RNA viruses of *Sphaeropsis sapinea* and *Diaporthe ambigua* and their possible use as biological control agents

by

Ntsane Moleleki

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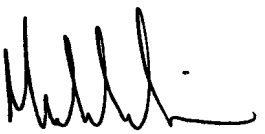
Supervisor:	**Prof. B. D. Wingfield**
Co-Supervisors:	**Prof. M. J. Wingfield**
	Dr O. Preisig

Declaration

I, the undersigned, declare that no portion of the work referred to in this thesis submitted for the degree Philosophiae Doctor to the University of Pretoria, has been submitted in support of an application for another degree or qualification of this or any other university or institution of learning.



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Preface

The forestry industry in South Africa and the Southern hemisphere in general is based primarily on the exotic *Eucalyptus*, *Pinus, Populus* and *Acacia* tree species. At present, *Eucalyptus*, *Pinus* and *Acacia* plantations cover about 1.5 million hectares of land in South Africa. The land suitable for establishing plantations is limited to less than 2 million hectares. Ecological concerns such as water preservation and conservation also limit the amount of land that can be allocated to forest plantations.

The production of wood is not only limited by land and ecological constraints but also by losses due to diseases. Fungal diseases cause losses that amount to millions of rands due to reduced wood quality, loss of volume and losses due to tree mortality. Meanwhile, there is an ever increasing demand for fiber and solid wood for making pulp and paper. While the constraints due to land and water requirements cannot be resolved, the impact of diseases can be alleviated by instituting viable disease management regimes.

Sphaeropsis sapinea and *Diaporthe ambigua* are serious pathogens in pine forest plantations and fruit orchards in South Africa, respectively. *S. sapinea* is not a serious pathogen on pines growing on their native range and growing under natural conditions. However, the fungus causes dramatic losses in exotic plantations in South Africa. *D. ambigua* is a serious orchard pathogen that causes diseases on pome and stone fruit trees.

At present diseases are controlled by chemical, cultural practices, and selection of disease-tolerant clones. There are many disadvantages associated with the use of chemical compounds to control fungal diseases. Some of the disadvantages include the fact that fungi like many other microorganisms have developed resistance to fungicides. This situation allows for the re-emergence of previously easily managed diseases. While selection of disease-tolerant clones and hybrids presents many advantages, there are some disadvantages. Selection of disease-tolerant trees is time consuming. Since disease tolerance is normally done using the most virulent strain of the pathogen, new strains of

the pathogens arise and overcome the tolerance thus leading to re-emergence of the disease.

The biological control of fungal diseases through hypovirulence represents another approach to disease control and management. Hypovirulence is mediated by mycoviruses that naturally occur in many species of fungi. The occurrence of a dsRNA mycovirus in the chestnut blight fungus, *Cryphonectria parasitica*, is responsible for the reduction of the impact of chestnut blight in Europe. The North American chestnut has virtually been wiped out from the American landscape by this pathogen. The natural transfer of the virus between strains of *C. parasitica* is controlled by vegetative compatibility groups (VCGs). The relatively small number of VCGs in the European population of *C. parasitica* has facilitated the rapid transfer of the virus within the population of the fungus. The American population of the fungus has a high VCG diversity and as such there has not been a transfer of the virus within the population of the fungus. The exploitation of mycoviruses for biological control is a promising method and a milestones to be achieved in disease control.

This thesis has been divided into two chapters. Chapter 1 is a literature review. The first part of this chapter deals with *S. sapinea* and *D. ambigua*. The impact of these two fungi in pine plantations and orchards is described. The second part of the review deals with biological control of fungal diseases through hypovirulence. Many fungi have been reported to be infected by dsRNAs. This review does not deal with all the literature dealing with fungal infections by mycoviruses but rather, it focuses on those systems that have shed some light toward a more complete understanding of the role that mycoviruses play in hypovirulence of their hosts. The molecular basis of fungal pathogenesis based on the hypovirus/*C. parasitica* model is reviewed.

Chapter 2 describes the cloning of the full-length cDNA copies of the genomes of *Sphaeropsis sapinea RNA virus 1* (SsRV1), *Sphaeropsis sapinea RNA virus 2* (SsRV2) and *Diaporthe ambigua RNA virus* (DaRV). In this chapter, the *in vitro* synthesis of strand-specific RNA using either T7 RNA polymerase or SP6 RNA polymerase is

described. Since there is no standard procedure for the production of fungal spheroplasts, a detailed explanation of the procedure to produce spheroplasts from *S. sapinea*, *Phomopsis* sp. and *D. ambigua* is described. This chapter further describes attempts to transfect spheroplasts of *S. sapinea*, *Phomopsis* sp. and *D. ambigua* with the *in vitro*-produced single-stranded positive strand RNA from SsRV1, SsRV2 and DaRV. The impact of the successfully transfected virus on its hosts is then discussed.

In order to simplify the reading of the multi-step clonings of the full-length cDNA copies of the genomes of the three mycoviruses, a flow diagram of the steps involved in cloning DaRV followed by *in vitro* transcription of RNA from the cloned cDNA and transfection of spheroplasts is presented below (Fig. 1). A similar procedure was followed to clone H6DaRV, SsRV1 and SsRV2. The cirles represent the plasmid used to clone the viral cDNAs (thick lines).

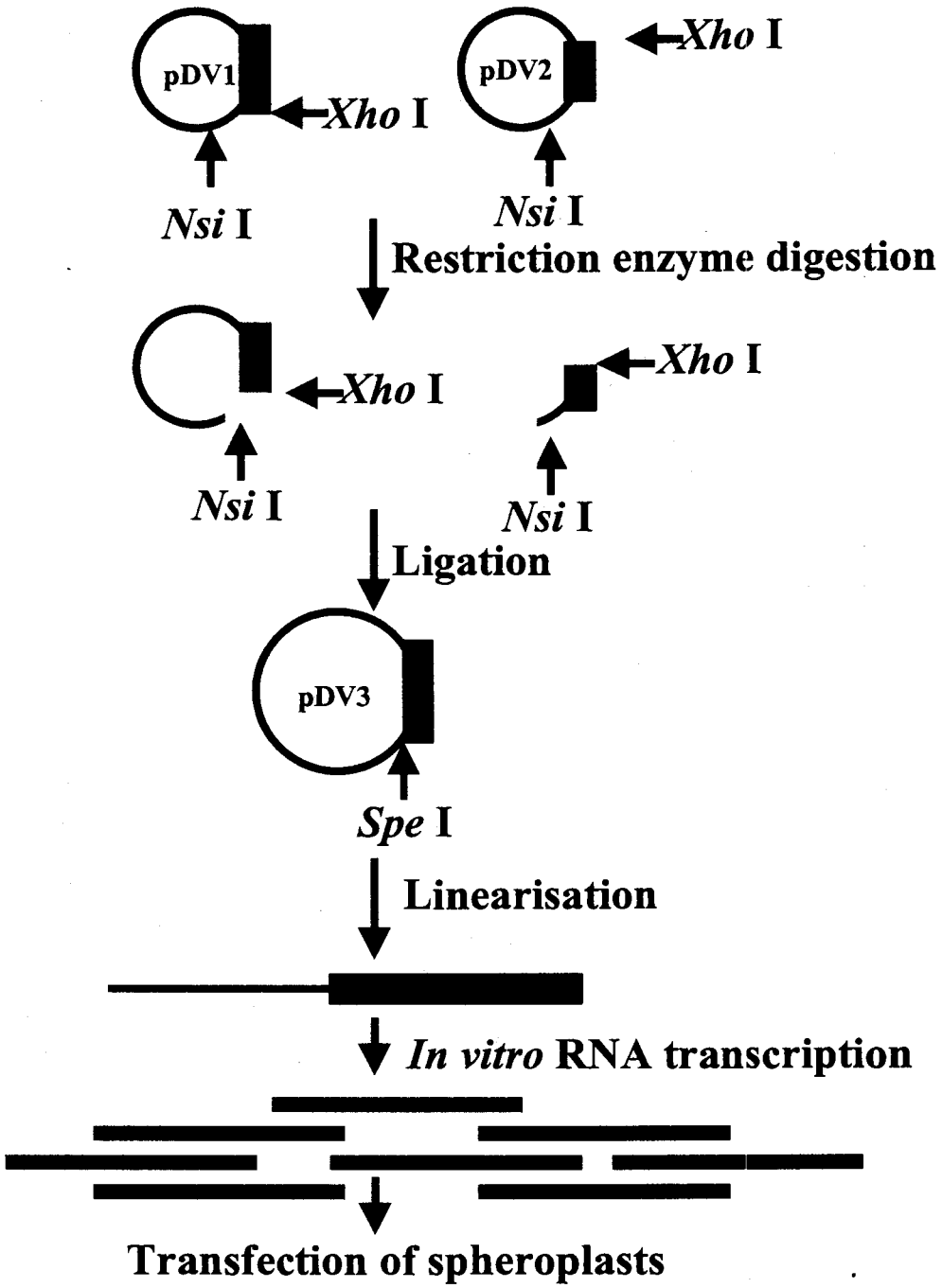

Fig. 1. A schematic drawing of the steps followed in cloning the full-length cDNA copy of the genome of DaRV. The restriction enzymes *Nsi* I and *Xho* I serve only as examples as different enzymes were used for different viruses.

Chapter 1

1. Introduction

1.1. *Sphaeropsis sapinea* and *Diaporthe ambigua*: Important pathogens in plantations and orchards

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton is an important pathogen of many species of *Pinus*. Diseases associated with *S. sapinea* have been reported in exotic pine plantations in many parts of the world including New Zealand, Australia, South Africa and the United States of America (Da Costa, 1955; Lückhoff, 1964; Buchanan, 1967; Punithalingam and Waterson, 1970, Currie and Toes, 1978; Gibson, 1979; Zwolinski *et al.*, 1994; Stanosz *et al.*, 1996). Although this fungus occurs worldwide, its most devastating effects have been reported in South Africa where it causes extensive infection and mortality in *Pinus radiata* D. Don and *P. patula* Schlechtend. & Cham. after hail injury (Laughton, 1937; Swart *et al.*, 1987b and 1988; Zwolinski *et al.*, 1990a,b; Swart and Wingfield, 1991a,b; Zwolinski *et al.*, 1994; Smith *et al.*, 1996).

In addition to shoot blight, cankers and tip die-back, other disease symptoms associated with *S. sapinea* include damping-off and collar rot of seedlings (Punithalingam and Waterson, 1970; Swart *et al.*, 1985 and 1987a,b; Stanosz and Carlson, 1996), sap stain (Laughton, 1937; Da Costa, 1955; Eldridge, 1961), root disease (Wingfield and Knox-Davies, 1980) as well as whorl cankers and crown wilt (Chou, 1984). Some of these symptoms are either unique to some parts of the world or occur only rarely elsewhere. For example, root disease of *Pinus elliottii* Engelm. and *P. taeda* L. has been reported only from South Africa (Wingfield and Knox-Davies, 1980; Swart *et al.*, 1987a,b) whereas collar rot of seedlings occurs in north-central USA (Palmer and Nicholls, 1985; Palmer *et al.*, 1988). Die-back resulting from hail damage occurs mainly in South Africa (Zwolinski *et al.*, 1990a,b; Swart and Wingfield, 1991a,b; Zwolinski *et al.*, 1994), with far fewer instances having been reported from Australia (Brown *et al.*, 1981; Eldridge, 1957).

S. sapinea occurs as a symptomless endophyte in healthy cones of *P. radiata* and *P. patula* (Smith *et al.*, 1996; Stanosz *et al.*, 1997). It has recently been argued that the application of the term endophytism to describe the association of *S. sapinea* and *Pinus* spp. obscures the intimate nature of the association of this fungus and its host. It has, therefore, been proposed that the term latency is a more appropriate term to describe the asymptomatic persistence of virulent strains of *S. sapinea* within pine tissues (Stanosz *et al.*, 1997).

Latency has been described as an appropriate designation for the *S. sapinea-Pinus* spp. association since it involves long periods of intimate association between the fungus and the tree without any incidence of disease. It has been proposed that latency is induced by the host until such time that the host's physiology shifts in a manner that permits the development and ultimate induction of disease (Stanosz *et al.*, 1997). Sources of physiological stress such as hail damage, droughts and wounding by pruning and by cambiophagus insects trigger the onset of disease symptoms (Haddow and Newman, 1942; Chou and Mackenzie, 1988; Zwolinski *et al.*, 1995; Stanosz and Carlson, 1996; Blodgett *et al.*, 1997a,b). In cases where stress is manifested as wounds, such as is the case after hail damage and insect infestation, the appearance of disease symptoms is a result of the stress itself and not due to new infection through the wounds. This view suggests that infection is caused by the fungus already existing within the living tissues of the trees (Smith *et al.*, 1996).

Diaporthe ambigua Nitschke is a pathogen of emerging importance in *Malus domestica* Borkh., *Pyrus communis* L. and *Prunus salicina* Lindl. in South African fruit orchards and those elsewhere in the world (Harris, 1988; Smit *et al.*, 1996a,b; 1997 and 1998). The disease caused by *D. ambigua* is not only restricted to orchards. The fungus also attacks soybean and rooibos tea, *Aspalathus linearis* (Burm. f.) R. Dahlg., a perennial leguminous woody plant used in making herbal tea in South Africa (Smit and Knox-Davies, 1989a,b; Fernández and Hanlin, 1996; Zhang *et al.*, 1997). In fact, the importance of *D. ambigua* as a plant pathogen in South Africa was first realised when this fungus destroyed an entire plantation of *A. linearis* in the Western Cape province in 1977 (Smit and Knox-Davies, 1989a,b). Smit *et al.* (1996a and 1998) later suggested that the *Diaporthe* strains responsible for the destruction of

A. linearis plantations were the same as those infecting the rootstocks of pome and stone fruits.

D. ambigua infection is associated with sunken, pointed lesions with marginal longitudinal cracks on affected trees. The disease can cause yellowing of foliage with or without wilting (Harris, 1988; Smit *et al.*, 1996a,b). *D. ambigua* readily kills nursery rootstocks of infected plant material, but it only kills mature rootstocks over a long period of time (Smit *et al.*, 1996b). The damage by this fungus varies from death of some parts of the tree to death of the whole tree (Harris, 1988). Diaporthe canker in South Africa is potentially serious since the causal agent is found on indigenous hosts that could serve as inoculum to apple, pear and plum (Smit *et al.*, 1998).

1.2. Literature Review: Hypovirulence induced by RNA viruses in plant pathogenic fungi with special reference to *S. sapinea* and *D. ambigua*

1.2.1. Disease control through hypovirulence

Biological control of plant pathogenic fungi through hypovirulence-mediating mycoviruses is increasingly being viewed as a possible disease management strategy (Nuss and Koltin, 1990; Enebak *et al.*, 1994; Smart and Fulbright, 1996; Nuss, 2000). The exploitation of mycoviruses for biological control of plant pathogenic fungi is a promising strategy and one of the remaining great challenges in plant pathology (Smart and Fulbright, 1996). Hypovirulence is mediated by non infectious dsRNA viruses that occur as virus-like particles in the cytoplasm of hosts (Ghabrial and Havens, 1992; Preisig *et al.*, 1998). In some cases, the dsRNA genome is not encapsidated in a virus-like particle but exists in host-derived vesicles (Hansen *et al.*, 1985; Fahima *et al.*, 1993; Preisig *et al.*, 2000). Mycoviruses are widespread in fungi and their effects on their hosts range from asymptomatic to severely debilitating diseases (Ghabrial, 1994 and 1998; Huang and Ghabrial, 1996).

1.2.2. Mycoviruses in plant pathogenic fungi

The presence of dsRNA viruses has been reported in many fungi. Some examples include *Cryphonectria parasitica* (Murrill) Barr (Day *et al.*, 1977; Anagnostakis, 1982a), *Helminthosporium victoriae* Meehan *et* Murphy (Lindberg, 1960; Sanderlin and Ghabrial, 1978), *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton (Preisig *et al.*, 1998; Steenkamp *et al.*, 1998), *Diaporthe ambigua* Nitschke (Smit *et al.*, 1996b), *Phytophthora infestans* (Montagne) de Bary (Tooley *et al.*, 1989), *Leucostoma persoonii* (Nits.) Höhn (Hammar *et al.*, 1989), *Ophiostoma ulmi* Brasier (Rogers *et al.*, 1986), *Rhizoctonia solani* Kühn (Castanho and Butler,1978; Castanho *et al.*, 1978) and many other fungi. The aim of this document is not to review all the literature dealing with dsRNA-harbouring fungi, but rather to examine those systems and studies, which might lead to a more complete understanding of the role that

mycoviruses play in the pathogenicity of fungi. The emphasis is on mycoviruses in filamentous fungi that are promising candidates for biological control of plant pathogenic fungi.

1.2.3. Hypovirulence: A historical perspective

The understanding of hypovirulence and its discovery early in the twentieth century requires a brief history of chestnut blight caused by *C. parasitica*, a disease that has virtually destroyed both European (*Castanea sativa* Mill.) and American (*C. dentata* (Marsh) Borkh.) chestnut (Anagnostakis, 1982a,b and 1987; Anagnostakis and Kranz, 1987). The disease caused by *C. parasitica* is known as chestnut blight and is a classic example of a plant disease epidemic resulting from an introduced fungus (Day *et al.*, 1977; Anagnostakis, 1982a,b and 1987; Fulbright *et al.*, 1983; MacDonald and Fulbright, 1991). It is a subject of keen interest to plant pathologists and the general population at large (Nuss, 1992). The chestnut blight epidemic is perhaps one of the best known plant diseases and this is evidenced by extensive treatment in widely read periodicals such as **American Scientist** (Burnham, 1988), **National Geographic** (Cochran, 1990) and **Scientific American** (Newhouse, 1990).

Chestnut blight was first observed in North America in 1905 (Merkel, 1905). However, in terms of this review, its discovery in Europe is of greater importance because it was in Europe that hypovirulence was discovered (Pavari, 1949; Biraghi, 1953; Nuss, 1992). Chestnut blight was discovered in Europe in Genoa, Italy in 1938 (Pavari, 1949). In this region, it was found that some chestnut trees were beginning to show signs of recovery even though they were severely blighted (Biraghi, 1953). A tree that is severely affected by chestnut blight has sunken cankers that result from necrosis and collapse of bark tissue. Stems and branches may become completely girdled by cankers. Girdling of stems and branches disrupt nutrient movement through the phloem and limits the capacity of the tree to generate new tissue. This results in wilting and death of the distal parts of the tree (Pavari, 1949; Biraghi, 1953; Anagnostakis, 1982a; Hebard *et al.*, 1984; Nuss, 1992).

Grente (1965) suggested the term hypovirulence to describe the reduced virulence in some isolates of *C. parasitica*. Grente and co-workers isolated two distinct forms of *C. parasitica* from the bark of the recovering Italian chestnut trees. While one form of isolates produced an orange pigmentation and displayed vigorous sporulation, the others were characterised by white mycelium and suppressed sporulation (Grente, 1965; Grente and Sauret, 1969; Grente and Berthelay-Sauret, 1978). It was further demonstrated that the inoculation of the Italian hypovirulent fungus on the cankers of the blighted French chestnut trees had a curative effect on the French chestnut trees (Grente and Sauret, 1969).

The discovery of the first American hypovirulent strain of *C. parasitica* was serendipitous. In her review on the biological control of chestnut blight in America, Anagnostakis (1982a) reported that:

> "in 1976, we heard from a woman in Michigan who had read a newspaper article about our work. She had been skiing on a golf course when she saw, in a small forest-island, blighted chestnut trees that looked as if they were healing. The trees were hardly beautiful, but they were surviving a massive infection. She sent us leaves that proved that the trees were American chestnuts, and pieces of bark from the gnarled and twisted trunks."

The Michigan hypovirulent strains of *C. parasitica* were distinct from the European strains. These hypovirulent American fungal strains of *C. parasitica* had the same curative effect on blighted chestnut trees as the European hypovirulent strains (Day *et al.*, 1977). Further occurrences of natural hypovirulence were later reported from other parts of the United States (Dodds, 1980a,b; Jaynes and Elliston, 1980).

Most of the European field isolates of *C. parasitica* are infected with the hypoviruses while only a small fraction of the North American isolates have been diagnosed with hypovirus infections (Anagnostakis *et al.*, 1986; Heiniger and Rigling, 1994). In nature, mycoviruses are transferred only among isolates that belong to the same or very closely related vegetative compatibility groups (VCGs) (Anagnostakis and Waggoner, 1981; Anagnostakis, 1983; Liu and Milgroom, 1996; Cortesi *et al.*, 1996 and 1997; Cortesi and Milgroom, 1998). This means that hypovirulence is conferred

on virulent strains only after the exchange of cytoplasm between the hypovirulent and the virulent isolates (Anagnostakis and Day, 1979; Kuhlman and Bhattacharyya, 1984).

It is known that there is a lower VCG diversity in European population of *C. parasitica* while the North American population has a high VCG diversity (Anagnostakis *et al.*, 1986; MacDonald and Fulbright, 1991). The low VCG diversity in the European population is responsible for the containment of chestnut blight in Europe. In contrast, the high VCG diversity is responsible for the epidemic in North America (Anagnostakis, 1977; Anagnostakis *et al.*, 1986; Milgroom, 1995; Liu and Milgroom, 1996). In some cases, vegetative incompatibility can be overcome so that the virus is transmitted across two strains that differ in one or more mycelial incompatibility genes (Anagnostakis and Waggoner, 1981; Kuhlman *et al.*, 1984; Melzer *et al.*, 1997).

1.2.4. Classification of mycoviruses

All mycoviruses that infect filamentous fungi reported thus far are RNA viruses. They have been classified into families *Totiviridae* (Diamond *et al.*, 1989; Icho and Wickner, 1989; Huang and Ghabrial, 1996; Park *et al.*, 1996; Preisig *et al.*, 1998), *Partitiviridae* (Ghabrial *et al.*, 1995), *Hypoviridae* (Shapira *et al.*, 1991; Hillman *et al.*, 2000) and *Narnaviridae* (Randles *et al.*, 2000; Wickner *et al.*, 2000a). With the exception of the family *Hypoviridae* and *Narnaviridae*, mycoviruses are associated with virions that exhibit isometric symmetry and are 30-40 nm in diameter (Ghabrial *et al.*, 1995; Ghabrial, 1998; Wickner *et al.*, 2000b). A list of all sequenced mycoviruses is given in Table I.

1.2.4.1. *Totiviridae*

The family *Totiviridae* includes the genera: *Totivirus*, *Giardiavirus* and *Leishmaniavirus*. The most studied members of this family are the *Saccharomyces cerevisiae virus L-A* (ScV-L-A), which is also the type species of this group and

Saccharomyces cerevisiae virus LBC (ScV-L-BC) [reviewed in Wickner (1992) and Ghabrial (1994)] (Wickner *et al.*, 2000b). The genus *Totivirus* exclusively infects fungi while *Giardiavirus* is associated with the protozoan *Giardia lamblia*, the causal agent of the waterborne enteric disease called giardiasis (Wang *et al.*, 1993). *Leishmaniavirus* is associated with the protozoa belonging to *Leishmania sp.*, the causal agents of leishmaniasis (Stuart *et al.*, 1992; Scheffter *et al.*, 1995a,b).

Totiviruses have monopartite dsRNA genomes that range from 4.6 to 6.7 kilobase pairs in size (Diamond *et al.*, 1989; Icho and Wickner, 1989; Stuart *et al.*, 1992; Bruenn, 1993; Wang *et al.*, 1993; Scheffter *et al.*, 1995a,b; Tai and Ip, 1995; Huang and Ghabrial, 1996; Park *et al.*, 1996; Preisig *et al.*, 1998). The dsRNA genome which has two open reading frames (ORFs), ORF A and ORF B, is replicated in a conservative fashion by a virion-associated RNA-dependent RNA polymerase (RDRP) end-to-end (Jacks *et al.*; 1988; Dinman *et al.*, 1991; Dinman and Wickner, 1992; Dinman, 1995; Wickner, 1996) (Fig. 2). The product of ORFB, the RDRP is expressed as a fusion protein with the product of ORFA, the coat protein by ribosomal frameshifting (Ghabrial, 1994; Ghabrial *et al.*, 1995). The exception to the rule is *Helminthosporium victoriae 190S virus* (Hv190SV), which expresses its RDRP as a separate non-fused protein by internal initiation (Huang and Ghabrial, 1996; Huang *et al.*, 1997; Soldevila and Ghabrial, 2000, Soldevila *et al.*, 2000). The dsRNA viruses of *S. sapinea*, SsRV1 and SsRV2, are also thought to translate their genomes into non-fused CP and RDRP (Preisig *et al.*, 1998).

1.2.4.2. *Partitiviridae*

The family *Partitiviridae* encompasses four genera: *Partitivirus*, *Chrysovirus*, *Alphacryptovirus* and *Betacryptovirus* (Ghabrial *et al.*, 1995; Ghabrial, 1998; Ghabrial *et al.*, 2000). *Partitivirus* and *Chrysovirus* exclusively infect fungi whereas *Alphacryptovirus* and *Betacryptovirus* are plant viruses (Ghabrial, 1998). The *Gaeumannomyces graminis virus 019/6-A* (GgV-019/6-A) and *Penicillium chrysogenum virus* (PcV) are the type species of genera *Partitivirus* and *Chrysovirus*, respectively (Ghabrial *et al.*, 2000). *Alphacryptovirus* and *Betacryptovirus* were formally classified in the family *Cryptoviridae* but the members of this family were

later included into the family *Partitiviridae* (Ghabrial, 1998). Partitiviruses differ from totiviruses in that they have bipartite genomes. The coat protein (CP) gene and the RDRP gene are carried on separate dsRNA segments. The two are expressed as separate proteins (Ghabrial *et al.*, 1995; Oh and Hillman, 1995). Unlike the totivruses, partititviruses are known to replicate their genomes in a semi-conservative fashion (Ghabrial *et al.*, 2000).

1.2.4.3. *Hypoviridae*

The family *Hypoviridae* encompasses only a single genus, the genus Hypovirus (Hillman *et al.*, 1994 and 1995; Hillman *et al.*, 2000). The hypovirus dsRNA genome contains two ORFs, ORFA and ORFB (Shapira *et al.*, 1991). There are no virion-associated particles in this family as the hypovirus does not encode a coat protein. The dsRNA genome is enclosed into host-derived vesicles (Hansen *et al.*, 1985; Fahima *et al.*, 1993; Hillman *et al.*, 1995).

The type species of the family *Hypoviridae*, the *Cryphonectria hypovirus 1-713* (CHV1-713), is the most studied mycovirus of a filamentous fungus to date. It is polyadenylated at the 3' end with a poly(A) tail of 20-24 residues (Shapira *et al.*, 1991). The 5' end is blocked by an unknown chemical group. The 5' end has a leader of some 495 bp within which there are several AUG codons that precede the AUG codon that serves as a start codon for ORFA (Rae *et al.*, 1989; Shapira *et al.*, 1991). The translation of ORFA is terminated at a **UAA**UG sequence in all hypoviruses examined. The AUG of UAAUG acts as the start codon for ORFB (Shapira *et al.*, 1991). While the product of ORFA may be autocatalytically cleaved depending on the virus, the N-terminus product from ORFB is always autocatalytically cleaved from the growing polypeptide chain (Choi *et al.*, 1991a,b; Craven *et al.*, 1993). This cleavage product is a papain-like cysteine protease (Choi *et al.*, 1991a,b; Shapira and Nuss, 1991; Craven *et al.*, 1993; Hillman *et al.*, 1995; Suzuki *et al.*, 1999).

1.2.4.4. *Narnaviridae*

The family *Narnaviridae* includes the genera: *Narnavirus* and *Mitovirus* (Wickner *et al.*, 2000a). The genus *Narnavirus* contains the *Saccharomyces cerevisiae 20S RNA narnavirus* (ScNV-20S) and *Saccharomyces cerevisiae 23S RNA narnavirus* (ScNV-23S) (Wickner *et al.*, 2000a) while the genus *Mitovirus* contains viruses that infect mitochondria of filamentous fungi (Polashock and Hillman, 1994; Hong *et al.*, 1998a,b, Hong *et al.*, 1999; Cole *et al.*, 2000). The type species of this genus is the *Cryphonectria parasitica mitovirus 1-NB631* (Polashock *et al.*, 1997; Wickner *et al.*, 2000a). Most of the characterised mitoviruses occur in *Ophiostoma novo-ulmi*, the well known causal agent of Dutch elm disease for which there is no control (Brasier, 1983; Brasier, 1986; Brasier, 1991).

A set of 12 dsRNA elements ranging in size from 0.33–3.5 kb have been discovered in a diseased isolate of *Ophiostoma novo-ulmi* (Cole *et al.*, 1998; Hong *et al.*, 1998a,b; Hong *et al.*, 1999; Cole *et al.*, 2000). This isolate is characterised by slow growth, amoeboid-like colonies, reduced sporulation and reduced mitochondrial cytochrome oxidase level (Brasier, 1983; Rogers *et al.*, 1987). Four of these 12 dsRNA elements (RNAs 3a, 4, 5, and 6) encode genomes of mitoviruses that replicate independently of each other (Hong *et al.*, 1999). On the other hand, RNAs 4, 7 and 10 are still unclassified. It has been suggested that RNAs 7 and 10 are defective RNAs that rely on RNA 4 for replication (Hong *et al.*, 1998b, Hong *et al.*, 1999).

1.2.4.5. Unassigned mycoviruses: *Agaricus bisporus* La France isometric virus

A disease of cultivated mushroom (*Agaricus bisporus*), has been diagnosed with 34-36 nm isometric virus-like particles which encapsidate dsRNAs ranging in size from 0.8 to 3.8 kb. There are about 9 disease-specific dsRNAs which may be packaged singly or in combinations (Gooding *et al.*, 1997; Ghabrial, 1998). The viral particles are believed to cause La France disease, a factor that limits the cultivation of mushrooms, worldwide (Ghabrial, 1994 and 1998; Gooding *et al.*, 1997). Analysis of the sequenced RDRP genes of five of the RNA segments reveal a closer affinity to the

genus *Partitivirus*. This close affinity to *Partitivirus* and the segmented nature of the genome of La France isometric virus has led to the conclusion that this genus originated from partitiviruses. This would have occurred by acquisition of additional genes through reassortment or recombination (Ghabrial, 1998).

1.2.5. Mechanisms of pathogenicity of dsRNA viruses

The mechanisms through which dsRNA viruses mediate the hypovirulence-associated traits are not fully understood. Some of the well known hypovirulence-associated traits include reduced virulence, diminished oxalate accumulation (Havir and Anagnostakis, 1983; Rigling *et al.*, 1989; Bennett and Hindal, 1990; Hillman *et al.*, 1990); reduced gallic and tannic acid oxidation (Bavendamm, 1928a,b), reduced laccase activity (Rigling *et al.*, 1989; Rigling and Van Alfen, 1991; Choi and Nuss, 1992a,b; Larson *et al.*, 1992), suppressed sporulation, altered colony morphology and reduced pigmentation (Elliston, 1985a,b; Rigling and Van Alfen, 1991). Existing evidence shows that these hypovirulence-mediated traits are not a result of non-specific response of the fungus to the physical presence of a replicating virus but rather, is a result of an interaction at gene level between the fungus and the virus (Choi and Nuss, 1992a).

The underlying mechanisms by which mycoviruses attenuate virulence in their hosts have been the subject of studies that have been done on the fungus-encoded cell wall-degrading enzymes (McCarrol and Thor, 1985; Varley *et al.*, 1992; Choi *et al.*, 1993; Gao and Shain, 1994 and 1995; Wang and Nuss, 1995; Gao *et al.*, 1996;). The expression of fungal-encoded enzymes which degrade cell walls or parts thereof have been studied in both hypovirus-infected and hypovirus-free isogenic strains of *C. parasitica*. These cell wall-hydrolysing enzymes include cellobiohydrolase (Wang and Nuss, 1995), cellulases (McCarrol and Thor, 1985), cutinases (Varley *et al.*, 1992), laccases (Powell and Van Alfen, 1987a,b; Rigling *et al.*, 1989; Hillman *et al.*, 1990), polygalacturonases (Gao and Shain, 1994 and 1995; Gao *et al.*, 1996) and the rennin-like protease endothiapepsin (Whitaker, 1970; Choi *et al.*, 1993). These studies show that the hypovirulent strains are unable to produce some or all of the cell wall-degrading enzymes. In addition, mycoviruses have been shown to suppress the

expression of pheromone precursor genes thus resulting in female sterility (Zhang *et al.*, 1993; 1994 and 1998).

Eukaryotes are known to sense and respond to external stimuli by using transmembrane signalling mechanisms. The best studied signal transduction pathway is mediated by GTP-binding proteins which perceive changes in the environment of the cells by activating a cascade of reactions that lead to changes in gene expression (Gilman, 1987). The phenotypic characteristics that are observed in hypovirus infected *C. parasitica* have been postulated to result from perturbation of signal transduction in the host by the hypovirus (Larson *et al.*, 1992; Choi *et al.*, 1995; Wang and Nuss, 1995; Chen *et al.*, 1996; Nuss, 1996; Gao and Nuss, 1996).

In an attempt to demonstrate that *C. parasitica* hypovirus perturbs signal transduction in its host, Choi *et al.* (1995) cloned two G-protein α-subunit genes, *cpg-1* and *cpg-2*. The authors showed that the hypovirus-infected fungus has a significant reduction in the accumulation of the *cpg-1* gene product, a G-protein α subunit named CPG-1. The same effect was observed when a sense *cpg-1* transgene was transformed into a virus-free strain of *C. parasitica*. This phenomenon was named transgenic co-suppression of CPG-1 accumulation. In a follow up study, hypovirus and transgenic co-suppression of accumulation of CPG-1 were shown to repress the cellulose-dependent induction of cellulases in *C. parasitica* (Wang and Nuss, 1995). The hypovirus and transgenic co-suppression of CPG-1 accumulation were also demonstrated to affect the cyclic AMP (cAMP)- and Ca^{2+}- dependent signalling pathways (Larson *et al.*, 1992; Gao and Nuss, 1996). Hypovirus infection and *C. parasitica* mutants with the disrupted *cpg-1* gene were found to have elevated levels of cAMP (Gao and Nuss, 1996). Other components involved in G-protein signalling pathways were also studied. The gene, *cpgb-1*, which codes for the β-subunit of G-protein was cloned and disrupted. *C. parasitica cpgb-1* mutants showed reduced mycelial pigmentation (Kasahara and Nuss, 1997).

1.2.6. Occurrence of mycoviruses in *S. sapinea* and *D. ambigua*

1.2.6.1. Occurrence of dsRNA in *S. sapinea*

The occurrence of dsRNA viruses in *S. sapinea* was first reported in 1989 from the North American isolates of the fungus (Wu *et al.*,1989). Steenkamp *et al.* (1995) produced the first report of dsRNAs in South African isolates of the fungus. This initial study suggested an association of dsRNA in *S. sapinea* with hypovirulence. Further studies showed that the presence of dsRNA viruses in *S. sapinea* is not associated with any observable hypovirulence (Steenkamp *et al.*, 1998).

The virus identified by Steenkamp *et al.* (1995 and 1998) has recently been further characterised. Two distinct dsRNA viruses belonging to the family *Totiviridae* were identified (Preisig *et al.*, 1998). This was the first report of co-infection of a filamentous fungus by two distinct dsRNA totiviruses. The dsRNA viruses, *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2) have similar-sized genomes of 5163 and 5202 bp, respectively. Each virus has two large overlapping open reading frames (ORFs). The ORF1 on the 5' half of the dsRNA genome encodes a coat protein while the 3' ORF2 gene product has the typical features of an RNA dependent RNA polymerase (RDRP). SsRV1 and SsRV2 are characterised by a GC content of 62% and 63%, respectively. The two viruses, SsRV1 and SsRV2 are closely related to *Helminthosporium victoriae 190S virus* (Hv190SV). Comparisons based on amino acid sequences further revealed that SsRV1 is more closely related to Hv190SV than to SsRV2 (Preisig *et al.*, 1998).

1.2.6.2. Occurrence of dsRNA in *D. ambigua*

Different isolates of *D. ambigua* display varying degrees of pathogenicity (Smit *et al.*, 1996b). This observation prompted the search for dsRNAs in these isolates. The dsRNA-harbouring hypovirulent strains display a number of hypovirulence-associated traits such as reduced gallic and tannic acid oxidation, reduced laccase activity, diminished oxalate accumulation, reduced virulence and suppressed sporulation.

Furthermore, the hypovirulent strains are able to convert compatible, virus-free strains of the same vegetative compatibility group (VCG) to hypovirulence (Smit *et al.*, 1996b).

All the hypovirulent strains of *D. ambigua* harbour a 4 kb dsRNA virus (Smit *et al.*, 1996b). The presence of a single dsRNA species of similar size and sequence homology within localised geographic area could suggest that there has been a spread of the virus within the *D. ambigua* population. This situation presents an ideal opportunity for the implementation of a biological control strategy of *D. ambigua* canker in South Africa (Smit *et al.*, 1996b; 1997 and 1998).

The complete sequence of the dsRNA element has recently been published (Preisig *et al.*, 2000). This mycovirus does not show sequence similarity to any known mycovirus. The genome of the virus, which has been named *Diaporthe ambigua RNA virus* (DaRV), contains two potential ORFs. Since these ORFs are in the same frame, it is likely that ORF2 is expressed as a fusion protein by reading through the amber stop codon of ORF1. The RDRP of DaRV shares homology with plant ssRNA viruses of the family *Tombusviridae*. Unlike these plant viruses, DaRV does not appear to code for a coat protein on the 3' half of its genome. DaRV occurs predominantly in the cells of *D. ambigua* as positive-strand RNA. This suggests that the genome of DaRV is positive-strand RNA. The N-terminus of ORF1 gene product has six hydrophobic transmembrane-like helices. This strongly suggests that the viral proteins are not encapsidated in a coat protein but are rather anchored on the membranes of the host (Preisig *et al.*, 2000).

1.2.7. Factors hindering effective biological control

Biological control of fungal pathogens through hypovirulence has been known for at least five decades. With the exception of natural hypovirulence that exists in *C. parasitica* in Europe, the application of mycoviruses to control plant pathogenic fungi has been more theoretical than practical. Even though a number of mycoviruses have been sequenced, only hypoviruses CHV1-EP713 and CHV1-Euro7 from *C. parasitica*

have been used in transfection and transformation studies (Choi and Nuss, 1992a,b; Chen *et al.*, 1993 and 1994b; Chen *et al.*, 1996; Chen and Nuss, 1999; Chen *et al.*, 2000; Suzuki *et al.*, 2000). Before any biological control programme can be implemented, the following aspects pertaining to both the fungus and the associated mycovirus will need to be carefully considered:

- Vertical transmission of mycoviruses often occurs only via asexual spores (Anagnostakis, 1982b and 1988; Chen *et al.*, 1993; Liu and Milgroom, 1996; Bissegger *et al.*, 1997). Since some fungi also have sexual stages in their life cycles, this would place a limitation on the spread of the virus.
- Horizontal transmission of mycoviruses is limited by vegetative incompatibility that operates in fungi (Anagnostakis and Waggoner, 1981; Anagnostakis, 1983; Liu and Milgroom, 1996; Cortesi *et al.*, 1996 and 1997; Cortesi and Milgroom, 1998). Thus in the field, the transfer of the virus would be limited only to the fungi within the same VCG as the donor fungus.
- The production of the asexual spores is adversely repressed by the resident mycovirus (Elliston, 1985b; Chen *et al.*, 1996; Bissegger *et al.*, 1997). This would reduce the number of propagules needed to spread the virus in a defined locality and as such, prolong the time needed to control a disease.

All the above-mentioned factors would negatively affect a biological control programme. The population diversity of the fungus being considered for biocontrol must also be known if biological control via hypovirulence is to be effectively implemented (Garbelotto *et al.*, 1992). In a diverse population with many VCGs, the virus-harbouring fungus used to disseminate the virus in a specific locality has to be in the dominating VCG (Anagnostakis, 1977; Anagnostakis *et al.*, 1986; Garbelotto *et al.*, 1992). This is important in the horizontal dissemination of the virus and it will also restrict the emergence of new VCGs by outcrossing (Cortesi *et al.*, 1998; Hoegger *et al.*, 1998).

The fungal strain used to disseminate the virus must also be genetically distinguishable from the strains in the field. Ahn and Lee (2001) were able to differentiate between the donor and recipient *Nectria radicicola* strains by marking

the recipient strain with antibiotic resistance gene. The ability to distinguish between the resident fungus and virus and the introduced fungus and virus, will help in measuring the temporal and spatial movement of the introduced fungus together with its associated virus. This would provide the easiest way of measuring the efficiency and success of the biological control strategy (Hoegger *et al.*, 1998).

1.2.8. Conclusions

Hypovirulence is caused by RNA viruses that are transmitted cytoplasmically between isolates that belong to the same or very closely related vegetative compatibility groups (VCGs), via hyphal anatomosis. Thus far only two variants of the same virus, CHV1-EP713 and CHV1-Euro7, both from *C. parasitica* have been successfully used in transfection and transformation studies.

In order to advance the field of biological control through hypovirulence further, the reverse genetics developed for *C. parasitica* viruses must be extended to unrelated viruses having different properties and occurring in other fungi. This should lead to the emergence of new patterns, which could lead to an increased understanding of the processes underlying hypovirulence. The emerging patterns may then be used in expanding host ranges of mycoviruses through genetic manipulation.

The studies presented in this thesis will focus on applying the reverse genetics of *C. parasitica* viruses to *Diaporthe ambigua RNA virus* (DaRV), *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2). It is expected that, since these viruses are different from the hypovirus of *C. parasitica*, new patterns will emerge that will expose new fields of study.

Table 1. A list of mycoviruses for which complete nucleotide sequences have been published.

