatgcaatcactgatagt
 I A H A L K L T D N A I T D S
 8579 gttacattgacaacagacattacaattgaggaagaagcaataatt
 V T L T T D I T I E E E A I I
 8624 agagacaacatacataaatgtaacctccataaatgtaaaccttta
 R D N I H K C N L H K C K P L
 8669 gagacgatgagtggttttgattaatacatggtgtggtgaacgtaaa
 E T M S V L I N T C C G E R K
 8714 agacactgtgtcattgggtgagacagaagagtggttacataatagtg
 R H C V I G E T E E C Y I I V
 8759 aattcaacacattaccctgatcacagaattgggtggaacagaaaa
 N S T H Y P D H R I G W N R K
 8804 caacatattgctatacaacccatcatgcccattccagttactgct
 Q H I A I Q P I M P I P V L R
 8849 ggtcattacgaaatagccactttgggttatcatgctcatggaatg
 G H Y E I A T L G Y H A H G M
 8894 tgtgaagcaaatagtgaaatcacctcctaacttttatataaaacct
 C E A N S E S P P N F Y I K P
 8939 aatcaagtatctgaatttaacaaatcgatagattgggattgattta
 N Q V S E F N K S I D W I D L
 8984 gttagattacacaatgggagatgggtcaaaccgaggagtggtcgga
 V R L H N G R W S N R G V F G
 9029 atcgaaccaaggacaggtatgtgcaattggtgtcacagatcaatg
 I E P R T G I C N C C H R S M
 9074 tggttattaagatcacaaaatgaaaagttgtgcacatcctgtcat
 W L L R S Q N E K L C T S C H
 9119 atacaaatcaattggcctcaattcactttcacagactccgtatcc
 I Q I N W P Q F T F T D S V S
 9164 accacaaaaccgataattttaagccaaatctagagcaggacac
 T T K T D N F K A K S R A G H
 9209 attatgttgagcttcgaagttacaagaatctcacgtgttatta
 I I F E L S K L Q E S H V L L
 9254 gatcctaaaggggaatagcaattgcggttcccaactgtttacgtgct
 D P K G N S N C V P N C L R A
 9299 gttgcaaagtatagtacaaaggtgtccagcagcatcaataatgca
 V A K Y S T K V S S T I N N A
 9344 tttattgattttgaaattgggtgagttcaccagtatagagaaaatt
 F I D F E I G E F T S I E K I
 9389 attcaaattgtcgattcatgagagtggtgacatacaagttgaccat
 I Q I C R F M R V D I Q V D H

9434 ccaaatgtaaaaactgggggacagattggttgcacaatttagagttt
 P N V K T G G R L L H N L E F
 9479 gataagactccaaaactgaggctgttaataacgggtgaaagagaa
 D K T P K L R L L I T G E R E
 9524 cagcactgcacattaaccgcccgtatcaaaaatacctgctgcacag
 Q H C T L T A A I K I P A A Q
 9569 aatcaataactttaatagaaaaccgaaaacccatgcgatgacatg
 K S I T L I E T E N P C D D M
 9614 aattcattgataactactgacttgattgacaatgcagacatggat
 N S L I T T D L I D N A D M D
 9659 ttcaatcaaattttaaaaacaaaccttaaaaccttcagggtcaaca
 F N Q I L K T N L K T F R S T
 9704 aagtacacagacgttatagcaaaaacaacagatggtaaatgaac
 K Y T D V I A K T T D G K L N
 9749 atttatggtagaaggaatttggtggggaagcaaatattgtattc
 I Y G R R N L L V E A N I V F
 9794 agaaagttcaaattctcatgaaatgtactttgggtgaattaccggag
 R K F K S H E M Y F G E L P E
 9839 tcatttaacagatttgaggacagaataactttgtgtcccgactaga
 S F N R F E D R I L C V P T R
 9884 accggtaaatggtattggggttagtcacaagattaaacaacacggaa
 T G N V I G L V T R L N N T E
 9929 aaacaactaattgttttgactggacaaagacctttacctgtggta
 K Q L I V L T G Q R P L P V V
 9974 aattatgtgatcgacgcaagtgtatcaattaccaaggtcaaaaaa
 N Y V I D A S V S I T K V K K
 10019 cacaaaaaacttattaaaaatgatactgttacagtggggcgcggtc
 H K K L I K N D T V T V G A F
 10064 aatgaaaaaagtagacgcgaattgtctaccatttaccagggtac
 N E K S R R E L S T I Y P G I
 10109 agtgtgaatagaaaactgccagaaagtagtgattagtagtctagac
 S V N R N C Q K V V I S S L D
 10154 actagaaatcatcacttgtatagtgagtggaaatgaaatgacact
 T R N H H L Y S E W N E M H T
 10199 ttgtaaataagaaacacaatggggtgaaacactcattgaattgtac
 L L N R N T M G E T L I E L Y
 10244 ggcaaaactggatgggtgcaacagtgccaacactgggtcgattcaaac
 G K L D G A T V P T L V D S N
 10289 ttaacacaaaagtatatgtgaacaattttctagaacgacaagggac
 L T Q S I C E Q F S R T T R D
 10334 caaattttcagatttggttgggacaaaaacctactggtgaaact
 Q I F R F G W D Q K P T V E T
 10379 tggaccctgataaaccaactggcttggctccttgaagagcaaggt
 W T R D K P T G L F L E E Q G
 10424 ttcacatcgccagaaaacgagaggtatgtattagattacgagcag
 F T S P E N E R Y V L D Y E Q