Genus	Virus Name	Reference
Hypovirus	*Cryphonectria hypovirus 1-EP713* (CHV1-EP713)	Shapira *et al.*, 1991
Totivirus	*Saccharomyces cerevisiae virus L-A* (ScV-L-A)	Icho and Wickner, 1989
	Saccharomyces cerevisiae virus L1 (ScVL1)	Diamond *et al.*, 1989
	Saccharomyces cerevisiae virus La (ScV-La)	Park *et al.*, 1996
	Saccharomyces cerevisiae virus L-BC (ScV-L-BC)	Park *et al.*, 1996
	Sphaeropsis sapinea RNA virus 1 (SsRV1)	Preisig *et al.*, 1998
	Sphaeropsis sapinea RNA virus 2 (SsRV2)	Preisig *et al.*, 1998
	Helminthosporium victoriae 190S virus (Hv190SV)	Huang and Ghabrial, 1996
Mitovirus	*Ophiostoma novo-ulmi mitovirus 3a-Ld* (OnuMV3a-Ld)	Hong *et al.*, 1998a
	Ophiostoma novo-ulmi mitovirus 4-Ld (OnuMV4-Ld)	Hong *et al.*, 1999
	Ophiostoma novo-ulmi mitovirus 5-Ld (OnuMV5-Ld)	Hong *et al.*, 1999
	Ophiostoma novo-ulmi mitovirus 6-Ld (OnuMV6-Ld)	Hong *et al.*, 1999
	Cryphonectria parasitica mitovirus 1 (CpMV1-NB631)	Polashock and Hillman, 1994
	Rhizoctonia solani M2 virus (RsM2-1A1)[#]	Lakshman *et al.*, 1998
Others[*]	*Ophiostoma novo-ulmi dsRNA7*	Hong *et al.*, 1998b

	Ophiostoma novo-ulmi dsRNA10	Hong *et al.*, 1998b
	Botrytis virus F (BVF)*	Howitt *et al.*, 2000
	Diaporthe ambigua RNA virus	Preisig *et al.*, 2000
Partitivirus	*Atkinsonella hypoxylon virus* (AhV)	Oh and Hillman, 1995
	Rhizoctonia solani virus	Strauss *et al.*, 2000
	Fusarium poae virus 1 (FpV1R2)	Compel *et al.*, 1998
	Fusarium solani dsRNA M1 (FusoVM1)	Nogawa *et al.*, 1996

The asterisk (*) represents a case where the dsRNA virus has not been formally assigned to a genus while the hash (#) represents a case where a dsRNA virus has been provisionally placed to a genus

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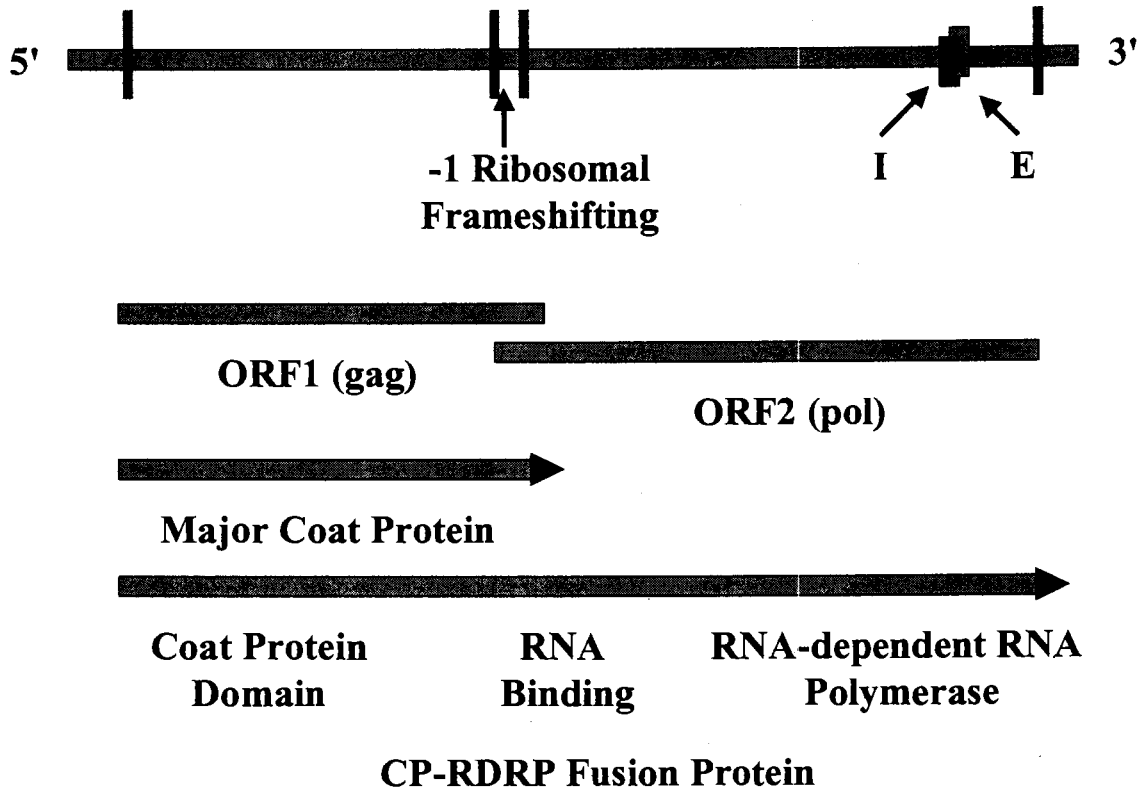


Fig. 2. Genome organisation of totiviruses. This figure is based on information obtained from ScV-L-A and complete nucleotide sequences of some of the sequenced totiviruses listed in Table 1. The figure shows that the ORFs overlap each other by a few nucleotides. Also shown is the position of the -1 ribosomal frameshifting site (Jacks *et al.*; 1988; Dinman *et al.*, 1991; Dinman and Wickner, 1992; Morikawa and Bishop, 1992; Dinman, 1995; Wickner, 1996). The letter I represents the region that encodes the information for internal replication enhancer while E represents the region encoding encapsidation signals (Ghabrial, 1994; Dinman, 1995).

Chapter 2

2. RNA viruses of *S. sapinea* and *D. ambigua* and their possible use as biological control agents

2.1. Introduction

Mycoviruses have been observed in many species of fungi (Buck, 1986; Nuss and Koltin, 1990; Smart and Fulbright, 1996; Ghabrial, 1998). Among these are several well known plant pathogens such as *Cryphonectria parasitica* (Day *et al.*, 1977; Dodds, 1980a,b; Shapira *et al.*, 1991), *Ophiostoma novo-ulmi* (Brasier, 1983; Brasier, 1991; Cole *et al.*, 1998; Hong *et al.*, 1998a,b and 1999), *Helminthosporium victoriae* (Sanderlin and Ghabrial, 1978), *Sphaeropsis sapinea* (Preisig *et al.*, 1998; Steenkamp *et al.*, 1988) and *Diaporthe ambigua* (Smit *et al.*, 1996b). With the exception of mycoviruses of *S. sapinea*, those infecting the fungi mentioned above mediate hypovirulence in their hosts. Hypovirulence was first used to describe the reduced virulence displayed by virus-infected *C. parasitica* isolates (Grente, 1965; Grente and Sauret, 1969; Grente and Berthelay-Sauret, 1978). In *C. parasitica*, it has been shown that the viruses, CHV1-EP713 and CHV1-Euro7 mediate hypovirulence to varying degrees (Choi and Nuss, 1992a,b; Chen *et al.*, 1994b; Chen *et al.*, 1996; Chen and Nuss, 1999; Chen *et al.*, 2000).

North American chestnut trees (*Castanea dentata*) have been reduced to infected understory shrubs that sprout from root-collar (Day *et al.*, 1977; Anagnostakis, 1982b). In contrast, many European chestnut trees (*Castanea sativa*) have recovered from chestnut blight because of the natural transmission of these hypoviruses (Heiniger and Rigling, 1994; Bissegger *et al.*, 1997). In these trees, the hypovirus is naturally transmitted through hyphal anastomosis (Van Alfen *et al.*, 1975; Anagnostakis and Day, 1979; Robin *et al.*, 2000). During this process, virus-free virulent isolates of *C. parasitica* are converted to the hypovirulent phenotype by the transfer of the virus.

Vegetative compatibility groups (VCGs) control the success of biological control through hypovirulence (Anagnostakis and Day, 1979; Anagnostakis, 1982b; Kuhlman *et al.*, 1984; Liu and Milgroom, 1996). The biological control of chestnut blight has been successful in Europe but not in North America because there are fewer VCGs in the populations of *C. parasitica* in Europe than in North America (Anagnostakis, 1977; Anagnostakis *et al.*, 1986; Milgroom, 1995; Liu and Milgroom, 1996).

Progress in developing mycoviruses as possible biological control agents has been slow. This is despite the fact that several mycoviruses have been sequenced and fully characterised. Transfection and transformation studies have mainly been carried out using the *C. parasitica* hypoviruses (Choi and Nuss, 1992a,b; Chen *et al.*, 1994b; Chen *et al.*, 1996; Chen and Nuss, 1999; Chen *et al.*, 2000; Nuss, 2000). Recently, it was reported that a previously virus-free isolate of *H. victoriae* displayed the diseased phenotype after being transformed with *Helminthosporium victoriae 190S virus* (Hv190SV) (Ghabrial, 2001). In order to advance this field further, the reverse genetics developed for *C. parasitica* hypoviruses and recently for Hv190SV should be extended to other mycoviruses with different properties than those of *C. parasitica* hypoviruses and Hv190SV. It is in view of this need that studies on the mycoviruses infecting *S. sapinea* and *D. ambigua* have been initiated (Preisig *et al.*, 1998; Preisig *et al.*, 2000).

The presence of dsRNA elements has been confirmed in many isolates of *S. sapinea*. However, mycovirus infection in *S. sapinea* does not seem to be associated with hypovirulence (Steenkamp *et al.*, 1998). Although these viruses do not confer hypovirulence in their natural hosts, there still exists a possibility that they may confer hypovirulence in other hosts (Steenkamp *et al.*, 1998). It has also been suggested that satellite dsRNAs conferring hypovirulent phenotype might be introduced into the same host with these viruses, so that the latter might act as helper viruses (Preisig *et al.*, 1998).

Two mycoviruses co-infecting a single isolate of *S. sapinea* have been sequenced and named *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2). The genetic organisation of these viruses has revealed that they belong to the genus *Totivirus* in the family *Totiviridae* (Ghabrial, 1994; Preisig *et al.*, 1998).

Members of this genus such as *Saccharomyces cerevisiae L-A* (ScV-L-A) and *Saccharomyces cerevisiae L-BC* (ScV-L-BC) viruses are characterised by isometric particles of 40 to 50 nm diameter that encapsidate the dsRNA genome (Ghabrial *et al.*, 1995; Wickner, 1996). The dsRNA genome encodes two ORFs. ORF1 encodes a coat protein while ORF2 encodes an RDRP. Since SsRV1 and SsRV2 appear to translate their ORF2 by internal initiation, they must produce the gene products of ORF1 and ORF2 as separate proteins and not as a fusion protein (Preisig *et al.*, 1998).

Isolates of *D. ambigua* display different morphology and virulence levels. Slow growing hypovirulent strains have been shown to harbour RNA viruses. Furthermore, these strains display other hypovirulence-associated traits such as reduced laccase activity and slow growth (Smit *et al.*, 1996b). The nucleotide sequence of this dsRNA has been established (Preisig *et al.*, 2000). This mycovirus has been named *Diaporthe ambigua RNA virus* (DaRV) and its genomic organisation and products are closely related to *Turnip crinkle virus* (TCV) and *Carnation mottle virus* (CarMV) both of which are plant viruses. These viruses belong to the genus *Carmovirus* of the family *Tombusviridae* (Guilley *et al.*, 1985; Carrington *et al.*, 1989).

Although DaRV is closely related to carmoviruses, the ORF encoding a coat protein is missing (Preisig *et al.*, 2000). The N-terminal part of the translation product of ORF1 codes for a potential six transmembrane anchor. Thus, this suggests that DaRV proteins like those from the hypovirus are associated with fungal membranes (Hansen *et al.*, 1985; Fahima *et al.*, 1993; Preisig *et al.*, 2000). The two ORFs of DaRV are likely translated by a readthrough mechanism into a single fusion protein containing the RDRP domain on the C-terminus. DaRV is a positive-strand RNA virus and has dsRNA as a replicative form (Preisig *et al.*, 2000).

This thesis represents an effort to extend the reverse genetics developed for *C. parasitica* hypoviruses to other viruses unrelated to it. I thus, report on the construction of full-length cDNA clones of DaRV, SsRV1 and SsRV2. The *in vitro* RNA production and the subsequent attempts to transfect *S. sapinea* will be discussed. Successful transfection of one isolate of *Phomopsis* sp. and three different isolates of *D. ambigua* with DaRV is then described. The results demonstrate that the different fungal isolates respond differently to the same virus. *C. parasitica* hypoviruses have

been successfully used previously in transfection studies. In my knowledge, this study represents the second successful transfection study done using a mycovirus.

2.2. Materials and methods

2.2.1. Fungal isolates and culture conditions

All fungal isolates used in this study were obtained from the fungal culture collection of the Tree Pathology Co-operative Programme (TPCP), housed in the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Table 2). The *S. sapinea* (CMW4254 [virus-infected] and CMW1184 [virus-free]) isolates were maintained on 2% malt extract agar (MEA). The *D. perjuncta* (CMW3407 and CMW5289 [both virus-infected]); *D. ambigua* (CMW5287; CMW5288; CMW5587) and *Phomopsis* sp. (CMW5588) isolates were maintained on potato dextrose agar (PDA).

2.2.2. Induction of sporulation in *Phomopsis* sp. and *D. ambigua* strains

Induction of sporulation in isolates of *Phomopsis* sp. and *D. ambigua* was achieved as described by Smit *et al.* (1996a,b) with minor modifications. Sterile apple twigs were placed at the centre of Petri dishes and 2 % water-agar was added so that the twigs were not fully covered with water-agar. Agar plugs of *Phomopsis* sp. and different *D. ambigua* strains were placed in separate Petri dishes in contact with the apple twigs and sealed with parafilm. The plates were incubated in the dark at 25 °C for one week. The plates were then exposed to a mixture of cool-white fluorescent and near-ultraviolet lights and checked regularly for sporulation.

2.2.3. Molecular biological techniques

2.2.3.1. Cloning and plasmid extraction

All cloning experiments of PCR fragments were done using pGEM T-Easy Vector (Promega) (Fig. 3). This vector makes use of adenine residues at the 3' ends of PCR products added by *Taq* polymerase. *Taq* polymerase has a template-independent terminal transferase activity, which has an exclusive preference for adenine. The

vector has 3' thymidine overhangs on both ends thus facilitating the cloning of *Taq* polymerase-amplified PCR products (Clark, 1988; Holton and Graham, 1990 Marchuk *et al.*, 1990; Hengen, 1995a). The recombinant plasmids were transformed in High Efficiency Competent *E. coli* strain JM109 (Promega). Mini-preparations of plasmid DNA were done by alkaline lysis (Sambrook and Russell, 2001).

2.2.3.2. Restriction enzyme digestions

With the exception of *Mse* I (Amersham), all the restriction enzyme digestions were performed with enzymes from Roch Molecular Biochemicals. Plasmid and genomic DNA digestions consisted of 0.1 - 1 µg DNA, 1 x buffer, 5 U restriction enzyme and 0.1 mg/ml RNase A.

2.2.3.3. Dephosphorylation of linearised plasmid DNA

When necessary linearised plasmids were dephosphorylated to prevent re-ligation during the subsequent subcloning steps (Sambrook and Russell, 2001). Dephosphorylation was done using calf intestinal alkaline phosphatase (CIP) (Roche Molecular Biochemicals). The reaction mixture (0.1 - 1 µg linearised plasmid DNA; 1 x dephosphorylation buffer; 1 U CIP) was incubated at 37 °C for 45 minutes. The reaction was stopped with 0.5 % SDS; 5 mM EDTA (pH 8) and 100 µg/ml Proteinase K (Roche Molecular Biochemicals) followed by incubation at 56 °C for 30 minutes. The dephosphorylated plasmid was purified using phenol/chloroform extraction and precipitated with absolute ethanol.

2.2.3.4. Agarose gel electrophoreses

All agarose gel electrophoreses were performed in 1 or 2 % agarose (Roche Molecular Biochemicals) dissolved in 1 x TAE (50 x TAE: 242 g Tris ; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA pH 8.0). All gels were stained with ethidium bromide to

visualise DNA and RNA. Occasionally, RNA gels were stained with SYBR Green II RNA gel stain to visualise single-stranded nucleic acids.

2.2.4. Extraction and purification of chromosomal DNA

The isolation of chromosomal DNA was achieved using a modification of the technique described by Raeder and Broda (1985). Fungal mycelia were grown in 2% malt extract broth in Erlenmeyer flasks at room temperature. The mycelia were harvested after two weeks and lyophilised in a freeze dryer (Dura-Dry μP, FTS Systems) overnight.

The lyophilised mycelia were ground with a pestle and mortar under liquid nitrogen. The ground mycelia were transferred to a 1.5 ml eppendorf tube into which 750 μl of DNA extraction buffer [200 mM Tris HCl (pH 8.5); 250 mM NaCl; 25 mM EDTA (pH 8.0); 0.5 % SDS] was added. The mixture was vortexed briefly and an equal volume of a 1:1 mixture of phenol/chloroform was added and vortexed. The DNA was repeatedly extracted with phenol/chloroform until the interface between the aqueous and organic phases disappeared. A final chloroform extraction was performed to remove traces of phenol. The DNA was precipitated with 2 volumes of ice-cold absolute ethanol in the presence of 10 % (v/v) of 3 M sodium acetate (pH 4.5) and pelleted by centrifugation in a bench-top centrifuge at 12000 rpm for 30 minutes at 4 °C. The DNA pellet was washed with 70 % ethanol and resuspended in 30 μl of double-distilled water. RNA was removed from the samples by digestion with RNase A (Roche Molecular Biochemicals) at the concentration of 0.1 mg/ml and stored at –20 °C.

2.2.5. Molecular identification and relatedness of fungal isolates

In order to identify and determine the relationship between the fungal isolates, the ITS regions and 5.8S rRNA gene of the ribosomal RNA (rRNA) gene operon (Fig. 4) were amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and

ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The Expand High Fidelity PCR System kit (Roche Molecular Biochemicals) was used for all PCR amplifications. Amplification conditions for PCR were 1 cycle at 96 °C for 2 min, 10 cycles at 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2 min followed by a final elongation step at 68 °C for 10 min. The reaction mixture consisted of about 5 ng DNA, 0.3 µM each primer, 0.2 mM (each) dNTPs, 1 x Expand buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA (pH 8.0) and 0.5 % Tween and 2.5 - 5 U Expand enzyme mix. The reaction volume was adjusted to 50 µl with double-distilled water. The amplified PCR products were separated on 1 % agarose gel in 1 x TAE. The PCR products were purified using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals).

The PCR products were analysed for Restriction Fragment Length Polymorphisms (RLFPs). Alternatively, their nucleotide sequences were determined and aligned using PAUP (Phylogenetic Analysis Using Parsimony) Version 4.0b1 (Swofford, 1998).

2.2.6. DNA sequencing and analysis

Sequencing reactions were composed of 0.2 µM primer, 2 µl BigDye mixture, 1 x dilution buffer, 50 to 250 ng plasmid DNA and made up to 10 µl with double-distilled water. Amplification conditions for the sequencing reactions were 25 cycles at 96 °C for 20 s, 50 °C for 20 s and 60 °C for 4 min. All the primers used for sequencing with the exception of ITS1, ITS4, DaRV-H6-FW and DaRV-H6-RV are listed in Table 3. The sequences of ITS1, ITS4, DaRV-H6-FW and DaRV-H6-RV are given in the text. All the PCR, RT-PCR and plasmid inserts were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). This sequencing method is based on the dideoxynucleotide chain termination method using fluorescence-labelled dye terminators (Sanger *et al.*, 1977; Lee *et al.*, 1992; Rosenblum *et al.*, 1997). The elongation products from the sequencing reactions were analysed using ABI PRISM 377 DNA sequencer (Perkin-Elmer). The DNA sequences were analysed using Sequence Navigator and analysis programs listed at the ExPASY home page (Translate, SIM/PRSS, PSORT, CLUSTALW and WU-BLAST). The

sequences were compared with deposited DNA and protein sequences in GenBank and SWISS-PROT databases.

2.2.7. Extraction and purification of dsRNA

Double-stranded RNA was extracted using the method of Valverde *et al.* (1990) and modified as described by Preisig *et al.* (1998). All the amino-free reagents were treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC) (Merck) to remove RNase contamination. The DEPC-treated reagents were autoclaved before use to destroy DEPC.

The lyophilised mycelia were ground using a pestle and mortar under liquid nitrogen. The ground mycelia (1 g) were transferred to a 50 ml centrifuge tube into which 10 ml of 2 x STE (0.1 M Tris HCl pH 8.5; 0.2 M NaCl; 2 mM EDTA pH 8.0; pH 6.8) and 1% SDS were added and vortexed to mix. This viscous mixture was incubated at 60 °C for 10 minutes. The mixture was cooled to room temperature and an equal volume of phenol was added and mixed by vortexing. The preparation was shaken at room temperature for 30 min followed by centrifugation in a Beckman JA25.50 rotor for 1 h at 4 °C. The aqueous layer was transferred to a new tube and extracted several times with an equal volume of a 1:1 mixture of phenol/chloroform. After extraction with phenol/chloroform another extraction with an equal volume of chloroform was performed to remove any traces of phenol. Absolute ethanol was added to a final concentration of 16 %. Some of the precipitated genomic DNA was removed by centrifugation at 5000 rpm for 5 min at 4 °C in a Beckman JA25.50 rotor.

The supernatant containing dsRNA was applied to a CF11 cellulose (Whatman) column packed in a 2 ml syringe (Promega) without a plunger. The cellulose-bound dsRNA was washed with 10 ml of 2 x STE containing 16 % ethanol. The dsRNA was eluted with 8 ml of 2 x STE and collected in 8 x 1 ml fractions. The dsRNA was precipitated with 0.6 volumes of isopropanol in the presence of 10 % (v/v) of 3 M sodium acetate (pH 4.5) and chilled at –20 °C for 2 hours. The dsRNA was pelleted by centrifugation using a bench-top centrifuge at 12000 rpm for 30 min at 4 °C and

washed with 70 % ethanol. The sample was dried and resuspended in 30 µl DEPC-treated water and stored at –20 °C.

2.2.8. One Tube RT-PCR amplification of viral RNA

cDNA synthesis and subsequent PCR amplification were performed using Titan One Tube RT-PCR System (Roche Molecular Biochemicals). An amount of 10-50 ng dsRNA was first denatured in 32.5 µl of water at 98 °C for 3 min. The denatured dsRNA was quickly transferred to ice. The reaction mixture (0.45 µM each primer; 5 mM DTT; 0.2 mM dNTPs ; 1 x RT-PCR buffer, 1 µl enzyme mixture of AMV reverse transcriptase and Expand *Taq* polymerase; 12 U RNase inhibitor) was added to the denatured dsRNA followed by a reverse transcription reaction at 50 °C for 1 hour. The reaction mixture was 50 µl but in some cases half reactions were set up. Amplification conditions for PCR were 1 cycle at 96 °C for 2 min, 10 cycles at 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2 min. This was followed by further 30 cycles at 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2 min with a cycle elongation of 5 s for each cycle. A final elongation step at 68 °C for 10 min was added. The RT-PCR products were separated on 1 % agarose gel. When any part of the cloned viral cDNAs was amplified the conditions outlined for RT-PCR above were followed without doing the reverse transcription step.

The RT-PCR products used for cloning the full-length cDNA clone of DaRV are shown in Table 4. The RT-PCR products used for cloning the full-length cDNA clone of SsRV1 and SsRV2 are shown in Table 5 and Table 6, respectively.

2.2.9. *In vitro* transcription of full-length cDNA clones

Strand-specific RNA was synthesised from the full-length cDNA clones of the viral genomes by using T7 RNA polymerase and SP6 RNA polymerase (Roche Molecular Biochemicals). In order to produce positive-stranded RNA, the plasmids containing the cDNA copies of the genomes of the viruses were linearised with *Sal* I (pDV3 and

pH6DV3) or with *Nsi* I (pNM1-5 and pNM2-5). Production of negative-stranded RNA from pDV3 was *in vitro* transcribed from *Nco* I-linearised plasmid. No suitable enzymes were found to linearise pNM1-5 and pNM2-5 for the production of negative-stranded RNA. This is because the enzymes that cut within the multiple cloning site (MCS) of the plasmids also cut within the viral cDNAs. RNA was synthesised from the cDNAs in the presence of ribonucleoside triphosphates (Roche Molecular Biochemicals) mix containing ATP, CTP, GTP and UTP. The reaction mix contained 150 ng linearised plasmid DNA, 1 mM of each NTP, 1 x transcription buffer, 10 U T7 RNA polymerase or SP6 RNA polymerase and 12 U RNase inhibitor. The volume was made up to 20 µl with RNase-free double-distilled water. The reaction was performed for 2 h at 30 °C for pDV3 and pH6DV3. In the case of pNM1-5 and pNM2-5, the reaction was allowed to run for 5-8 h. Transcription products were analysed on a 1 % agarose gel.

2.2.10. Preparation of fungal spheroplasts

The preparation of spheroplasts was done using the method of Royer and Yamashiro (1999) with minor modifications. Fungal mycelia were grown in 5 ml of 2 % ME broth in 14 ml culture bottles for 5-10 days. Mycelia were harvested from the culture broth with a pair of tweezers and placed in a 60 mm Petri dish. Mycelia were then thoroughly drained of the broth using a pipette. Chitinase (0.5 % w/v) (Fluka) and cellulase (1 % w/v) (Sigma) were dissolved in 6 ml of 1 M Magnesium Sulphate ($MgSO_4.7H_2O$). Mycelia were immersed in this cell wall-degrading solution and left at room temperature overnight. Mycelia were then gravity-filtered through a 120 micron flour gauze (Swiss Milling Company). An equal volume of ice-cold 1 M sorbitol was then added to the resuspended spheroplasts. The spheroplast solution was centrifuged in Eppendorf tubes in a bench-top centrifuge at 5000 rpm for 5 min at 4 °C. The resulting pellet was washed with 500 µl of ice-cold sorbitol and centrifuged at 5000 rpm for 5 min at 4 °C. The pellet was resuspended in 500 µl of STC [1 M sorbitol; 50 mM Tris HCl (pH 8); 50 mM $CaCl_2$] and centrifuged as described above. The spheroplasts were finally resuspended in a solution of spheroplast storage buffer. The spheroplast storage buffer contained STC, PTC (40 % PEG 6000; 50 mM Tris

HCl, pH 8; 50 mM $CaCl_2$) and DMSO in the ratio of 8:2:0.1. The spheroplasts were used immediately or stored at –80 °C.

2.2.11. Transfection of fungal spheroplasts with *in vitro*-produced viral RNA

Freshly prepared fungal spheroplasts or defrosted spheroplasts were transfected by electroporation using an Eppendorf multiporator (Hamburg, Germany). The electroporator was switched to the mode for electroporating bacteria and yeasts. The method of transfection was essentially a modification of that described by Chen *et al.* (1993). If spheroplasts were frozen, they were first thawed on ice followed by resuspension in 500 µl ice-cold sorbitol and centrifuged in a bench-top centrifuge at 5000 rpm for 5 min at 4 °C. The supernatant was removed from the Eppendorf tube and the spheroplasts were gently resuspended in 85 µl of ice-cold sorbitol and placed on ice. RNase inhibitor (12 U) was added to 15 µl transcription reaction containing the viral *in vitro*-produced positive stranded RNA transcripts. The viral transcripts were added to the spheroplast solution and placed on ice for 5 minutes. The spheroplast/RNA solution was transferred to a pre-chilled 1 mm gap width, 100 µl cuvette (Eppendorf).

The spheroplasts were pulsed 3-5 times at 1.8 – 2.5 kV. A volume of 500 µl ice-cold sorbitol was added to the cuvette and placed on ice for 10 minutes. A volume of 200 µl of the transfected spheroplasts was pipetted onto the center of a 90 mm Petri dish. Regeneration medium (0.1 % casein hydrolysate; 0.1 % yeast extract; 34.2 % sucrose; 1.6 % agar) at 48 °C was added to the Petri dish around the spheroplasts from the edge to the center. The plates were allowed to solidify in a laminar flow bench. The Petri dishes were sealed with parafilm and transferred to bench top and left overnight. The plates were transferred to 25 °C for 48 hours followed by 5-10 days on bench top at room temperature. Three blocks of agar were randomly excised from the edges of the culture and placed on PDA in the case of *Phomopsis* sp. and *D. ambigua* or MEA in the case of *S. sapinea*.

2.2.12. Sequence determination of distal ends of viral genomes from transfected fungi

Double-stranded RNA was isolated from transfected fungi as described in section 2.2.7 above. The distal ends of the viral genomes were amplified by 5' RACE (Rapid Amplification of cDNA Ends) (Frohman, 1994) as described by Preisig *et al.* (1998, 2000) using a 5'/3' RACE kit (Roche Molecular Biochemicals). The 5' and 3' ends of DaRV were reverse transcribed with the primers Oli73 and Oli75 (Table 3) respectively, using AMV reverse transcriptase. Approximately 50 ng dsRNA in 11 µl H_2O was denatured at 98 °C for 3 minutes and quickly transferred to ice. A reaction mixture consisting of 0.75 µM Oli73 or Oli75 (Table 3); 1 x cDNA synthesis buffer; 1 mM dNTPs (each); 20 U AMV reverse transcriptase and 12 U RNase inhibitor was added to the denatured dsRNA.

Conditions for cDNA synthesis were 50 °C for 15 min, 55 °C for 45 min and 65 °C for 10 min. The cDNA was purified using High Pure PCR product purification kit (Roche Molecular Biochemicals). A poly(A) tail was added to the cDNA product using terminal transferase in the presence of dATP. A reaction mix consisting of 19 µl purified cDNA; 1 x reaction buffer; 0.2 mM dATP was incubated at 94 °C for 3 min. The mixture was cooled on ice followed by addition of 10 U terminal transferase. The tailing reaction was performed at 37 °C for 20 min and stopped at 70 °C for 10 min.

A nested PCR was done using Oli78 and Oli81 (Table 3) for the 5' and 3' ends, respectively. The reaction mix consisting of 1 µl tailed cDNA; 0.75 µM Oli78 or Oli81 (Table 3); 0.75 µM oligo dT-anchor primer; 0.2 mM dNTPs (each); 1 x reaction buffer; 0.5 µl Expand enzyme mix was set up in a total volume of 50 µl. Amplification conditions for PCR were 1 cycle at 96 °C for 2 min, 10 cycles at 94 °C for 30 s, 50 °C for 30 s and 75 °C for 45 s. This was followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s with a cycle elongation of 5 s for each cycle. A final elongation step at 72 °C for 10 min was added. The PCR amplification products were analysed on a 1 % agarose gel in 1 x TAE and sequenced.

2.2.13. Phenotypic characterisation of test fungi

2.2.13.1. Virulence tests on Golden Delicious apples

The virulence of fungal isolates was tested using an apple-based test developed for *Cryphonectria* spp. (De Lange *et al.*, 1998). The test was carried out using Golden Delicious apples at room temperature. The apples were surface sterilised with 70 % ethanol. Holes of about 15 mm deep were punched on the surface-sterilised apples using a 5 mm diameter cork borer. Ten apples were each inoculated with similar sized disks of mycelia from the naturally-infected, DaRV-transfected, negative control (water transfected) and wild-type fungi of each isolate (Table 7). The wounds on the apples were then covered with masking tape to reduce desiccation. Sterile PDA disks were inoculated onto 10 apples and these served as negative controls. The inoculated apples were kept at room temperature. Each transfected fungal isolate and its associated isogenic negative control and the wild-type fungi were treated as different experiments. After 6 days, the masking tape was removed from inoculated apples and the necrotic lesions were measured. Differences in lesion size caused by different isolates on apples were analysed using a one way analysis of variance (ANOVA) (SYSTAT version 7.0). Differences among isolates were analysed using post-hoc Bonferroni pairwise comparisons.

2.2.13.2. Virulence tests on apple trees (Golden Delicious/M793 rootstock)

Apple trees were inoculated after leaf fall at the end of May, 2001. Inoculation of apple trees was performed in a manner similar to that described for the apple test on cultivar Golden Delicious crafted on M793 rootstock. Trees were wounded by removing disks of bark with a 5 mm diameter cork borer to expose the cambium. Similar sized disks of each fungal test isolate (Table 7) were used to inoculate the trees. Ten trees were inoculated with each isolate. Ten additional trees were inoculated with sterile PDA disks to serve as controls. The inoculated wounds were covered with masking tape to reduce desiccation. Lesion sizes were measured 12 weeks after inoculation. Differences in lesion size on apple trees were analysed using

a one way analysis of variance ANOVA (SYSTAT version 7.0). Differences among isolates were analysed using post-hoc Bonferroni pairwise comparisons.

2.2.13.3. Growth studies of fungi

Mycelial plugs from the edges of actively growing one-week old fungal isolates of the naturally-infected, DaRV-transfected, negative control (water transfected) and wild-type fungi were transferred to 2% PDA plates. Five plates per isolate were incubated in the dark at 15 °C, 20 °C, 25 °C and 30 °C. The growth rates of isolates were determined by measuring colony diameters after 2, 3, 4 and 5 days. The growth rates of the different fungal isolates were analysed using a one way ANOVA (SYSTAT version 7.0).

2.2.13.4. Bavendamm's phenol oxidase tests

Fungi have the ability to oxidise tannic acid and gallic acid (Bavendamm, 1928a,b). Many studies have shown that due to the repression of expression of enzymes responsible for the oxidation of these substances, virus-infected fungi do not discolourise medium containing either tannic acid or gallic acid (Rigling *et al.*, 1989; Hillman *et al.*, 1990; Rigling and Van Alfen, 1991; Choi *et al.*, 1992). Virus-free fungi readily produce colour on Bavendamm medium containing either gallic acid or tannic acid. Fungal isolates (Table 7) were grown on Bavendamm's medium containing tannic acid (1.5 % malt extract; 2 % agar; 0.5 % tannic acid with pH adjusted to 4.5 with 10 N NaOH). Each fungal isolate was replicated 5 times on the medium. Mycelium plugs were cut from the edges of the actively growing fungal cultures and transferred to Petri dishes containing Bavendamm's medium. The plates were incubated in the dark at 25 °C for one week (Smit, 1996a,b). The plates were exposed to light for short time periods daily to monitor colour development on the plates.

2.2.13.5. Bavendamm's gallic oxidase tests

Fungal isolates (Table 7) were grown on Bavendamm's medium containing gallic acid (1.5 % malt extract; 2 % agar, 0.5 % gallic acid with pH adjusted to 4.5 with 10 N NaOH). The experiment was carried out as explained for the Bavendamm's phenol oxidase tests in the preceding section (Smit, 1996a,b).

2.2.14. Hybridisation techniques

2.2.14.1 Southern blot hybridisations

Total chromosomal DNA from *Phomopsis* sp. and *D. ambigua* isolates was digested using the restriction enzymes *Eco* RI, *Hin*d III and *Eco* RI/*Hin*d III. The digested DNA samples were separated in 1 % agarose gel at 40 V overnight. The DNA in the agarose gel was then depurinated by submerging the gel in 250 mM HCl. After a brief rinse in double distilled water, the DNA was denatured by treating the gel 2 x 15 min with denaturation solution (0.5 N NaOH; 1.5 M NaCl). The gels were rinsed and the DNA neutralised 2 x 15 min in neutralisation solution (3 M NaCl; 0.5 M Tris HCl pH 7.5) and blotted by capillary action onto a positively charged nylon membrane (Roche Molecular Biochemicals). The DNA was cross-linked onto the membrane by a 2 min exposure to UV light on both sides.

The non-specific nucleic acid binding sites on the nylon membrane were blocked by pre-hybridising the membrane in hybridisation buffer (5 x SSC; 0.1 % [w/v] N-lauroyl-sarcosine; 0.02 % [w/v] SDS; 1 % [w/v] blocking reagent) at 65 °C for 1 h. The prehybridisation solution was discarded. The DIG-labelled DNA probe was denatured by heating at 98 °C for 10 min followed by rapid cooling on ice and diluted in 5 ml hybridisation buffer. This probe was added to the membrane and allowed to hybridise overnight in a roller hybridiser Hb-1D (Techne, UK). After the hybridisation step, the hybridisation buffer with the probe was discarded. The nylon membrane was then washed 2 x 5 min in 2 x SSC/0.1 % SDS at room temperature followed by another 2 x 15 min stringency washes in 0.1 x SSC/0.1 % SDS at 65 °C.

2.2.14.2. Preparation of DIG-labelled DNA probes

DNA fragments for DIG labelling were amplified from the plasmid pDV3 by PCR using the primers DaRV5' and DaRV3'. The PCR product was gel-purified followed by purification using High Pure Purification kit. For labelling, 16 µl of the PCR product was denatured at 98 °C for 10 min. The denatured DNA was immediately transferred to ice followed by the addition of 4 µl DIG-High Prime mixture (Roche Molecular Biochemicals). The random labelling was allowed to proceed overnight at 37 °C. The reaction was stopped by heating at 65 °C for 10 min. The probe was stored at –20 °C until use.

2.2.14.3. Northern blot hybridisations

Total nucleic acids were extracted and purified as described in section 2.2.7 above with the following modifications. The supernatant containing total nucleic acids was not applied to a CF11 cellulose (Whatman, UK) column but was immediately precipitated with 2 volumes of absolute ethanol. As control, positive and negative stranded RNA of the viruses were produced *in vitro* with either T7 or SP6 RNA polymerase using conditions described in the following section.

The RNA was separated on a 1 % agarose gel in 1 x TAE buffer. The agarose gel was denatured in 50 mM NaOH and 150 mM NaCl for 30 min followed by neutralisation for 15 min in 1 M Tris HCl (pH 7.5) and 1.5 mM NaCl. The neutralisation step was repeated twice. The gel was placed on Whatman 3M paper (Whatman, UK), wetted with 20 x SSC and overlaid with a positively charged nylon membrane (Roche Molecular Biochemicals) pre-wetted in 2 x SSC. The nylon membrane was overlaid with several Whatman 3M papers over which several absorbent papers were placed. The nucleic acids were transferred onto the positively charged nylon membrane by capillary action for 6 hrs. The membrane was UV-crosslinked for 2 min on each side. The crosslinked membrane was rinsed briefly in double distilled water and allowed to dry at room temperature.

The membrane was prehybridised in DIG Easy Hyb Buffer (Roche Molecular Biochemicals) for 1 h with gentle shaking at 68 °C. DIG-labelled RNA probe was added to pre-heated DIG Easy Hyb. The pre-hybridisation solution was discarded and the RNA probe/hybridisation mixture was added to the membrane and incubated overnight at 68 °C . The membrane was washed 2 x 5 min in excess 2 x SSC and 0.1 % SDS at room temperature followed by 2 x 15 min washes in 0.5 x SSC and 0.1 % SDS at 68 °C.

2.2.14.4. Preparation of DIG-labelled RNA probes

The positive and negative strand RNA were transcribed from *Sal* I or *Nco* I linearised pDV3 using T7 RNA polymerase (Fig. 5A) and SP6 RNA polymerase (Fig. 5B), respectively. In the case of SsRV1, pNM1-5 was linearised with *Nsi* I for producing positive strand RNA (Fig. 5A). For SsRV2, positive strand RNA was produced from *Nsi* I-linearised pNM2-5 (Fig. 5A). No suitable restriction enzymes were found to restrict both SsRV1 and SsRV2 for the production of negative-stranded RNA. The enzymes that could be used to linearise the plasmid also cut within the viral cDNAs. The reaction was carried out as described in section 2.2.9 above, except that DIG-11-UTP instead of UTP was used. The reaction mix contained 150 ng linearised plasmid DNA, 1 x DIG RNA labelling mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM TTP, 3.5 mM DIG-11-UTP), 1 x transcription buffer, 12 U RNase inhibitor, 10 U T7 RNA polymerase or SP6 RNA polymerase. The volume was adjusted to 20 µl using RNase-free double-distilled water. The reaction was run for 2 h at 30 °C. The reactions were allowed to proceed for 5-8 h in the cases of SsRV1 and SsRV2.

2.2.15. Immunological detection of hybridised labelled DNA and RNA probes

The detection was carried out following the protocol supplied with the DIG High Prime Labelling and Detection Kit I. The hybridised membrane was washed 2 x 5 min in excess 2 x SSC and 0.1 % SDS at room temperature followed by two washes for 15 min in 0.5 x SSC and 0.1 % SDS at 68 °C. The membrane was rinsed for 5 min in

maleic acid buffer (0.1 M maleic acid; 0.15 M NaCl; adjusted to pH 7.5 with NaOH pellets). The membrane was then incubated in 100 ml of blocking solution (10 x blocking solution from the kit diluted 1:10 in maleic acid buffer).

The membrane was incubated for 30 min in 20 ml of anti-DIG-AP conjugate, diluted 1:5000 in blocking solution, followed by washing 2 x 15 min in maleic acid buffer. The washed membrane was equilibrated in 20 ml of detection buffer (0.1 M Tris HCl; 0.1 M NaCl; 50 mM $MgCl_2$; pH 9.5) for 5 min, followed by incubation in 15 ml of freshly prepared colour solution, in the dark for up to 16 h without shaking. The reaction was stopped by washing the membrane in 50 ml of distilled water for 5 min.

Table 2. Isolates used for isolating viruses and virus-free isolates used in transfection studies.

Isolate	Species	Host	Area of isolation	Remarks	Collector
CMW4254	*S. sapinea*	*Pinus roxburghii*	Gauteng	Virus-infected	W. J. Swart
CMW1184	*S. sapinea*	*P. radiata*	Jonkershoek, Stellenbosch	Virus-free	W. J. Swart
CMW3407	*D. perjuncta* (formely *D. ambigua*)	*Malus domestica*	Simondium, Stellenbosch	Virus-infected	W. A. Smit
CMW5589	*D. ambigua*	*Malus domestica*	Simondium, Stellenbosch	Virus-infected	W. A. Smit
CMW5287	*D. ambigua*	*Malus domestica*	Simondium, Stellenbosch	Virus-free	W. A. Smit
CMW5288	*D. ambigua*	*Malus domestica*	Simondium, Stellenbosch	Virus-free	W. A. Smit
CMW5587	*D. ambigua*	*Malus domestica*	Simondium, Stellenbosch	Virus-free	W. A. Smit
CMW5588	*Phomopsis* sp. (formely *D. ambigua*)	*Prunus persica*	Simondium, Stellenbosch	Virus-free	W. A. Smit

Table 3. Primers used to sequence and clone the three cDNA copies of the genomes of the *S. sapinea* RNA viruses, SsRV1 and SsRV2 and the *Diaporthe ambigua RNA virus*, DaRV.

SsRV1	SsRV2	DaRV
T7 (5'-TAATACGACTCACTATAGGG-3')[1]	**T7** (5'-TAATACGACTCACTATAGGG-3')[1]	**T7** (5'-TAATACGACTCACTATAGGG-3')[1]
SP6 (5'-TATTTAGGTGACACTATAG-3')[2]	**SP6** (5'-TATTTAGGTGACACTATAG-3')[2]	**SP6** (5'-TATTTAGGTGACACTATAG-3')[2]
SsRV1-5' (5'-GGATTTCACGTACAACGTAGGGTTGTC-3')	**SsRV2-5'** (CCAGGGACCCCTGCAGCCC-3')	**DARV-5'** (5'-GGGAAATTTGTGAGATTATCGCC-3')
SsRV1-3' (5'-CTGCAGTTTGGCCCCAGCGG-3')	**SsRV2-3'** (5'-GGCATTTGGGGCCCGTAGGC-3')	**DARV-3'** (5'-GGGCCACAGGATCCGGAGAAC-3')
Oli3 (5'-ATCCTCCGGTACTCACGCG-3')	**2-RDRP-5'** (5'CCGCAACTAGTAACGACAGACTCC-3')[b]	**Oli62** (5'-GCTGCGTAATCTGCGCCTTGC-3')
Oli4 (5'-CTTGTCTTAAGGCCTGCGG-3')	**Oli5** (5'-TGATCTTCGGATTGCGGTCG-3')	**Oli63** (5'-CCAGCACAGGTTCAAGAGAGG-3')
Oli12 (5'-GCGGGCATCTTGGCATATCTCGACG-3')	**Oli6** (5'-AAGTAGGGCGCGACGGTC-3')	**Oli64** (5'-GTCGCATCTCACAGCCGAGCG-3')
Oli14 (5'-TGTAGACGGGGCCGTCGTCGAGTGCC-3'	**Oli9** (5'AGTTACTGACACCTGAACGGGGACC-3')	**Oli65** (5'-AACCTCGAGCACAGCGCAACG-3')
Oli18 (5'-CGCGTGCTCTGATCAATCGATCAGG-3')	**Oli10** (5'-CGGCTGAGTTTATGAACACTCAGTCG-3')	**Oli72** (5'-CTTGCCGACATCAAGAGGC-3')
Oli19 (5'-GCGGCGAGGCGATAACCGCGGCGAGG-3')	**Oli13** (5'-GATTACGATGACTTCAACTCCCACC-3'	**Oli73** (5'-GTGCCCTGCACAAACAACTC-3')
Oli20 (5-GCGGCTTATCCGTCCTCCGCGGCC-3)'	**Oli15** (5'-CGTCTTACGGTAACTCGCCTCCGCC-3')	**Oli74** (5'-CTGGTCTCCCAGGTCACGG-3')
Oli23 (5'-CATCAGGAGCGCGTCCACGTAGCAG-3')	**Oli16** (5'-CGGGACGCGGAAAGCTTCATGGCGG-3')[c]	**Oli75** (5'-TCCATCTCACCGGAGCGGCAG-3')
Oli21 (5'-CCCGAATTCTTAGGGACGGCGGCAG-3')[a]	**Oli17** (5'-CTGGCCAGCTTGCCCTCGAGAACGG-3')	**Oli76** (5'-CCGGGTCTTGGTTCTTCCTGG-3')
Oli25 (5'-CTACCTACTATCGGTTGTCGAACGC-3')	**Oli22** (5'-TCAGCAACGGCAGTCGTAGTCCTGG-3')	**Oli77** (5'-AACTGGCCTGCAGGTTGC-3')
Oli26 (5'-GCTTCTGATCTACTGCACCGTGA-3')	**Oli24** (5'-GCTGCCGTAGCGTCGTCCGACTTCG-3')	**Oli78** (5'-CCTGGGTGACGGTTGTTACAC-3')
Oli28 (5'-GTTCCTGAACTTGACCG-3')	**Oli27** (5'-CGCCGAGGCAGGTGGCAATCCG-3')	**Oli79** (5'-AAGAGCGAAAGAGCGTAGGC-3')
Oli29 (5'-CCACCACTACTGGGGCCAG-3')	**Oli32** (5'-AAGGTTGGGTACTCGATGGCGTG-3')	**Oli80** (5'-CTCACCAGCCTCCAACCG-3')
Oli30 (5'-CTGCGCCACCACGGCCAG-3')	**Oli34** (5'-ACGTGTTCGCGGGCAACC-3')	**Oli81** (5'-TTGAACGATGGGTGTAGGTGG-3')
Oli31 (5'-CATTGACAGGGCCGAACAATTCG-3')	**Oli36** (5'-TCTCCATGACCGGTGATGAAGCC-3')	**Oli82** (5'-CTGACGGGCTGTGTTACC-3')
Oli33 (5'-TCGCACGGTAGCGCCGGTAAGTC-3')	**Oli37** (5'-CTTAGCCGCGAGCTCAGG-3')	**Oli83** (5'-CACGTCGACCTACACCCATCGTTC-3')[d]
Oli35 (5'-CCTCCATCGGGTTTACGG-3')	**Oli44** (5'-GTGCTCAAGGCCGTAGGC-3')	**Oli84** (5'-GGTGCATGCTTGTGCCATTTCC-3')[e]

SsRV1	SsRV2	DaRV
Oli38 (5'-CCGCGACATCTTCCGGGTCTCCG-3')	**Oli58** (5'-ATTCTAGAGGCACAAGCGATCG-3')	**Oli85** (5'-GACACCTCTAGTTCCCTGC-3')
Oli39 (5'-GAGCCTTGATAGCCGTCGAGATGC-3')	**Oli59** (5'-CTCTAGAATTAGCGGCGGTTGTCTC-3')	**Oli86** (5'-CCGCATGCGTTTCTTCAACGAG-3')[f]
Oli40 (5'-ACACTGCCCGTTTCCGCG-3')	**Oli90** (5'-CGCTCCGCATCCGTGCTC-3')	**Oli87** (5'-CACGAAAGTCGACTGGGCATCC-3')[g]
Oli41 (5'-GCACCGAAGTCATGGGCGTTCGC-3')	**Oli94** (5'-TGGTCGGCGCAAACATGG-3'	**Oli88** (5'-TTGAGGTTTGTGGAGTGG-3')
Oli42 (5'-GTCGGACATACTGACGAGGCTGG-3')		**Oli95** (5'-GCAAACGCTCCACGAGATC-3')
Oli91 (5'-ATATGGCTAACCGTAACC-3')		
Oli92 (5'-ACTCTTGACCGCACCGAC-3')		
Oli93 (5'-TGGACATAGCACGCAACCG-3')		

[1] T7 and [2] SP6 primers are universal primers that bind to the promoter sequence of the T7 and SP6 polymerases, respectively.

Restriction enzyme sites were introduced into some of the primers (underlined sequences) to facilitate cloning. The introduced restriction enzyme sites are as follows:

[a] *Eco* RI (G↓AATTC), [b] *Spe* I (A↓CTAGT), [c] *Hin*d III (A↓AGCTT), [d,g] *Sal* I (G↓TCGAC), and [e,f] *Sph* I (GCATG↓C)

All the primers were synthesised by MWG Biotech (Gemany).

Table 4. Plasmids containing RT-PCR products derived from the genome of the *Diaporthe ambigua RNA virus*, DaRV, as inserts.

Plasmid	Insert	Fragment size (kb)
pDV1	DaRV-5'/Oli65 RT-PCR product	2.8
pDV2	Oli64/ DaRV-3' RT-PCR product	1.4
pDV3	DaRV-5'/Oli5 - Oli64/ DaRV-3' RT-PCR products (full-length cDNA clone)	4.1

Table 5. Plasmids containing RT-PCR products derived from the genome of the *Sphaeropsis sapinea RNA virus 1*, SsRV1, as inserts.

Plasmid	Insert	Fragment size (kb)
pNM1-1	SsRV1-5'/Oli23 RT-PCR product	1.2
pNM1-2	Oli25/SsRV1-3' RT-PCR product	2.4
pNM1-3	Oli20/Oli39 RT-PCR product	2.1
pNM1-4	SsRV1-5'/Oli23-Oli25/SsRV1-3' construct	4.5
pNM1-5	SsRV1-5'/Oli23- Oli20/Oli39-Oli25/SsRV1-3' RT-PCR products (full-length cDNA clone)	5.1

Table 6. Plasmids containing RT-PCR products derived from the genome of the *Sphaeropsis sapinea RNA virus 2*, SsRV2, as inserts.

Plasmid	Insert	Fragment size (kb)
pNM2-1	SsRV2-5'/Oli36 RT-PCR product	2.2
pNM2-2	Oli10/Oli9 RT-PCR product	0.8
pNM2-3	2-RDRP-5'/SsRV2-3' RT-PCR product	2.6
pNM2-4	SsRV2-5'/Oli36 - Oli10/Oli9 construct	2.8
pNM2-5	SsRV2-5'/Oli36- Oli10/Oli9-2-RDRP-5'/SsRV2-3' (full-length cDNA clone)	5.2

Table 7. *Diaporthe* spp. used to inoculate Golden Delicious apples and apple trees

Isolate	Transfection
CMW5588-DaRV	DaRV
CMW5588-H_2O	H_2O
CMW5588-WT	-
CMW5587-DaRV	DaRV
CMW5587-H_2O	H_2O
CMW5587-WT	-
CMW5287-DaRV	DaRV
CMW5287-H_2O	H_2O
CMW5287-WT	-
CMW5288-DaRV	DaRV
CMW5288-H_2O	H_2O
CMW5288-WT	-
CMW3407	+

Transfection: During transfection, spheroplasts were mixed with viral transcripts (DaRV) or water (H_2O). The sign (-) denotes the wild-type isolate of the fungus while (+) denotes the naturally-infected fungus. Sterile PDA disks were included in the experiments as inocula.

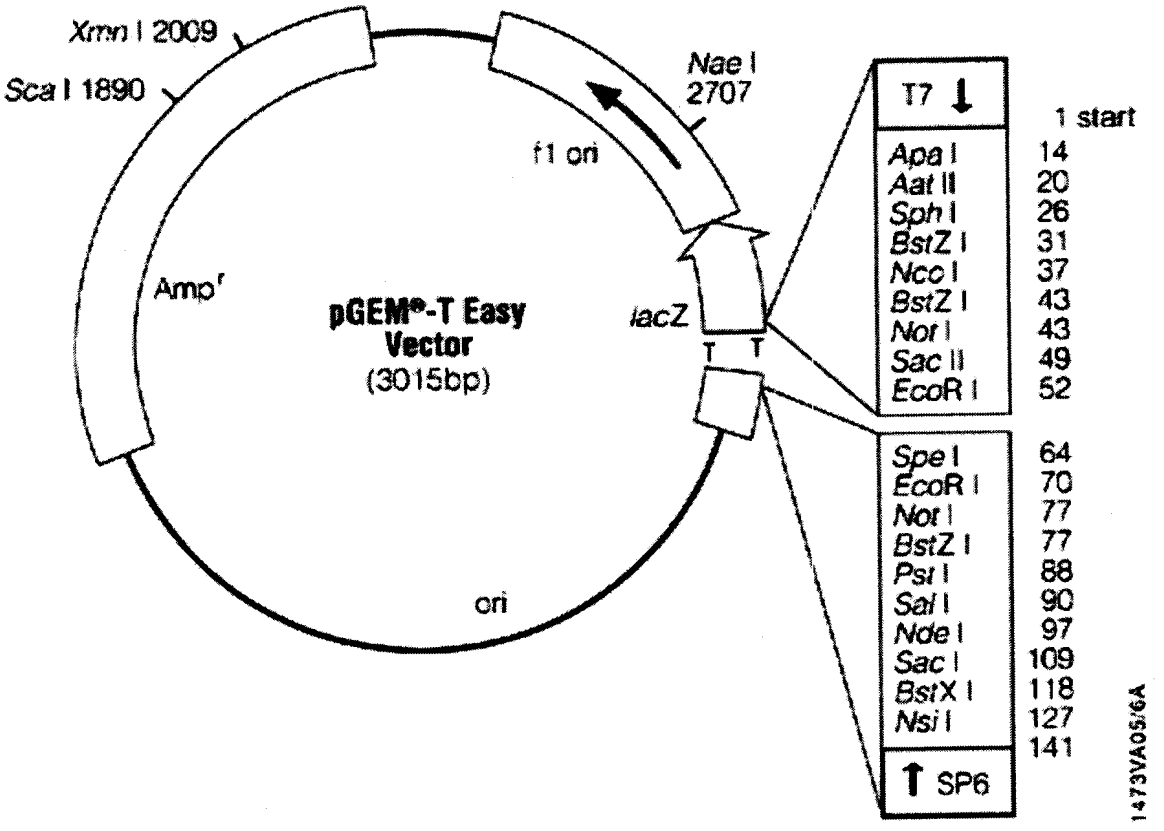


Fig. 3. Schematic representation of pGEM-T Easy Vector which was used in all the cloning experiments (downloaded from http\\:www.promega.com). The multiple cloning site is shown on the right. The added 3' thymidine overhangs which facilitates the cloning of *Taq* polymerase-generated PCR products are also shown (Marchuk *et al.*, 1990).

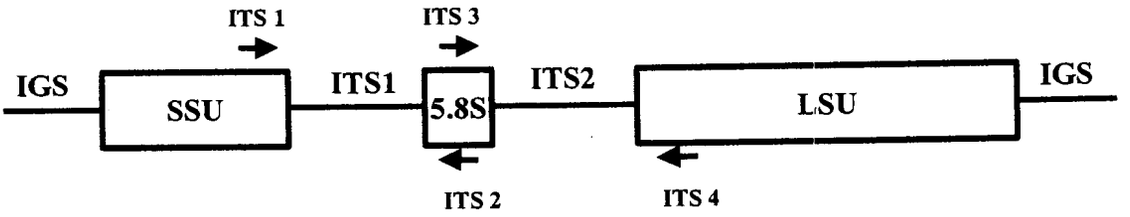


Fig. 4. A schematic representation of rRNA gene operon. The primer pair ITS1/ITS4 was used to amplify *ca.* 450 bp PCR product from the *Diaporthe* spp. used in this study. The positions of other commonly used primers, ITS2 and ITS3 are shown. SSU = small subunit, LSU = large subunit, ITS = internal transcribed spacer and IGS = intergenic spacer region.

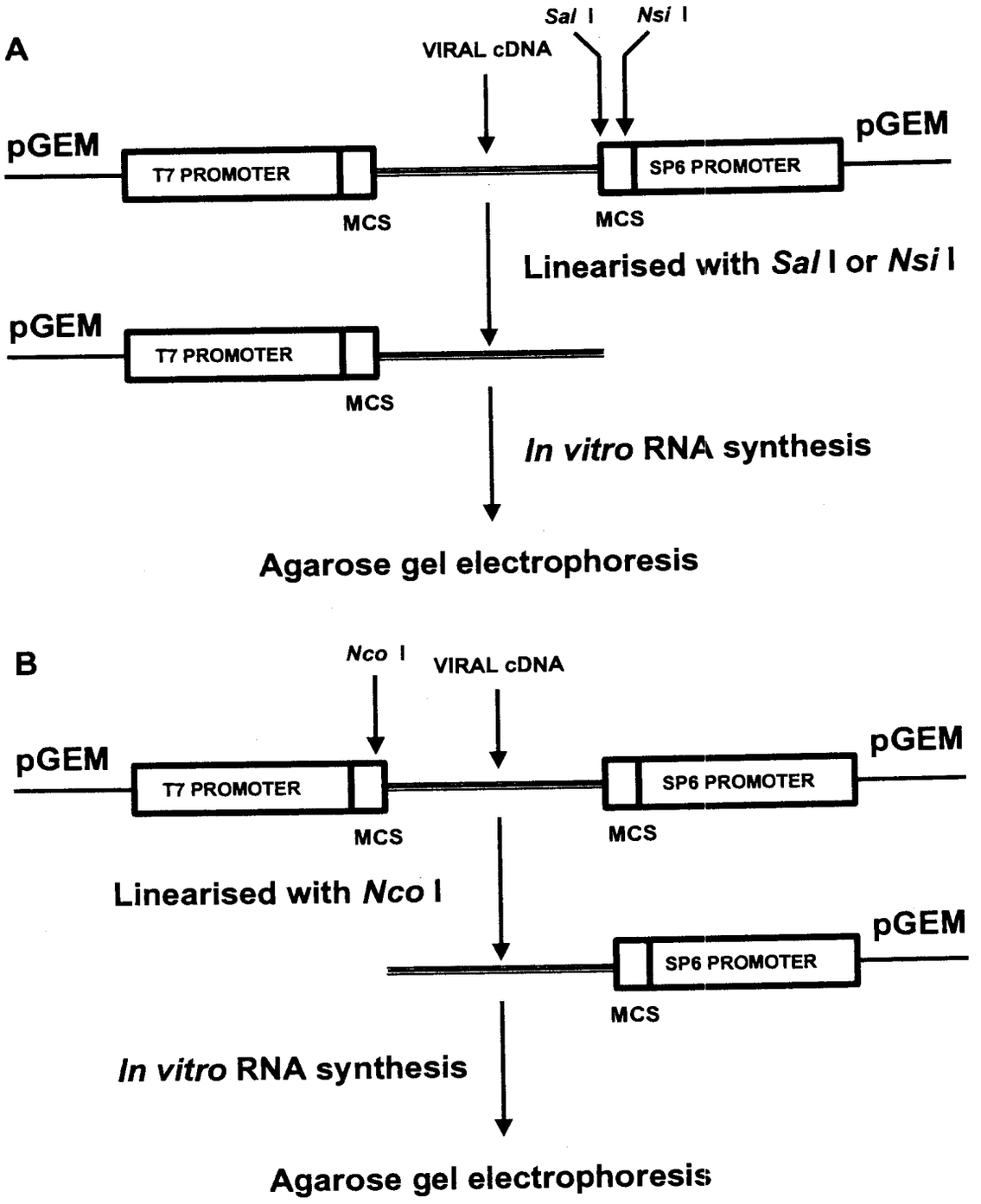


Fig. 5. A simplified multiple cloning site (MCS) and the flanking T7 and SP6 promoters of pGEM-T Easy Vector. (A): Positive-stranded RNA was produced *in vitro* from the *Sal* I-linearised pDV3 and the *Nsi* I-linearised pNM1-5 or pNM2-5 using T7 RNA polymerase. (B): Negative-stranded RNA was produced from *Nco* I-linearised pDV3 using SP6 RNA polymerase. There were no appropriate enzymes to linearise pNM1-5 and pNM2-5 for producing full-length negative-stranded RNA.

2.3. Results

In order to investigate the possibility of using *Sphaeropsis sapinea RNA virus 1* (SsRV1), *Sphaeropsis sapinea RNA virus 2* (SsRV2) and *Diaporthe ambigua RNA virus* (DaRV) as biological control agents for pathogenic fungi, their genomes had to be cloned as full-length cDNA copies. The cloned viruses would then be used to produce strand-specific RNA for the transfection of spheroplasts from *Diaporthe* spp. and *S. sapinea*. The hypovirus infecting the chestnut blight fungus, *C. parasitica*, has been shown by transfection (Chen *et al.*, 1994b; Chen *et al.*, 1996; Chen and Nuss, 1999) and transformation (Choi and Nuss, 1992b) studies to be a possible biological control agent for chestnut blight. The possibility of using mycoviruses to control plant pathogenic fungi has been further strengthened by the report that a virus-free *H. victoriae* isolate transformed with a full-length cDNA of *Helminthosporium victoriae 190S virus* (Hv190SV) displayed the diseased phenotype (Ghabrial, 2001).

2.3.1. Cloning of the full-length copies of the genomes of DaRV, H6DaRV, SsRV1 and SsRV2

The viral genomes were cloned in the multiple cloning site of pGEM T-Easy Vector which is flanked by a T7 and SP6 RNA polymerase promoters on opposite sides (Fig. 3). The flanking promoters could then be used to produce, *in vitro*, strand-specific RNA that would be used to transfect fungal spheroplasts. In addition to the three cDNA clones, a His-tagged mutant clone of DaRV, H6DaRV was constructed. The tagged gene product of ORF1 would allow for the purification of the ORF1 gene product and to determine the localisation of these proteins in the fungal cells. This section of results describes how the cDNA clones of these viruses were constructed.

2.3.1.1. Cloning of a full-length cDNA copy of DaRV

The linear full-length sequence of the genome of DaRV (Fig. 6) was reported to be 4113 nucleotides with a GC content of 53 % (Preisig *et al.*, 2000). The virus is related

to viruses in the family *Tombusviridae*, a virus family of viruses known to infect plants. The analysis of the sequences flanking the stop codon (amber) of ORF1 of DaRV showed that these sequences are similar to consensus sequences flanking the readthrough codons of carmoviruses. Therefore, it was suggested that ORF2 which codes for RDRP of DaRV, is translated by a readthrough mechanism as a fusion protein with the ORF1 gene product (Preisig *et al.*, 2000). A cDNA copy of this genome was subsequently constructed in order to investigate the possibility of using this virus in biological control of Diaporthe canker, a serious disease caused by *D. ambigua* in pome and stone fruit orchards in South Africa (Smit *et al.*, 1996a).

To clone the full-length cDNA copy of the genome of the *Diaporthe ambigua RNA virus*, DaRV, two large overlapping partial RT-PCR products of the viral genome were generated (Fig. 7). The intermediate RT-PCR products (Table 4) were cloned in pGEM T-Easy Vector. The RT-PCR product amplified using the primer pair DaRV-5' and Oli65 was cloned into pDV1. The clone pDV2 contained as an insert the RT-PCR product amplified with the primers Oli64 and DaRV-3'. Both clones were selected so that the 5' ends of the cDNA of the viral genome were located on the T7 promoter site of the pGEM T-Easy Vector. The plasmids pDV1 and pDV2 were restricted using the restriction enzymes, *Xho* I and *Nsi* I. The former restriction enzyme cuts in the DaRV coding region while the latter cuts in the multiple cloning site of pGEM T-Easy Vector. The 1.4 kb *Xho* I/*Nsi* I cDNA fragment of pDV2 was gel-purified and then ligated into the *Xho* I/*Nsi* I linearised pDV1. This resulted in the plasmid pDV3, which represents the full-length cDNA clone of the genome of DaRV.

The authenticity of the full-length cDNA copy of the genome of DaRV was confirmed by restriction digest analysis (Fig. 8) and sequencing. The subcloning strategy and the nucleotide positions of the primers are depicted in Fig. 7 and Fig. 9, respectively.

2.3.1.2. Construction of a mutant DaRV genome containing a histidine tag insertion

Since no viral particles were found for DaRV, it was proposed that this virus might exist as nucleoproteins anchored on the fungal membranes by the six potential

transmembrane helices at the N-terminus of the ORF1 gene product as well as the ORF1/ORF2 readthrough protein (Preisig *et al.*, 2000). It was, therefore, desirable to attempt to isolate DaRV nucleoproteins and to investigate the expression of ORF2. In order to facilitate this process, six codons coding for histidine (CAC CAT)$_3$ residues were inserted immediately downstream of the potential start codon for the translation of the first ORF of DaRV at position 578 (Fig 8). The inserted histidine residues would allow for the easy purification of the viral proteins on Ni^{2+} columns. The localisation of the virus within fungal cells and the viral translational products containing the His-tag would be detected using the commercially available antibodies against the histidine tag. The cDNA fragments were amplified from the plasmid pDV3 (Fig. 7) and cloned in pGEM T-Easy Vector.

The PCR fragment cloned in pH6DV1, was amplified from pDV3 using DaRV-5' as a forward primer and DaRV-H6-RV (5'-GAAATGCATAAGGGAGCGCTG-3') as a reverse primer. The second clone, pH6DV2, contained the PCR product amplified from pDV3 using DaRV-H6-FW (5'-TTATGCAT**CACCATCACCATCAC**CGTTTCTTCAACGAGTTAATGATG-3') as a forward primer and DaRV-3' as a reverse primer. A restriction site for *Nsi* I (ATGCA↓T) was introduced into the primers DaRV-H6-RV and DaRV-H6-FW by substituting guanine by adenine in the codon downstream of the start codon as shown in Fig. 11. The His-tag was introduced in the primer DaRV-H6-FW (boldfaced). The plasmid pH6DV1 was linearised with *Nsi* I. The 3.5 kb *Nsi* I fragment of pH6DV2 was excised out and ligated into the *Nsi* I site of pH6DV1. The resulting plasmid pH6DV3, represents the His_6-tagged mutant cDNA clone of the genome of DaRV. The authenticity of the mutant DaRV genome containing six codons for histidine residues was confirmed by digestion with *Nsi* I (Fig. 8) and sequencing. The subcloning strategy and the nucleotide positions of the primers are depicted in Fig. 12 and Fig. 13, respectively.

2.3.1.3 Cloning of a full-length cDNA copy of SsRV1

The genome of SsRV1 (Fig. 14) was sequenced and the ability to sequence the ends of the virus confirmed that the genome is a linear molecule of 5163 nucleotides. This viral genome has 62 % GC content and it belongs to the family *Totiviridae* (Preisig *et al.*, 1998). The virus has two ORFs. ORF1 codes for a coat protein and ORF2 codes for RDRP. The translation of ORF2 has been proposed to be internally-initiated. In this case, the coat and RDRP proteins would be produced as two separate proteins (Preisig *et al.*, 1998). In order to use this virus in fungal transfection studies, the cDNA copy of the genome of this virus was cloned.

The full-length cDNA copy of the genome of *Sphaeropsis sapinea RNA virus 1* (SsRV1) was constructed from three large intermediate overlapping clones (Fig. 15 and Table 5). As in the case of DaRV, the clones were selected so that the 5' regions of the viral genome copy were on the T7 promoter site of pGEM T-Easy Vector. The first clone, pNM1-1, contains as an insert the RT-PCR product amplified from SsRV1 RNA using the primers SsRV1-5' and Oli23. The second clone, pNM1-2, contains the RT-PCR product amplified using the primers Oli25 and SsRV1-3'. The cloning of the RT-PCR product amplified with the primer pair Oli38 and Oli39 yielded the plasmid pNM1-3. The SsRV1 cDNA has two *Eco*R V restriction sites at positions 1103 and 3177. These sites at 1103 and 3177 are present in pNM1-1 and pNM1-2, respectively. Both the sites are present at the distal ends of pNM1-3 insert. The plasmid pNM1-1 was linearised using *Eco*R V and *Nsi* I. The 2.4 kb *Eco*R V/*Nsi* I fragment of pNM1-2 was excised out of the plasmid. This RT-PCR fragment was subcloned into the *Eco*R V/*Nsi* I site of pNM1-1. This resulted in the plasmid pNM1-4 that was then linearised using *Eco*R V. The linearised plasmid was dephosphorylated to prevent religation during the subsequent subcloning step. The 2.1 kb *Eco*R V fragment from pNM1-3 was subcloned into the dephosphorylated pNM1-4. This step resulted into pNM1-5 which represents the full-length cDNA clone of the genome of SsRV1.

The authenticity of the full-length cDNA copy of the genome of SsRV1 was confirmed by restriction digest analysis (Fig. 16) and sequencing. The subcloning strategy and the nucleotide positions of the primers are depicted in Fig. 15 and Fig. 17, respectively.

2.3.1.4. Cloning of a full-length cDNA copy of SsRV2

SsRV2 (Fig. 14) has been found to co-exist with SsRV1 in the cells of an isolate of *S. sapinea*. The virus has a linear genome of 5202 and a GC content of 63 %. This virus belongs to the family *Totiviridae*. It has the same genome organisation as SsRV1. Therefore, translation of the coat and RDRP ORFs most probably results in two separate proteins, due to the fact that the translation of OFR2 is internally-initiated (Preisig *et al.*, 1998).

The full-length cDNA copy of the genome of SsRV2 was constructed from three large intermediate overlapping RT-PCR products that were cloned into pGEM T-Easy Vector (Fig. 18 and Table 6). As in the cases of DaRV and SsRV1, the orientation of all clones was selected so that the 5' regions of the viral genome copy were on the T7 promoter site of the vector. The first clone, pNM2-1, contains a 2.2 kb RT-PCR product amplified from the 5' end of the genome of SsRV2 using the primers SsRV2-5' and Oli36. The second clone, pNM2-2, contains a 0.8 kb RT-PCR product from SsRV2 amplified using the primer pair Oli10 and Oli9. The third clone, pNM2-3, contains as an insert the 2.6 kb RT-PCR product amplified from the 3' end of the genome of SsRV2 using the primer pair RDRP2-5' and SsRV2-3'.

The plasmid, pNM2-1, was linearised using *Cla* I and *Nsi* I. *Cla* I cuts once in the SsRV2 cDNA insert while *Nsi* I cuts only in the pGEM T-Easy Vector's multiple cloning site. The 0.8 kb *Cla* I/*Nsi* I cDNA insert from SsRV2 cloned in pNM2-2 was excised out of agarose gel after restriction digestion of pNM2-2 plasmid DNA. This cDNA fragment was then subcloned into the *Cla* I/*Nsi* I site of pNM2-1. The resulting plasmid was named pNM2-4. The 2.6 kb *Xba* I/*Nsi* I fragment of pNM2-3 was excised out of the plasmid and subcloned into the *Xba* I/*Nsi* I site of pNM2-4. This cloning step resulted in pNM2-5 which represents the full-length cDNA clone of the genome of SsRV2.

The authenticity of the full-length cDNA was confirmed by restriction digest analysis (Fig. 19) and sequencing. The subcloning strategy and the nucleotide positions of the primers are depicted in Fig. 18 and Fig. 20, respectively.

2.3.2. Sequence analysis of the full-length cDNA copies of the genomes of DaRV, SsRV1 and SsRV2

2.3.2.1. Sequence analysis of the full-length cDNA copy of the genome of DaRV

The published sequence of DaRV (Preisig *et al.*, 2000) and the cDNA clone sequence were compared. Two sequence variations were recognised. As shown in Table 8, the sequence variations occurred at positions 389 and 1743 in which guanines were substituted for by adenines. The base change at position 389 occurred outside the coding region of the virus in the 5' untranslated region (UTR). The guanine substitution at position 1743 in ORF1 occurred in the first position of the codon for glutamate (GAG). This resulted in a substitution of glutamate (GAG) by lysine (AAG).

2.3.2.2. Sequence analysis of the full-length cDNA copy of the genome of SsRV1

A total of 14 sequence variations were recognised between the published sequence of SsRV1 (Preisig *et al.*, 1998) and the corresponding cDNA clone sequence (Table 9). Four base sequence variations occurred in the first position of the codon, 5 in the second position and 4 in the third position of the codons of the amino acids. Two of the base sequence variations did not result in the change of the amino acids while 11 sequence variations resulted in amino acid changes. Of the fourteen codon changes, one was located in the 5' UTR, nine in the coat protein ORF while four were in the RDRP ORF.

All 4 base changes in the first position of the codons resulted in changes in amino acids of the gene products. The first sequence variation was at position 809 where a guanine was replaced by cytosine. In this position, aspartate (GAC) was substituted by histidine (CAC). In position 1475, guanine was substituted by adenine. This change substituted alanine (GCG) with threonine (ACG). In the third base change, cytosine was replaced by thymine. In this case, arginine (CGT) was replaced by cystein (TGT). The fourth base change in the first codon of the amino acid involved

the replacement of cytosine by thymine resulting in proline (CCA) being replaced by serine (TCA).

The base changes in the second position of the 5 codons resulted in changes of amino acids in all the positions where the base changes occurred. At position 1026, thymine was replaced by guanine resulting in leucine (CTC) being replaced by arginine (CGC). At position 2454, thymine was replaced by cytosine. This base change resulted in leucine (CTT) being replaced by proline (CCT). Another second codon base variation occurred in position 2505, resulting in a substitution of cystein (TGT) by serine (TCT). The last base change in the second codon was observed in position 3913 where guanine was replaced by adenine. Glycine (GGT) was thus replaced by aspartic acid (GAT).

Two of the 4 base changes in the wobble position of the codons did not result in the replacement of the original amino acid by another while two of the base changes resulted in amino acids replacement. The base change at positions 1579 (arginine → guanine), 2855 (adenine → thymine) did not result in a change of the amino acids at these positions. The base changes at positions 2512 and 2515 both guanine to cytosine, resulted in the replacement the amino acids. At position 2512, glutamine (CAG) was replaced by histidine (CAC). At position 2515, glutamate (GAG) was replaced by aspartate (GAC).

2.3.2.3. Sequence analysis of the full-length cDNA copy of the genome of SsRV2

A total of 145 sequence variations were recognised between the published sequence of SsRV2 (Preisig *et al.*, 1998) and the cloned cDNA sequence (Table 10). Of these 145 sequence variations, 107 are located in the coat protein ORF while 38 are in the RDRP ORF. One hundred of the 107 base changes within the coat protein did not result in amino acid changes while 7 caused amino acid changes. In contrast, 12 base changes in the RDRP ORF did not result in amino acid changes while 26 base changes resulted in the changes of the encoded amino acids. Most of the changes in amino acids were caused by more than one change in the same codon.

A total of 22 amino acids were substituted by different ones because of the base changes. Of the 22 amino acid changes, 8 occurred within the coat protein ORF while 14 were in the RDRP ORF. A total of 5 amino acid changes were caused by base changes in all the three positions of the codons and another 5 amino acid changes were caused by two base changes in the same codons.

The cDNA sequence was found to have a cytosine insertion at position 2484 close to the 3' end of ORF1. This insertion introduced a frameshift in ORF1. Preisig *et al.* (1998) reported that the translation of ORF1 terminates at a stop codon (UAA) at position 2663 while the translation of ORF2 starts at a start codon (AUG) at position 2658. This implied that ORF1 and ORF2 overlapped by eight nucletides (**AUGAGUAA**). The insertion at position 2484 terminates the translation of ORF1 at a stop codon (UGA) at postion 2660 while ORF2 starts at an AUG codon at position 2659. This means that ORF1 and ORF2 overlap by four nucleotides (AUGA). This makes the genomic organisation of the full-length cDNA sequence of SsRV2 similar to that of *Helminthosporium victoriae 190S virus* (Hv190SV) (Huang and Ghabrial, 1996). The amino acid changes due to the insertion are shown in Fig. 21. Unlike the other amino acid changes reported in this work, these changes were due to frameshift and not due to base changes in the codons.

2.3.3. Molecular identification of *Diaporthe* spp. isolates and their relatedness

Since the morphology of the *Diaporthe* spp. isolates used in this study was diverse, a decision was made to consider the relationships among these isolates. The internal transcribed spacer (ITS) region of the ribosomal DNA operon was used to determine the relationships among the *Diaporthe* spp. isolates chosen for transfection studies as well as the two naturally-infected isolates. The ITS1/ITS4 (White *et al.*, 1990) primer pair was used to amplify and sequence fragments of *ca.* 450 bp from the DNA of each fungal isolate (Fig. 22). A total of 10 taxa were included in the analysis. These taxa included three recently published reference taxa, namely *D. perjuncta* STE-U2655, *D. ambigua* STE-U2657 and *Phomopsis* sp. MCK6 STE-U2680 (Mostert *et al.*, 2001).

The sequences were aligned using Clustalw (Expasy) and the alignment was analysed using maximum parsimony. A heuristic search from the aligned sequences produced four most parsimonious trees of 178 steps (Fig. 23). The trees did not differ in topology. The trees were rooted on *C. cubensis* ITS sequence. Out of the 508 characters of the sequences, 374 were constant. Of the remaining characters, 53 variable characters were parsimony-uninformative while 81 were parsimony-informative. The trees were evaluated with 1000 bootstrap replications and decay indices for clade stability. The phylogenetic tree topology showed three different clades. Different alignments of the ITS sequence data did not affect the topology of the trees.

One of the isolates used in this study previously identified as *D. ambigua* CMW5588 grouped together with *Phomopsis* sp. MCK6 STE-U2680. This grouping was supported by a bootstrap value of 100 %. Three of the isolates, namely *D. ambigua* CMW5288, *D. ambigua* CMW5587 and *D. ambigua* CMW5287 grouped together with *D. ambigua* STE-U2657. This group was also supported by a bootstrap value of 100 %. The isolate (CMW3407), believed to be *D. ambigua* at the beginning of this study, which is the strain from which DaRV was isolated, grouped together with *D. perjuncta* STE-U2655. A bootstrap value of 100 % supported this group. Therefore, the virus was isolated from *D. perjuncta* and not from with *D. ambigua* as originally assumed by Preisig *et al.* (2000).

Restriction fragment length polymorphisms (RFLPs) have been used extensively to distinguish between fungal species within a genus (Harrington and Wingfield, 1995; Harrington *et al.*, 2001). Unlike sequencing, RFLPs provide a rapid technique that gives results within a single day (Harrington *et al.*, 2001). Restriction digests of the ITS region fragments amplified with ITS1/ITS4 using *Mse* I produced unique restriction fragment patterns that distinguished the naturally-infected *D. perjuncta* isolates (CMW3407 and CMW5289) from the virus-free *D. ambigua* (CMW5587, CMW5288 and CMW5287) and *Phomopsis* sp. (CMW5588) isolates (Fig. 24). Manual examination of the aligned sequences showed that the virus-infected *D. perjuncta* isolates have an additional *Mse* I site (T↓TAA) at around position 150 (Fig. 22). Therefore, the RFLPs using *Mse* I produced two bands (*ca.*150 bp and 300 bp) in

the case of *D. perjuncta* CMW3407 while the other three isolates had only one detectable band as the restriction enzyme cuts the PCR products from these isolates at their extreme 3' ends (Fig. 22 and Fig. 24).

2.3.4. Transfection of *Phomopsis* sp., *D. ambigua* and *S. sapinea* isolates

2.3.4.1. Production of fungal spheroplasts

Spheroplasts were produced from the *Phomopsis* sp., *D. ambigua* and *S. sapinea* isolates using a mixture of chitinase and cellulase as described in materials and methods (Royer and Yamashiro, 1999). This method was successful because the spheroplasts were easily regenerated using a sucrose-based medium. However, it is important to note that there is no standard procedure for producing spheroplasts (Jang *et al.*, 1993). In most cases, specific procedures must be found for each species and in some cases, for each strain of the species of interest (Jang *et al.*, 1993).

2.3.4.2. *In vitro* RNA transcription from pDV3, pH6DV3, pNM1-5 and pNM2-5

In order to carry out hybridisation studies and transfections, strand-specific RNA was transcribed from linearised plasmids using either T7 RNA polymerase or SP6 RNA polymerase. Positive-stranded RNA was transcribed from *Sal* I-linearised pDV3 and pH6DV3 to electroporate *D. ambigua* and *Phomopsis* sp. spheroplasts (Fig. 22). It is thought that *in vivo*, positive-stranded RNA is produced from the dsRNA and it is from this single-stranded positive-strand RNA that the negative-stranded RNA strands are synthesised to form new viral molecules (Ghabrial, 1994, Yao *et al.*, 1997).

The manufacturers of the *in vitro* transcription kit (Roche Molecular Diagnostics) recommend that plasmids must be linearised using restriction enzymes that create 5'-overhangs. In this case, the use of the 3'-overhang-creating *Nsi* I did not affect the yields of RNA transcripts. No restriction enzyme creating a 5'-overhang was found that cut only within the multiple cloning site of the vector without cutting within the viral cDNA for both pNM1-5 and pNM2-5. As such, the 3'-overhang-creating *Nsi* I

was used to linearise these plasmids in order to synthesise positive-stranded RNA for transfection (Fig. 26 and 27). No restriction enzyme could be found to linearise pNM1-5 and pNM2-5 for the *in vitro* RNA production of negative-stranded RNA. The restriction enzymes that cut in the vector's multiple cloning site upstream of the 5' end of the viral cDNAs also cut within the viral sequences.

While the *in vitro* RNA transcription of *Nsi* I-cut pNM1-5 resulted in multiple bands (Fig. 26), the *in vitro* transcription of pNM2-5 resulted in a single band (Fig. 27). The multiple bands could be due to RNA transcripts of different lengths. Conversely, the multiple bands could be due to different conformations of the single-stranded RNA products. The latter explanation is more probable since SsRV1 has 62 % GC content. Different stretches of GCs could result in hairpin structures. These hairpin structures would, therefore, result in different mobilities of the RNA in native agarose gel electrophoresis.

2.3.4.3. Electroporation of *Phomopsis* sp. and *D. ambigua* spheroplasts with positive-stranded RNA from DaRV and H6DaRV

Transfection of *Phomopsis* sp. and *D. ambigua* spheroplasts was performed over a range of voltages and different number of pulses. Transfections were done using the mode for electroporating bacteria and yeasts on Eppendorf multiporator which combines very short pulses with relatively high voltages. The mode for electroporating eukaryotic cells did not result in successful electroporations. This was probably due to low voltages that can be achieved using this mode. It must be noted that the Eppendorf multiporator model used in these studies regulates all the parameters automatically. Only the voltage can be set when using the mode for electroporating bacteria and yeasts, while both voltage and time constant (τ) can be set when using the mode for electroporating eukaryotic cells. RNA transcribed from the plasmid pDV3 was used successfully to transfect the spheroplasts of three isolates of *D. ambigua* (CMW5587, CMW5288 and CMW5287) and one isolate of *Phomopsis* sp. (CMW5588) while transfection with *in vitro*-produced pH6DV3 RNA failed even after many attempts using different combinations of conditions.

Transfections were attempted with voltages ranging from 800-2500 V. Transfection voltage of 2000 V with 5 pulses at 4 seconds intervals was found to be optimal for *D. ambigua* and *Phomopsis* sp. isolates. Transfection was not found to be a reliable procedure for introducing the viral RNA into fungal spheroplasts because the success rate was less than 50 %.