10469 tttaaagatataagtgagttgaatgatacagaaaaccatgaacaag
 F K D I S E L N D T E T M N K
 10514 ttaataagttgCGgatttgatcggatacatttcactcaagaattgt
 L I S C D L I G Y I S L K N C
 10559 gtgaaatatttcgaaatcattaaatcgactgaggatattgtcgcga
 V K Y F E I I K S T E D I V A
 10604 ttacgggtgtggataaaaaatgaattaaatcgtgaattgattgat
 F T G V D K N E L N R E L I D
 10649 tatgatgaggctagcttaacagtgacttgtgggCCgatctggaaa
 Y D E A S L T V T C G P I W K
 10694 ggcggattgccccgagataacgacgatggattctcttaacaggact
 G G L P E I T T M D S L N R T
 10739 gtctttgtgagaccagaagatattaaggagtcgaacggtgaattg
 V F V R P E D I K E S N G E L
 10784 acgctagtttggttcaggtctaattgtttaattttgacaaccttt
 T L V W F R S N C L I L T T F
 10829 gtatggaaaaagaaagtaagaggtggttcttatggtcaaaaacag
 V W K K K V R G G S Y G Q K Q
 10874 gatctcgaggatagaaaacaaaatattgacagtgacaacaacaaa
 D L E D R K Q N I D S D N N K
 10919 ctttattggtttaatgaggagattgaagaaagattagaacaccgc
 L Y W F N E E I E E R L E H R
 10964 ggtgccgaatcctggatagacaaaaggatcaaaaccaaaaaaacact
 G A E S W I D K G S K P K N T
 11009 caattaccagcttttagctgccaatgaagttgaactcgatgcctta
 Q L P A L A A N E V E L D A L
 11054 tgtagtgttttcaatgatggaatggagggttacgacgggaaaacg
 C S V F N D G M R G Y D G K T
 11099 tttggtgcttctgaacctgctaataaaaagtttttaatggtttact
 F V A S E P A N K K F L M F T
 11144 gaaccagacacgccaactttggttgccatgaaacgggagaagga
 E P D T P N F V C H E T G E G
 11189 ccagaaataggaacggaagtgatggatataattgacgatgttgac
 P E I G T E V M D I I D D V D
 11234 ttggccgatcacgtcacaagactagctccgaaaactggaataatt
 L A D H V T R L A P K T G I I
 11279 aagtcacacgaagttccaggtaggattgtcactaacactaagaca
 K S H E V P G R I V T N T K T
 11324 actatgggttaaatatccagagatgttctaaacctgttataacgaaa
 T M V K Y P E M S K P V I T K
 11369 ggggtctaccaaacattcaatgctgtttcatcaagactgcatagt
 G V Y Q T F N A V S S R L H S
 11414 gtcgtgaaatatcgcaaacataaaaatggatgttgtgtctgagttt
 V V K Y R K H K M D V V S E F
 11459 gacagatgtgctaattgttattttgtgcctaattatcaacatgta
 D R C A N C Y F V P N Y Q H V
 11504 ttggatgaatttaagaatgatcccataacctacaatgtaacaac
 L D E F K N D P I T Y N V N N