2.3.4.4. Electroporation of *S. sapinea* spheroplasts with positive-stranded RNA from SsRV1 and SsRV2

The same conditions used for electroporating *D. ambigua* and *Phomopsis* sp. spheroplasts were used to electroporate *S. sapinea* spheroplasts with *in vitro*-produced positive-stranded RNA from SsRV1 and SsRV2. Several attempts under these conditions did not result in success. Increasing voltage to the maximum level (2500 V) and increasing the number of pulses did not improve the situation. Transfections using an equimolar amount of transcripts from SsRV1 and SsRV2 also failed to give successful transfection. The spheroplasts of *D. ambigua*, *Phomopsis* sp. and *S. sapinea* remained viable for regeneration after electroporation at high voltages using multiple pulses.

2.3.5. Confirmation of successful transfection of *Phomopsis* sp. and *D. ambigua* isolates with DaRV

2.3.5.1. Isolation of dsRNA from transfected mycelia

In order to confirm the presence of DaRV in transfected fungi, dsRNA was isolated from freeze-dried mycelia using CF11 cellulose chromatography. Bands of 4 kb were found in the dsRNA preparation from one *Phomopsis* sp. (CMW5588-DaRV) isolate and the three *D. ambigua* (CMW5587-DaRV, CMW5288-DaRV and CMW5287-DaRV) isolates transfected with DaRV. The bands were not found in negative (CMW5588-H_2O, CMW5587-H_2O, CMW5288-H_2O and CMW5287-H_2O) control and wild-type (CMW5588-WT, CMW5587-WT, CMW5288-WT and CMW5287-WT) isolates (Fig. 28A).

2.3.5.2. RT-PCR using DaRV-specific primers

The dsRNA isolated from section 2.3.5.1 was used in RT-PCR using the primer pair Oli64/Oli80. RT-PCR fragments of 600 bp were amplified from the transfected isolates but not from the negative control and wild-type isolates (Fig. 28B). These fragments were sequenced and their sequences were 100 % identical to the DaRV sequence bordered by these primers. This confirmed that the 4 kb bands are DaRV.

2.3.5.3. Northern blot analysis

Freeze-dried mycelia from transfected *D. ambigua* (CMW5587, CMW5288 and CMW5287) and *Phomopsis* sp. (CMW5588) were used to purify dsRNA. The dsRNA was used in Northern blot analyses using DIG-labelled negative-stranded RNA of DaRV as a probe. This single-stranded RNA was transcribed from *Nco* I-linearised pDV3 with SP6 RNA polymerase. As shown in Fig. 29, the dsRNA from all the transfected isolates could be detected using this technique. This result corroborated the results from RT-PCR using the primer pair Oli64/Oli80 (Fig. 28B) and confirmed the presence of DaRV in these isolates.

2.3.5.4. Analysis of the 5' and 3' ends of the viruses

When pDV3 was linearised with *Sal* I for the production of strand-specific RNA, the transcribed RNA used for transfection had 35 vector-derived nucleotides on its 3' end. The 5' end of the *in vitro* produced RNA also had 61 vector-derived nucleotides because T7 RNA polymerase starts transcription within the T7 RNA promoter 61 nucleotides away from the cDNA insert. In order to determine whether the transfected replicating DaRV genome had extra nucleotides on its 5' and 3' ends, RACE (rapid amplification of cDNA ends) reactions were performed (Frohman, 1994). The 5' and 3' ends of DaRV were reverse-transcribed with the primers Oli73 and Oli75, respectively. After "tailing" the products with a poly(A) tail, a nested PCR was performed on these fragments using Oli78 and Oli81, respectively. An oligo(dT) primer that bound on the introduced poly(A) tail was used as a reverse primer in the

nested PCR. The PCR products were column-purified and sequenced. The analysis of the PCR products originating from the 5' and 3' ends revealed that the virus was DaRV and the 5' and 3' termini were identical to wild-type DaRV ends (data not shown).

2.3.6. Analysis of transfected *Phomopsis* sp. and *D. ambigua* isolates

2.3.6.1. Morphological characterisation of *Phomopsis* sp. and *D. ambigua* isolates used in transfection experiments

In this study, three isolates of *D. ambigua* and one isolate of *Phomopsis* sp. were successfully transfected with DaRV. Slight changes in morphology could be observed between the transfected and wild-type isolates (Fig. 30). During the first 2-4 days of growth, there were no observable differences in morphology between the transfected (CMW5588-DaRV) and wild-type (CMW5588-WT) isolates of *Phomopsis* sp. CMW5588. However, as the colonies aged, some differences became apparent. The mycelia of the DaRV-transfected fungus became more woolly and the mycelium clumped together into woolly tufts. The bundled mycelial tufts became so pronounced that the agar underneath was ultimately exposed. This was not observed for the negative control and wild-type isolates (Fig. 30A).

The transfected (CMW5587-DaRV) isolate of *D. ambigua* CMW5587 was more vigorous in growth than the wild type (CMW5587-WT) and grew evenly over the whole surface of the agar plate. Furthermore, this strain had a mycelial mat that appeared more dense on the plate than the wild-type fungus. The negative control (CMW5587-H_2O) and the wild-type isolates were fluffy with some sectors on the plates appearing fluffier that other sectors (Fig. 30B).

The transfected (CMW5287-DaRV) isolate of *D. ambigua* CMW5287 was also different from the negative control (CMW5287-H_2O) and the wild-type (CMW5287-WT) isolates (Fig. 30C). The colonies resembled those of isolate CMW5588 of *Phomopsis* sp. The transfected isolate grew into woolly aerial tufts of mycelia. As

observed for *Phomopsis* sp. CMW5588, the clumping of aerial mycelia into tufts exposed the agar underneath.

The transfected (CMW5288-DaRV) isolate of *D. ambigua* CMW5288 was morphologically different from the wild-type (CMW5288-WT) isolate (Fig. 30D). The transfectant has a fluffy appearance and is almost white in colour. This is in contrast to the other two isolates which had some yellowish pigmentation. Additionally, this isolate seemed to grow in zones with mycelia growing even fluffier between the zones. The wild-type fungus had some orange pigmentation and its mycelium was thick and densely appressed to the agar.

2.3.6.2. Growth rates of *Phomopsis* sp. and *D. ambigua* isolates

In order to assess the impact of DaRV on its new transfected hosts, the growth rates of all the transfected fungal isolates was measured at 15, 20, 25 and 30 °C. In addition, growth within the first two days was compared between the fungi. The growth rate data did not meet assumptions of parametric testing because the data were not normally distributed but heavily left skewed. These data had to be log(10) transformed to normalise their distribution. Significant differences in growth rates were observed among the different isolates.

The growth studies demonstrated that the naturally-infected *D. perjuncta* CMW3407 generally grows faster within the first two days than the transfected, the negative control and wild-type isolates at 15 °C and 20 °C (Table 11). At these temperatures, the negative control isolates of *D. ambigua* (CMW5587-H_2O, CMW5287-H_2O and CMW5288-H_2O) showed less growth after two days and slower growth rate than the isogenic virus-infected and the wild-type isolates. No growth was observed for the naturally-infected *D. perjuncta* CMW3407 isolate at 30 °C.

The DaRV-transfected (CMW5588-DaRV) isolate of *Phomopsis* sp. CMW5588 grew significantly faster after two days than both the negative control (CMW5588-H_2O) and the wild-type (CMW5588-WT) isolates at all temperatures (Table 11). This isolate (CMW5588-DaRV) also had a higher growth rate than the isogenic negative

control and the wild-type isolates at 15 °C to 20 °C. However, at 25 °C to 30 °C, the wild-type isolate grew faster than both the virus-infected and the negative control isolates.

The negative control (CMW5587-H_2O) isolate of *D. ambigua* CMW5587 had the slowest growth after two days and a lower growth rate than both the transfected (CMW5587-DaRV) and the wild-type (CMW5587-WT) isolates at all temperatures (Table 11). At 15 °C to 20 °C, both the transfected and the wild-type isolates had more or less the same growth after two days and growth rates. At 25 °C to 30 °C, the transfected isolate had higher growth after two days and higher growth rate than the wild-type isolate.

As was observed for the negative control (CMW5587-H_2O) isolate of *D. ambigua* CMW5587, the negative control isolate (CMW5287-H_2O) of *D. ambigua* CMW5287 had a slower growth after two days than both the transfected (CMW5287-DaRV) and the wild-type (CMW5287-WT) isolates at all temperatures (Table 11). These three isolates had more or less the same growth rate over all the temperatures. At 25 °C and 30 °C the transfected isolate showed a nominally slower growth rate than the negative control and the wild-type isolates.

The wild-type isolate (CMW5288-WT) of *D. ambigua* CMW5288 achieved a higher growth after two days than both the negative control (CMW5288-H_2O) and the transfected (CMW5288-DaRV) isolates at 15 °C, 20 °C and 25 °C. At 30 °C, the three isolates had more or less the same growth after two days (Table 11). The wild-type isolate had a significantly higher growth rate than the negative control and the transfected isolates at 15 °C.

2.3.6.3. Virus transfer to spores

One of the effects of hypoviruses on their hosts is reduction of sporulation (Elliston, 1985a,b). Additionally, it has been shown that since the virus-infected isolates have the replicating virus in their cytoplasm and not in their nuclei, the ascopore progeny of a sexual cross do not harbour the virus (Chen *et al.*, 1996). Sporulation experiments

were carried out to investigate if the transfected isolates could sporulate. Furthermore, if the isolates sporulated it was of importance to investigate if the spore cultures still contained the virus. After three months of inoculation, perithecia could be observed on the surface of the pieces of apple stems on water agar. The DaRV-transfected *D. ambigua* isolates were not found to sporulate while the DaRV-transfected *Phomopsis* sp. CMW5588 sporulated. The black perithecia of this isolate was broken with a sharp sterile needle. The spore mass at the tip of the needle was transferred onto PDA media. Isolation of dsRNA from the mycelia resulting from the spore mass of this isolate (CMW5588-DaRV) did not show a distinct dsRNA band similar in size to DaRV. RT-PCR amplification using the primer pair Oli64/Oli80 did not result in a fragment of around 600 bp which is characteristic of DaRV.

2.3.6.4. Virulence tests on Golden Delicious apples

Necrotic lesions were observed on all the apples inoculated with different *Phomopsis* sp. and *D. ambigua* isolates (Fig. 31-34). However, there were no necrotic lesions on apples inoculated with sterile PDA agar block which served as negative control. Since the lesion area data did not meet assumptions of parametric testing as the data sets were heavily left skewed, the data were log (10) transformed to normalise their distribution. Significant differences were observed for the different isolates inoculated ($F=40.34$, $dF=13$, $p<0.01$).

The naturally-infected *D. perjuncta* CMW3407 produced the smallest necrotic lesions of all the isolates (Table 12). The DaRV-transfected (CMW5588-DaRV) isolate of *Phomopsis* sp. CMW5588 caused significantly larger lesions (919 ± 107.8 mm^2) than the isogenic negative control (169 ± 30.4 mm^2) or the wild-type (455.5 ± 116.5 mm^2) isolates on the apples ($F=21.57$, $dF=4$, $p<0.01$) (Fig. 31 and 35). A similar result was observed for the DaRV-transfected (CMW5587-DaRV) isolate of *D. ambigua* CMW5587 ($F=17.61$, $dF=4$, $p<0.01$) (Fig. 32 and 36). The lesion caused by CMW5587-DaRV was 297.7 ± 22.3 mm^2 while the lesions caused by CMW5587-H_2O and CMW5587-WT were 171.2 ± 2.5 mm^2 and 265.7 ± 33.0, respectively. In these two cases, it was observed that there was no significant difference in virulence

between the negative control fungus and the naturally-infected *D. perjuncta* CMW3407. In the case of *D. ambigua* CMW5287, there were no significant differences in virulence between the DaRV-transfected (CMW5287-DaRV), the negative control (CMW5287-H_2O) and the wild-type (CMW5287-WT) isolates ($F=25.74$, $dF=4$, $p<0.01$) (Fig. 33 and 37). These three isolates were significantly more virulent than the naturally-infected *D. perjuncta* CMW3407. The transfected *D. ambigua* CMW5288 did not differ significantly in virulence from the isogenic wild-type (CMW5288-WT) isolate (Fig. 34 and 38). The negative control (CMW5288-H_2O) isolates, however, formed significantly larger lesions than the DaRV-transfected (CMW5288-DaRV) and the wild-type (CMW5288-WT) isolates.

2.3.6.5. Virulence tests on apple trees

Apple trees inoculated with *Phomopsis* sp. and *D. ambigua* isolates did not display necrotic lesions on the bark three months after inoculation. When the bark was stripped off to expose the cambium, extensive discolouration of the underlying cambial and cortical tissues was observed. The discolouration extended in both the distal and proximal directions from the point of inoculation. The lesion size data set did not meet assumptions of parametric testing because the data set was heavily left skewed. Therefore, the data was log (10) transformed to normalise its distribution. There were significant differences among the different isolates ($F=28.14$, $dF=13$, $p<0.01$) (Table 13; Fig. 39).

The wild-type (CMW5588-WT) isolate of *Phomopsis* sp. CMW5588 did not form significantly larger lesions (40.2 ± 10.2 mm) than the DaRV-transfected (36.4 ± 13.6 mm) isolate (CMW5588-DaRV). The wild-type (CMW5588-WT) isolate formed significantly larger lesions than the negative control (CMW5588-H_2O) isolate (27.1 ± 2.9 mm). There were no significant differences between the virus-transfected (CMW5587-DaRV) [40.3 ± 10.2 mm], the negative control (CMW5587-H_2O) [35.0 ± 9.7 mm] and the wild-type (CMW5587-WT) [39.1 ± 13.1 mm] isolates of *D. ambigua* CMW5587.

D. ambigua CMW5287 showed a different pattern from *Phomopsis* sp. CMW5588 and *D. ambigua* CMW5587. The DaRV-transfected (CMW5287-DaRV) *D. ambigua* CMW5287 formed significantly larger lesions (47.7 ± 5.7 mm) than the negative control (CMW5287-H_2O) (23.0 ± 8.7 mm) and the wild-type (CMW5287-WT) isolates (22.7 ± 16.4 mm). There were no significant differences in lesions sizes formed by the negative control (CMW5287-H_2O) and the DaRV-transfected (CMW5287-DaRV) isolates.

The wild-type (CMW5288-WT) isolate of *D. ambigua* CMW5288 formed the largest lesions (71.2 ± 22.2 mm) on apple tree stems when compared with all the other isolates. The lesions formed by the DaRV-transfected (CMW5288-DaRV) (23.1 ± 4.3 mm) and the negative control (CMW5288-H_2O) isolates (25.7 ± 8.8 mm) did not differ significantly. The naturally-infected *D. perjuncta* CMW3407 formed the smallest lesions of all the isolates. The ranking of the lesion data set showed that the lesions associated with this isolate ranked only higher than the lesions observed on trees inoculated with PDA agar disks.

The comparison of the data sets from the apple and tree inoculation techniques does not show any correlation except in one case. The DaRV-transfected (CMW5288-DaRV) isolate of *D. ambigua* CMW5288 displayed reduced virulence when compared to the wild-type (CMW5288-WT) isolate on both apples and apple trees. The DaRV-transfected (CMW5588-DaRV)isolate of *Phomopsis* sp. CMW5588 was more virulent on apples than the wild-type (CMW5588-WT) isolate. The virulence levels of these two isolates did not vary significantly on apple trees. An opposite pattern was observed for *D. ambigua* CMW5287. The isolate transfected with DaRV (CMW5287-DaRV) was more virulent on apples trees than the wild-type isolate (CMW5287-WT). There were no significant differences in virulence between these two isolates on apples. The DaRV-transfected (CMW5587-DaRV) and the wild-type (CMW5587-WT) isolates of *D. ambigua* CMW5587 did not differ significantly in virulence on both apple and apple trees.

2.3.6.6. Re-isolation of *Phomopsis* sp. and *D. ambigua* isolates from inoculated trees

The inoculated trees were characterised by discolouration of cambial and cortical tissues. To confirm the causative agent of the discolouration, fungal re-isolations were performed. Fungi were isolated from the edges of the inoculation points and from the farthest points of the streaks. Morphological examination confirmed that these isolated fungi were those used to inoculate the trees. DNA was isolated from these isolates. The primer pair ITS1 and ITS4 were used in a PCR to amplify fragments of about 450 bp. RFLPs with *Mse* I on these ITS1/ITS4 fragments produced the same RFLP profiles (Fig. 40A) as those shown in Fig. 24. This result provides clear evidence that the fungi used to inoculate the trees were the same as those responsible for the discolouration on the inoculated trees.

2.3.6.7. Isolation and purification of dsRNA from re-isolated fungi

After satisfying Koch's postulates, it was necessary to examine the four transfected isolates and the naturally-infected isolate for the presence of DaRV. Double-stranded RNA was isolated from these transfected isolates but not from the negative control and the wild-type isolates (Fig. 40B). RT-PCR using the DaRV-specific primer pair Oli64/Oli80 amplified fragments of 600 bp from the dsRNA preparation of the transfected and the naturally-infected isolates but not from the negative control and the wild-type isolates (Fig. 40C).

2.3.6.8. Phenol oxidase reaction/Gallic acid oxidation

The naturally-infected *D. perjuncta* CMW3407 did not show any colour reaction when tested on both Bavendamm media containing tannic acid and gallic acid. The transfected (CMW5288-DaRV) isolate of *D. ambigua* CMW5288 did not give a colour reaction on these media. The transfected (CMW5588-DaRV) isolate of *Phomopsis* sp. CMW5588 and the other two transfected (CMW5287-DaRV and CMW5587-DaRV) *D. ambigua* isolates gave a weak colour reaction. The wild-type

virus-free isolates of *Phomopsis* sp. (CMW5588-WT) and the *D. ambigua* isolates (CMW5287-WT, CMW5587-WT and CMW5288-WT) gave weak colour reactions on the two media.

2.3.7. Southern blot analysis of the genomic DNA of *Phomopsis* sp. and *D. ambigua* isolates

The naturally-infected isolate of *D. perjuncta* CMW3407 displayed the typical symptoms of mycovirus-infected fungi. However, the transfected isolates did not display such symptoms. Homologous Southern blot hybridisations were carried out in order to confirm the absence of any DNA copy of DaRV in the genome of the naturally-infected isolate (Fig. 41). The low and high stringency hybridisations did not reveal any bands on the membrane-blotted DNA of the fungus. The DIG-labelled cDNA of DaRV readily hybridised to the isolated dsRNA at all stringencies. This was clear evidence that DaRV does not originate from the genome of the naturally-infected fungus but that it is a typical RNA virus without DNA stage as it is also true for all plant tombusviruses.

Table 8. Sequence differences between the published sequence of the *Diaporthe ambigua RNA virus* (DaRV) and the corresponding full-length cDNA clone of DaRV genome constructed from independently extracted dsRNA.

Base change	DaRV sequence (Preisig *et al.*, 2000)	DaRV cDNA sequence (pDV3 sequence)	Amino acid change
G^{389}-A			UTR*
G^{1743}-A	GAG	AAG	E-K

*This base change occurred in the 5' UTR.

Table 9. Sequence differences between the published sequence of the *Sphaeropsis sapinea RNA virus 1* (SsRV1) and the corresponding full-length cDNA clone of SsRV1 genome constructed from independently extracted dsRNA.

Base change	SsRV1 sequence (Preisig *et al.*, 1998)	SsRV1 cDNA sequence (pNM1-5 sequence)	Amino acid change
T^{30}-C			UTR*
G^{809}-C	GAC	CAC	D-H
T^{1026}-G	CTC	CGC	L-R
G^{1475}-A	GCG	ACG	A-T
C^{2111}-T	CGT	TGT	R-C
A^{1579}-G	TTA	TTG	L
T^{2454}-C	CTT	CCT	L-P
G^{2505}-C	TGT	TCT	C-S
G^{2512}-C	CAG	CAC	Q-H
G^{2515}-C	GAG	GAC	E-D
A^{2855}-T	GTA	GTT	V
A^{3832}-G	AAA	AGA	K-R
G^{3913}-A	GGT	GAT	G-D
C^{4011}-T	CCA	TCA	P-S

* This base change occurred in the 5' UTR.

Table 10. Sequence differences between the published sequence of the *Sphaeropsis sapinea RNA virus 2* (SsRV2) and the corresponding full-length cDNA clone of SsRV1 genome constructed from independently extracted dsRNA.

Base change	SsRV2 sequence (Preisig *et al.*, 1998)	SsRV2 cDNA sequence (pNM2-5 sequence)	Amino acid change
C^{328}-T	TAC	TAT	Y
T^{382}-C	GCT	GCC	A
T^{405}-C	CGT	CGC	R
T^{520}-C	CCT	CCC	P
T^{562}-C	AAT	AAC	N
C^{652}-T	GCC	GCT	A
C^{675}-G	GCG	GGG	A-G
C^{691}-T	TAC	TAT	Y
T^{701}-C	TTA	CTA	L
T^{736}-C	ATT	ATC	I
T^{760}-C	CTT	CTC	L
G^{766}-A	TCG	TCA	S
G^{775}-A	ACG	ACA	T
G^{778}-T	GCG	GCT	A
C^{814}-T	GAC	GAT	D
C^{845}-T	CTA	TTA	L
C^{856}-T	GCC	GCT	A
T^{868}-C	GCT	GCC	A
A^{880}-C	GTA	GTC	V
C^{886}-T	ACC	ACT	T
C^{892}-T	GTC	GTT	V
G^{928}-A	GTG	GTA	V
C^{946}-G	GCC	GCG	A

Base change	SsRV2 sequence (Preisig *et al.*, 1998)	SsRV2 cDNA sequence (pNM2-5 sequence)	Amino acid change
C^{988}-T	GGC	GGT	G
A^{991}-T	GCA	GCT	A
T^{1021}-C	GTT	GTC	V
C^{1039}-A	ACC	ACA	T
T^{1045}-C	GCT	GCC	A
A^{1055}-G	ATC	GTC	I-V
C^{1087}-T	GGC	GGT	G
T^{1090}-C	GGT	GGC	G
G^{1102}-A	GGG	GGA	G
T^{1120}-C	GCT	GCC	A
T^{1159}-C	CGT	CGC	R
G^{1180}-A	CCG	CCA	P
C^{1195}-T	GCC	GCT	A
T^{1201}-C	AGT	AGC	S
A^{1231}-G	GCA	GCG	A
C^{1282}-T	GCC	GCT	A
G^{1288}-A	GGG	GGA	G
A^{1297}-G	CCA	CCG	P
C^{1303}-T	GTC	GTT	V
T^{1318}-A	GGT	GGA	G
T^{1330}-C	GCT	GCC	A
G^{1348}-A	GAG	GAA	E
G^{1349}-A	GGC	AGC	G-S
C^{1372}-T	GCC	GCT	A
C^{1399}-T	GCC	GCT	A
C^{1414}-T	TTC	TTT	F
T^{1422}-C	ATG	ACT	M-T

Base change	SsRV2 sequence (Preisig *et al.*, 1998)	SsRV2 cDNA sequence (pNM2-5 sequence)	Amino acid change
G^{1423}-T	"	"	"
A^{1453}-G	GGA	GGG	G
G^{1462}-A	TCG	TCA	S
A^{1495}-G	ACA	ACG	T
T^{1510}-C	TAT	TAC	Y
C^{1528}-T	CAC	CAT	H
T^{1552}-C	CAT	CAC	H
G^{1564}-A	GCG	GCA	A
C^{1579}-T	GTC	GTT	V
C^{1594}-T	CTC	CTT	L
T^{1621}-C	GCT	GCC	A
A^{1624}-T	GCA	GCT	A
T^{1633}-C	GCT	GCC	A
T^{1636}-C	GAT	GAC	D
C^{1639}-T	TAC	TAT	Y
T^{1645}-C	GCT	GCC	A
T^{1657}-C	CCT	CCC	P
T^{1663}-C	GGT	GGC	G
C^{1699}-T	CGC	CGT	R
T^{1711}-C	CAT	CAC	H
T^{1720}-C	ACT	ACC	T
C^{1729}-T	CAC	CAT	H
T^{1756}-C	TCT	TCC	S
C^{1774}-T	TTC	TTT	F
C^{1786}-T	TAC	TAT	Y
T^{1804}-C	GTT	GTC	V
C^{1807}-T	CTC	CTT	L

Base change	SsRV2 sequence (Preisig *et al.*, 1998)	SsRV2 cDNA sequence (pNM2-5 sequence)	Amino acid change
T^{1810}-C	GGT	GGC	G
T^{1840}-C	GCT	GCC	A
G^{1843}-A	TCG	TCA	S
T^{1870}-C	CCT	CCC	P
C^{1876}-T	AAC	AAT	N
C^{1882}-T	ACC	ACT	T
G^{1900}-A	GCG	GCA	A
T^{1912}-C	CCT	CCC	P
T^{1913}-C	TTG	CTG	L
A^{1936}-C	CGA	CGC	R
C^{1939}-T	GGC	GGT	G
G^{1942}-A	CAG	CAA	Q
T^{1969}-C	TTT	TTC	F
C^{1975}-T	AAC	AAT	N
C^{1993}-T	GGC	GGT	G
G^{2119}-A	GGG	GGA	G
C^{2161}-T	ACC	ACT	T
G^{2212}-A	GGG	GGA	G
G^{2224}-A	TCG	TCA	S
A^{2239}-T	ATA	ATT	I
A^{2251}-G	CCA	CCG	P
T^{2257}-C	GAT	GAC	D
T^{2296}-C	AAT	AAC	N
T^{2305}-C	TTT	TTC	F
C^{2353}-T	GCC	GTC	A-V
C^{2355}-T	GCC	GTC	A-V
G^{2468}-A	GCC	ATG	A-M

Base change	SsRV2 sequence (Preisig *et al.*, 1998)	SsRV2 cDNA sequence (pNM2-5 sequence)	Amino acid change
C^{2469}-T	"	"	"
C^{2470}-G	"	"	"
Insertion^{2484}	CTC	CCT	L-P
T^{2726}-C	CTT	CTC	L
T^{2760}-C	TTG	CTG	L
A^{2913}-C	AAC	CAC	N-H
A^{3014}-T	CCA	CCT	P
A^{3148}-C	CTA	CTC	L
A^{3244}-C	GAT	GCT	D-A
G^{3275}-C	GCG	GCC	A
A^{3545}-T	TCA	TCT	S
T^{3572}-C	CAT	CAC	H
T^{3581}-C	GCT	GCC	A
C^{3587}-T	CCC	CCT	P
G^{3618}-C	GCG	CGG	A-R
C^{3619}-G	"	"	"
G^{3621}-T	GTC	TCA	V-S
T^{3622}-C	"	"	"
C^{3623}-A	"	"	"
A^{3624}-T	ATG	TGG	M-W
T^{3625}-G	"	"	"
G^{3627}-C	GCT	CTC	A-L
C^{3628}-T	"	"	"
T^{3629}-C	"	"	"
C^{3630}-G	CGA	GAG	R-E
G^{3631}-A	"	"	"
A^{3632}-G	"	"	"

Base change	SsRV2 sequence (Preisig *et al.*, 1998)	SsRV2 cDNA sequence (pNM2-5 sequence)	Amino acid change
G^{3633}-A	GAG	AGA	E-R
G^{3634}-A	"	"	"
G^{3635}-A	"	"	"
A^{3636}-G	AGT	GTT	S-V
G^{3637}-T	"	"	"
T^{3689}-A	TCT	TCA	S
G^{3706}-A	GGG	GAG	G-E
C^{3888}-T	CTG	TTG	L
G^{4075}-A	CGC	CAC	R-H
G^{4096}-A	AGC	AAC	S-N
G^{4317}-A	GGT	AGT	G-S
A^{4932}-C	AGG	CGG	R
C^{4948}-T	TCT	TTC	S-F
T^{4949}-C	"	"	"

Table 11. Growth achieved within the first two days (mm) and growth rates (mm/day) of virus-transfected (DaRV), negative control (H_2O) and wild-type (WT) *Phomopsis* sp. and *D. ambigua* isolates. The naturally-infected isolate of *D. perjuncta* was also included in the experiment.

Isolate	Growth after 2 days	Growth rate 15 °C	Growth after 2 days	Growth rate 20 °C	Growth after 2 days	Growth rate 25 °C	Growth after 2 days	Growth rate 30 °C
CMW5588-DaRV	7.62 ± 0.28	7.06 ± 0.33	12.05 ± 0.77	12.37 ± 0.38	27.95 ± 0.45	15.86 ± 0.54	31.28 ± 0.83	11.70 ± 0.37
CMW5588-H_2O	6.8 ± 0.01	3.69 ± 0.33	11.28 ± 2.31	6.33 ± 1.11	18.05 ± 1.87	3.10 ± 0.75	12.13 ± 1.87	2.60 ± 0.64
CMW5588-WT	5.0 ± 0.01	3.48 ± 0.58	5.05 ± 0.01	9.77 ± 0.41	12.63 ± 2.50	17.97 ± 1.55	15.18 ± 1.49	14.78 ± 1.67
CMW5587-DaRV	8.78 ± 0.57	8.78 ± 0.24	10.89 ± 0.41	12.18 ± 0.26	16.20 ± 0.89	21.40 ± 0.33	19.95 ± 1.46	24.58 ± 0.98
CMW5587-H_2O	7.00 ± 0.01	5.64 ± 0.54	7.37 ± 0.36	9.72 ± 0.56	11.92 ± 0.28	14.59 ± 0.25	11.91 ± 0.27	16.50 ± 0.71
CMW5587-WT	8.32 ± 1.03	9.45 ± 0.23	9.75 ± 0.47	13.27 ± 0.26	14.01 ± 0.77	13.27 ± 0.26	12.73 ± 0.90	23.07 ± 0.36
CMW5287-DaRV	14.22 ± 1.62	10.68 ± 0.75	21.20 ± 1.20	14.36 ± 0.35	35.98 ± 4.70	20.33 ± 1.53	50.92 ± 4.01	14.46 ± 1.45
CMW5287-H_2O	10.00± 0.01	9.59 ± 0.81	14.56 ± 1.73	15.56 ± 0.79	29.53 ± 0.49	23.87 ± 0.73	41.36 ± 2.01	19.16 ± 1.61
CMW5287-WT	12.53 ± 1.28	10.26 ± 0.60	16.83 ± 2.28	14.94 ± 0.98	30.30 ± 1.80	22.45 ± 0.60	43.40 ± 1.82	18.42 ± 1.76
CMW5288-DaRV	12.98 ± 0.65	3.75 ± 0.32	16.42 ± 1.20	7.68 ± 0.85	19.51 ± 10.6	9.33 ± 0.99	29.85 ± 3.79	16.55 ± 1.66
CMW5288-H_2O	9.40 ± 0.45	3.43 ± 0.33	11.79 ± 1.22	9.61 ± 0.63	21.22 ± 1.40	20.54 ± 0.59	28.31 ± 1.04	22.63 ± 0.68
CMW5288-WT	14.66 ± 1.46	6.91 ± 0.36	19.63 ± 1.86	9.58 ± 0.69	27.56 ± 0.73	12.95 ± 0.91	27.56 ± 0.73	15.41 ± 0.63
CMW3407	17.22 ± 1.00	6.93 ± 0.34	22.76 ± 2.17	8.73 ± 0.65	26.06 ± 1.04	8.64 ± 0.70	0.0	0.0

The isolate numbers followed by DaRV are the transfected isogenic strains of the wild-type (isolate numbers followed by WT) and water controls (isolate numbers followed by H_2O). CMW3407 represents the naturally-infected *D. perjuncta* CMW3407.

Table 12. Virulence of *Phomopsis* sp. and *D. ambigua* isolates assessed by apple inoculation technique. Mean lesion areas on Golden Delicious apples after inoculation with *Phomopsis* sp. and *D. ambigua* isolates.

Isolate	Transfection	Mean lesion area (mm^2)
CMW5588-DaRV	DaRV	919.8 ± 107.8
CMW5588-H_2O	H_2O	169.9 ± 30.4
CMW5588-WT	-	455.5 ± 116.5
CMW5587-DaRV	DaRV	297.7 ± 22.3
CMW5587-H_2O	H_2O	171.2 ± 2.5
CMW5587-WT	-	265.7 ± 33.0
CMW5287-DaRV	DaRV	1188.9 ± 100.6
CMW5287-H_2O	H_2O	1027 ± 171.6
CMW5287-WT	-	1004.1 ± 107.2
CMW5288-DaRV	DaRV	267.1 ± 17.7
CMW5288-H_2O	H_2O	675.1 ± 180.7
CMW5288-WT	-	359.9 ± 39.9
CMW3407	+	194.6 ± 12.2

The isolate numbers followed by DaRV are the transfected isogenic strains of the wild-type (isolate numbers followed by WT) and water controls (isolate numbers followed by H_2O). CMW3407 represents the naturally-infected *D. perjuncta* CMW3407. The sign (-) denotes the wild-type isolate of the fungus while (+) denotes the naturally-infected *D. perjuncta* CMW3407. In addition to these 13 fungal isolates shown in the table, sterile PDA disks were also used to inoculate apples.

Table 13. Virulence of *Phomopsis* sp. and *D. ambigua* isolates assessed by apple tree inoculation technique. Mean lesion lengths on Golden Delicious apple trees crafted on M793 rootstocks three months after inoculation with *Phomopsis* sp. and *D. ambigua* isolates.

Isolate	Transfection	Mean lesion length (mm)
CMW5588-DaRV	DaRV	36.4 ± 13.6
CMW5588-H_2O	H_2O	27.1 ± 2.9
CMW5588-WT	-	40.2 ± 10.2
CMW5587-DaRV	DaRV	40.3 ± 10.2
CMW5587-H_2O	H_2O	35.0 ± 9.7
CMW5587-WT	-	39.1 ± 13.1
CMW5287-DaRV	DaRV	47.7 ± 5.7
CMW5287-H_2O	H_2O	23.0 ± 8.7
CMW5287-WT	-	22.7 ± 16.4
CMW5288-DaRV	DaRV	23.1 ± 4.3
CMW5288-H_2O	H_2O	25.7 ± 3.8
CMW5288-WT	-	71.2 ± 22.2
CMW3407	+	21.7 ± 2.9
Agar		19.2 ± 2.6

The isolate numbers followed by DaRV are the transfected isogenic strains of the wild-type (isolate numbers followed by WT) and water controls (isolate numbers followed by H_2O). CMW3407 represents the naturally-infected *D. perjuncta* CMW3407. The sign (-) denotes the wild-type isolate of the fungus while (+) denotes the naturally-infected *D. perjuncta* CMW3407. In addition to these 13 fungal isolates shown in the table, sterile PDA disks were also used to inoculate apple trees.

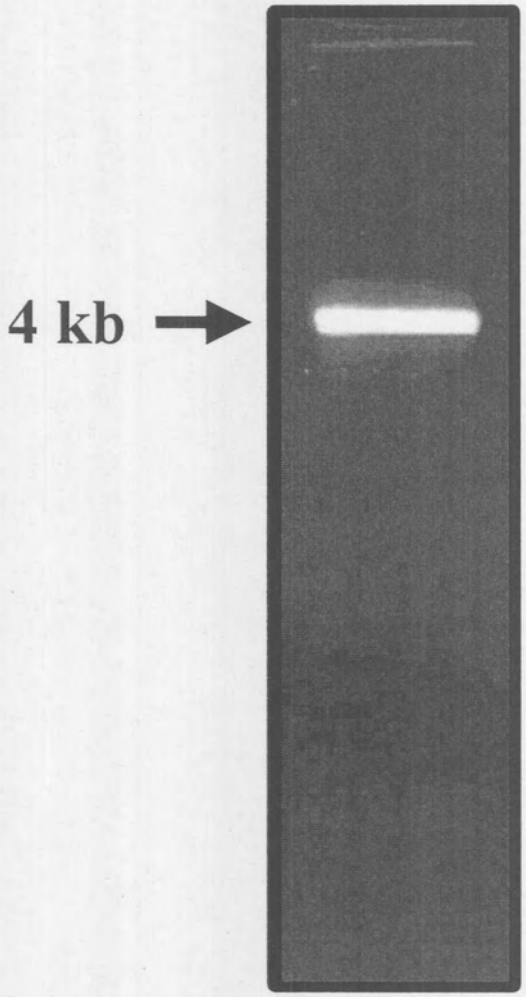


Fig. 6. Agarose gel electrophoresis of the single dsRNA element of the *Diaporthe ambigua RNA virus* (DaRV). The dsRNA of the virus was purified on CF 11 column chromatography. The 1 % agarose gel was stained with ethidium bromide.

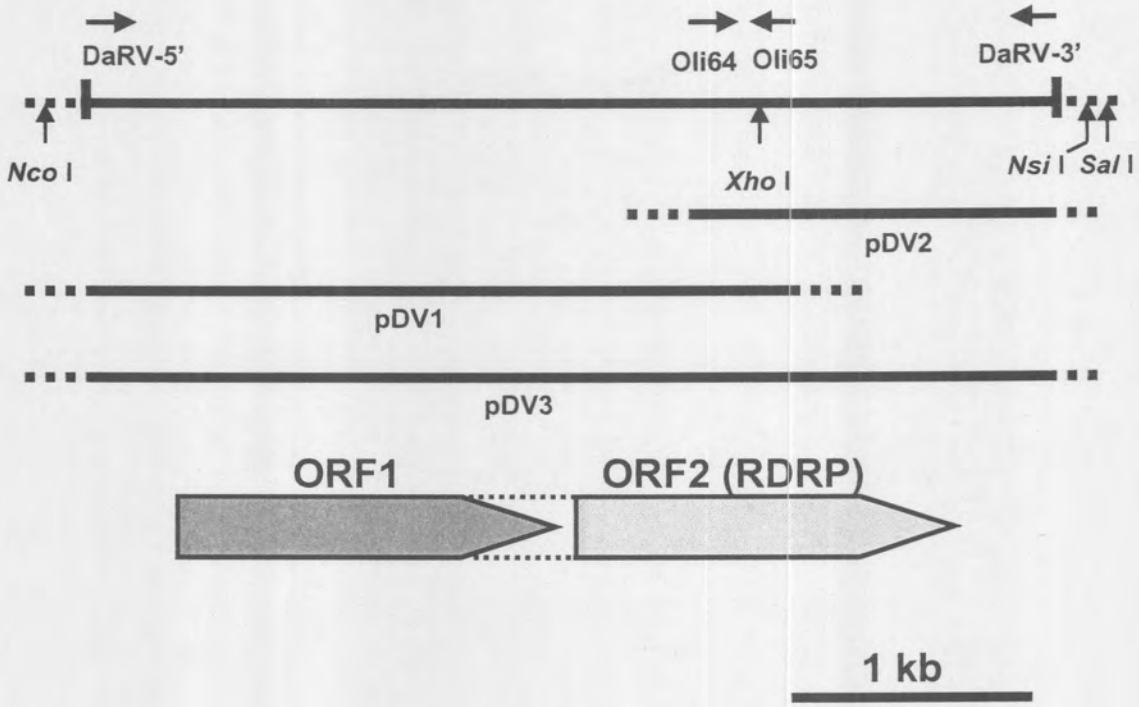


Fig. 7. Construction scheme for the full-length cDNA clone of the genome of the *Diaporthe ambigua RNA virus*, DaRV. The full-length cDNA clone was achieved by obtaining two subclones, pDV1 with cloned DaRV-5'/Oli65 RT-PCR product and pDV2 with cloned Oli64/DaRV-3' RT-PCR product. The clone pDV1 was linearised with *Xho* I and *Nsi* I. The 1.46 kb *Xho* I/*Nsi* I fragment was excised from pDV2 and cloned into the linearised pDV1 resulting in pDV3. The dotted lines at the ends of the thick lines represent the multiple cloning site of pGEM T-Easy Vector. The restriction enzymes *Nco* I and *Nsi* I or *Sal* I were used to linearise the plasmid for the *in vitro* RNA production experiments. The thin dotted lines joining ORF1 and ORF2 indicate that the resulting protein is a ribosomal readthrough protein.

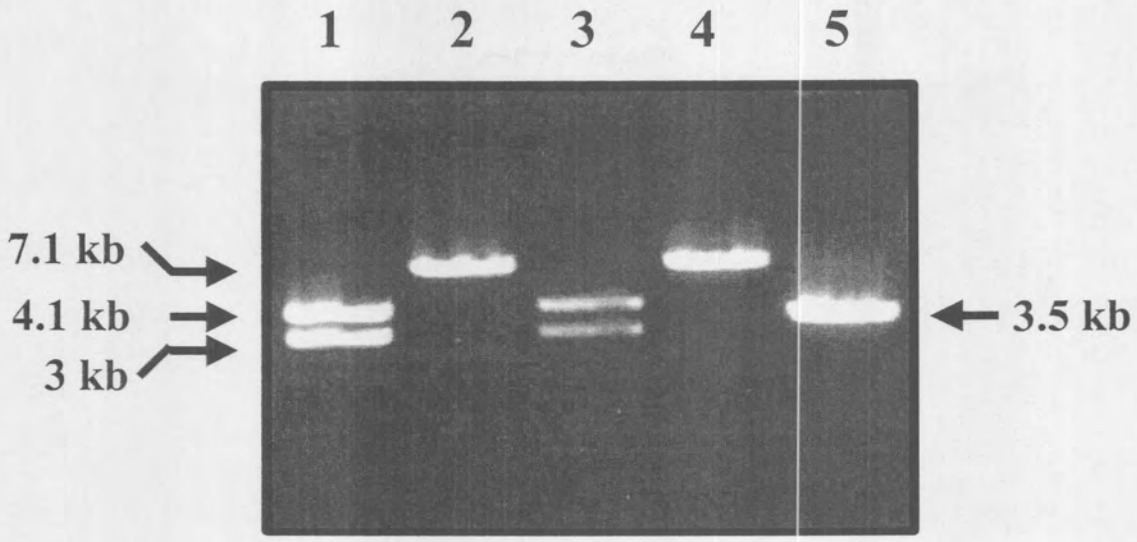


Fig. 8. Restriction digest analysis of pDV3 (lanes 1, 2 and 4) containing the wild-type *Diaporthe ambigua RNA virus* (DaRV) cDNA and pH6DV3 (lanes 3 and 5) containing the His-tagged full-length cDNA copy of the DaRV genome. Lane 1: pDV3 cut with *Eco* RI. This digestion liberates the 4.1 kb cDNA copy of DaRV from the 3 kb pGEM T-Easy Vector. Lane 2: pDV3 linearised with *Sal* I. Lane 3: pH6DV3 cut with the *Eco* RI. Lane 4: pH6DV3 linearised with *Sal* I. Lane 5: pH6DV3 cut with *Nsi* I. This digestion resulted in a double band at 3.5 kb. An *Nsi* I site (ATGCA↓T) was introduced by a His tag insertion at position 578 on the genome of DaRV. The 1 % agarose gel was stained with ethidium bromide.