11549 atagtagattgggtgaataggagaaaaaacagttctgtcgtagct
 I V D W V N R R K N S S V V A
 11594 gccgaattgattgaactgatgcaaactgggtgggttaatcatcca
 A E L I E L M Q T G W F N H P
 11639 atcaacagcgtgaaagtgcacagtaaacttgaatcattgttghaa
 I N S V K V H S K L E S L L K
 11684 gaagatgaaattggttatcaatatggacaacaaaaaatatcgatt
 E D E I G Y Q Y G Q Q K I R I
 11729 atcgtgcaccaaacatagcgtatgtgcatatgtgcagatata
 I V H Q T Y A I C A I F A D I
 11774 tttttgcaagcaaaagaaagatttaaacactcctgggtgaaaaa
 F L Q A K E R F K Q L L G E K
 11819 tgcgtgtatgccgacggcttcacacttgaagaattggcagccaga
 C V Y A D G F T L E E L A A R
 11864 atgagattaatcaaactcagacaaaccattatgggtatgtgagcgt
 M R L I K S D K P L W V F E R
 11909 gacttcacaaaacaagacagacaaacagacgaagatttaataaat
 D F T K Q D R Q T D E D L I N
 11954 tttgaaatgtacgtttacaaacaacttggagttcatgacgacgta
 F E M Y V Y K Q L G V H D D V
 11999 ataagtttttggcgcaagacacatgagaattgggttctacaaaggt
 I S F W R K T H E N W F Y K G
 12044 actgacttgagtggttcgtaagttggatgctcactggacaa
 T D L S G S L S W M R L T G Q
 12089 tgtacaacggcattaggaaatgctataactaatatgggttgtgaat
 C T T A L G N A I T N M V V N
 12134 agcagaacttatcaagaatatgaacaaaacattaaaattatgctg
 S R T Y Q E Y E Q N I K I M L
 12179 ttgctgggtgatgataattcaatgatcagtgactgtgaaataaat
 L L G D D N S M I S D C E I N
 12224 gttcacaatagtagaaaaattagtagggattattacaacatgggag
 V H N S R K I S R D Y Y N M E
 12269 tcaaaagatttttgtaaaaaacacactggcatatttttgagaatg
 S K D F C K K H T G I F L R M
 12314 atattacaccaacaacaagacggcagtatgtctgtctgtcctgac
 I L H Q Q Q D G S M S V C P D
 12359 tttttacgtttacgaagaagataggaagttactaacgggagtgac
 F L R L R R R Y E V T N G V H
 12404 aaaatgggtgacgaagaacttaactacgtgggttaagttatgtg
 K M G D E E L K L R G L S Y L
 12449 tacatggttgggtcgtaacaaagttactgaagaagcagtgacatct
 Y M L G R N K V T E E A V T S
 12494 ttgggttataacgacgaattattgccgacgtattacaattttgaa
 L G Y N D E L L P T Y Y N F E
 12539 gaagcagttatggctgtggcaactaacaatgggatgacgaatgat
 E A V M A V A T N N G M T N D

```
12584 gcagcaagaaatgagtttaatctattgattgatatgatcaaagca
      A A R N E F N L L I D M I K A
12629 aacaagtcttacgatgtagactatgtggttaccagtagtcaaatt
      N K S Y D V D Y V V T S S Q I
12674 cgttaa 12679
      R *
```

ANNEX 3.3

PAIRWISE IDENTITY OF ENDORNAVIRUS HELICASE AND RdRp SEQUENCES

Alignment of endornavirus RdRp (motifs III-VI) and helicase amino acid sequences were obtained using the MegAlign package via ClustalW algorithm. Pairwise identities are indicated in %. The highest match for the ^{MVX}14.4 helicase sequence was with PEV1, whereas for the ^{MVX}14.4 RdRp was with OSV

		HELICASE						
	RdRp	ORV	OSV	VFV	PEV1	GaEV	HmEV1-670	^{MVX} 14.4
	ORV	*	82.7	27.4	26.6	12.1	28.6	25.0
	OSV	98.4	*	27.5	27.1	12.6	28.7	26.3
	VFV	61.8	62.0	*	30.8	11.6	26.0	27.6
	PEV1	58.1	57.5	61.2	*	11.2	24.1	30.5
	GaEV	36.6	36.6	38.6	33.4	*	9.3	11.7
	HmEV1-670	53.0	53.0	56.5	53.0	40.3	*	24.6
	^{MVX} 14.4	58.9	59.2	58.1	55.3	35.8	51.1	*

ORV: *Oryza rufipogon endornavirus*; OSV: *Oryza sativa endornavirus*; VFV: *Vicia faba endornavirus*; PEV1: *Phytophthora endornavirus 1*; GaEV: *Gremmeniella abietina endornavirus*; HmEV1-670: *Helicobasidium mompa endornavirus 1-670*; ^{MVX}14.4: ^{MVX}14.4

ANNEX 3.4

AMINO ACID SEQUENCE PAIRWISE IDENTITY OF VIRAL, FUNGAL AND PLANT UGTs (MOTIF IV)

Alignment of viral, fungal and plant motif IV- UGT amino acid sequences were obtained using the MegAlign package via ClustalW algorithm. Pairwise identities are indicated in %. The highest match was with PEV1, followed by *Ustilago maydis*

	Mb	Ms	Gc	Mg	Sc	Cc	Cn	Um	At	Os	Bm virus	Mb virus	^{MVX} 14.4	ORV	OSV	PEV1
Mb	*															
Ms	68.1	*														
Gc	47.9	45.8	*													
Mg	48.5	54.5	25.0	*												
Sc	19.1	21.3	14.6	27.3	*											
Cc	44.7	42.6	72.9	30.3	13.7	*										
Cn	55.6	48.9	48.9	35.6	22.2	53.3	*									
Um	50.0	45.5	45.8	42.4	20.5	52.3	71.1	*								
At	53.2	44.7	62.5	39.4	13.3	64.4	64.4	56.8	*							
Os	46.8	44.7	58.3	42.4	13.7	67.4	57.8	61.4	82.2	*						
Bm virus	34.0	25.5	27.1	18.2	9.8	22.9	33.3	31.8	26.7	25.0	*					
Mb virus	34.0	25.5	27.1	18.2	24.5	22.9	33.3	31.8	26.7	25.0	100.0	*				
^{MVX}14.4	36.2	34.0	31.2	27.3	15.1	26.4	35.6	38.6	33.3	26.4	24.5	24.5	*			
ORV	25.5	21.3	25.0	24.2	9.8	30.2	31.1	34.1	28.9	34.9	25.0	25.0	24.5	*		
OSV	29.8	27.7	31.2	27.3	15.7	28.6	35.6	36.4	33.3	32.7	26.5	27.1	26.4	69.4	*	
PEV1	36.2	25.5	47.1	27.3	11.9	38.1	42.2	50.0	40.5	40.5	23.8	23.8	40.5	31.0	28.6	*