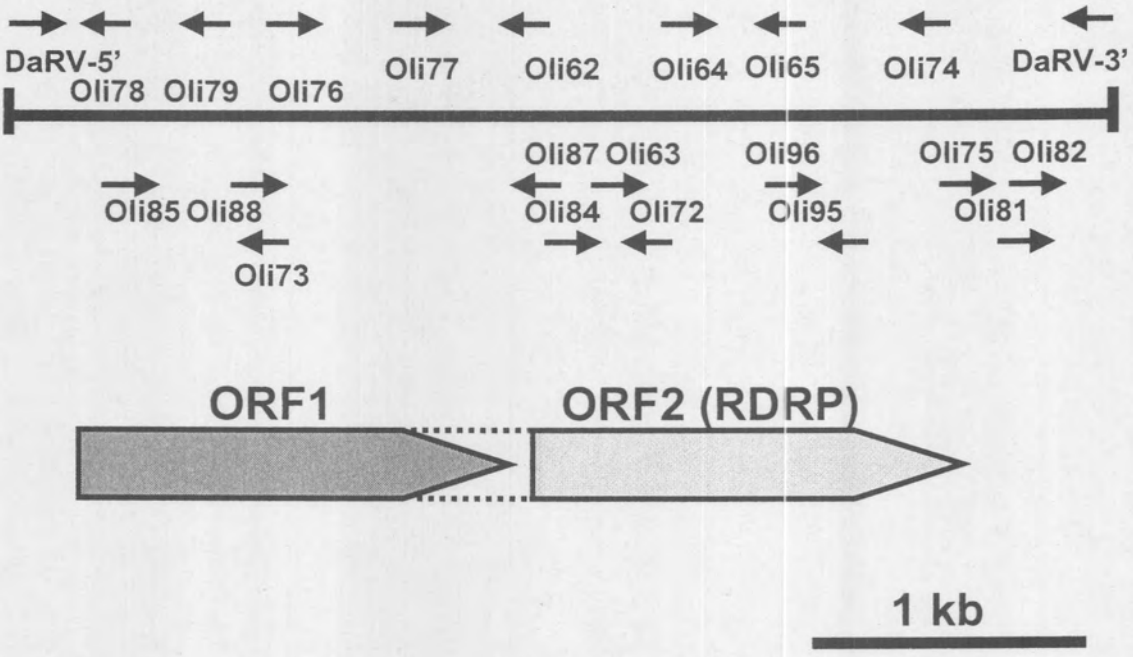


Fig. 9. A schematic representation of the genome of the *Diaporthe ambigua RNA virus*. The arrows indicate the position and orientation of the primers that were used in sequencing and cloning of the DaRV genome.

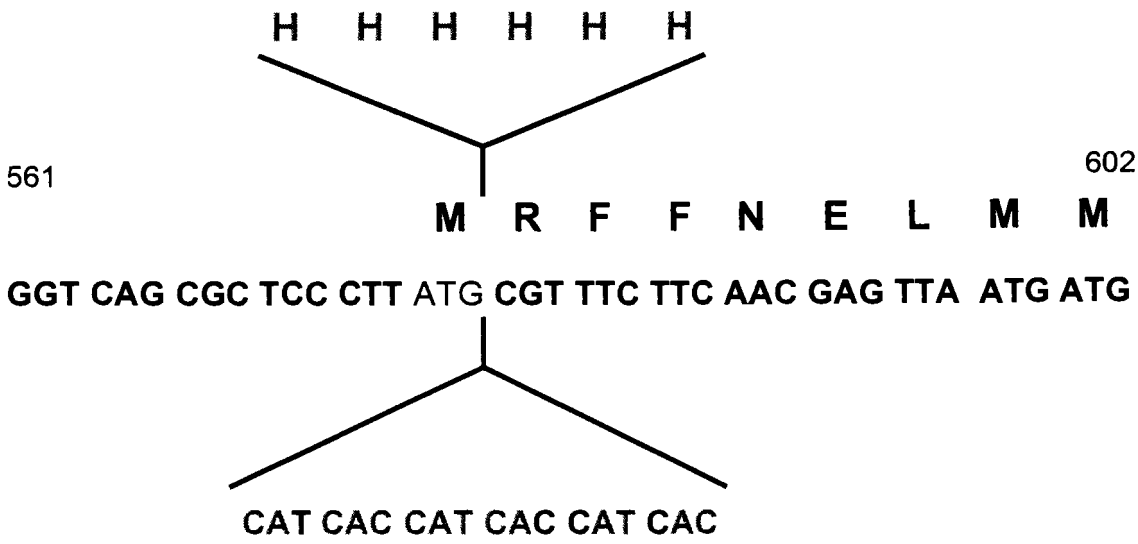
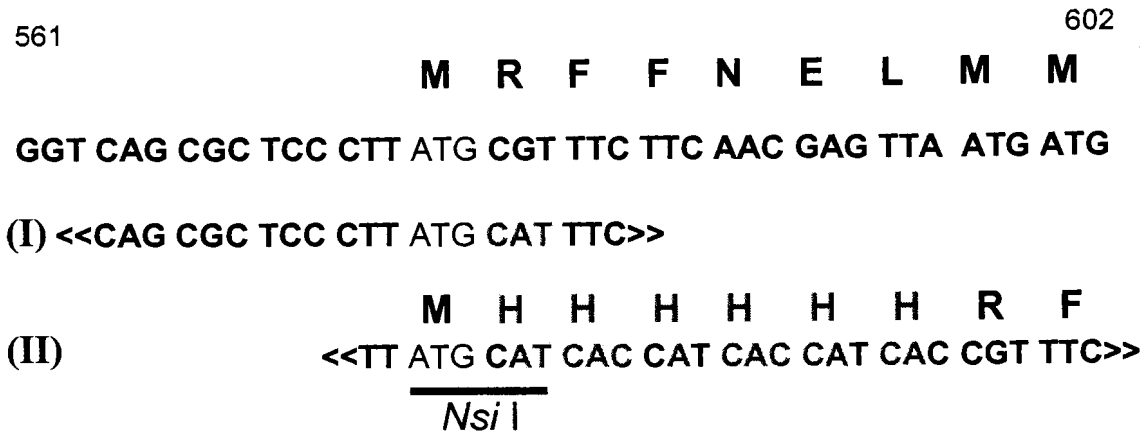


Fig. 10. A diagrammatic scheme showing the nucleotide position into which the His_6-tag was inserted into the cDNA copy of the genome of the *Diaporthe ambigua RNA virus* (DaRV). The construction gave rise to the plasmid pH6DV3. The six histidine codons were inserted immediately downstream of the putative start codon for ORF1 at nucleotide position 576-578. The new clone became 18 nucleotides longer than the wild-type DaRV.



DaRV-H6-FW

5'TT ATG CAT CAC CAT CAC CAT CAC CGT TTC TTC AAC GAG TTA ATG ATG 3'

DaRV-H6-RV

5'GAA ATG CAT AAG GGA GCG CTG 3'

Fig. 11. A diagrammatic scheme showing the primer design and PCR approach to insert a His_6-tag to the N-terminus of ORF1 gene product of the *Diaporthe ambigua RNA virus* (DaRV) genome. The reverse primer (DaRV-H6-RV) was designed so that the second base of arginine codon was changed from guanine to adenine (G→A) thus introducing an *Nsi* I (ATGCA↓T) site into the primer. The forward primer, DaRV-H6-FW incorporated six histidine codons (in grey print) at its 5' end. I: shows the 3' end of the PCR product amplified with DaRV-5' and DaRV-H6-RV. II: shows the 5' end of the PCR product amplified with DaRV-H6-FW and DaRV-3'. The sequences of the primers are shown under DaRV-H6-FW and DaRV-H6-RV.

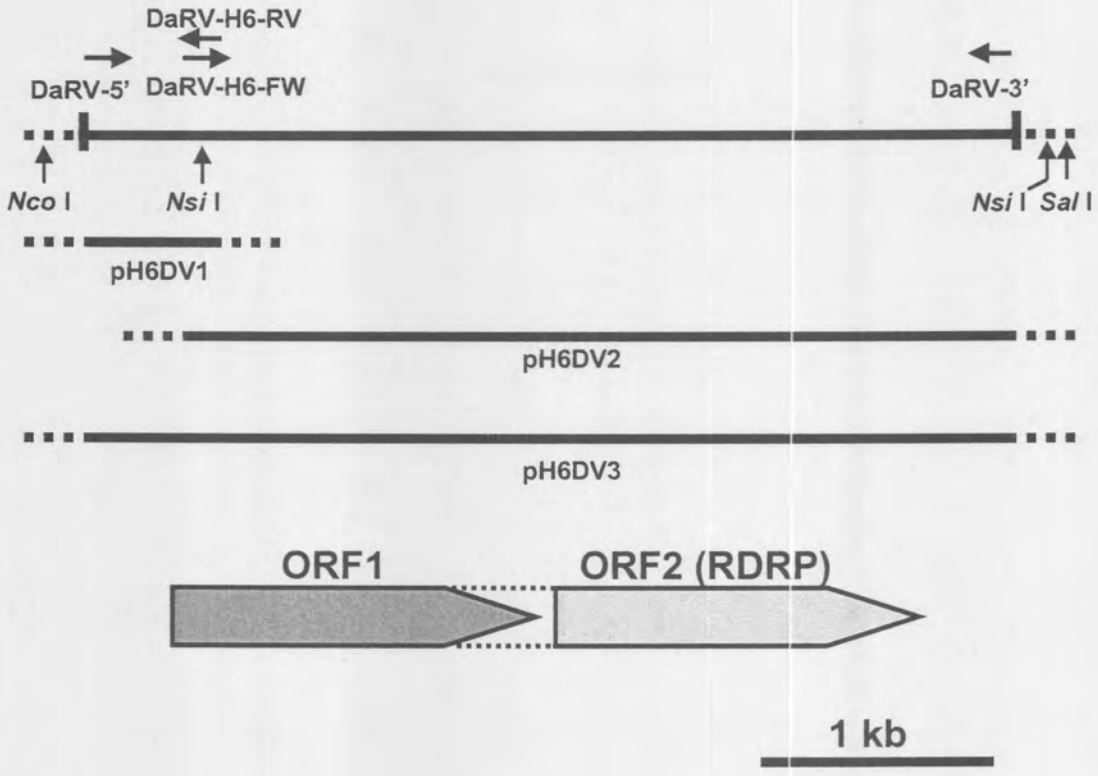


Fig. 12. Construction scheme of a His_6-tag mutant of the *Diaporthe ambigua RNA virus* (DaRV). The cloning was achieved by amplifying two large overlapping fragments from pDV3. The 570 bp DaRV-5'/DaRV-H6-RV PCR product was cloned into pH6DV1. The second clone, pH6DV2, contained the DaRV-H6-FW/DaRV3' PCR product as an insert. The sequence of the two primers DaRV-H6-RV and DaRV-H6-FW were altered so that an *Nsi* I site was created into their sequences. In addition to the *Nsi* I site, DaRV-H6-FW contained six histidine codons. The 3.6 kb *Nsi* I fragment was excised from pH6DV2 using *Nsi* I and subcloned into the *Nsi* I-linearised pH6DV1. This resulted in pH6DV3, the full-length cDNA copy of DaRV with introduced six histidine codons. The dotted lines at the end of the thick line represent the multiple cloning site of pGEM T-Easy Vector. The restriction enzymes *Nco* I or *Sal* I were used to linearise the plasmid for *in vitro* RNA production. The thin dotted lines joining ORF1 and ORF2 indicate that the resulting protein is a readthrough protein.

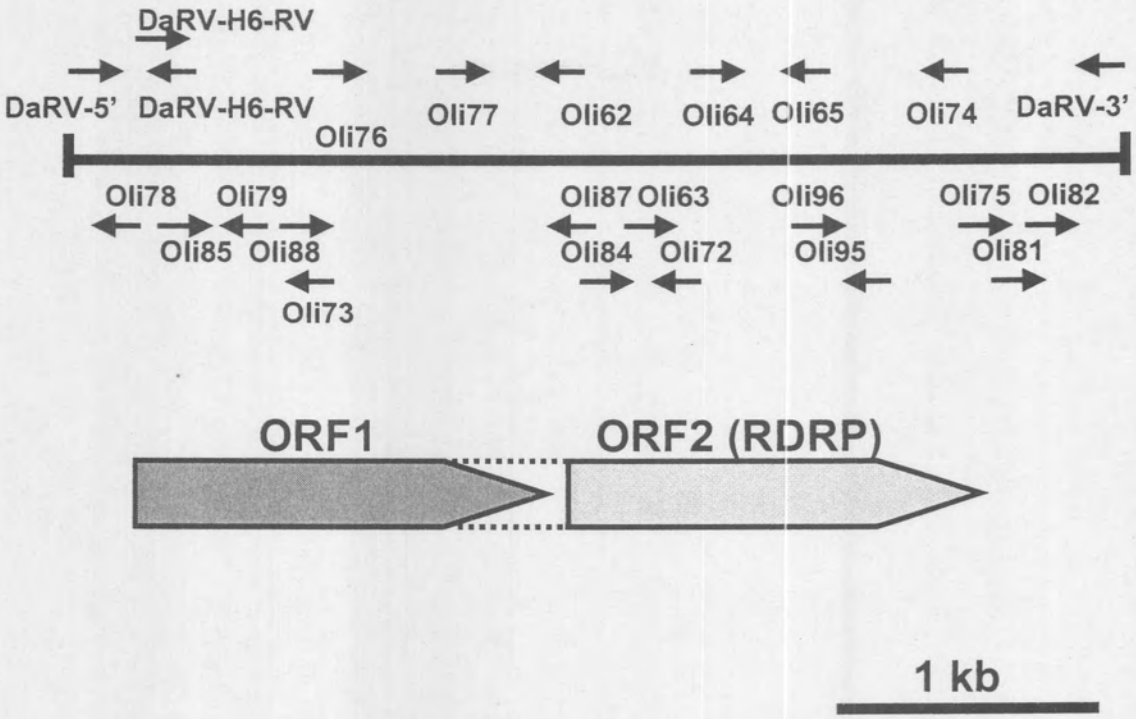


Fig. 13. A schematic representation of the genome of the *Diaporthe ambigua RNA virus* modified with a His_6-tag. The arrows indicate the position and orientation of the primers that were used in sequencing and cloning of the DaRV genome.

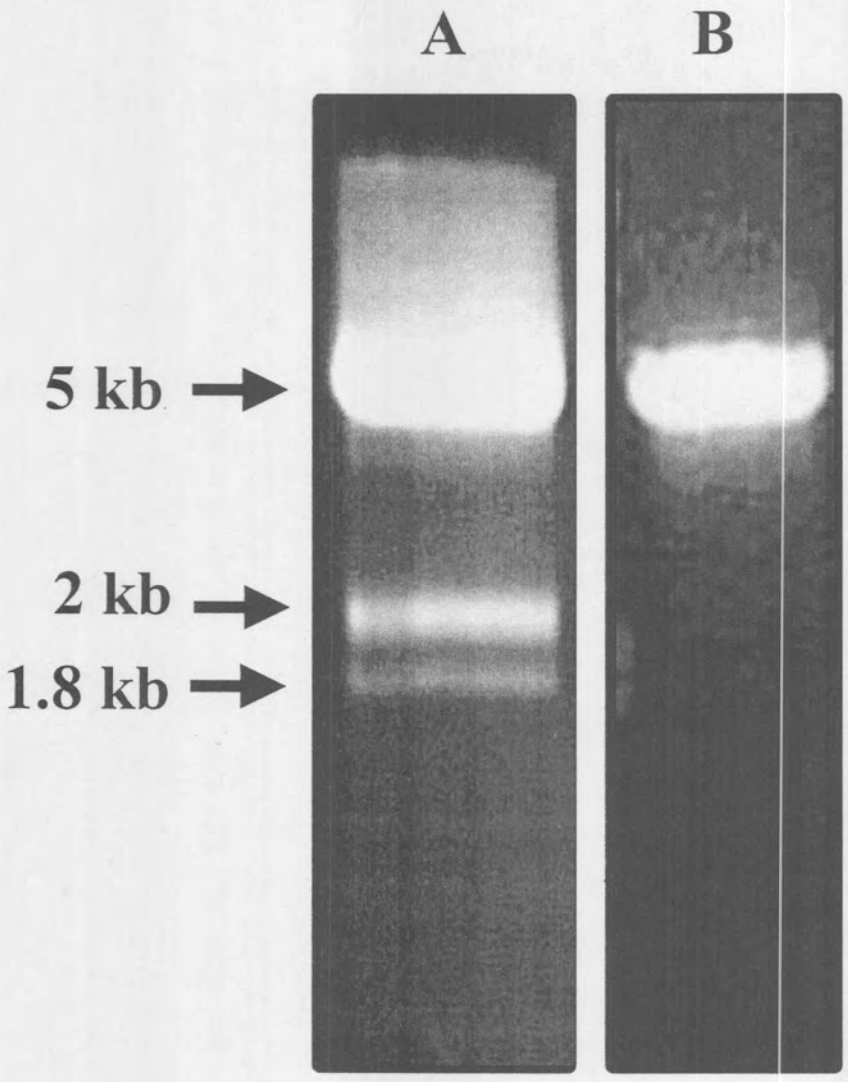


Fig. 14. The dsRNA elements isolated from *Sphaeropsis sapinea* CMW4254. The 5 kb band represents the mixture of the genomes of *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2). Double-stranded RNA was purified by CF 11 cellulose column chromatography and was separated on 1 % agarose gel stained with ethidium bromide. The origin of the additional bands of about 2 kb and 1.8 kb is unknown. A: a crude dsRNA extract and B: gel purified dsRNA.

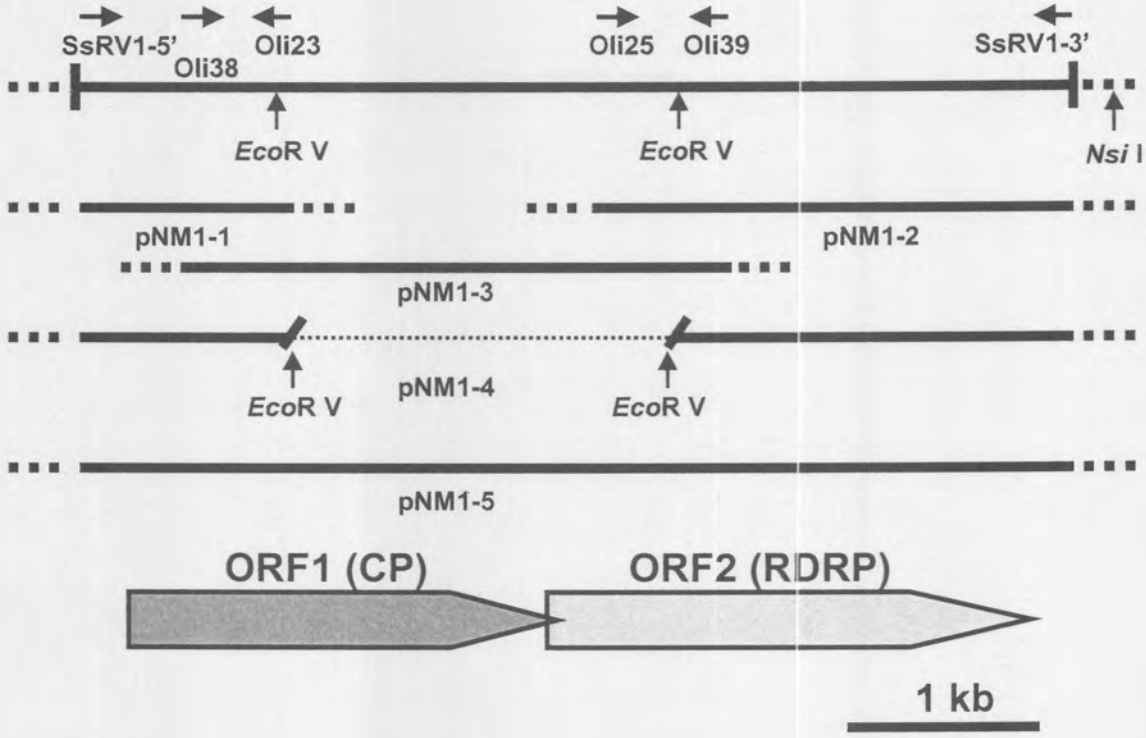


Fig. 15. A schematic representation of the cloning of the full-length cDNA copy of the genome of the *Sphaeropsis sapinea RNA virus 1*, SsRV1. The cloning was achieved using three different primary clones: pNM1-1, pNM1-2 and pNM1-3. The plasmid pNM1-1 contains the SsRV1-5'/Oli23 RT-PCR product while pNM1-2 contains the Oli25/SsRV1-3' RT-PCR product as an insert. The third clone, pNM1-3, was constructed from the Oli38/Oli39 RT-PCR product. The fourth clone, pNM1-4, was obtained by cloning the 2.4 kb *Eco*R V/*Nsi* I fragment from pNM1-2 into the *Eco*R V/*Nsi* I site of pNM1-1. The plasmid pNM1-4 was linearised with *Eco*R V followed by dephosphorylation. The full-length cDNA clone, pNM1-5, was obtained by excising the 2.1 kb *Eco*R V fragment from pNM1-3 and subcloning it into the *Eco*R V site of pNM1-4. The dotted lines at the end of the thick lines represent the multiple cloning site of pGEM T-Easy Vector. The thin dotted lines in pNM1-4 indicate the missing portion from SsRV1 genome.

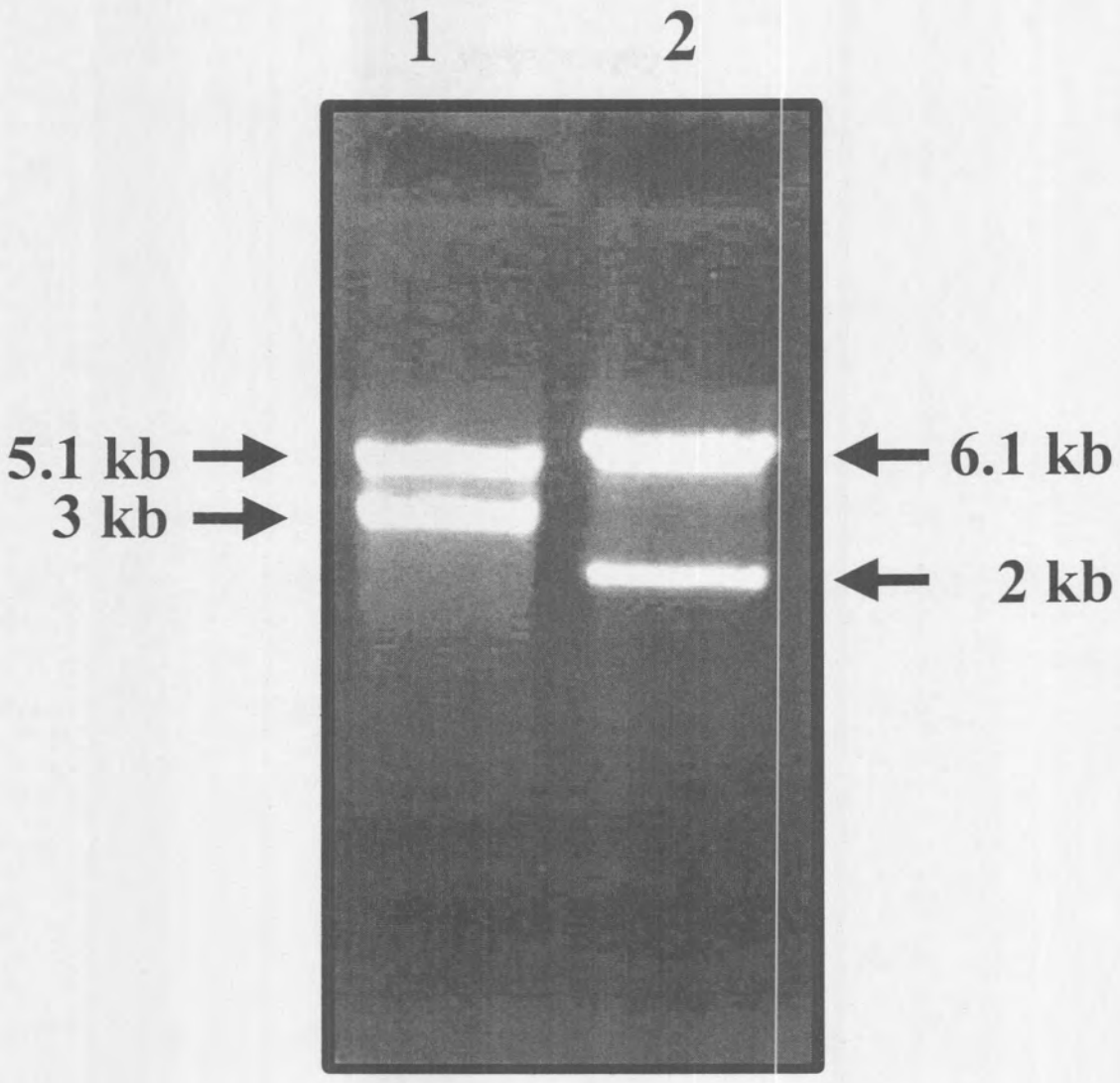


Fig. 16. Restriction digest analysis of the full-length cDNA clone of the *Sphaeropsis sapinea RNA virus 1* (SsRV1) genome. Lane 1: pNM1-5 cut with *Eco* RI. This plasmid contains the full-length cDNA copy of the genome of SsRV1. This digestion liberates the full-length cDNA copy of the SsRV1 genome from pGEM T-Easy Vector. Lane 2: pNM1-5 cut with *Eco*R V. The digestion of pNM1-5 with *Eco*R V results in two fragments of 2 kb and 6.1 kb. This enzyme has sites at position 1103 and 3177 in the cDNA copy of the genome of SsRV1. The 1 % agarose gel was stained with ethidium bromide.

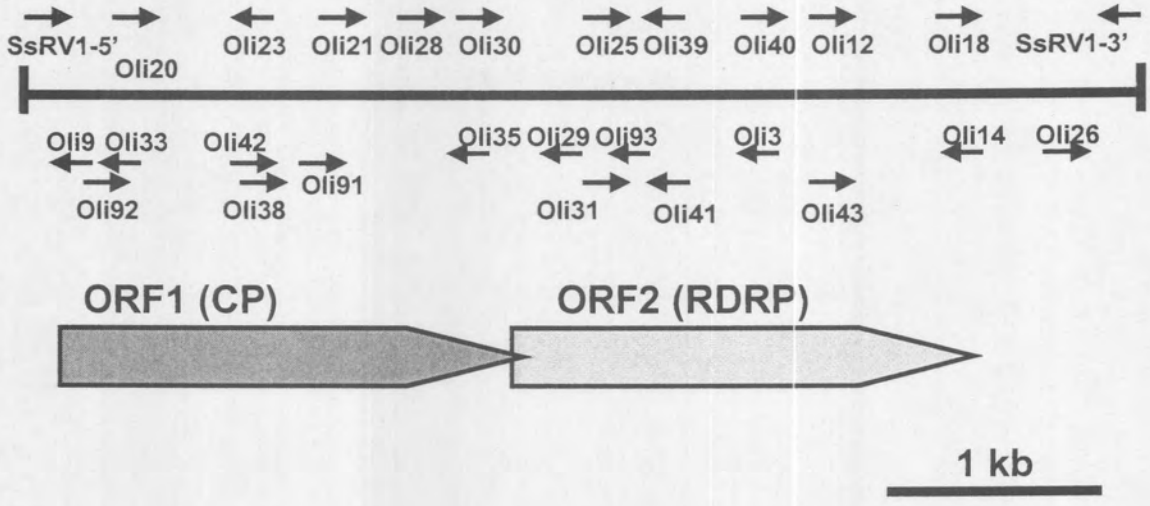


Fig. 17. A schematic representation of the genome of *Sphaeropsis sapinea RNA virus 1* (SsRV1). The arrows indicate positions and orientation of primers used in sequencing and cloning the viral genome. The high GC content of the SsRV1 genome made the sequencing more difficult. Primers were designed to cover short distances over the genome. This allowed the sequencing of this genome by use of sequences shorter than 400 bp in most cases. Longer sequences were not possible at certain regions of the genome.

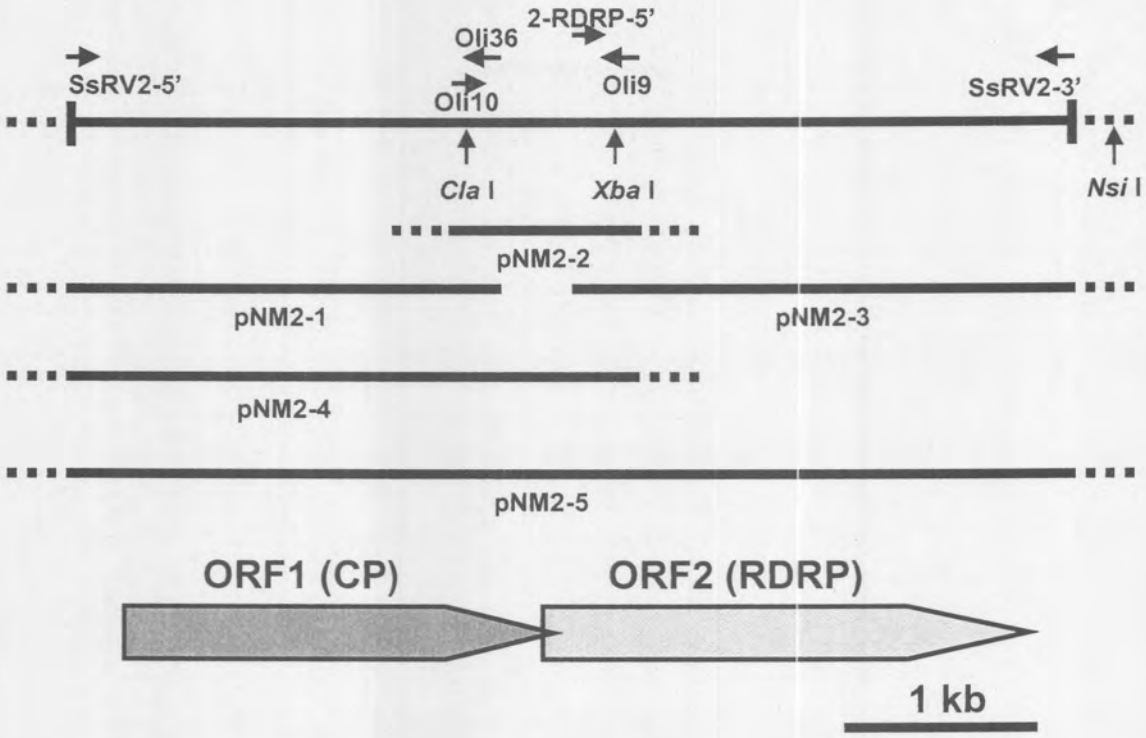


Fig. 18. A schematic representation of the plasmids used in cloning the full-length cDNA copy of the genome of the *Sphaeropsis sapinea RNA virus 2*, SsRV2. The cloning was based on *Cla* I and *Xba* I restriction sites. The cloning was achieved by using three primary clones: pNM2-1, pNM2-2 and pNM2-3. The first pNM2-1 contains the SsRV2-5'/Oli36 RT-PCR product while pNM2-2 contains the Oli10/Oli9 RT-PCR product as an insert. The clone pNM2-3 was constructed from the 2-RDRP-5'/SsRV2-3' RT-PCR product. The fourth clone, pNM2-4 was constructed by subcloning the 0.8 kb *Cla* I/*Nsi* I fragment from pNM2-2 into the *Cla* I/*Nsi* I site of pNM2-1. The final clone, pNM2-5 was constructed by excising the 2.6 kb *Xba* I/*Nsi* I fragment from pNM2-3 and sub-cloning it into the *Xba* I/*Nsi* I site of pNM2-4. The dotted lines at the end of the thick line represent the multiple cloning site of pGEM T-Easy Vector.

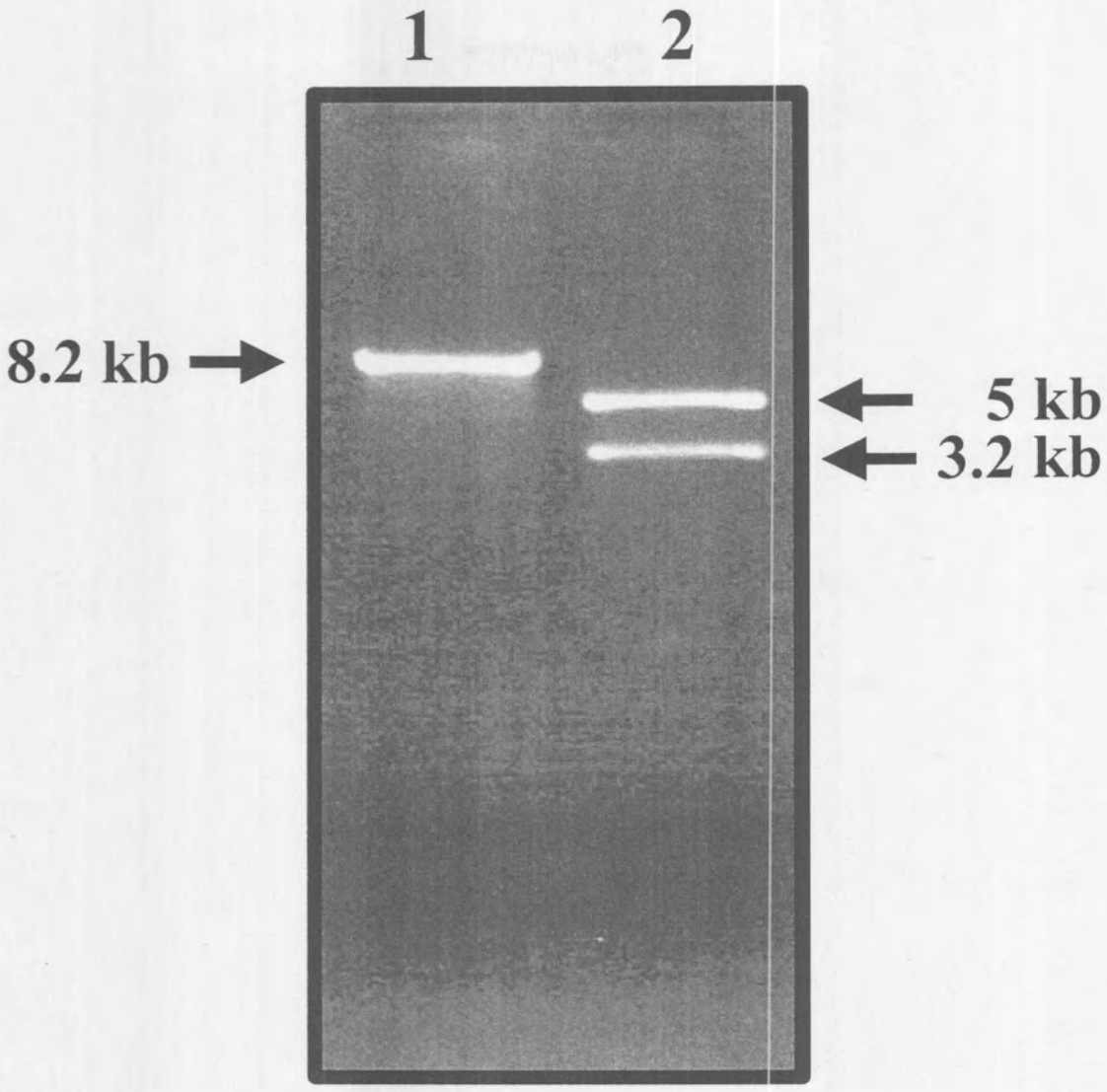


Fig. 19. Restriction digest analysis of the full-length cDNA clone of the *Sphaeropsis sapinea RNA virus 2* (SsRV2) genome. Lane 1: pNM2-5 linearised with *Cla* I. The plasmid pNM2-5 contains the full-length cDNA copy of the genome of SsRV2. In lane 2: pNM2-5 cut with *Cla* I and *Nsi* I. *Cla* I cuts within the genome of SsRV2 at position 2078 while *Nsi* I cuts in the multiple cloning site of the vector. This digestion results in two bands of 3.2 kb and 5 kb. The 1 % agarose gel was stained with ethidium bromide.

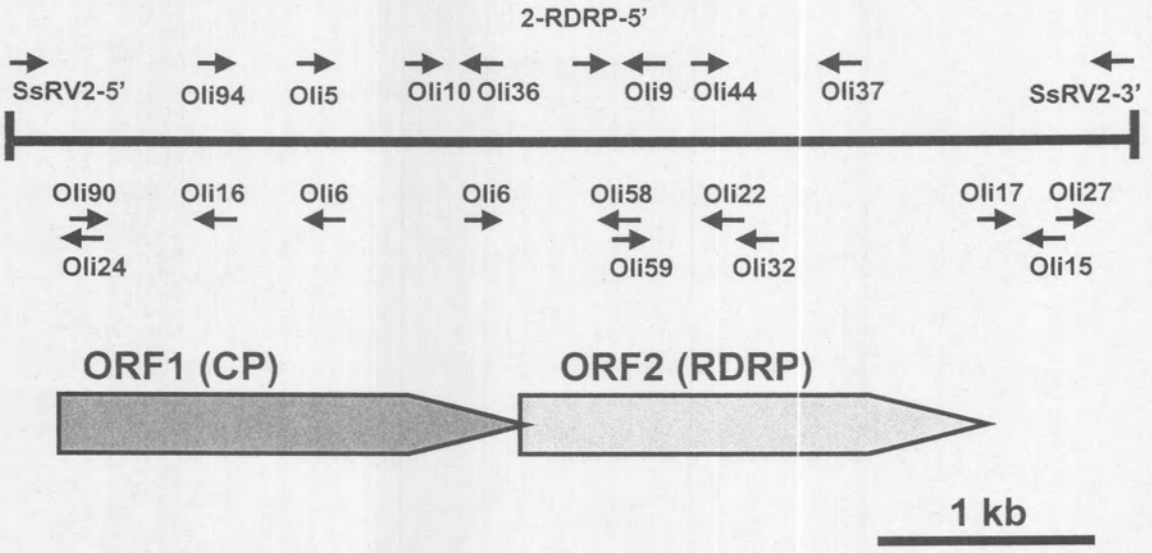


Fig. 20. A schematic representation of the genome of *Sphaeropsis sapinea RNA virus 2* (SsRV2). The arrows indicate the positions and orientation of primers used in sequencing and cloning the virus. As in the case of SsRV1, the high GC content of SsRV2 made it difficult to sequence the genome by using primers more than 400 bp apart.

```
1) P  P  P  P  P  G  G  N  P  F  P  P  P  P  P  P  G  G  N  N  D  G
      *     *  *                 *  *  *              *  *
2) P  L  P  P  L  V  A  T  P  F  P  F  F  R  R  P  P  E  E  T  M  T  A

1) P  G  G  P  P  P  P  G  P  P  P  V  D  P  L  P  I  P  H  N  P  P  A
         *     *              *              *     *     *
2) L  A  A  R  P  L  P  F  R  L  P  L  T  R  S  P  S  P  T  T  P  L  P

1) A  E  G  A  D  G  N  V  P  P  A  P  P  Q  *
                                   *
2) L  K  A  P  T  V  K  R  Y  R  R  P  R  N  E  *
```

Fig. 21. Amino acid changes caused on the C-terminus of the ORF1 gene product of SsRV2 by an insertion at genome position 2484. The amino acid changes are caused by a frameshift and not by base changes. (1): Amino acid sequence of the published coat protein of SsRV2 (Preisig *et al.*, 1998). (2): Amino acid sequence of the coat protein derived from the SsRV2 cDNA. The first boldfaced proline (P) is an amino acid immediately upstream of the insertion. All the other boldfaced amino acids are downstream of the insertion but were not affected by the insertion. All the other amino acids downstream of the insertion were changed as shown. The asterisk at the end of each amino acid sequence denotes the stop codon of ORF1.