Mb: *Mycobacterium bovis* (bacterium); Ms: *Mycobacterium smegmatis* (bacterium); Gc: *Glomerella cingulata* (Ascomycete); Mg: *Magnaphorte grisea* (Ascomycete); Sc: *Saccharomyces cerevisiae*; (Ascomycete); Cc: *Coprinus cinereus* (Homobasidiomycete); Cn: *Cryptococcus neoformans* (Heterobasidiomycete); Um: *Ustilago maydis* (Heterobasidiomycete); At: *Arabidopsis thaliana* (plant); Os: *Oryza sativa* (plant); Bm virus: *Bombix mori nuclear polyhedrosisvirus* (virus); Mb virus: *Mamestra brassicae nucleopolyhedrovirus* (virus); ^{MVX}14.4: ^{MVX}14.4; ORV: *Oryza rufipogon endornavirus*; OSV: *Oryza sativa endornavirus*; PEV1: *Phytophthora endornavirus* 1

ANNEX 5.1

PAIRWISE ALIGNMENT OF ^{ARP250}14.4 AND AbEV1 NUCLEOTIDE SEQUENCES IN THE HELICASE REGION

The alignment was constructed using the MegAlign package and displayed via GENEDOC program. The sequences share 98% identity. Identities are highlighted in blue, while dissimilarities are highlighted in green (arrow). ARP250: ^{ARP250}14.4

```

      *           20           *           40
ARP250 : CCGAGAGCTAAAGAAATGAAGAAAATTGTAAACGTTAAATCTTTAGAGA : 49
AbEV1  : CCGAGAGCTAAAGAAATGAAGAAAATTGTAAACGTTAAATCTTTAGAGA : 49

      *           60           *           80           *           1
ARP250 : AATTCTTGAGTGAGGGGAAGAAATACAAGACTGTGTACTTGGATGAGTG : 98
AbEV1  : AATTCTTGAGTGAGGGGAAGAAATACAAGACTGTGTACTTGGATGAGTG : 98

      00           *           120           *           140
ARP250 : TACTTTAGTTAAACCATTTGACGGAGTGTTGGTTCTCACATTGTGCGAC : 147
AbEV1  : TACTTTAGTTAAACCATTTGACGGAGTGTTGGTTCTCACATTGTGCGAC : 147

      *           160           *           180           ↓           *
ARP250 : AAAATGTATGCGTATGGTGACAACAATCAAATTCATTGTCGATATGT : 196
AbEV1  : AAAATGTATGCGTATGGTGACAACAATCAAATTCATTGTCGATATGT : 196

      200           *           220           *           240
ARP250 : CGCAGTCAGCTGGGACTAGGGAAGTTAAAAATATTACTGACGTTGTCAA : 245
AbEV1  : CGCAGTCAGCTGGGACTAGGGAAGTTAAAAATATTACTGACGTTGTCAA : 245

      *           260           ↓           *           280           *
ARP250 : ACCCGAAAAGATTGAGACCAAATTAATCTATCTAGAAGATTTGGACCTTCA : 294
AbEV1  : ACCCGAAAAGATTGAGACCAAATTAATCTATCTAGAAGATTTGGACCTTCA : 294

      300           *           320           ↓           *           340
ARP250 : TTAGTTTCTGAGTTGTCCCGTTAATCCCCTGGAATTATGTGTCATCCAT : 343
AbEV1  : TTAGTTTCTGAGTTGTCCCGTTAATCCCCTGGAATTATGTGTCATCCAT : 343

      *           360           ↓           *           380           *
ARP250 : CTGTTGAACATGACACTGATATTAGATTTGCAAATATGAGAAAAATTC : 392
AbEV1  : CTGTTGAACATGACACTGACATTAGATTTGCAAATATGAGAAAAATTC : 392

      400           ↓           *           420           *           440
ARP250 : TATACAAGTGATGTCAGAAATAGAAAATAACGTCAGGAACCTTTAAACCT : 441
AbEV1  : TATACAAGTGACTGTCAGAAATAGAAAATAACGTCAGGAACCTTTAAACCT : 441

      *           ↓           ↓           *           460           *           480
ARP250 : AATGTAGTGTTGGTGTGTTTTCCAAGCGAAGAAAGCATTTTG : 482
AbEV1  : AATGTAGTGTTAGTATTTTTCCAAGCGAAGAAAGCATTTTG : 482

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ANNEX 5.2

PAIRWISE ALIGNMENT OF ^{ARP250}14.4 AND AbeV1 NUCLEOTIDE SEQUENCES IN THE RdRp REGION

The alignment was constructed using the MegAlign package and displayed via GENEDOC program. The two sequences share 97% identity. Identities are highlighted in red, while dissimilarities are highlighted in blue (arrow). ARP250: ^{ARP250}14.4

```

                *           20           *           40
ARP250 : GGATTGTCACCTAACAACCTAAGACAACCATGGTTAAATACCAGAGATGT : 48
AbeV1  : GGATTGTCACCTAACAACCTAAGACAACCTATGGTTAAATATCCAGAGATGT : 48

                *           60           *           80           *
ARP250 : CTAAACCTGTTATAACGAAAGGGGTTTACCAAACATTCAATGCTGTTT : 96
AbeV1  : CTAAACCTGTTATAACGAAAGGGGCTTACCAAACATTCAATGCTGTTT : 96

                100           *           120           *           140
ARP250 : CATCAAGACTGCATAGTGTCTGCGTAAATATCGCAAACATAAAAATGGATG : 144
AbeV1  : CATCAAGACTGCATAGTGTCTGCGTAAATATCGCAAACATAAAAATGGATG : 144

                *           160           *           180           *
ARP250 : TTGTATCTGAGTTTGACAGATGCGCTAATTGTTATTTTGTACCTAATT : 192
AbeV1  : TTGTGCTCTGAGTTTGACAGATGCTGCTAATTGTTATTTTGTGCTAATT : 192

                200           *           220           *           240
ARP250 : ATCAACATGTATTGGATGAATTTAAGAATGATCCCATAACTTACAATG : 240
AbeV1  : ATCAACATGTATTGGATGAATTTAAGAATGATCCCATAACCTACAATG : 240

                *           260           *           280
ARP250 : TTAACAATATAGTAGATTGGGTGAATAGAGGAAAAACAGTTCCTGTCTG : 288
AbeV1  : TTAACAACATAGTAGATTGGGTGAATAGGAGAAAAACAGTTCCTGTCTG : 288

                *           300           *           320           *
ARP250 : TAGCTGCCGAATTGATTGAATTTGATGCAAACCTGGTTGGTTTAAATCATC : 336
AbeV1  : TAGCTGCCGAATTGATTGAACCTGATGCAAACCTGGTTGGTTTAAATCATC : 336

                340           *           360           *           380
ARP250 : CAATCAACAGCGTGAAAGTACACAGTAAACTTGAATCATTGTTGAAAG : 384
AbeV1  : CAATCAACAGCGTGAAAGTGCACAGTAAACTTGAATCATTGTTGAAAG : 384

                *           400           *           420           *
ARP250 : AAGATGAAATTGGTTATCAATATGGACAACAAAAAATACGCATTATCG : 432
AbeV1  : AAGATGAAATTGGTTATCAATATGGACAACAAAAAATACGCATTATCG : 432

                440           *           460           *           480
ARP250 : TGCACCAAACATATGCAATTTGTGCAATATTTGCAGATATATTTTTGTC : 480
AbeV1  : TGCACCAAACATATGCGAATTTGTGCGAATATTTGCAGATATATTTTTGTC : 480

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```

*           500           *           520
ARP250 : AAGCAAAAAGAAAGATTTAAACAACCTCCTGGGTGAAAAATGCGTGTATG : 528
AbEV1  : AAGCAAAAAGAAAGATTTAAACAACCTCCTGGGTGAAAAATGCGTGTATG : 528

*           540           *           560           *
ARP250 : CCGACGGCTTCACACTTGAAGAATTGGCAGCTAGAATGAGATTAATTA : 576
AbEV1  : CCGACGGCTTCACACTTGAAGAATTGGCAGCCAGAATGAGATTAATCA : 576

580           *           600           *           620
ARP250 : AATCAGACAAACCATTATGGGTATTTGAGCGTGACTTCACAAAACAAG : 624
AbEV1  : AATCAGACAAACCATTATGGGTATTTGAGCGTGACTTCACAAAACAAG : 624

*           640           *           660           *
ARP250 : ACAGACAAACAGACGAAGATTTAATAAATTTTCAAATGTACGTTTACA : 672
AbEV1  : ACAGACAAACAGACGAAGATTTAATAAATTTTCAAATGTACGTTTACA : 672

680           *           700           *           720
ARP250 : AACAACTTGGAGTTCATGACGACGTAATAAGTTTTTGGCGCAAGACAC : 720
AbEV1  : AACAACTTGGAGTTCATGACGACGTAATAAGTTTTTGGCGCAAGACAC : 720

*           740           *           760
ARP250 : ATGAGAATTGGTTCCTACAAAGGTAAGTTTGGAGTGGGTCATTAAGTT : 768
AbEV1  : ATGAGAATTGGTTCCTACAAAGGTAAGTTTGGAGTGGGTCGTTAAGTT : 768

*           780           *           800           *
ARP250 : GGATGCGTCTCACTGGACAATGTACAACGGCATTAGGAAATGCTATAA : 816
AbEV1  : GGATGCGTCTCACTGGACAATGTACAACGGCATTAGGAAATGCTATAA : 816

820           *           840           *           860
ARP250 : CTAATATGGTTGTAATAGCAGAACTTATCAAGAATATGAACAAAACA : 864
AbEV1  : CTAATATGGTTGTAATAGCAGAACTTATCAAGAATATGAACAAAACA : 864