Fig. 22. Aligned DNA sequence data from the ITS region of rRNA gene operon of 9 selected isolates of *Phomopsis* sp. and *Diaporthe* spp. *C. cubensis* was used as an outgroup taxon. The restriction sites for *Mse* I, an enzyme used in RFLPs to distinguish between the naturally-infected *D. perjuncta* and the virus-free *Phomopsis* sp. and *D. ambigua* isolates are bold-faced and boxed.

```
STE-U2680       ---------------AACCCTTTGTGAACTTATACCT---TACTGTTGCCTCGGCGCTAG 42
CMW5588         ---GGCGCACCCAGAAACCCTTTGTGAACTTATACCT---TACTGTTGCCTCGGCGCTAG 54
CMW3407         GGCGCACCTCCAGACAACCCTTTGTGAACTTATACCTA--TACTGTTGCCTCGGCGTCAG 58
CMW5289         GGCGCACCTCCAGACAACCCTTTGTGAACTTATACCTA--TACTGTTGCCTCGGCGTCAG 58
STE-U2655       ---------------AACCCTTTGTGAACTTATACCTA--TACTGTTGCCTCGGCGTCAG 43
CMW5288         ---GGCGCACCCAGAAACCCTTTGTGAACTTATACCT----ATCGTTGCCTCGGCGA-AG 52
CMW5587         ---GGCGCACCCAGAAACCCTTTGTGAACTTATACCT----ATCGTTGCCTCGGCGA-AG 52
CMW5287         ---GGCGCACCCAGAAACCCTTTGTGAACTTATACCT----ATCGTTGCCTCGGCGA-AG 52
STE-U2657       ---------------AACCCTTTGTGAACTTATACCT----ATCGTTGCCTCGGCGA-AG 40
CRY0140         ----------CCAGATACCCTTTGTGAACTTATACCTTTTTATCGTTGCCTCGGCGCCGA 50
                                ********************    *  ************

STE-U2680       -CTGGT--CCCTC----GGGGCCCCTCACCCTCG-GGT-GTTGAGACAGCCCGTCGGCGG 93
CMW5588         -CTGGT--CCCTC----GGGGCCCCTCACCCTCG-GGT-GTTGAGACAGCCCGTCGGCGG 105
CMW3407         GCTGGC--CCCCCTCGGGGGGTCCCTCACCATCTCGGT-GAGGAGCAGGCCCGCCGGCGG 115
CMW5289         GCTGGC--CCCCCTCGGGGGGTCCCTCACCATCTCGGT-GAGGAGCAGGCCCGCCGGCGG 115
STE-U2655       GCTGGC--CCCCCTCGGGGGGTCCCTCACCATCTCGGT-GAGGAGCAGGCCCGCCGGCGG 100
CMW5288         GCCGGC--CTCCCCACCGAGGCCCCTTG-----GGAAC-AAGGAGC-AGCCCGCCGGCGG 103
CMW5587         GCCGGC--CTCCCCACCGAGGCCCCTTG-----GGAAC-AAGGAGC-AGCCCGCCGGCGG 103
CMW5287         GCCGGC--CTCCCCACCGAGGCCCCTTG-----GGAAC-AAGGAGC-AGCCCGCCGGCGG 103
STE-U2657       GCCGGC--CTCCCCACCGAGGCCCCTTG-----GGAAC-AAGGAGC-AGCCCGCCGGCGG 91
CRY0140         GCCGGGAGTGCTCTTCTGTGCTCCCCCACCGCGCAAGCAGTGGAGCAGGCCCGCCGGCGG 110
                 * **     * *    * *  ***                  ***   ***** ******

STE-U2680       CCAACCTAACTCTT-GTTTTTACACTGAAACTCT-----GAG--CACAAAACATAAATGA 145
CMW5588         CCAACCTAACTCTT-GTTTTTACACTGAAACTCT-----GAG--CACAAAACATAAATGA 157
CMW3407         CCAAGTTAACTCTT-GTTTTTACACTGAAACTCT-----GAG--AATAAAACAAAAATGA 167
CMW5289         CCAAGTTAACTCTT-GTTTTTACACTGAAACTCT-----GAG--AATAAAACAAAAATGA 167
STE-U2655       CCAAGTTAACTCTT-GTTTTTACACTGAAACTCT-----GAG--AATAAAACAAAAATGA 152
CMW5288         CCAACCAAACTCTT-GTTTCT-TAGTGAATCTCT-----GAGTAAAAAAAACATAAATGA 156
CMW5587         CCAACCAAACTCTT-GTTTCT-TAGTGAATCTCT-----GAGTAAAAAAAACATAAATGA 156
CMW5287         CCAACCAAACTCTT-GTTTCT-TAGTGAATCTCT-----GAGTAAAAAAAACATAAATGA 156
STE-U2657       CCAACCAAACTCTT-GTTTCT-TAGTGAATCTCT-----GAGTAAAAAAAACATAAATGA 144
CRY0140         CCCACCAAACTCTTTGTTTTTAGAACGTATCTCTTCTGAGTGTTTATAACAAACAAATGA 170
                ** *   ******* **** *  *  * * ****     * *   * ** * * ******

STE-U2680       ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 205
CMW5588         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 217
CMW3407         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 227
CMW5289         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 227
STE-U2655       ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 212
CMW5288         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 216
CMW5587         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 216
CMW5287         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 216
STE-U2657       ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 204
CRY0140         ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG 230
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STE-U2680       CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 265
CMW5588         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 277
CMW3407         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 287
CMW5289         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 287
STE-U2655       CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 272
CMW5288         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 276
CMW5587         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 276
CMW5287         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 276
STE-U2657       CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG 264
CRY0140         CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC- 289
                ************************************************************

STE-U2680       CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 324
CMW5588         CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 336
CMW3407         CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 346
CMW5289         CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 346
STE-U2655       CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 331
CMW5288         CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 335
CMW5587         CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 335
CMW5287         CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 335
STE-U2657       CCCTCTGGTATTCCGGAGGGCAT-GCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 323
CRY0140         ----CGTGAATTCC--ACGGCATCGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGC 343
                    *  * *****  * ***** ************************************
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STE-U2680       TTGGTGATGGGGCACTGCTTCTTACCCAAGAAGCAGGCCCTGAAATTCAGTGGCGAGCTC 384
CMW5588         TTGGTGATGGGGCACTGCTTCTTACCCAAGAAGCAGGCCCTGAAATTCAGTGGCGAGCTC 396
CMW3407         TTGGTGATGGGGCACTGCCT-TCGGTAAGAAGGCAGGCCCTGAAATTCAGTGGCGAGCTC 405
CMW5289         TTGGTGATGGGGCACTGCCT-TCGGTAAGAAGGCAGGCCCTGAAATTCAGTGGCGAGCTC 405
STE-U2655       TTGGTGATGGGGCACTGCCT-TCGGTAAGAAGGCAGGCCCTGAAATTCAGTGGCGAGCTC 390
CMW5288         TTGGTGATGGGGCACTGCTT--CCGAGAGGGAGCAGGCCCTGAAATCTAGTGGCGAGCTC 393
CMW5587         TTGGTGATGGGGCACTGCTT--CCGAGAGGGAGCAGGCCCTGAAATCTAGTGGCGAGCTC 393
CMW5287         TTGGTGATGGGGCACTGCTT--CCGAGAGGGAGCAGGCCCTGAAATCTAGTGGCGAGCTC 393
STE-U2657       TTGGTGATGGGGCACTGCTT--CC-AGAGGGAGCAGGCCCTGAAATCTAGTGGCGAGCTC 380
CRY0140         TTGGTGTTGGGGCACTACCT-GTTCACAGCGGGTAGGCCCTGAAATTTAATGGCGGGCTC 402
                ****** ********* * *       *    * ************  * ***** ****

STE-U2680       GCCAGGACCCCGAGCGCAGTAGTT---AAACCCTCGCTCTGGAAGGCCCTGGCGGTGCCC 441
CMW5588         GCCAGGACCCCGAGCGCAGTAGTT---AAACCCTCGCTCTGGAAGGCCCTGGCGGTGCCC 453
CMW3407         GCCAGGACCCCGAGCGCAGTAGTT---AAACCCTCGCTCTGGAAGGTCTTGGTGCGGCCC 462
CMW5289         GCCAGGACCCCGAGCGCAGTAGTT---AAACCCTCGCTCTGGAAGGTCTTGGTGCGGCCC 462
STE-U2655       GCCAGGACCCCGAGCGCAGTAGTT---AAACCCTCGCTCTGGAAGGTCTTGGTGCGGCCC 447
CMW5288         GCCAGGACCCCGAGCGTAGTAGTT---ATATC-TCGCTCCGGAAGGCCCTGGCGGTGCCC 449
CMW5587         GCCAGGACCCCGAGCGTAGTAGTT---ATATC-TCGCTCCGGAAGGCCCTGGCGGTGCCC 449
CMW5287         GCCAGGACCCCGAGCGTAGTAGTT---ATATC-TCGCTCCGGAAGGCCCTGGCGGTGCCC 449
STE-U2657       GCCAGGACCCCGAGCGTAGTAGTT---ATATC-TCGCTCCGGAAGGCCCTGGCGGTGCCC 436
CRY0140         GCTAAGACTCTGAGCGTAGTAGTTTTTATCACCTCGCTTTGGAAGGA-TTAGCGGTGCTC 461
                ** * *** * ***** *******   *   * *****  ******   * * *  ** *

STE-U2680       T-GCCGTTAAACCCCCAACTTCTGAAAATT 470
CMW5588         T-GCCGTTAAACC----------------- 465
CMW3407         T-GCCGTTAAACC----------------- 474
CMW5289         T-GCCGTTAAACC----------------- 474
STE-U2655       T-GCCGTTAAACCCCCAACTTCTGAAAATT 476
CMW5288         T-GCCGTTAAACC----------------- 461
CMW5587         T-GCCGTTAAACC----------------- 461
CMW5287         T-GCCGTTAAACC----------------- 461
STE-U2657       T-GCCGTTAAACCCCCAACTTCTGAAAATT 465
CRY0140         TTGCCGTAAAACC----------------- 474
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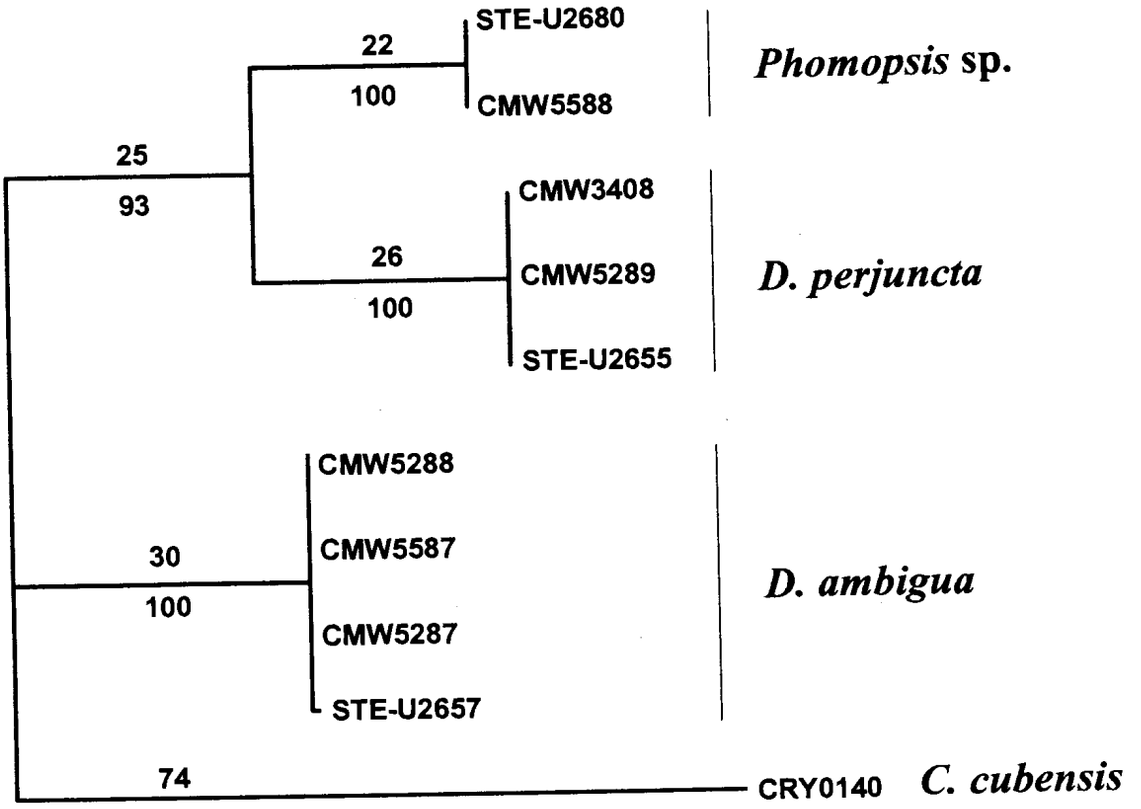


Fig. 23. Phylogenetic relatedness of *Phomopsis* sp., *D. perjuncta* and the different *D. ambigua* isolates used in this study. This is one of four most parsimonious trees (tree length = 178, CI = 0.949, RI = 0.958, RC = 0.910 and HI = 0.051) resulting from maximum parsimony analysis of the aligned ITS sequence data of the respective isolates. Bootstrap values are indicated below the branches while the number of base substitutions are indicated above the branches. The naturally-infected *D. perjuncta* isolates (CMW3407 and CMW5289) form a clade of their own together with the reference *D. perjuncta* STE-U2655. The tree was rooted on *C. cubensis*. The *D. ambigua* isolates (CMW5288, CMW5587 and CMW5287) and the *Phomopsis* sp. isolate (CMW5588) were those used in transfection studies.

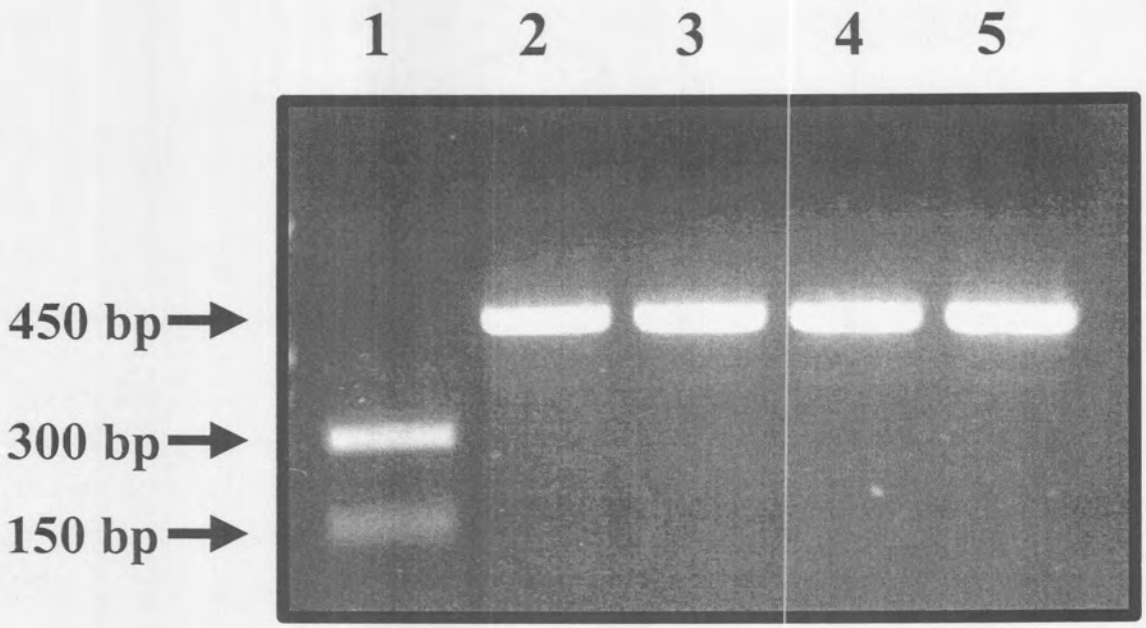


Fig. 24. RFLP technique to distinguish *Phomopsis* sp., *D. perjuncta* and different *D. ambigua* isolates used in the transfection studies. *Mse* I digested PCR products amplified from the ITS region of the rRNA gene operon using the primer pair ITS1/ITS4. Lane 1: *D. perjuncta* CMW3407, Lane 2: *Phomopsis* sp. CMW5588, Lane 3: *D. ambigua* CMW5587, Lane 4: *D. ambigua* CMW5287 and Lane 5: *D. ambigua* CMW5288. The enzyme *Mse* I has an additional restriction site in the sequences of *D. perjuncta* CMW3407 and *D. perjuncta* CMW5289 that makes the RFLP profiles of these virus-infected isolates different from the virus-free isolates. The DNA restriction fragments were separated on 2 % agarose gel stained with ethidium bromide.

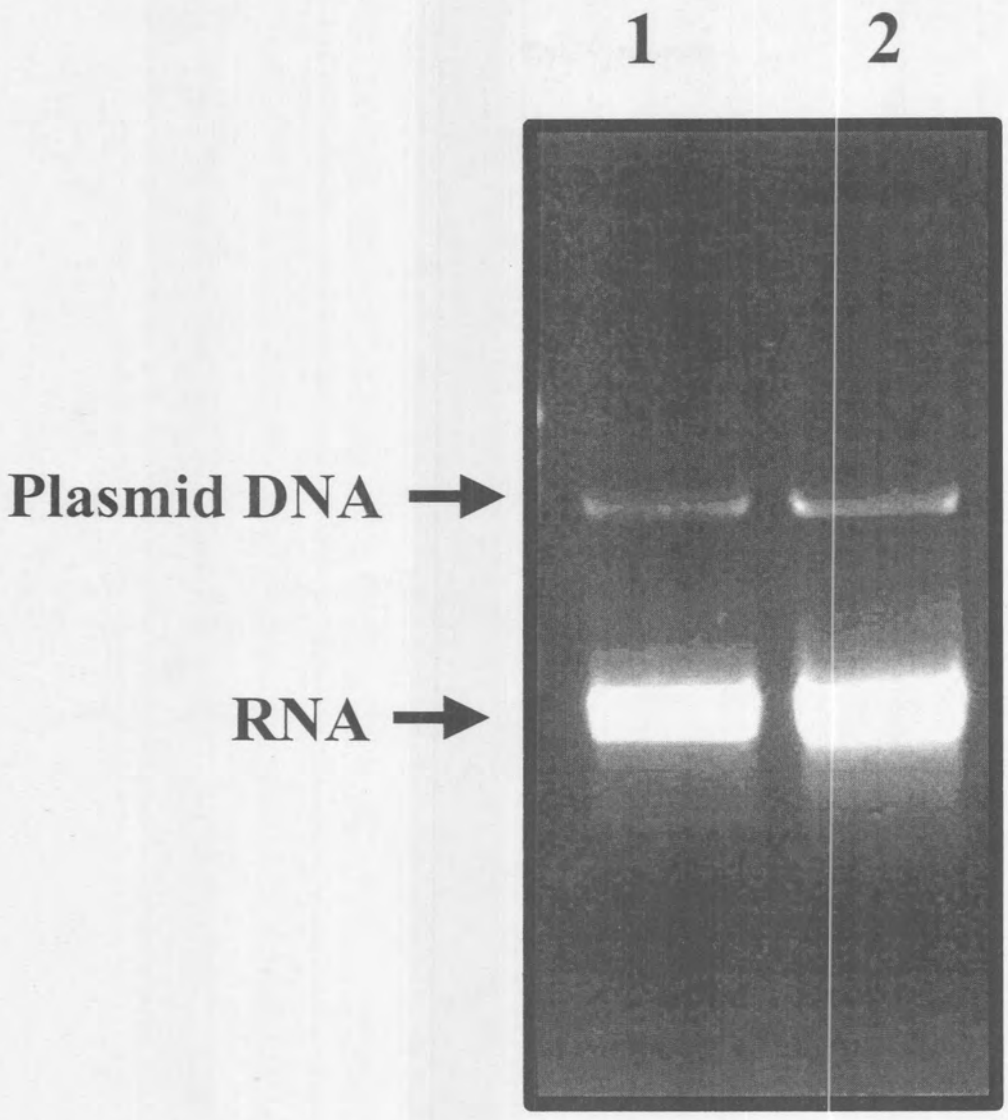


Fig. 25. *In vitro* RNA transcription products transcribed from the cDNA clone of *Diaporthe ambigua RNA virus* (DaRV) using T7 RNA polymerase. RNA transcripts were produced from *Sal* I linearised pH6DV3 (lane 1) and *Sal* I linearised pDV3 (lane 2). The RNA transcripts were used directly in electroporation of *D. ambigua* spheroplasts by transfection. The RNA transcripts were separated on 1 % agarose gel stained with ethidium bromide.

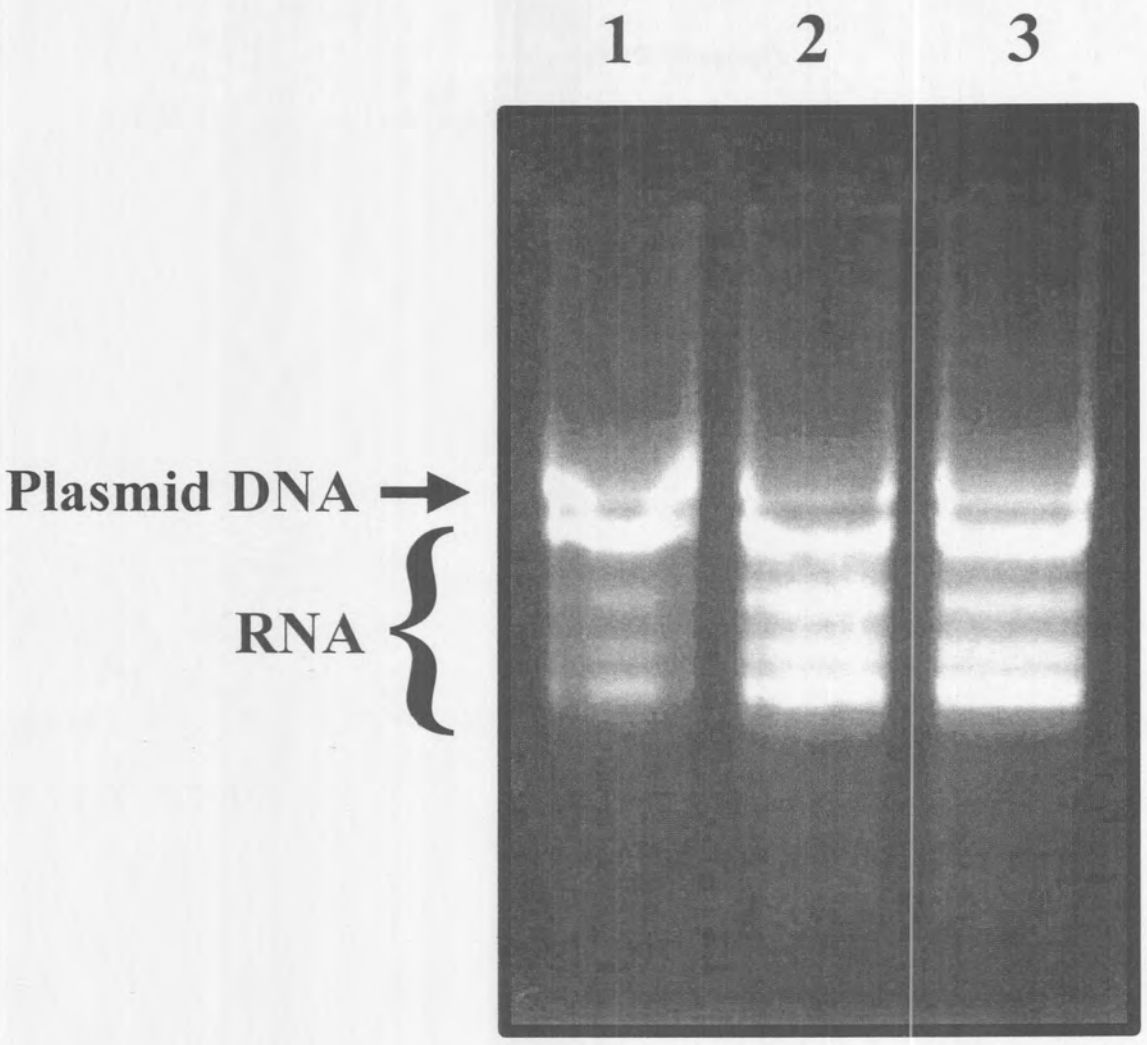


Fig. 26. *In vitro* RNA transcription products from the cDNA of SsRV1 using T7 RNA polymerase. RNA transcripts were produced from *Nsi* I-linearised pNM1-5. Lanes 1-3 represent different template concentrations of the same *Nsi* I-linearised pNM2-5. Lane 2 and 3 had two and three times the concentration of template DNA in lane 1, respectively. RNA transcripts of different banding patterns were produced. The different banding patterns may represent RNA transcripts of different sizes or RNA transcripts of the same size but with different conformations. The RNA transcripts were separated on 1 % agarose gel stained with ethidium bromide.

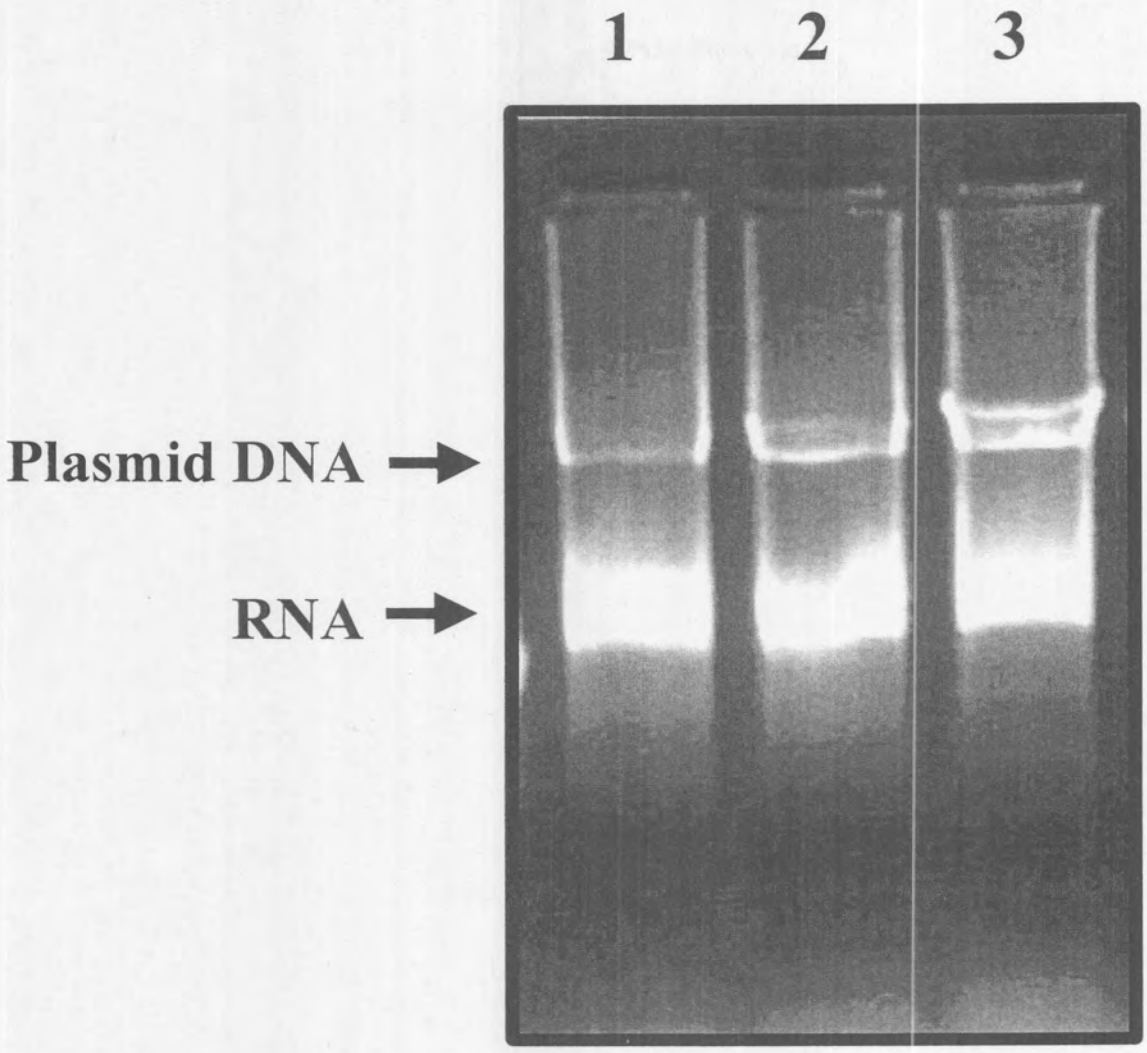


Fig. 27. *In vitro* RNA transcription products from the cDNA of SsRV2 using T7 RNA polymerase. RNA transcripts were produced from *Nsi* I-linearised pNM2-5. Lanes 1-3 represent different concentrations of the template pNM2-5. Transcription products separated in lanes 2 and 3 had two and three times the concentration of template in lane 1, respectively. The RNA transcripts were separated on 1 % agarose gel stained with ethidium bromide.

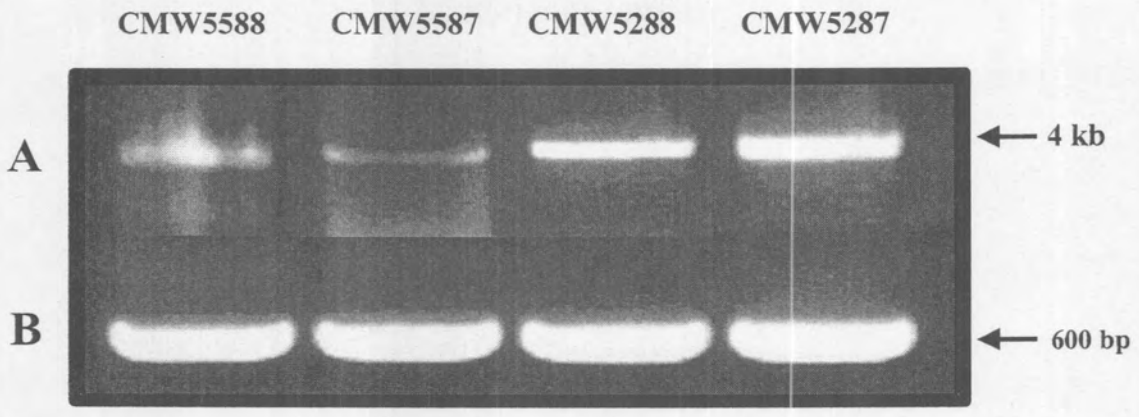


Fig. 28. Confirmation of successful transfection of *Phomopsis* sp. and *D. ambigua* isolates with DaRV. Agarose gel electrophoresis of: (A) single dsRNA elements isolated from transfected isolates and (B) RT-PCR products amplified from each dsRNA using the primers Oli64/Oli80. Lane 1: *Phomopsis* sp. CMW5588; Lane 2: *D. ambigua* CMW5587; Lane 3: *D. ambigua* CMW5288 and Lane 4: *D. ambigua* CMW5287. The dsRNA of the viruses were purified on CF 11 column chromatography. The 1 % agarose gel was stained with ethidium bromide.

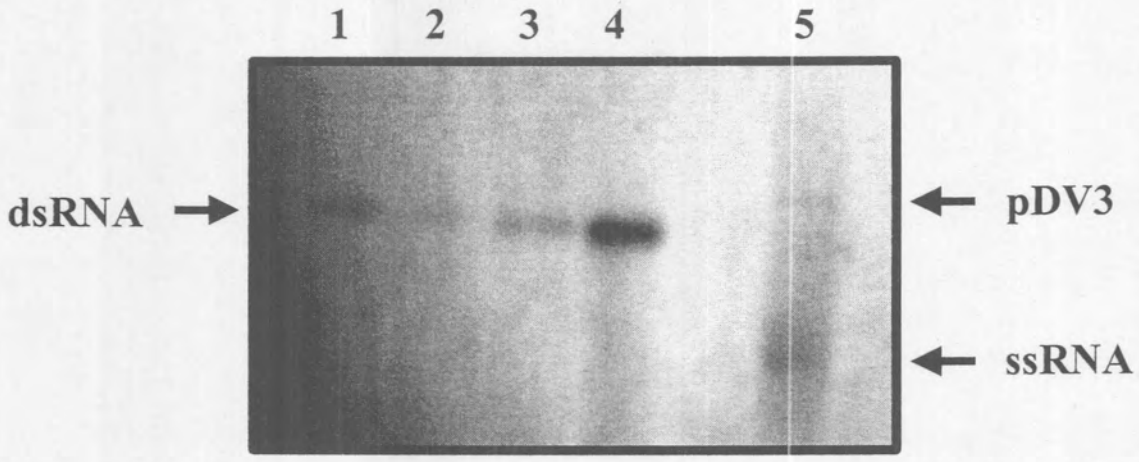
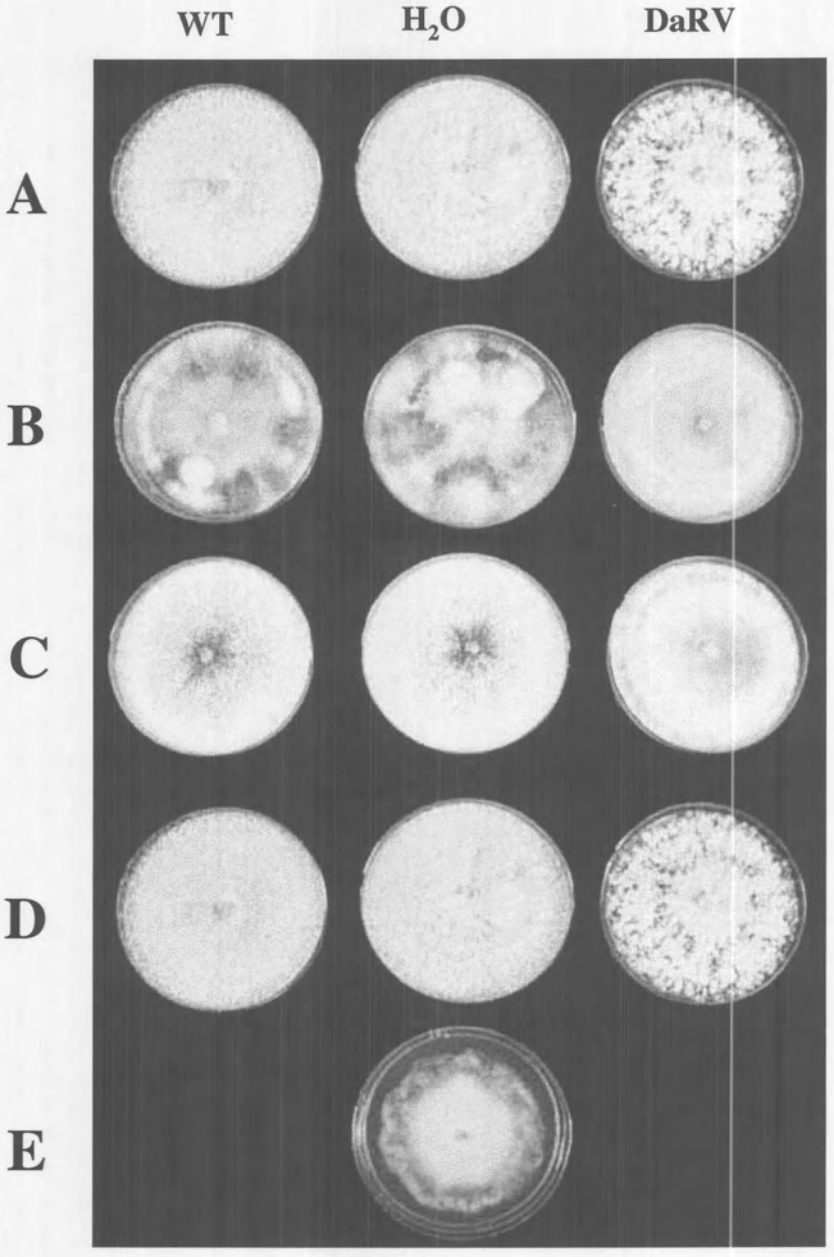
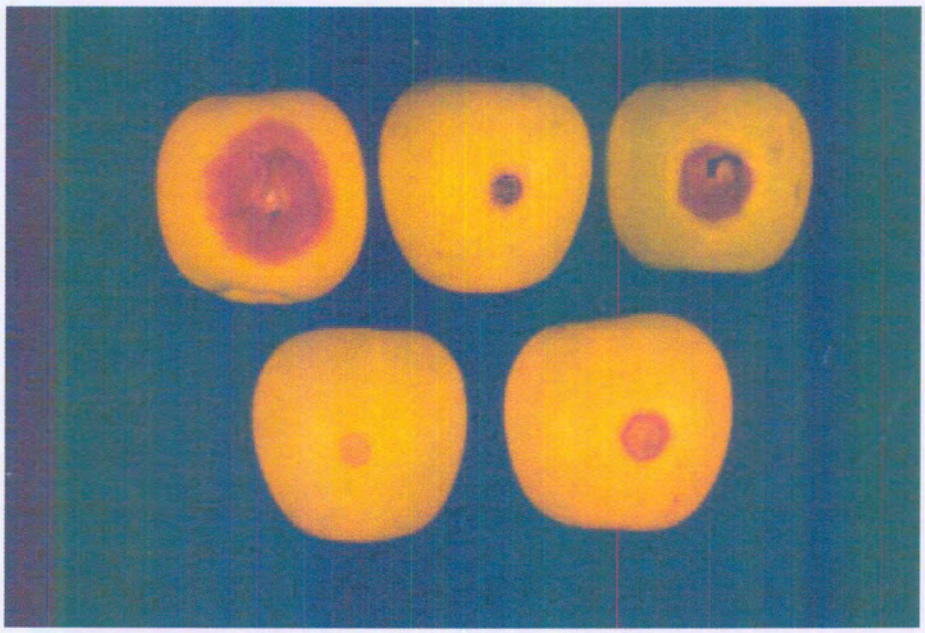


Fig. 29. Northern blot analysis of dsRNA isolated from transfected *Phomopsis* sp. and *D. ambigua* isolates. Lane 1: *D. ambigua* CMW5288, Lane 2: *D. ambigua* CMW5287, Lane 3: *D. ambigua* CMW5587, Lane 4: *Phomopsis* sp. CMW5588 and Lane 5: Positive strand RNA (T7 RNA polymerase-produced RNA) from pDV3 as positive control. The purified dsRNA and the positive-stranded RNA were separated at 80 V on 1 % agarose gel for 40 min, blotted on nylon membrane and probed at 68 °C with DIG-labelled negative-stranded (SP6 RNA polymerase-produced) RNA. Colorimetric detection with NBT/BCIP was performed in the dark for 4 hours.

Fig. 30. Colony morphology of wild-type (WT), water transfected (H_2O) (negative control), DaRV transfected (DaRV) *Phomopsis* sp., *D. ambigua* isolates and the naturally-infected *D. perjuncta* isolates grown on potato dextrose agar (PDA). The fungal cultures were photographed on day 7. A: *Phomopsis* sp. CMW5588. B: *D. ambigua* CMW5587. C: *D. ambigua* CMW5287. D: *D. ambigua* CMW5288. E: *D. perjuncta* CMW3407 (naturally-infected isolate).



A B C



D E

Fig. 31. Representative examples of lesions on Golden Delicious apples caused by (A) DaRV-transfected *Phomopsis* sp. CMW5588-DaRV, (B) water-transfected *Phomopsis* sp. CMW5588-H_2O, (C) wild-type *Phomopsis* sp. CMW5588-WT, (D) agar and (E) naturally-infected *D. perjuncta* CMW3407. The lesions were photographed 6 days after inoculation. The apples inoculated with the transfected isolate were totally covered with the lesion 10 days after inoculation.

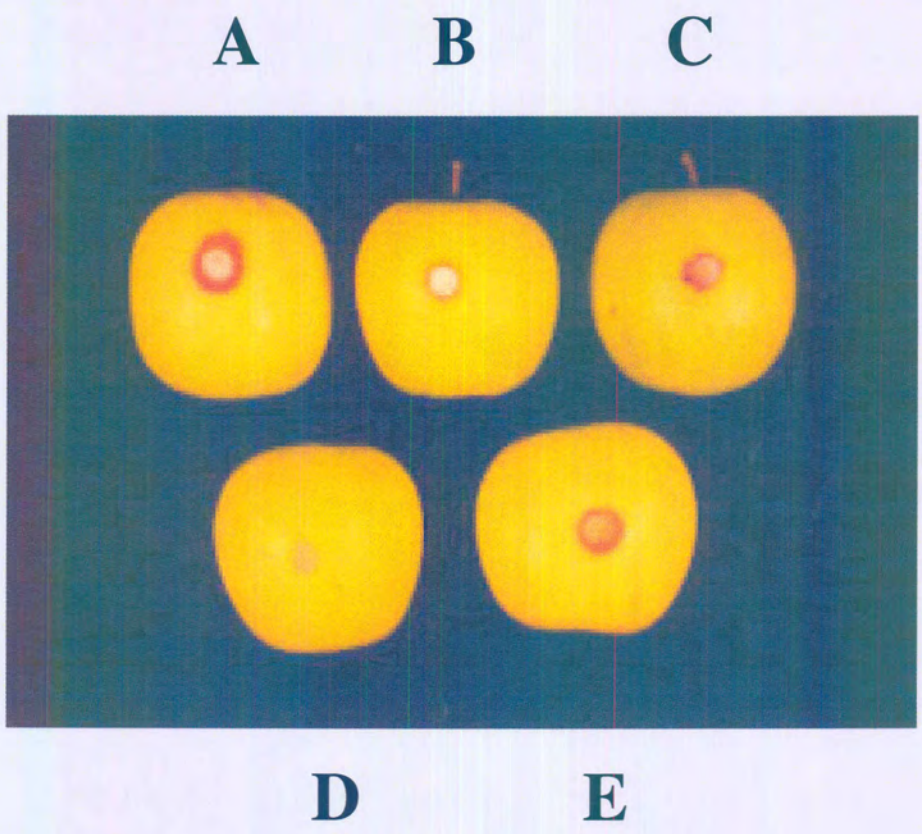


Fig. 32. Representative examples of lesions on Golden Delicious apples caused by (A) DaRV-transfected *D. ambigua* CMW5587-DaRV, (B) water-transfected *D. ambigua* CMW5587-H_2O, (C) wild-type *D. ambigua* CMW5587-WT, (D) agar and (E) naturally-infected *D. perjuncta* CMW3407. The lesions were photographed 6 days after inoculation. The lesions were characterised by white mycelium in the middle of the lesions. The lesions did not increase significantly with time.

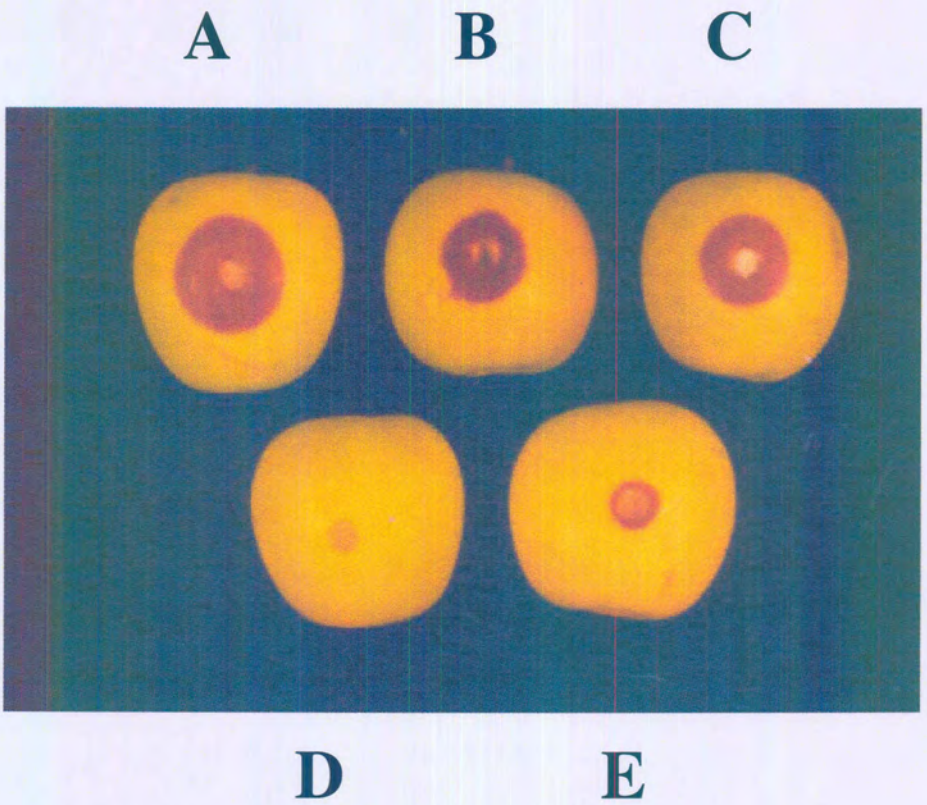


Fig. 33. Representative examples of lesions on Golden Delicious apples caused by (A) DaRV-transfected *D. ambigua* CMW5287-DaRV, (B) water-transfected *D. ambigua* CMW5287-H_2O, (C) wild-type *D. ambigua* CMW5287-WT, (D) agar and (E) naturally-infected *D. perjuncta* CMW3407. The lesions were photographed 6 days after inoculation. The lesions on the apples inoculated with (A) DaRV-transfected *D. ambigua* CMW5287 and (B) water-transfected *D. ambigua* CMW5287 covered over 90% of the surface of the apples10 days after inoculation.

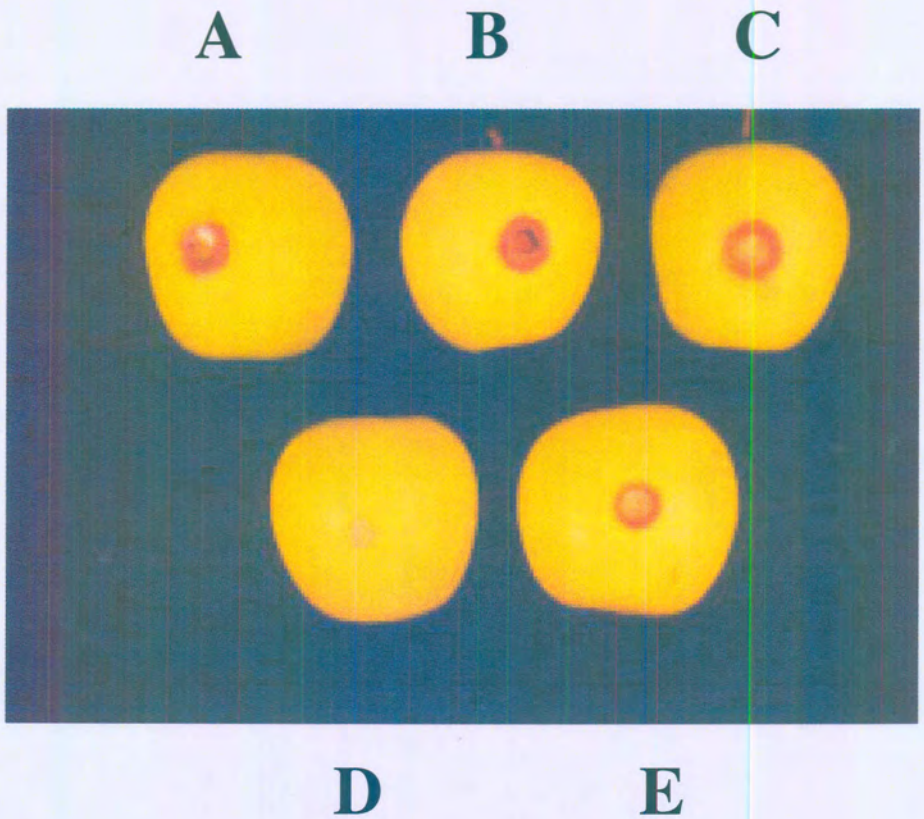


Fig. 34. Representative examples of lesions on Golden Delicious apples caused by (A) DaRV-transfected *D. ambigua* CMW5288-DaRV, (B) water-transfected *D. ambigua* CMW5288-H_2O, (C) wild-type *D. ambigua* CMW5288-WT, (D) agar and (E) naturally-infected *D. perjuncta* CMW3407. The lesions were photographed 6 days after inoculation. The lesions due to (A) DaRV transfected *D. ambigua* CMW5288, (B) water transfected *D. ambigua* CMW5288 and (C) wild-type *D. ambigua* CMW5288 grew slowly with time and covered almost 50% of the apple surface 10 days after inoculation.

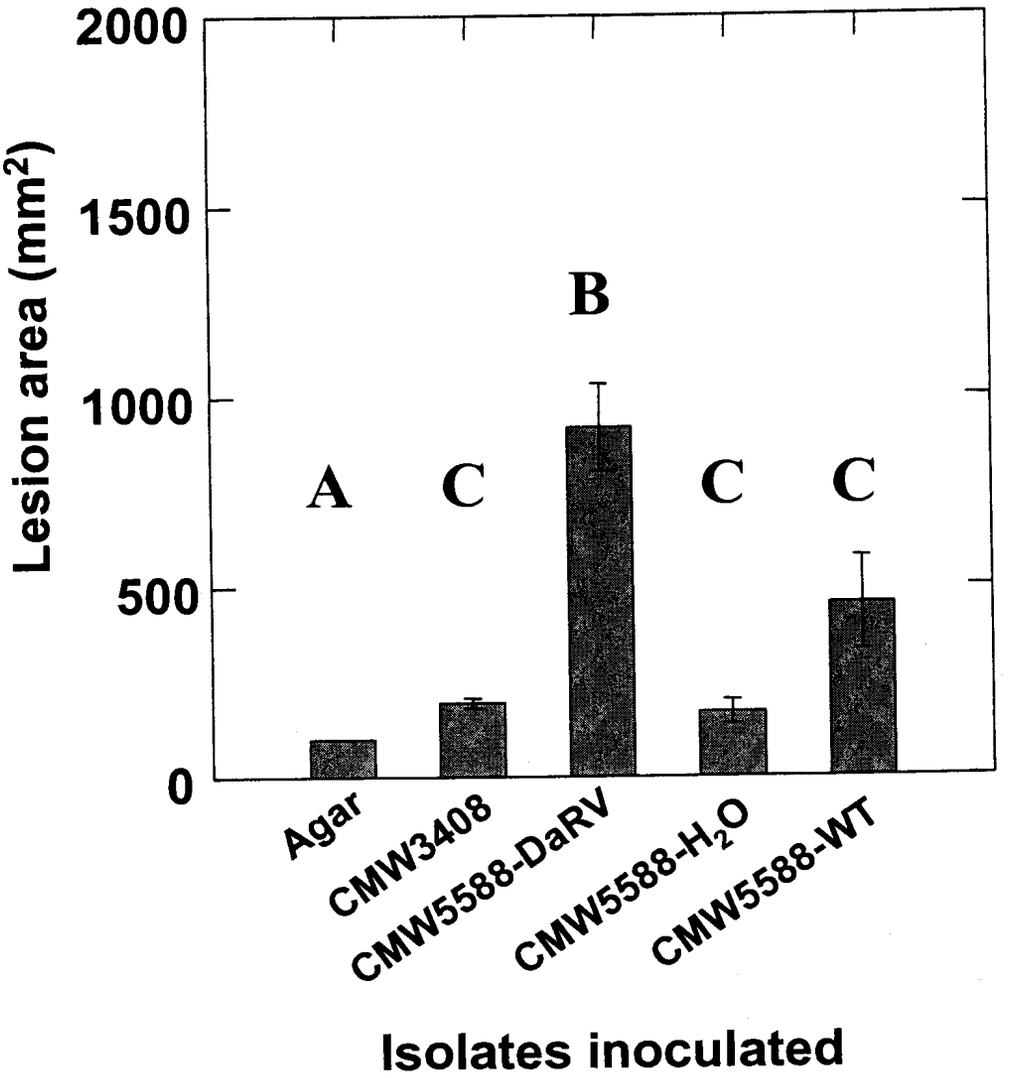


Fig. 35. Mean lesion area (±S.E.M) after inoculation of Golden Delicious apples with *Phomopsis* sp. CMW5588 transfected with DaRV (CMW5588-DaRV), negative control (CMW5588-H_2O) and wild type isolate of the fungus (CMW5588-WT). The naturally-infected *D. perjuncta* CMW3407 and agar disks were included for comparison. Lesion areas differed significantly between the transfected isolate and all the other isolates. Columns annotated with the same letter do not differ significantly from each other.

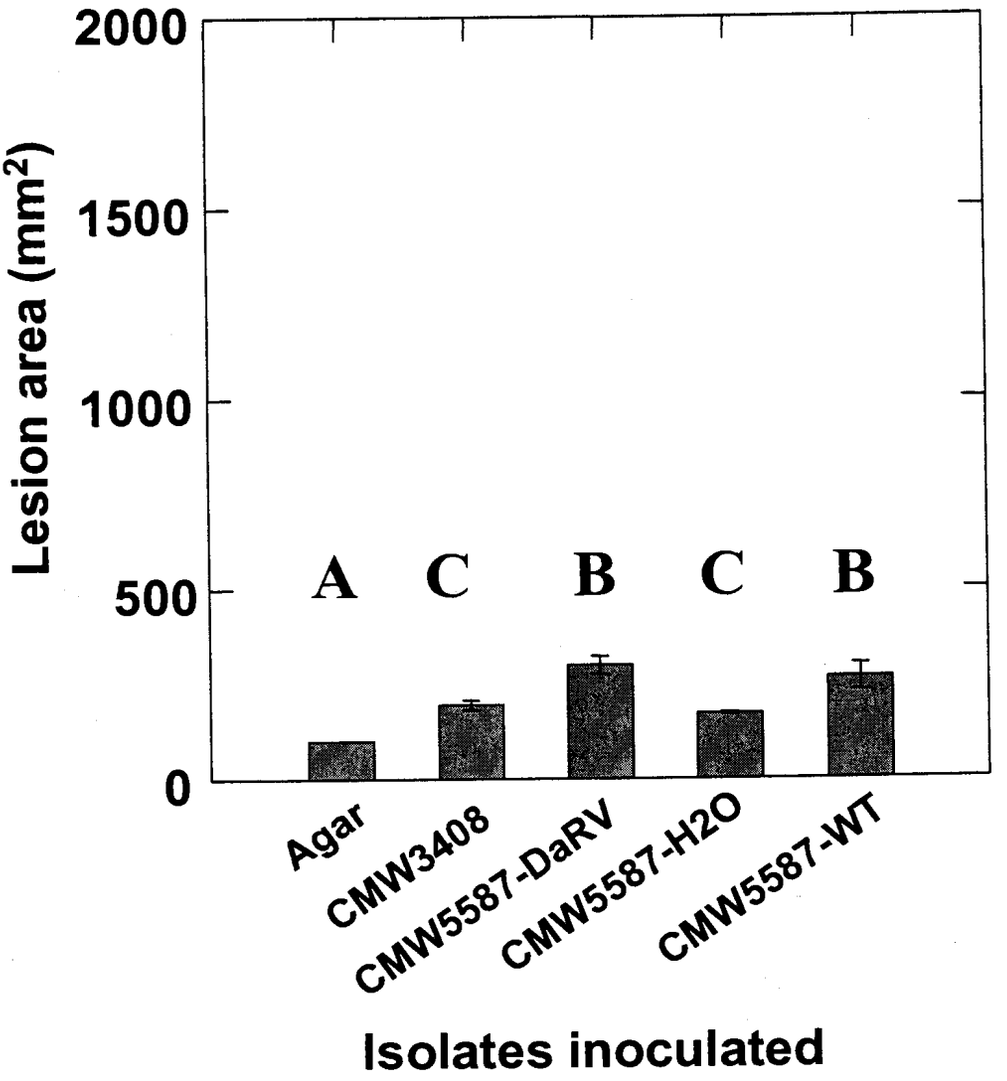


Fig. 36. Mean lesion area (±S.E.M) after inoculation of Golden Delicious apples with *D. ambigua* CMW5587 transfected with DaRV (CMW5587-DaRV), negative control (CMW5587-H_2O) and wild type isolate of the fungus (CMW5587-WT). The naturally-infected *D. perjuncta* CMW3407 and agar disks were included for comparison. Lesion areas did not differ significantly between the transfected isolate and the wild-type isolate. Columns annotated with the same letter do not differ significantly from each other.

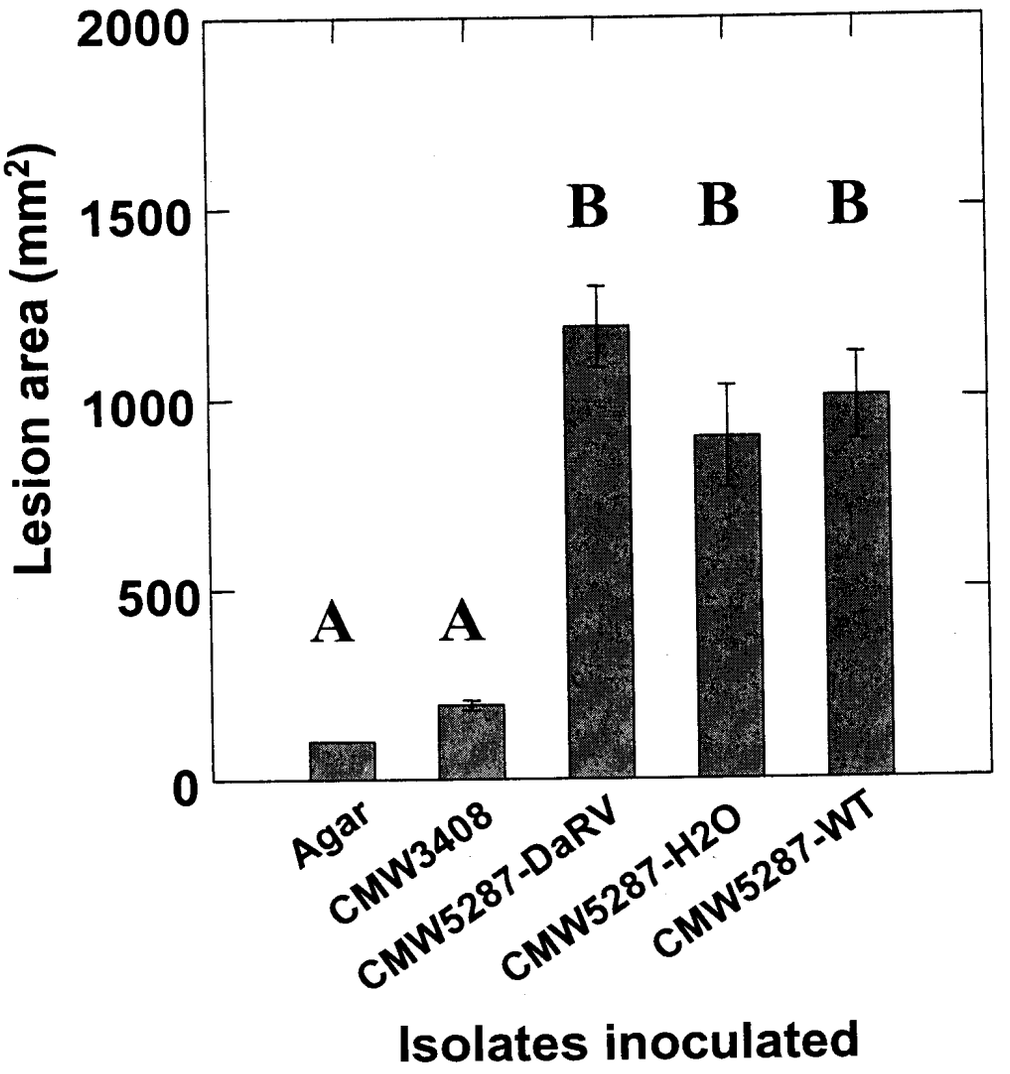


Fig. 37. Mean lesion area (±S.E.M) after inoculation of Golden Delicious apples with *D. ambigua* CMW5287 transfected with DaRV (CMW5287-DaRV), negative control (CMW5287-H_2O) and wild type isolate of the fungus (CMW5287-WT). The naturally-infected *D. perjuncta* CMW3407 and agar disks were included for comparison. Lesion areas did not differ significantly between the transfected isolate and the wild-type isolate. Columns annotated with the same letter do not differ significantly from each other.

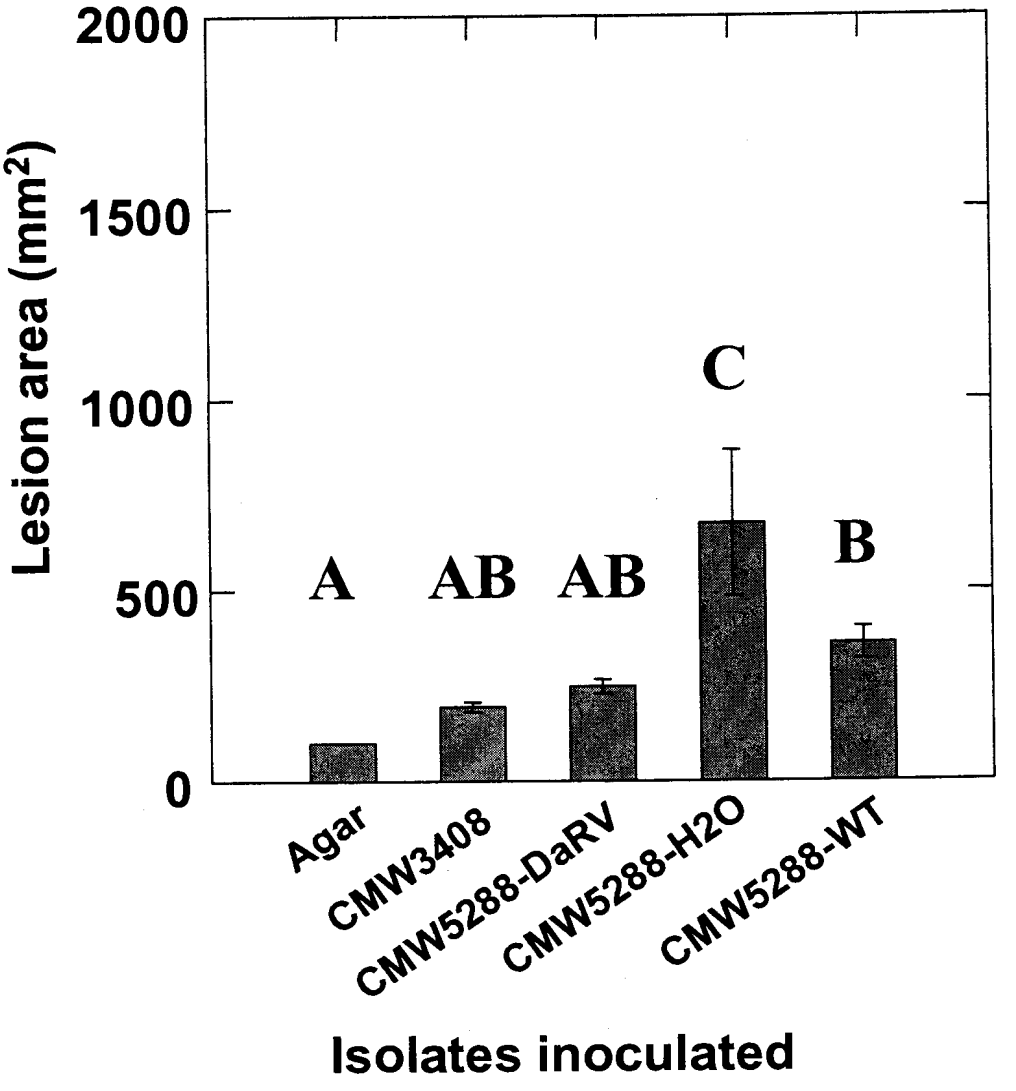


Fig. 38. Mean lesion area (±S.E.M) after inoculation of Golden Delicious apples with *D. ambigua* CMW5288 transfected with DaRV (CMW5288-DaRV), negative control (CMW5288-H_2O) and wild type isolate of the fungus (CMW5288-WT). The naturally-infected *D. perjuncta* CMW3407 and agar disks were included for comparison. Lesion areas did not differ significantly between the transfected isolate and the wild-type isolate. Columns annotated with the same letter do not differ significantly from each other.

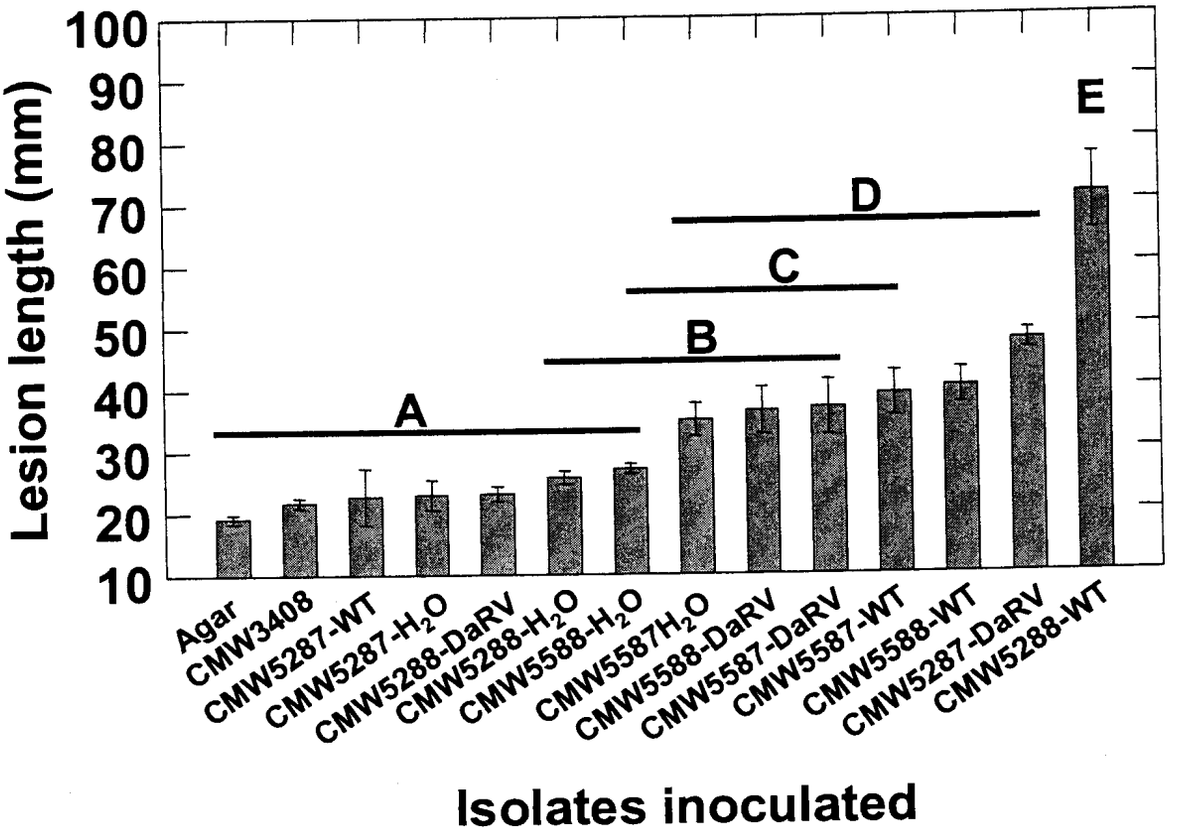
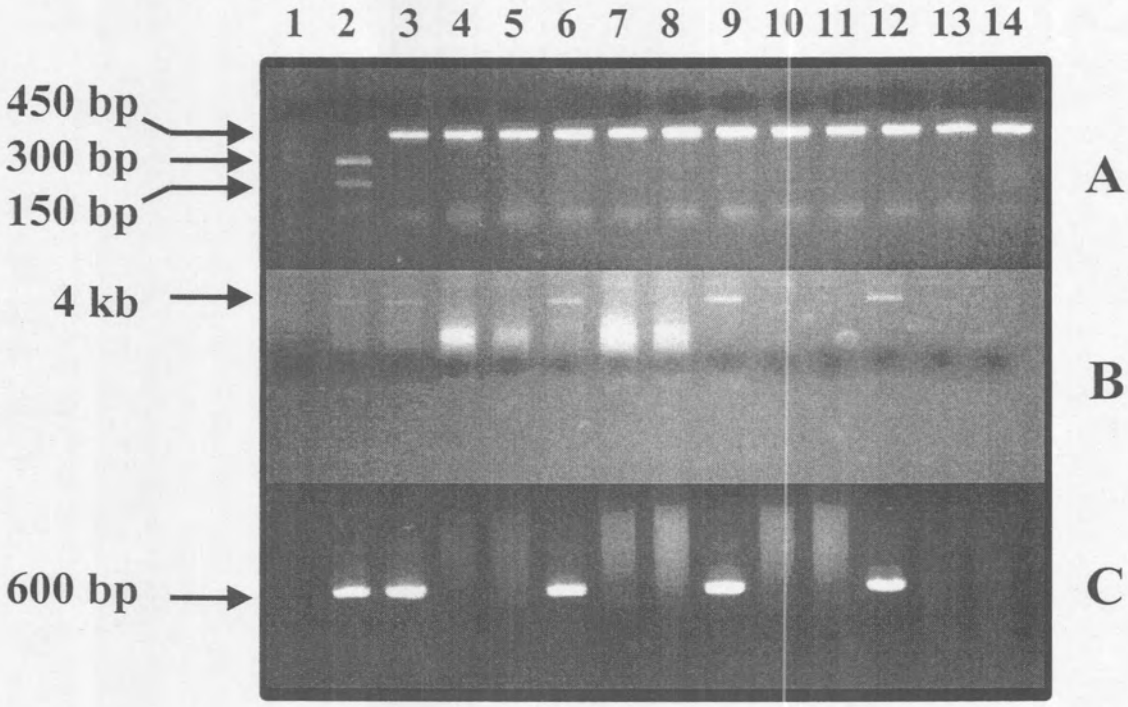


Fig. 39. Mean lesion lengths (±S.E.M) after inoculation of apple trees (Golden Delicious/M793) with *Phomopsis* sp. transfected with DaRV (CMW5588-DaRV), negative control (CMW5588-H_2O) and wild type (CMW5588-WT) isolates. Three isolates of *D. ambigua* transfected with DaRV (CMW5587-DaRV, CMW5287-DaRV and CMW5288-DaRV), negative control (CMW5587-H_2O, CMW5287-H_2O and CMW5288-H_2O) and wild type (CMW5587-WT, CMW5287-WT and CMW5288-WT) were also used. The naturally-infected *D. perjuncta* CMW3407 and sterile agar disks (Agar) were included for comparison. Lesions were measured three months after inoculation and differed significantly among and between isolates. Columns annotated with the same letter do not differ significantly from each other.

Fig. 40. Confirmation of identity of virus infection of fungal isolates re-isolated from inoculated apple trees. Isolations were made from the edges of the inoculum wounds and along the streaking on the stems of the trees. (A). RFLP identification of isolates. DNA was isolated from all the isolated fungi. The primer pair ITS1/ITS4 was used to amplify the ITS region of rRNA gene operon. The PCR products were digested with *Mse* I. The RFLP profiles proved that the fungi were those used for inoculations. (B): Double-stranded RNA was isolated from all the transfected and the naturally-infected isolates but not from the negative control and wild-type isolates. (C): The primer pair Oli64/Oli80 was used in RT-PCR to confirm that the isolated dsRNA was DaRV. Amplification of 600 bp fragments was observed from all the transfected isolates but not from the negative control and wild-type isolates. Lane 1: negative control; Lane 2: *D. perjuncta* CMW3407; Lane 3: *Phomopsis* sp. CMW5588-DaRV; Lane 4: *Phomopsis* sp. CMW5588-H_2O; Lane 5: *Phomopsis* sp. CMW5588-WT; Lane 6: *D. ambigua* CMW5587-DaRV; Lane 7: *D. ambigua* CMW5587-H_2O; Lane 8: *D. ambigua* CMW5587-WT; Lane 9: *D. ambigua* CMW5287-DaRV; Lane 10: *D. ambigua* CMW5287-H_2O; Lane 11; *D. ambigua* CMW5287-WT; Lane 12: *D. ambigua* CMW5288-DaRV; Lane 13: *D. ambigua* CMW5288-H_2O; Lane 14: *D. ambigua* CMW5288-WT. The 2 % (A) and 1 % (B,C) agarose gels were stained with ethidium bromide.



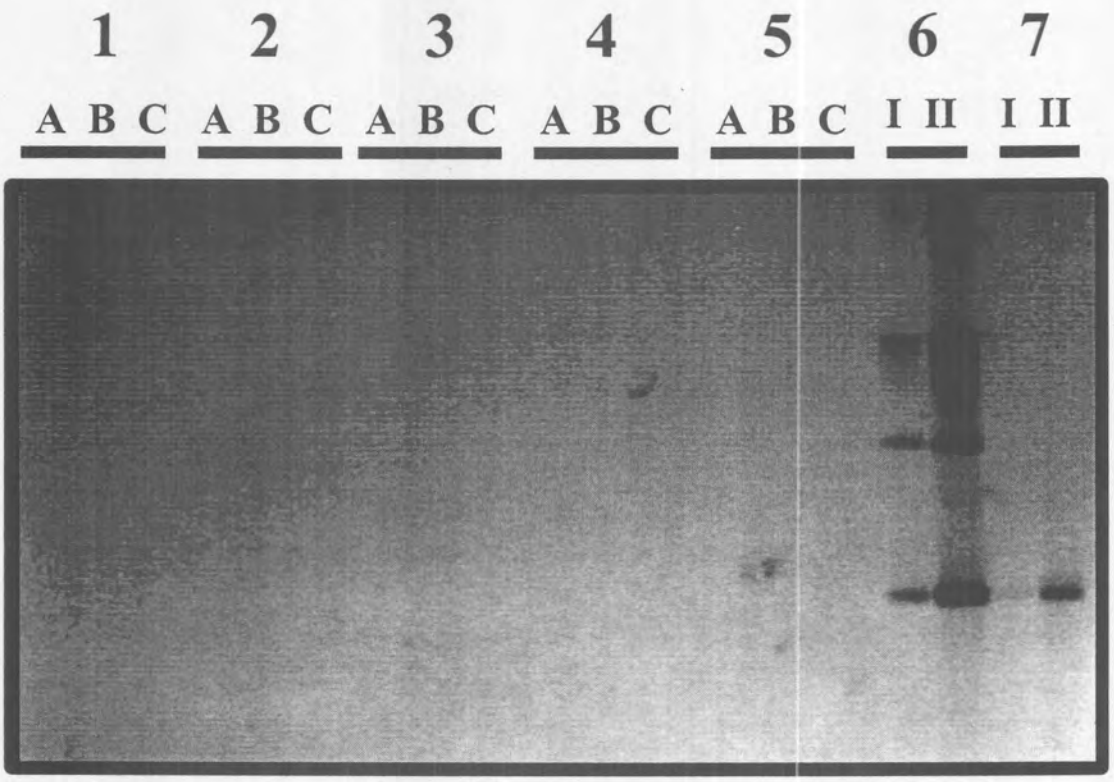


Fig. 41. Homologous Southern blot analysis of restricted genomic DNA of different isolates of *D. ambigua*, *Phomopsis* sp. and the naturally-infected *D. perjuncta* (lanes 1-5), pDV3 (lane 6) and the full-length cDNA of DaRV (lane 7) obtained from *Eco* RI digested pDV3. The different isolates were: Lane 1: *D. ambigua* CMW5288, Lane 2: *D. ambigua* CMW5287, Lane 3: *D. ambigua* CMW5587, Lane 4: *Phomopsis* sp. CMW5588 and Lane 5: *D. perjuncta* CMW3407. Lane 6 is (I) 1:1000 and (II) 1:100 of dilution of undigested pDV3. Lane 7 is (I) 1:1000 and (II) 1:100 dilution of the full-length cDNA of DaRV obtained from *Eco* RI digested pDV3. The genomic DNA of the isolates was digested with (A) *Eco* RI, (B) *Hin*d III and (C) *Eco* RI/*Hin*d III. The digested DNA was separated overnight at 40 V on 1 % agarose gel, blotted on a nylon membrane and probed at 68 °C with DIG-labelled cDNA of DaRV. Colorimetric detection with NBT/BCIP was performed in the dark overnight.

2.4. Discussion

The aim of this study was to construct full-length cDNA copies of the genomes of *Diaporthe ambigua RNA virus* (DaRV), *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2). The intention was to use these plasmids to synthesise *in vitro*, viral RNA. The *in vitro*-produced RNA would be subsequently used in transfection studies of *S. sapinea*, *Phomopsis* sp. and *D. ambigua* in order to study the potential of these viruses to be used as biological control agents. Transfections using *in vitro*-produced RNA from both SsRV1 and SsRV2 were unsuccessful. Transfection with RNA from DaRV was successful. Transfection and the production of cDNA clones from SsRV1, SsRV2 and DaRV genomes are discussed in this section.

2.4.1. Construction of cDNA clones of three mycoviruses DaRV, SsRV1 and SsRV2

The three mycoviruses of interest in this study have RNA genomes. It has been proposed that dsRNA mycoviruses replicate by synthesising positive-stranded RNA from the dsRNA (Ghabrial, 1994; Yao *et al.*, 1997). This single-stranded positive-stranded RNA is then used as a template to synthesise negative-stranded RNA and in this way generate the dsRNA form of the virus (Ghabrial, 1994; Yao *et al.*, 1997). The cDNA copies of the RNA genomes of DaRV, SsRV1 and SsRV2 were successfully constructed. The cDNA copies were cloned between T7 and SP6 RNA promoters of pGEM T-Easy Vector. The T7 RNA promoter could then be used to produce, *in vitro*, single-stranded positive-stranded RNA that could subsequently be used to transfect fungal spheroplasts.

Attempts to directly clone the viral genomes from single full-length cDNAs produced by RT-PCR using primers on the most distal ends of the viral genomes were not successful. In the case of DaRV, RT-PCR using primers designed from the 5' and 3' ends of DaRV genome produced the full-length cDNA in low amounts but ligation into pGEM-T Easy Vector failed. This was probably due to a low ratio of plasmid to

insert cDNA which must be in the ratio of 1:3 for this cloning system. Amplification with the most distal primers designed from the ends of the SsRV1 and SsRV2 genomes produced many different truncation products of shorter length. This could have been due to inefficient denaturing or strong secondary structures of the viral genomes. SsRV1 and SsRV2 are characterised by high GC contents of 62 % and 63 %, respectively (Preisig *et al.*, 1998). The GC content of DaRV is 53 % (Preisig *et al.*, 2000). The copies of the viral genomes were, therefore, cloned from large overlapping RT-PCR fragments. The full-length cDNA clone of the genome of *Diaporthe ambigua RNA virus* (DaRV) was named pDV3. Additionally, a cDNA copy with six codons for a histidine tag introduced immediately downstream of the putative start codon of ORF1 of this virus was also constructed. The latter cDNA clone was named pH6DV3. Two additional cDNAs were constructed from the genomes of *Sphaeropsis sapinea RNA virus 1* (SsRV1) and *Sphaeropsis sapinea RNA virus 2* (SsRV2). These cDNA clones are known as pNM1-5 and pNM2-5, respectively.

2.4.2. Sequence variations between the published viral sequences and their independently-derived cDNA clones

The sequences of the full-length cDNA copies of the genomes of DaRV, SsRV1 and SsRV2 have many base variations to those published for these genomes (Table 8, Table 9 and Table 10). Sequencing some of the regions containing these base variations in both directions confirmed that these are not due to human error but that they might represent mutations. It is accepted that RNA viruses have the highest rate of mutation in nature (Worobey and Holmes, 1999). Replication errors by RDRP in viral genomes are between 10^{-3} and 10^{-4} substitutions per nucleotide copied (Holland *et al.*, 1982; Domingo *et al.*, 1996). On the other hand, replication error rates of DNA polymerases in eukaryotic and prokaryotic DNA are in the range of 10^{-8} and 10^{-11} misincorporations per base per replication (Drake, 1991). The high rate of mutation in viral RNA genomes is due to the lack of the editing 3' → 5' exonuclease activity found in eukaryotic and prokaryotic replication complexes (Holland *et al.*, 1982; Domingo *et al.*, 1996). This high evolution in viruses is driven by mutations,

reassortment and recombination (Domingo and Holland, 1997, Nagy and Simon, 1997; Roossinck, 1997; Aaziz and Tepfer, 1999).

All positive-stranded RNA viruses encode an RDRP (Lai, 1998). Since RDRPs play a central role of replicating new viral genomes in conjunction with other host- or virus-encoded proteins or on their own, it was expected that there would be fewer mutations in the RDRP coding region than in the ORF encoding the structural protein (García-Cuéllar *et al.*, 1997; Poch *et al.*, 1989). The comparison of the published sequence of SsRV1 with its cDNAs shows that there are fewer changes at both nucleic acid and protein levels in the RDRP ORF than in the coat protein ORF. While there were fewer base variations in the RDRP ORF than the coat protein ORF of SsRV2, there were more amino acid changes in the RDRP ORF than the coat protein ORF. This was because most of the base changes in the RDRP ORF of SsRV2 were in the first codon of the amino acids. These resulted in many amino acid substitutions. Only two nucleotide alterations are present between the published sequence (Preisig *et al.*, 2000) and the cDNA clone sequence of DaRV.

DaRV, SsRV1 and SsRV2 depend on the host's machinery for the replication of their genomes. RDRPs of these viruses are responsible for the task of replicating these viruses. Conservation of sequence in RDRP of these viruses can, therefore, be ascribed to the fact that RNA-dependent RNA polymerisation processes are rare if not foreign to the eukaryotic and prokaryotic hosts of RNA viruses. Many host's cellular factors are recruited for the replication of the viral genomes (Lai, 1998). In order for the viruses to survive, they must ensure sequence conservation in their RDRP genes if their gene products are to preserve their enzymatic activity and their interaction with the highly conserved cellular proteins (Bruenn, 1993).