*           880           *
ARP250 : TTAAAATTATGCTGTTGCTGGGTGATGATA : 894
AbEV1  : TTAAAATTATGCTGTTGCTGGGTGATGATA : 894

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ANNEX 5.3

PAIRWISE ALIGNMENT OF ^{ARP250}14.4 AND AbEV1 NUCLEOTIDE SEQUENCES IN THE GLYCOSYLTRANSFERASE REGION

The alignment was constructed using the MegAlign package and displayed via GENEDOC program. The two sequences share 98% identity. Identities are highlighted in dark green, while dissimilarities are highlighted in purple (arrow). ARP250:
^{ARP250}14.4

```

      *           20           *           40
ARP250 : AAGAGAAAGGAGCATTACTTTATAATGATTTTGATTACAAGATT : 44
AbEV1  : AAGAGAAAGGAGCATTACTTTATAATGATTTTGATTACAAGATT : 44

      *           60           *           80
ARP250 : TTGAAACCAGACTTGGTTGTTGGAAGTTGTTTGACTGTACAGGG : 88
AbEV1  : TTGAAACCAGACTTGGTTGTTGGAAGTTGTTTGACTGTACAGGG : 88

      *           100          *           120          *
ARP250 : ACCAATAATCGCCAAAAAGTTCAATGCAAACCTACCTTGAATTGA : 132
AbEV1  : ACCAATAATCGCCAAAAAGTTCAATGCAAACCTACCTTGAATTGA : 132

      140          *           160          *
ARP250 : ATGCATTACCTTGAATTTAGTAAGAGCTGAAAATAAAAAGATTC : 176
AbEV1  : ATGCATTACCTTGAATTTAGTAAGAGCAGAAAATAAAAAGATTC : 176
      ↓
      180          *           200          *           220
ARP250 : GACTCACTTAAAAATGTATTGATATCGGGGTTACAGAGCAATGCA : 220
AbEV1  : GACTCACTTAAAAATGTATTATATATCGGGGTTACAGAGCAATGCA : 220
      ↓
      *           240           *
ARP250 : ACTAGAAACCATCATAAAACAATACGCCATTGCCGG : 256
AbEV1  : ACTAGAAACCATCATAAAGCAATACGCCATTGCCGG : 256

```

ANNEX 5.4

PAIRWISE ALIGNMENT OF ^{ARP250}14.4 AND AbEV1 AMINO ACID SEQUENCES IN THE GLYCOSYLTRANSFERASE REGION

The alignment was constructed using the MegAlign package and displayed via GENEDOC program. The two sequences share 100% identity. ARP250:
^{ARP250}14.4

```

      *           20           *           40
ARP250 : EKGALLYNDFDYKILKPDLVVGTCLTVQGPIIAKKFNANYLE : 42
AbEV1  : EKGALLYNDFDYKILKPDLVVGTCLTVQGPIIAKKFNANYLE : 42

      *           60           *           80
ARP250 : LNALPWNLVRAENKRFDSLKNVLYRGYRAMQLETIIKQYATA : 84
AbEV1  : LNALPWNLVRAENKRFDSLKNVLYRGYRAMQLETIIKQYATA : 84

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ANNEX 6.1

Relative quantification of the hairpin construct transcript using RT-qPCR. Relative quantification was obtained normalising the targeted insert (F1) of biological replicates with 18S rRNA. RNAi_F1U plasmid was used as reference for the relative quantification of transformants containing the RNAi_F1U construct, while the control transformant NC_1 (containing only the hyg^R cassette) was used as negative control. Transformants of *Agaricus* strain A15 were screened either before the dual-culture challenge with AbEV1 (transformant F1U_1, F1U_2, F1U_3, and F1U_4) or after (transformants F1U_2 x SSI61 and F1U_4 x SSI61). All transformants showed no detectable transcript of the hairpin cassette compared to the reference (RNAi_F1U plasmid). PCR efficiency was determined from the standard curve slope generated from serial dilutions. Reaction efficiency (RE) was calculated with the following formula $(-3.32/-\text{slope value}) \times 100$.

Sample	Quantity F1 (slope= -3.21) (RE ≈ 100%)	Quantity 18S (slope= -3.26) (RE ≈ 100%)	Normalised Target	Mean	Standard Error
RNAi_F1U plasmid	3975.458	3064.1685	1.297401889	0.730301392	0.567100497
RNAi_F1U plasmid	3702.5444	22687.035	0.163200894		
F1U_2 x SSI61	0.23970653	61868.906	3.87443E-06	4.01271E-06	1.38282E-07
F1U_2 x SSI61	0.23277473	56076.91	4.15099E-06		
F1U_4 x SSI61	2.3282213	66594.355	3.49612E-05	2.36429E-05	4,55665E-06
F1U_4 x SSI61	2.6878927	60985.153	4.40745E-05		
F1AU_1	0.19731024	61442.695	3.21129E-06	3.59487E-06	3.83584E-07
F1AU_1	0.27683145	69582.61	3.97846E-06		
F1AU_2	0.121332005	45970.215	2.63936E-06	3.61432E-06	9.74962E-07
F1AU_2	0.21122792	46026.324	4.58929E-06		
F1AU_3	0.51875126	33348.773	1.55553E-05	1.57292E-05	1.73822E-07
F1AU_3	0.6144053	38634.61	1.5903E-05		
F1AU_4	5.772473	66186.336	8.72155E-05	9.6185E-05	8.9695E-06
F1AU_4	6.2432904	59372.562	0.000105154		
NC_1	0	52205.668	0	0	0
NC_1	0	44686.324	0		

ANNEX 6.2

Relative quantification of AbEV1 using RT-qPCR. Relative quantification was obtained normalising the AbEV1 helicase transcript with 18S rRNA (see normalised target) in biological replicates (blue and purple) of transformants strain A15. The control transformant NC_1 was used as reference for the virus quantification in transformants (F1U_1, F1U_2, F1U_4, and F5D_1) after the *in vitro* dual-culture challenge with AbEV1 donor. AbEV1 RNA was also quantified in the AbEV1 donor (SSI 61) and in the strain A15 (negative control). PCR efficiency was determined from the standard curve slope generated from serial dilutions. Reaction efficiency (RE) was calculated with the following formula $(-3.32/-\text{slope value}) \times 100$. Each mean was normalised against the control transformant mean (transformant/NC_1; see normalised mean). Transformants F1U_1 and F5D_1 showed a silencing percentage close to the negative control (A15).

Sample	Quantity AbEV1 (slope= -3.19) (RE ≈ 100%)	Quantity 18S (slope= -3.14) (RE ≈ 100%)	Normalised Target	Mean	Standard Error	Normalised Mean (%)	Silencing Percentage
NC_1	186.68872	261.91797	0.71277553	0.736845274	0.024069744	100.00%	100.00%
NC_1	237.28867	311.84648	0.760915018				
F1U_1	0.11822892	165.46497	0.000714525	0.000446164	0.000268362	0.06%	99.94%