There were a total of 23 base variations between the published and the cDNA sequences of SsRV1. In this case, 71 % of the sequence variations were in the ORF coding for the coat protein and 29 % were in the ORF coding for the RDRP. A total of 145 base variations were found between the published and the cDNA sequences of SsRV2. The ORF coding for the coat protein constituted 75 % of the sequence variations while the ORF coding for the RDRP constituted only 25 % of those sequence alterations. There is a possibility that the observed sequence variations in

SsRV1 and SsRV2 represent quasi-species of these viruses. It has been suggested that the cDNA sequences of viruses do not represent a single virus, but must be considered as part of a swarm or a quasi-species of mutants that "vary around a consensus sequence" (Domingo *et al.*, 1996; Roossinck, 1997). In order to obtain the sequence of the consensus sequence, direct sequencing of the RNA would be required (Roossinck, 1997). The new viral mutants might display decreased vigour that might be compensated for over time (Worobey and Holmes, 1999). However, the driving force for any sequence variations would be to better adapt the virus to its environmental selective pressure (Domingo *et al.*, 1996; Aaziz and Tepfer, 1999).

2.4.3. Attempts to transfect *S. sapinea* with SsRV1 and SsRV2

2.4.3.1. Failed transfection of *S. sapinea* with SsRV1 and SsRV2

Numerous attempts were made to electroporate *S. sapinea* spheroplasts with *in vitro*-produced single-stranded positive-strand RNA of SsRV1 and SsRV2. Despite these attempts, there was no success. A number of factors might have contributed to the failure to transfect *S. sapinea* with SsRV1 and SsRV2 positive-stranded RNA transcripts.

SsRVs are associated with dsRNA elements of estimated sizes of 1.8 kb and 2 kb in addition to the 5 kb dsRNA band (Fig. 14A). The 5 kb band represents a mixture of genomic dsRNAs of SsRV1 and SsRV2. In order to produce full-length cDNAs of the genomes of these viruses, this 5 kb band was isolated from agarose gel thus effectively separating it from the smaller bands that normally co-purify with it (Fig. 14B).

In transfection studies, the low molecular weight dsRNA elements with unknown functions were excluded. Potential gene products encoded by the low molecular weight dsRNA elements of SsRVs might be essential in some as yet unknown biological interaction with the viral components. The interactions of these components might be essential for the replication cycle of the SsRV1 and SsRV2. The

exclusion of these low molecular weight nucleic acids may therefore deprive the SsRVs of some function that is needed in their replication cycle or in their assembly.

The low molecular weight nucleic acids might play a role similar to the one played by the satellite RNA (sat-RNA) of the groundnut rosette umbravirus (GRV). In the case of the GRV, sat-RNA is essential for the encapsidation of GRV by groundnut assistor luteovirus (GRAV) (Robinson *et al.*, 1999). Until the function of the low molecular weight dsRNA elements of SsRVs is established, it will remain unclear why transfection studies with SsRV1 and SsRV2 failed. The low molecular weight nucleic acids must be sequenced and cloned. Then they must be used to transfect spheroplasts of *S. sapinea* in concert with the SsRVs. The results from these transfections might provide some insight into why transfection of *S. sapinea* with SsRV1 and SsRV2 failed. Such studies might also shed some light on the function of these nucleic acid species.

Thus far, successful transfection of fungal spheroplasts has been reported only for the *C. parasitica* hypovirus (Chen *et al.*, 1996; Chen and Nuss, 1999; Van Heerden *et al.*, 2001). This study reports on the transfection of a *Phomopsis* sp. and three different isolates of *D. ambigua* with DaRV. It is interesting to note that both the *hypovirus* and DaRV do not have ORFs coding for coat proteins (Shapira *et al.*, 1991; Preisig *et al.*, 2000). SsRV1 and SsRV2 both have ORFs that code for a coat protein. In fact, these mycoviruses have been demonstrated to occur as viral particles in their host (Preisig *et al.*, 1998). In their new host, gene products translated from the introduced SsRV1 and SsRV2 must go through a process of assembly of viral particles while *hypovirus* and DaRV only have to be associated with host's membranes (Fahima *et al.*, 1993, Preisig *et al.*, 2000). It is possible that the amount of electroporated single-stranded RNA from the SsRVs is not sufficient for the initiation of the assembly of viable viral particles.

As has been demonstrated for all totiviruses, SsRV1 and SsRV2 have two large open reading frames coding for a coat protein and an RDRP (Ghabrial, 1994 and 1998; Preisig *et al.*, 1998). The need to assemble a capsid protein by both these viruses might be the limiting factor that caused all the transfections to fail. Even though the capsid protein of *Helminthosporium victoriae 190S virus* (Hv190S) is encoded by a

single gene, it is composed of three different polypeptides (Ghabrial and Havens, 1992; Huang *et al.*, 1997; Soldevila *et al.*, 1998). Two of the proteins, p88 and p83 are phosphorylated while p78 is not. The assembly of a capsid is quite a complicated process that includes phosphorylation and posttranslational modification depending on the host (Huang *et al.*, 1997; Soldevila *et al.*, 1998). Interference with any of these processes could have led to failure to assemble viable SsRV1 and SsRV2.

Since the translation of the RDRP ORF has been proposed to be internally-initiated, both SsRV1 and SsRV2 would have two separate proteins for the capsid and replication (Preisig *et al.*, 1998). For proper viral assembly, these proteins would have to be produced in certain optimal ratios. It has been shown for example, that the overproduction of ScVL1 capsid and capsid-RDRP protein interferred with viral replication and effectively resulted in curing of the virus (Valle and Wickner, 1993). Additionally, if the capsid of ScVLa is overproduced in relation to the capsid-RDRP protein, it interferes with viral replication and ultimately results in curing (Yao *et al.*, 1995). Therefore, if the same principle holds for the SsRVs, the proteins for both the capsid and the RDRP would need to be produced in optimal amounts for the successful resurrection of the viruses in their new host. It has been shown in this work that the *in vitro* RNA production of pNM1-5 resulted in multiple bands. These bands could result from secondary structures in full-length RNA transcripts, or they could be a result of truncation products. If these are truncation products, transfection might result in overproduction of short viral fragments either from the capsid or from the RDRP. The overproduction of N-terminal capsid fragments of ScVLa interferes with replication of the virus (Yao and Bruenn, 1995). Many of these concepts will have to be investigated in future transfection studies with SsRV1 and SsRV2.

2.4.3.2. Possible need for a cap structure on RNA transcripts from SsRV1 and SsRV2

The *in vitro*-produced RNA from DaRV, SsRV1 and SsRV2 was not capped prior to transfection. The 5' ends of mRNAs and heterogeneous nuclear RNA (hnRNA) are blocked by 7-methylguanosine (m^7G) to form the structure 3'-G-5'ppp5'-N-3'p. This structure, known as a cap, directs ribosomes to attach to mRNA or hnRNA so that

translation is initiated on the correct AUG (Perry and Kelley, 1976; Kozak, 1978; Kozak, 1991). The cap also stabilises the mRNA (Drummond *et al.*, 1985; Mead *et al.*, 1985). This could have contributed to transfection failures with *in vitro* transcripts from pNM1-5 and pNM2-5. Uncapped RNA transcripts of globin mRNA have been shown not to direct protein synthesis when injected into frog oocytes. In contrast, capped mRNA was able to initiate protein synthesis (Krieg and Melton, 1984). In another experiment it was shown that capped polyadenylated mRNAs coding for chicken lysozyme, calf preprochymosin and *Xenopus* globin were not only efficiently translated but that these mRNAs were more stable than the uncapped mRNAs (Drummond *et al.*, 1985). The importance of a cap structure in translation has also been demonstrated in reoviruses. Thus, the removal of the cap on reovirus mRNA abolishes its translation (Furuichi *et al.*, 1975). Chen *et al.* (1994b) showed that both capped and uncapped RNA of *C. parasitica* hypovirus give successful transfection. In this case, capping was not necessary for the translation of the viral transcripts into the mature virus. Since SsRV1 and SsRV2 are different from the hypovirus, a cap might be essential for translation.

2.4.4. Failed transfections of *Phomopsis* sp. and *D. ambigua* isolates with His-tagged DaRV

Many attempts to transfect *Phomopsis* sp. and different isolates of *D. ambigua* with the His-tagged mutant DaRV failed. The reasons for the failure to transfect the fungal isolates that were successfully transfected with the wild-type DaRV with the engineered His-tagged DaRV are not obvious. As can be seen in Fig. 10 and Fig. 11, the his-tag was introduced immediately downstream of the putative start codon for ORF1 at position 578. It could be that this additional coding sequence for 6 histidine residues interferes with initiation of translation by ribosomes. Even if the *in vitro*-produced RNA was introduced into the spheroplasts, it could not be sufficiently translated by the ribosome of the cell. This could result in the failure of the cell to produce viral proteins that are important to initiate replication of the RNA genome. Chen *et al.* (1996) also reported that despite repeated attempts, *C. cubensis* could not be transfected with a *hypovirus* deletion mutant. This was despite the fact that the same fungus could be transfected with the full-length virus. In the same study, *C.*

parasitica, *C. radicalis*, *C. havanensis* and *E. gyrosa* could be transfected with both the full-length and the deletion mutant *hypovirus*.

The gene product of the first ORF of DaRV is thought to anchor the viral proteins on the host membranes by six potential transmembrane helices at its N terminus (Preisig *et al.*, 2000). Insertion of six histidine residues in this region might affect the ability of the proteins to insert themselves correctly in the host's membranes. This might provide an alternative explanation why the His-tagged mutant DaRV RNA might not initiate replication of the mutant virus.

2.4.5. Relatedness of the transfected *Diaporthe* spp. isolates and the DaRV natural host

The *Diaporthe* spp. used in this study were isolated by Dr W. A. Smit (ARC Infruitec-Nietvoorbij, Stellenbosch). The virus-free and virus-infected isolates had all initially been identified as *D. ambigua* (Smit *et al.*, 1996a,b). This work preceded the recent emergence of DNA sequencing techniques now commonly used in fungal taxonomy. With the exception of *Phomopsis* sp. CMW5588, all the isolates originated from apple. *Phomopsis* sp. CMW5588 was isolated from peach.

At the start of this study, the focus was believed to be on the transfection of different *D. ambigua* isolates with DaRV. However, it was realised that the isolates differed in morphological characteristics. The reason for the classification of the two virus-infected isolates into *D. ambigua* is most probably due to the fact that these isolates do not sporulate in culture (Smit *et al.*, 1996b). Without spores it is very difficult if not impossible to identify *Diaporthe* species. The comparison of the sequences of the ITS regions from these isolates and the analysis of the data showed that the isolates resided in three different clades. These clades represent three different species of *Diaporthe*. The isolates resided in three clades together with *Phomopsis* sp., *D. ambigua* and *D. perjuncta* recently sequenced by Mostert *et al.* (2001). The fact that these isolates are different species is supported by bootstrap values of 100 % within each clade including a reference sequence from the work of Mostert *et al.* (2001) (Fig. 23).

The phylogentic analysis based on ITS sequence data in this study clearly showed that the two virus-infected isolates (CMW3407 and CMW5289) are more closely related to *D. perjuncta* (STE-U2655) while the peach isolate (CMW5588) is more closely related to *Phomopsis* sp. Therefore, this represents evidence that the virus used in these transfections did not originate from *D. ambigua* as reported by Preisig *et al.* (2000) but most likely from *D. perjuncta*. This has far reaching consequences. It implies that a virus originating from a species of *D. perjuncta* was used to transfect different species of the same genus. Certain characteristics of the virus-infected *D. perjuncta* isolate might, therefore, be species-specific and not necessarily due to infection by DaRV.

The transfected *Phomopsis* sp. and *D. ambigua* isolates displayed different morphological characteristics than those of the naturally-infected *D. perjuncta*. In a study where different *Cryphonectria* sp. were transfected, it was recognised that the different species responded differently to the viral infection. It was discovered that while transfected isolates of *C. radicalis*, *C. cubensis* and *C. havanensis* sporulated, the transfected *C. parasitica* could not sporulate (Chen *et al.*, 1996). In another study, it has been reported that the *C. parasitica* hypovirus causes hypovirulence in a South African isolate of *Cryphonectria cubensis* (Van Heerden *et al.*, 2001). The transfected *C. cubensis* isolate displayed hypovirulence-associated traits including inability to sporulate, slow growth and production of orange pigmentation (Van Heerden *et al.*, 2001). The latter phenotype was the opposite of what was observed for hypovirus-infected *C. parasitica*.

No virus-free isolates of *D. perjuncta* were available for this study. Transfection of virus-free *D. perjuncta* isolates will clearly demonstrate the effects of DaRV in these isolates in comparison to the natural host. The lack of hypovirulence in the *Phomopsis* sp. and *D. ambigua* isolates transfected with DaRV in study cannot provide a definitive answer as to whether this virus can be used in biological control of *Diaporthe* spp. closely related to the natural host. However, the study shows that DaRV can replicate in other fungal species related to *D. perjuncta*. In the future, it will be interesting to investigate the possible host range of DaRV.

2.4.6. Transfection of *Phomopsis* sp. and *D. ambigua* isolates with DaRV

Full-length DaRV RNA transcripts were successfully *in vitro*-transcribed from pDV3 and used successfully to transfect one isolate of *Phomopsis* sp. and three isolates of *D. ambigua* strains. Transfection of single-stranded positive sense RNA of DaRV into *Phomopsis* sp. and *D. ambigua* strains spheroplasts was achieved by electroporation at 2000 V. Single pulses resulted in no transfection. After increasing the number of pulses to 5 pulses at 4 seconds intervals, fungi transfected with DaRV were detected. In the past, successful transfections have been reported only for the mild (Chen and Nuss, 1999) and aggressive (Chen and Nuss, 1994b; Chen *el al.*, 1996) variants of the *C. parasitica* hypovirus. To the best of my knowledge this is only the second time that a mycovirus has been used to successfully transfect fungal spheroplasts.

2.4.7. Possible influence on transfection by GDN in motif C of DaRV's RDRP

The genome sequence of DaRV has been found to encode a modified sequence at the highly conserved GDD of motif C of RDRPs. In this motif, DaRV has the altered sequence GDN, wherein the aspartic acid residue is replaced by an asparagine residue. Motif C is thought to contain a β-strand, turn, β-strand structure in which the two aspartic acid residues are positioned on the turn (Argos, 1988). These three amino acid residues are conserved in all RDRPs (Kamer and Argos, 1984). This region is thought to chelate divalent ions at or near the catalytic active site of the enzyme (Argos, 1988; Delarue *et al.*, 1990). In addition to the conserved GDD motif in viral RDRPs, there are 8 other regions which show striking sequence conservation (Koonin, 1991; Bruenn, 1993; Koonin and Dolja, 1993; Routhier and Bruenn, 1998). In fact it has been shown that four of these eight conserved motifs are conserved across all polymerases (Poch *et al.*, 1989). Additionally, it has been shown by computer prediction of secondary structure that although specific regions are different, the palm structure that contains the catalytic site in RDRPs is conserved in all the polymerases that were included in the study (O'Reilly and Kao, 1998). The ability of DaRV with the altered GDN instead of the consensus GDD in motif C to

infect *Phomopsis* sp. and *D. ambigua* spp. means that this mutant is replication competent.

The ability of the RDRP of DaRV to direct replication of the virus despite the mutation in motif C represents an interesting observation. The importance of motif C in the function of RNA-dependent RNA polymerases has been demonstrated in many point mutation experiments in which the glycine and the two aspartic acid residues were replaced by different amino acids. In the first ever study involving motif C, the glycine residue of bacteriophage Qβ RNA replicase was replaced by alanine, serine, proline, methionine or valine. These single amino acid substitutions resulted in complete loss of enzyme activity (Inokuchi and Hirashima, 1987). In another study involving motif C, the glycine residue of poliovirus RDRP was replaced by alanine, cysteine, methionine, proline, serine and valine in different genomic constructs (Jablonski *et al.*, 1991). With the exception of the alanine and serine mutant polymerases, all the other mutants lost their *in vitro* RNA polymerase activity (Jablonski *et al.*, 1991). The studies that followed used RDRPs of such varying viruses as poliovirus (Jablonski and Morrow, 1995), hepatitis C virus NS5B (Lohmann *et al.*, 1997), *rabbit hemorrhagic disease virus* (Vázquez *et al.*, 2000), potato virus X (Longstaff *et al.*, 1993) and encephalomyocarditis virus (Sankar and Porter, 1992) to demonstrate the importance of the two aspartic acid residues in the function of RDRPs.

The first aspartic acid residue is highly essential for function since point mutations in this position result in the abolition of *in vivo* RNA replication and/or *in vitro* RNA synthesis (Longstaff *et al.*, 1993; Jablonski and Morrow, 1995; Lohmann *et al.*, 1997; Vázquez *et al.*, 2000). It was also found that point mutations at the second aspartic acid residue were tolerated even though the resulting polymerases functioned only at the fraction of the wild-type polymerase (Sankar and Porter, 1992; Jablonski and Morrow, 1995; Vázquez *et al.*, 2000). In contrast to the above observations, Vázquez *et al.* (2000) reported total loss of activity of *rabbit hemorrhagic disease virus* RDRP when the second aspartic acid was replaced by asparagine residue. Furthermore, these authors reported that a mutant enzyme in which an aspartic acid residue was replaced by glutamic acid residue regained *in vitro* activity when Mg^{2+} was replaced by either

Mn^{2+} or Fe^{2+}. It would be of interest to investigate the influence of Mg^{2+}, Mn^{2+} and Fe^{2+} on the ability of DaRV RDRP to initiate *in vitro* RNA polymerisation.

The effects of the presence of an asparagine residue instead an aspartic acid residue in mortif C on the activity of this RDRP may be studied if a DaRV mutant with GDD sequence in motif C is constructed and used in transfection studies. Future work must, therefore, concentrate on transfecting *Phomopsis* sp. and *D. ambigua* isolates with the DaRV having the asparagine codon of GDN in motif C replaced by an aspartic acid codon. The successful transfection clearly demonstrates the activity of the GDN RDRP. If the full-length cDNA clone had been derived from a defective RNA, the transfected positive-stranded RNA would not have been able to initiate virus replication. However, there are possibilities that its replication activity is somehow impaired thus only functioning at basal levels.

Impairment of replication of DaRV may be responsible for the general lack of hypovirulence in all the transfected isolates. The hypovirulent naturally-infected *D. perjuncta* CMW3407 might contain a mixed population of DaRV molecules in which there are fewer RNA molecules encoding a motif C with GDD than the ones encoding a mortif C with GDN. The GDD RDRP may be responsible for replicating DaRV RNA *in vivo* and in turn may result in the observed hypovirulence in the naturally-infected *D. perjuncta* CMW3407. When the virus is transmitted via hyphal anastomosis, the recipient fungus would acquire all the viral species that exist in the donor. In transfection studies, only the virus with GDN sequence in motif C was transmitted.

The occurrence of GDN instead of the consensus GDD in motif C of RDRP of DaRV RDRP could be accounted for in two ways. Firstly, this could be a result of an error introduced by RT-PCR amplification. *Taq* DNA polymerase like AMV (avian myeloblastosis virus) reverse transcriptase is known for its infidelity during transcription and reverse transcription, respectively (Saiki *et al.*, 1988; Barnes, 1992). Since these enzymes lack the 3' to 5' exonuclease proofreading activity, they cannot excise mismatched nucleotides (Tindal and Kunkel, 1988). Error rates associated with *Taq* DNA polymerase have been estimated at 2.1×10^{-4} to 1.6×10^{-6} errors per nucleotide per extension (Keohavong and Thilly, 1989; Eckert and Kunkel, 1990;

Lundberg *et al.*, 1991; Barnes, 1992; Hengen, 1995b). It is because of the infidelity of *Taq* DNA polymerase that alternative DNA polymerases able to replicate DNA with high fidelity have been sought. A thermostable DNA polymerase from *Pyrococcus furiosus* has the 3' to 5' exonuclease proofreading activity and has 11- to 12-fold greater replication fidelity than *Taq* DNA polymerase (Lundberg *et al.*, 1991; Barnes, 1994).

The possibility that the sequence variation in motif C of RDRP of DaRV could be due to the infidelity of *Taq* DNA polymerase or AMV reverse transcriptase was ruled out by carrying out more than 10 independent RT-PCRs using the primers Oli64 and Oli80 which amplify the genome from position 2647 to position 3247 (Preisig *et al.*, 2000). These repetitions were necessary to confirm the sequence (Hengen, 1995b). The sequence of the RT-PCR products sequenced in both directions confirmed that the sequence is GDN.

Another way to explain the presence of GDN in motif C of RDRP of DaRV is that the cDNA could have been amplified from a defective RNAs in the population of viral RNAs of DaRV (Preisig *et al.*, 2000). Since there is a possibility that the defective RNAs occur in far larger proportions than the normal RNAs in the fungal cells, the RT-PCR might not be able to detect the RNAs producing an active GDD polymerase. Thus, the possibility that GDN exists on defective RNAs cannot be completely ruled out (Preisig *et al.*, 2000). However, this work shows clearly that such defective RNAs are able to initiate replication in the new hosts.

The RDRP of DaRV shows significant homology to the nonstructural proteins of the carmovirus-like *Turnip crinkle virus* (TCV) and *Carnation mottle virus* (CarMV) (Preisig *et al.*, 2000). Both these viruses belong to the family *Tombusviridae* and infect plants (Carrington *et al.*, 1989; Guilley *et al.*, 1985). Besides the homology of the non-structural proteins of DaRV and these carmovirus-like viruses, their genome organisation is similar. The exception is that the genome of DaRV does not contain an ORF for a movement protein and a coat protein at its 3' end (Preisig *et al.*, 2000). Therefore, DaRV might be a degenerate plant virus. Due to this apparent similarity between DaRV and these plant viruses, it will be important to investigate if this virus can replicate and/or infect plants or plant protoplasts. If DaRV would infect plants

and/or plant protoplasts, this would raise questions about the origins of this virus. Since the *D. perjuncta* from which this virus was isolated is a plant pathogen, this would raise the question of whether this fungus was accidentally infected by a virus from a plant host. Studies to infect *Hibiscus cannabinus* plants and *Arabidopsis thaliana* protoplasts with the infectious DaRV cDNA clone are underway by two research groups elsewhere.

2.4.8. Loss of vector-derived nucleotides at the ends of transfected DaRV

The RNA used for transfection was obtained from an *in vitro* transcription reaction using a *Sal* I-linearised pDV3 (Fig. 25). T7 RNA polymerase starts transcribing 61 nucleotides away for the start of the cloned cDNA. Thus, this introduces 61 vector-derived nucleotides on the 5' end of the wild-type DaRV genome. Since the restriction enzyme *Sal* I was used to linearise pDV3 for *in vitro* RNA production, 35 vector-derived nucleotides were introduced on the 3' end of the wild-type DaRV genome. These vector-derived flanking nucleotides were not present on the replicating RNA of DaRV in the transfected isolates as determined by 5'-RACE for both ends of the genome. Therefore, it was concluded that the vector sequences were trimmed off from the viral sequence after initiation of virus replication within the fungal cells. This phenomenon in which the flanking vector-derived sequences are trimmed off from the viral genome has been observed with the *C. parasitica* hypovirus (Chen *et al.*, 1994a). The authors suggested that the pre-mRNA is subjected to RNA splicing during trafficking from the nucleus to the cytoplasm. Although in this case the virus replication was initiated from a plasmid construct integrated into the fungal genome, such a mechanism might also apply for the introduced *in vitro*-produced viral RNA. It is also possible that the viral RDRP recognises specific sequences at the ends of the viral genome to initiate replication and as such does not allow for the replication of the additional nucleotides.