F1U_1	0.023192678	130.44127	0.000177802				
F1U_2	51.165348	222.92795	0.229515177	0.117997578	0.111517599	16.01%	83.99%
F1U_2	2.068887	319.27374	0.006479979				
F1U_4	14.252535	409.25293	0.034825737	0.136583087	0.10175735	18.54%	81.46%
F1U_4	52.682365	221.0383	0.238340437				
F5D_1	4.6608047	774.3264	0.006019173	0.003009587	0.003009587	0.41%	99.59%
F5D_1	0	449.90247	0				
SSI 61	138.73688	174.72444	0.794032478	0.781237744	0.012794733	106.02%	-6.02%
SSI 61	134.288002	174.753365	0.768443011				
A15	0.5482433	2025.7786	0.000270633	0.000151898	0.000118735	0.02%	99.98%
A15	0.022072103	665.5543	3.31635E-05				

ANNEX 6.3

NUCLEOTIDE SEQUENCE OF THE PHLEOMYCIN RESISTANCE CASSETTE

Nucleotide sequence generated by direct sequencing and resulting in 2060 bp sequence encompassing the phleomycin resistance cassette

FHJ53_consensus.seq : SEQUENCE		Position: 1	2.060kb																			
CCC	CNC	GCT	TCC	CGA	AGG	GAG	AAA	GGC	GGG	CAG	GTA	TCC	GGT	NAG	CGG	CAG	GGT	CGG	AAN	CAG	GAG	66
AGC	GCA	CGA	GGG	AGC	TTC	CAG	GGG	NAA	ACG	CCT	GGT	ATC	TTT	ATA	GTC	CTG	TCG	GGT	TTC	GCC	ACN	132
TCT	GAC	TTG	AGC	GTC	GAT	TTT	TGT	GAT	GCT	CGT	CAG	GGG	GGC	GGG	GCC	TAT	GGA	AAA	ACG	CCA	GCA	198
ACG	CGG	CCT	TTT	TAC	GGT	TCC	TGG	CCT	TTT	GCT	GGC	CTT	TTG	CTC	ACA	TGT	TCT	TTC	CTG	CGT	TAT	264
CCC	CTG	ATT	CTG	TGG	ATA	ACC	GTA	TTA	CCG	CCT	TTG	AGT	GAG	CTG	ATA	CCG	CTC	GCC	GCA	GCC	GAA	330
CGA	CCG	AGC	GCA	GCG	AGT	CAG	TGA	GCG	AGG	AAG	CGG	AAG	AGC	GCC	CAA	TAC	GCA	AAC	CGC	CTC	TCC	396
CCG	CGC	GTT	GGC	CGA	TTC	ATT	AAT	GCA	GCT	GGC	ACG	ACA	GGT	TTC	CCG	ACT	GGA	AAG	CGG	GCA	GTG	462
AGC	GCA	ACG	CAA	TTA	ATG	TGA	GTT	AGC	TCA	CTC	AAT	AGG	CAC	CCC	AGG	CTT	TAC	ACT	TTA	TGC	TTC	528
CGG	CTC	GTA	TGT	TGT	GTG	GAA	TTG	TGA	GCG	GAT	AAC	AAT	TTC	ACA	CAG	GAA	ACA	GCT	ATG	ACC	ATG	594
ATT	ACG	AAT	TCC	CGG	GGG	TCC	ACG	CGT	CTT	AAG	GCG	GCC	GCG	GTA	CCG	GGC	CCG	TCG	AGG	CTT	GCG	660
TCA	TTC	TGT	GTC	AGG	CTA	GCA	GTT	TAT	AAG	CGT	TGA	GGA	TCT	AGA	GCT	GCT	GTT	TCC	GCG	TCT	CGA	726
ATG	TTC	TCG	GTG	TTT	AGG	GGT	TAG	CAA	TCT	GAT	ATG	ATA	ATA	ATT	TGT	GAT	GAC	ATC	GAT	AGT	ACA	792
AAA	ACC	CCA	ATT	CCG	GTC	ACA	TCC	ACC	ATC	TCC	GTT	TTC	TCC	CAT	CTA	CAC	ACA	ACA	AGC	TTA	TCG	858
CCA	TGG	CCA	AGT	TGA	CCA	GTG	CCG	TTC	CGG	TGC	TCA	CCG	CGC	GCG	ACG	TCG	CCG	GAG	CGG	TCG	AGT	924
TCT	GGA	CCG	ACC	GGC	TCG	GGT	TCT	CCC	GGG	ACT	TCG	TGG	AGG	ACG	ACT	TCG	CCG	GTG	TGG	TCC	GGG	990
ACG	ACG	TGA	CCC	TGT	TCA	TCA	GCG	CGG	TCC	AGG	ACC	AGG	TGG	TGC	CGG	ACA	ACA	CCC	TGG	CCT	GGG	1056
TGT	GGG	TGC	GCG	GCC	TGG	ACG	AGC	TGT	ACG	CCG	AGT	GGT	CGG	AGG	TCG	TGT	CCA	CGA	ACT	TCC	GGG	1122
ACG	CCT	CCG	GGC	CGG	CCA	TGA	CCG	AGA	TCG	GCG	AGC	AGC	CGT	GGG	GGC	GGG	AGT	TCG	CCC	TGC	GCG	1188
ACC	CGG	CCG	GCA	ACT	GCG	TGC	ACT	TCG	TGG	CCG	AGG	AGC	AGG	ACT	GAC	CGA	CGC	CGA	CCG	ACA	CCG	1254
CCG	GTC	CGA	GCG	GGC	CCG	ACG	GGT	CCG	AGG	AAT	TCA	GCT	TAG	CCC	GAA	CTT	TGT	CAA	GCT	GAT	TGC	1320
TTG	GTA	CGA	TAA	CGA	ATG	GGG	ATA	CTC	GCG	CCG	TGT	TTG	CAA	CCT	CCT	CCA	ATA	CGT	TGC	AAA	GGA	1386
GGA	GCG	CAA	GGC	TGG	CAT	TTA	GAT	AGT	TGC	TTG	AAT	GCG	CCG	CTC	GTC	AAA	AAA	GAA	AAT	CGA	ACT	1452
TTT	TAT	AGT	GTA	ATG	GTA	TCA	AGT	TTA	GAA	TAT	GCG	CTG	TTC	TGT	GAT	TTC	ATT	TGT	GTT	TAG	AAG	1518
TGC	GTT	AAG	GGA	TGA	TAT	ATT	GAT	ACA	TTG	ATG	GAT	GTC	AGA	AAT	GCA	TGA	ATC	AAC	GTG	CTT	TTT	1584
TGA	ATG	GCG	TGA	AGT	TTT	CCA	GTA	CCC	GGG	ACC	TGG	CCG	CGG	GCA	TGG	ACA	TTG	GAC	TCG	GAG	ATC	1650
CGA	CAG	AAA	GTC	ACG	GGG	GTG	TTG	TGT	GTA	GTC	ACG	TGG	GCA	ACG	CGA	GCG	ACG	CGG	TTG	ACG	GGC	1716
GTC	CAT	TTT	TGA	CTT	TCT	TGT	TCT	CAT	CAC	ACT	CCA	ACC	ATC	TTC	TTC	CAT	CGC	CGT	TGC	CTC	CCC	1782
TAT	TTT	ACC	CGT	CTT	TTT	CTG	GCA	GAC	TTT	GAA	CTA	TTT	GAA	CCA	GGA	TGT	GGG	CAA	AGT	TCA	GTT	1848
CCG	CAT	TGA	AAT	CGA	CAA	CGA	CGA	GGC	CTC	AAG	AGC	CTG	AAG	AGA	CAA	GAT	CCA	CAA	CAC	CGG	TGC	1914
ATT	TCG	GTC	CGT	GCG	GGG	CTG	AAA	CAC	GCG	ANA	GTA	TCG	GGC	GTA	GCC	AGG	CAG	AAG	TTA	CCG	CTC	1980
ACT	TAC	TGG	GAG	AAA	GTA	GTT	CTG	ACT	CGA	CTG	CTT	TCG	GCG	TTC	CGA	GTA	CTT	CAC	ACA	TCT	CAC	2046
CAC	TAC	CAC	ACC	CC																		2060