2.4.9. Characterisation of the DaRV-transfected *Phomopsis* sp. and *D. ambigua* isolates and their isogenic virus-free strains

2.4.9.1. Effects of DaRV on growth of transfected isolates

One of the effects of the *hypovirus* on *C. parasitica* as well as on *C. cubensis* is that the transfectants grow slower than the isogenic virus-free isolates (Chen *et al.*, 1994b; Choi and Nuss, 1992a,b; Chen *et al.*, 1996; van Heerden *et al.*, 2001). The presence of the introduced virus into *Phomopsis* sp. and *D. ambigua* isolates had no general effect. The different fungal isolates responded differently to DaRV. The genetic background of the new host might have a strong influence on the response of each isolate to DaRV. In a similar study, all *Cryphonectria* hypovirus-infected isolates displayed reduced sporulation and laccase accumulation (Chen *et al.*, 1996). The same virus seemed to cause different changes in other hypovirulence-associated traits such as colony morphology, growth rate and pigmentation. The expression of these different phenotypic characters has, therefore, been suggested to be modulated by the host's genetic background (Chen *et al.*, 1996).

An interesting observation was that at low temperatures, the naturally infected isolate grew faster than all the other isolates in the first two days. At 15 °C , this isolate grew to the diameter of 17.22±1.00 mm/day in the first two days of growth. This is more than two times the growth achieved by *Phomopsis* sp. CMW5588 and *D. ambigua* CMW5587 within the same time period and temperature (Table 11). The same trend was also observed at 20 °C. However, the naturally-infected *D. perjuncta* CMW3407 did not grow at 30 °C. It would be interesting to determine if this behaviour is in any way due to the infection by DaRV or only a species-specific characteristic. This question will be partially addressed by transfecting virus-free isolates of *D. perjuncta* with DaRV.

2.4.9.2. Relative virulence of *Phomopsis* sp. and *D. ambigua* isolates using Golden Delicious apples

The apples inoculated with *Phomopsis* sp. and *D. ambigua* isolates (Table 12) developed brown sunken lesions. The lesions were measured after 6 days. Golden Delicious apples have been used successfully to assess virulence of different isolates of fungi (Fulbright, 1984; Elliston, 1985a,c; Enebak *et al.*, 1994; De Lange *et al.* 1998). Fulbright (1984) was able to differentiate between virus-infected and virus-free isolates of *Endothia parasitica* by the lesion areas formed on Golden Delicious apples. In the latter study, it was reported that 3 weeks was the optimal time for measuring the lesions. In another study, the same variety of apples was used to distinguish virus-infected and virus-free isolates of *E. parasitica* (Elliston, 1985a,c). Some of the virus-infected isolates of this fungus did not cause lesions at all on apples. It was reported that 15 days was the optimal time for the test. In another study, it was reported that virus-infected and virus-free isolates of *C. parasitica* did not differ in pathogenicity on Golden Delicious apples. It was, therefore, concluded that the dsRNA viruses occurring in those particular isolates did not induce any observable reaction on the infected fungi (Enebak *et al.*, 1994).

De Lange *et al.* (1998) showed that relative virulence of *C. cubensis* isolates could be rapidly assessed using an apple-based inoculation technique. This test was able to discriminate between virulent and hypovirulent isolates of *C. cubensis*. The accuracy of the apple test was later confirmed when it was demonstrated that the isolates that were more virulent on Golden Delicious apples were also more virulent on *Eucalyptus grandis* trees (Van Heerden *et al.*, 2001). Some of the advantages of the apple inoculation technique are that apples are much cheaper than trees, they are readily available and can be used without the need for a greenhouse. Furthermore, the technique can be applied to fungi under quarantine (De Lange *et al.*, 1998).

The increased virulence of *Phomopsis* sp. CMW5588 transfected with DaRV is not unique as it has been documented that some mycoviruses mediate increased virulence (hypervirulence) in their hosts. Some examples of mycoviruses mediating hypervirulence in their hosts include the viruses of *Phytophthora infestans* (Tooley *et al.*, 1989). It has been reported that virus-infected isolates of *Phytophthora infestans*

are more virulent than virus-free isolates. Additionally, these virus-infected isolates have higher dry weight than the virus-free isolates. The isolate *Phomopsis* sp. CMW5588-DaRV also had a faster growth rate than *Phomopsis* sp. CMW5588-WT. Therefore, growth rate seems to correlate with virulence on Golden Delicious apples since *Phomopsis* sp. CMW5588-DaRV formed larger lesions than *Phomopsis* sp. CMW5588-WT.

The increased virulence due to the presence of a mycovirus in a fungus is not only limited to *Phomopsis* sp. CMW5588-DaRV and *Phytophthora infestans*. It was originally thought that the dsRNA viruses of *Rhizoctonia solani* mediate hypovirulence (Castanho and Butler, 1978). This view was strengthened by the observation that the virus-infected isolates of this fungus were weakly pathogenic while the virus-free isolates were highly pathogenic (Castanho and Butler, 1978; Castanho *et al.*, 1978). Furthermore, the diseased state of the fungus could be transmitted by hyphal anastomosis between virus-infected and virus-free isolates (Castanho *et al.*, 1978). Later evidence showed that the presence of dsRNAs in this fungus was not necessarily associated with hypovirulence (Bharathan and Tavantzis, 1990 and 1991). It was later shown that a dsRNA fragment of 6.4 kb was associated with virulence in *R. solani*. On the other hand, a dsRNA fragment of 3.6 kb was shown to reverse the effects of the 6.4 kb dsRNA (Jian *et al.*, 1997 and 1998).

In a recent study, it has been shown that a dsRNA element occurring in *Nectria radicicola* increases the virulence of this fungus on ginseng (*Panax ginseng*) plants (Ahn and Lee, 2001). *N. radicicola* harbours 4 dsRNA elements of 6.0, 5.0, 2.5 and 1.5 kb. Four isolates each harbouring a single dsRNA element were used in curing studies. The curing studies showed that the isolates cured from the 5.0, 2.5 and 1.5 kb dsRNA were not different in virulence from the non-cured strains. However, isolates cured from the 6.0 kb dsRNA lost their virulence, their ability to sporulate, their ability to discolour Bavendamm medium and their intracellular and extracellular laccase activities. Virulence and the hypovirulence-associated traits were restored on the cured strains after the virus was re-introduced to them by hyphal anastomosis.

In my study, DaRV was introduced into *Phomopsis* sp. CMW5588 by transfection. This means that no other cytoplasmic factors from the donor fungus were introduced

together with this virus into this new host. Therefore, the virulence on apples can be ascribed exclusively to the presence of the replicating genome of DaRV in *Phomopsis* sp. CMW5588. However, the evidence suggesting that mycoviruses in *P. infestans*, *R. solani* and *N. radicicola* mediate increased virulence in their hosts was obtained by curing and re-introducing the viruses into the fungi by hyphal anastomosis. During virus transfer by hyphal anastomosis, the dsRNAs are transferred together with many other cytoplasmic factors. In order to conclusively confirm the effect of these dsRNA elements on these hosts either transfection or transformation studies with the individual viruses will have to be done. The effects manifested by the recipient fungus would therefore be the sole result of the virus.

2.4.9.3. Virulence of *Phomopsis* sp. and *D. ambigua* isolates on apple trees

In this study, apple trees were inoculated in late May during leaf cessation and the results were read in late August just as the trees were showing signs of sprouting. This implies that the inoculation was performed in Autumn and read at the onset of spring. It has been suggested that in Michigan (USA), the peach canker pathogen *Leucostoma persoonii* only causes lesion development on inoculated peach trees when inoculations are done in Autumn (Gerald Adams, personal communication). The development of lesions has been linked to the fact that due to dormancy, the invading pathogens are able to establish themselves without any defence mechanisms being switched on in the tree (Gerald Adams, personal communication). The cankers form during the time when the temperatures allow fungal growth while the trees are in dormancy (Chang *et al.*, 1989). It was further reported that Autumn inoculations result in significantly larger lesions than those formed when the trees were inoculated in Spring. Furthermore, it was shown that Autumn inoculations allowed for the assessment of levels of virulence of different isolates of *L. persoonii* among hosts with different susceptibilities (Chang *et al.*, 1989). It was also shown that peach cultivars with lower cold-hardiness had much larger cankers than the cultivars with higher cold-hardiness (Chang *et al.*, 1989; Jones and Luepschen, 1971). The trees in this present study were, therefore, inoculated in Autumn and infection allowed to continue during the cold season so as to encourage the establishment of lesions.

The tree inoculation experiments showed that within some isogenic isolates (wild-type, transfected and negative control), there were no differences in lesion sizes (Fig. 39). For example there were no significant differences in lesions caused by *D. ambigua* CMW5587-WT and *D. ambigua* CMW5587-DaRV. The same observation was made for *D. ambigua* CMW5287-H_2O and *D. ambigua* CMW5287-DaRV. This could mean that the transfected isolates do not differ in virulence from the wild-type isolates. Such a conclusion may be wrong. There are examples of studies where inoculation of trees with *Diaporthe* failed to result in lesion development (Harris, 1988). In some cases, it has also been observed that even on already infected plants, current and biennial shoots do not show disease symptoms (Fujita *et al.*, 1988). The observation has been made that even though a tree may be infected or inoculated with a virulent *Diaporthe* isolate, lesions only appear after two years (Nakatani *et al.*, 1981; Fujita *et al.*, 1988). If the virus infection of the transfected isolates in this study is stable, the differences in virulence between the DaRV-transfected and wild-type isolates will only be observed after two years.

The growth studies conducted as part of this investigation revealed that the naturally-infected *D. perjuncta* CMW3407 had a higher growth rate at lower temperatures than the virus-free isolates while it did not grow at all at 30 °C (Table 11). Since the inoculation experiments were done in winter when temperatures may be as low as 5 °C, it would be expected that the naturally-infected *D. perjuncta* CMW3407 could at least form the same lesion sizes as the virus-free isolates. The results show that the lesions caused by the naturally-infected *D. perjuncta* CMW3407 did not differ significantly from those formed by *D. ambigua* CMW5287 (Table 13 and Fig. 39). If the ability to cause disease correlates with the growth rate of the fungus, then it should be expected that *D. ambigua* CMW5287 would cause significantly bigger lesions than the naturally-infected *D. perjuncta* CMW3407 in warmer seasons. However, the trees would not be dormant during this period and the formation of lesions might not occur.

In these studies, it was observed that *Phomopsis* sp. CMW5588, *D. ambigua* CMW5288 and *D. ambigua* CMW5287, gave contrasting results on the tree inoculation technique and the apple inoculation technique. It was found that the DaRV-transfected (CMW5588-DaRV) isolate of *Phomopsis* sp. CMW5588 formed

significantly larger lesions on apples than the wild-type (CMW5588-WT) and negative control (CMW5588-H_2O) isolates. The tree inoculation technique showed that even though the lesions caused by the wild-type fungus and the DaRV-transfected fungus were not statistically different, the wild-type fungus formed slightly bigger lesions than the DaRV-transfected fungus. This isolate originated from peach. Perhaps differences between the DaRV-transfected and the wild-type isolates would be observed if the virulence tests were performed on peach trees.

The apple inoculation technique did not reveal any differences in lesion size between the DaRV-transfected (CMW5288-DaRV) and the wild-type (CMW5288-WT) isolates of *D. ambigua* CMW5288 while the tree inoculations showed that the wild-type isolate formed significantly larger lesions than the DaRV-infected isolate. The negative control (CMW5288-H_2O) isolate of this fungus formed significantly larger lesions than the wild-type isolate on apples. The reasons for this behaviour are not known but they may be related to stress due to the transfection process. There were no significant differences in lesion sizes on apple trees. In the case of *D. ambigua* CMW5587, the tree inoculation results corroborated the apple inoculation results. There were no significant differences between lesion sizes formed by the DaRV-transfected (CMW5587-DaRV) and wild-type (CMW5587-WT) isolates of *D. ambigua* CMW5587 on both apples and apple trees. This might suggest that DaRV has no observable impact on this particular isolate of *D. ambigua*.

In order to confirm Koch's postulates, re-isolations of inoculated fungi were done. All the fungi used to inoculate the trees were recovered. PCR amplification of the internal transcribed spacer (ITS) followed by restriction enzyme digestion of these PCR products using *Mse* I conclusively confirmed the identity of the fungi. Double-stranded RNA corresponding to DaRV was isolated from the transfected isolates. This means that the viral infection of these isolates is stable.

2.4.9.4. Phenotypic changes induced by DaRV

The morphological differences between transfected and wild-type isolates of the *Phomopsis* sp. and *D. ambigua* isolates were not as striking as those observed in

hypovirus-transfected *C. parasitica* and *C. cubensis* isolates (Chen *et al.*, 1994b; Choi and Nuss, 1992a,b; Chen *et al.*, 1996; van Heerden *et al.*, 2001). In this study, the morphological differences between the DaRV-transfected and virus-free isolates were not clear. In *Phomopsis* sp. CMW5588 and *D. ambigua* CMW5287, it was discovered that the viral infection enhances mycelial growth. Anomalies in transfected fungi have also been reported to occur in *C. havanensis* (Chen *et al.*, 1996). While the transfected isolates of *C. parasitica*, *C. cubensis* and *E. gyrosa* are characterised by reduced production of aerial mycelia, it was found that the transfected *C. havanensis* had increased production of aerial mycelia (Chen *et al.*, 1996).

2.4.9.5. Virus transfer to spores

The three transfected isolates of *D. ambigua* and the naturally-infected *D. perjuncta* isolate did not sporulate. However, their isogenic wild-type and negative control isolates sporulated. Chen *et al.* (1996) reported that transfected *C. parasitica* and *E. gyrosa* did not sporulate. However, *C. parasitica* was able to sporulate when exposed to high intensity light. In the same study, it was reported that hypovirus-transfected *C. radicalis* and *C. havanensis* sporulated and that 5 % and 86 % of the spore cultures still contained the virus, respectively. Since the transfected isolates harbour the viral RNA in their cytoplasm and not in the genome, they do not have the ability to transmit the virus to ascospore progeny (Chen *et al.*, 1996).

Phomopsis sp. CMW5588-DaRV sporulated both on bench top and when exposed to a mixture of cool-white fluorescent and near-ultraviolet lights. Under both conditions the spore cultures did not contain the virus. These results are consistent with those of Chen *et al.* (1996) and Van Heerden *et al.* (2001). The hypovirus-transfected *C. cubensis* sporulated but the spore cultures did not contain the virus (Chen *et al.*, 1996; Van Heerden *et al.*, 2001).

The fact that the isolates of *D. ambigua* lost their ability to sporulate upon transfection with DaRV while *Phomopsis* sp. CMW5588 isolate retained its ability to sporulate suggests that the same virus can cause different phenotypic changes in different isolates of different species of *Diaporthe*. This means that the host genetic

background has an influence on these phenotypic changes (Chen *et al.*, 1996). It has been suggested that the differences in processing of the infecting hypovirus by the recipient isolates of *Cryphonectria* sp. might influence the phenotypic changes observed in each isolate and the ability of each isolate to support hypovirus infection upon transfection (Chen *et al.*, 1996). However, it is important to recognise that *Phomopsis* sp. is an anamorph of the genus *Diaporthe* while *D. ambigua* is the teleomorph of the genus. The two sexual states of this fungus seem to react differently to the virus.

2.4.9.6. Bavendamm phenol oxidase reaction/gallic acid oxidation

Smit *et al.* (1996b) showed that the virus-harbouring *D. perjuncta* (formerly *D. ambigua*) isolates do not give a colour reaction on the phenol oxidase reaction and gallic acid oxidation media while virus-free isolates produced a colour reaction. In the present study, it was found that some of the transfected isolates (*Phomopsis* sp. CMW5588-DaRV, *D. ambigua* CMW5587-DaRV and *D. ambigua* CMW5287-DaRV) showed a weak colour reaction on the two media. This implies that the virus infection reduces the expression of the enzymes responsible for the reactions but does not abolish their expression. The production of colour by virus-free fungi and the lack of colour production by virus-infected fungi does not seem to be universal. Steenkamp (1996) showed that both the virus-infected and cured isolates of *S. sapinea* produced a strong colour reaction on these media. In a recent study, it has also been shown that cured and virus-harbouring *Nectria radicicola* isolates did not produce different colour intensities on Bavendamm medium (Ahn and Lee, 2001).

2.4.10. Anomalies in growth rate and pathogenicity of negative control fungi

It was expected that the isogenic negative control and wild-type isolates would show exactly the same pattern of pathogenicity and growth rates. However, it was observed that with the exception of *D. ambigua* CMW5288, the negative control isolates showed decreased levels of pathogenicity on apples and decreased growth rates.

Conversely, *D. ambigua* CMW5288-H_2O showed an increased level of pathogenicity and increased growth rate when compared to the wild-type isolate.

In electroporation, cell membranes are charged by applying an electric pulse on their surfaces (Lucas, 2000; Chand *et al.*, 1988). There are two stages to the electric pulse. The first part involves a short pulse at high voltage. During this step, the cell membrane becomes porous. The second part of the pulse is associated with "soft" voltage which increases the sizes of the pores on the cell membrane (Lucas, 2000). These pores serve as entry channels for nucleic acids and other macromolecules into the cell (Lucas, 2000; Chand *et al.*, 1988).

During electroporation, water is electrolysed. At the anode, the pH becomes acidic ($6H_2O \rightarrow O_2 + 4H_3O^+ + 4e^-$) while at the cathode the pH becomes basic ($4H_2O + 4e^- \rightarrow 2H_2 + 4OH^-$) (Lucas, 2000). Since spheroplasts are used in electroporation, buffers that maintain turgor pressure of the spheroplasts are used. In this way, many potentially toxic substances and radical intermediates are produced from the chemical components of the buffer. These substances would, therefore, find their way into the cells with the nucleic acids and may induce reactions on the regenerated fungus. This might be one reason why the negative control fungi showed decreased levels of pathogenicity on apples and decreased growth rates.

The cuvettes used in these studies have electrodes made from aluminium. Electroporation causes dissolution of Al^{3+} ions from the electrodes into the buffer. High concentrations of Al^{3+} ions are cytotoxic (Loomis-Husselbee *et al.*, 1991; Chand *et al.*, 1988). In plants, however, it has been recognised that low amounts of Al^{3+} ions stimulate protoplast division, colony formation and shoot regeneration (Chand *et al.*, 1988; Gupta *et al.*, 1988; Loomis-Husselbee *et al.*, 1991). This phenomenon has not been recorded for fungi. It is possible that Al^{3+} ions are responsible for increased levels of pathogenicity on apples and increased growth rates of the negative control (CMW5288-H_2O) isolate of *D. ambigua* CMW5288. In order to test this phenomenon in this specific isolate, plasma-optical-emission spectroscopic studies would be useful (Loomis-Husselbee *et al.*, 1991). If the effects by Al^{3+} ions are long lasting, then they

might be responsible for the anomalies observed for the growth of the negative control isolates.

2.5. Conclusions

In this study, one isolate of *Phomopsis* sp. and three isolates of *D. ambigua* were successfully transfected with *Diaporthe ambigua RNA virus* (DaRV). This is, to the best of my knowledge, only the second report of transfection using a mycovirus other than the *C. parasitica* hypovirus. The other transfections have been carried out using both the mild (CHV1-Euro7) and aggressive (CHV1-EP713) strains of *C. parasitica* hypovirus (Chen *et al.*, 1994a; Chen *et al.*, 1996; Van Heerden *et al.*, 2001).

An important finding in this study pertained to the identity of the fungus from which *Diaporthe ambigua RNA virus* (DaRV) was first isolated. It was shown that this fungus is *D. perjuncta* and not *D. ambigua* as previously thought by Preisig *et al.* (2000). Therefore, the acronym DaRV should rather be used for ***Diaporthe RNA virus***. In this present study, this virus was transfected into two other species of *Diaporthe*, *D. ambigua* and a *Phomopsis* sp. for which the *Diaporthe* state has not yet been found.

It was expected that since the naturally-infected *D. perjuncta* was hypovirulent, all the transfected isolates would show reduced degrees of virulence. This view was further strengthened by an earlier observation that when the virus was transmitted to virus-free *Diaporthe* isolates belonging to the same VCG by hyphal anastomosis, the resulting fungi became hypovirulent (Smit *et al.*, 1996b). However, the studies in which the virus was transmitted by hyphal anastomosis gave different results from those obtained by transfection in this study. When the virus was transmitted by hyphal anastomosis, the recipient strains became hypovirulent and displayed the hypovirulence-associated traits (Smit *et al.*, 1996b). This suggests that during viral transfer by anastomosis, some other cytoplasmic factors might be transferred along with the virus. These factors might be responsible for the hypovirulence observed in the naturally-infected *D. perjuncta* isolates. The lack of hypovirulence in the transfected isolates could imply that the host genetic background influences the expression of hypovirulence-associated traits as suggested by Chen *et al.* (1996). It is also possible that the duration of the inoculation experiment on trees was too short to assess virulence levels between transfected and virus-free isolates.

Since the phylogenetic analysis shows that the virus does not originate from *D. ambigua* as originally published (Preisig *et al.*, 2000) but instead originates from *D. perjuncta*, it would be of importance to isolate and transfect virus-free isolates of *D. perjuncta*. This would give a more balanced comparative study of the effects of the virus on its natural host. Such a study would also help to establish if there are other factors that might be responsible for the hypovirulence of the naturally-infected isolate.

Up to the present time only the *C. parasitica* hypovirus (CHV1-EP713 and CHV1-Euro7) and the *Diaporthe ambigua RNA virus* (DaRV) have been shown to be infective when transfected into fungal hosts that are closely related to their natural hosts. Both these viruses lack a coat protein and have been suggested to be associated with fungal membranes (Shapira *et al.*, 1991; Fahima *et al.*, 1993; Preisig *et al.*, 2000). The relative ease to transfect fungal spheroplasts with these two viruses and the failure to transfect with the SsRVs, which are known to have capsid proteins, might be related to the fact that the former are much easier to assemble in new hosts than the latter. It can be speculated that in the case of DaRV and hypovirus, fewer *in vitro*-produced viral RNA transcripts are needed to initiate viral replication.

Analysis of the published sequences of DaRV, SsRV1 and SsRV2 and the independently-derived cDNA clones of these viruses revealed some differences at both nucleotide sequence level and to a lower degree at the derived amino acid sequence level. This comparison showed that at nucleotide sequence level, there were more variations in the ORF coding for the coat protein than there were in the ORF coding for RDRP for all the three viruses. However, at the derived amino acid level, the published sequence of SsRV2 and the cDNA clone had more differences between each other in the RDRP ORF than in the coat protein ORF. The reason was that the cDNA sequence had more base changes in the first codon of the amino acids than there were in the second or the wobble positions of the amino acids. The lack of proofreading activity by viral RDRPs in combination with the short replication times give rise to new mutant viral populations known as quasi-species (Holland and Domingo, 1998). It might as well be that the sequence variants observed for SsRV1 and SsRV2 represent quasi-species of these viruses of *S. sapinea*.

RDRP of positive-stranded RNA viruses studied thus far are known to contain the consensus sequence GDD in their motif C. Mutations in this region have been shown to have drastic effects on the replication of the viruses. DaRV has GDN in motif C of its RDRP. The transfected *in vitro*-produced positive-stranded RNA has been demonstrated to be able to give rise to replication-competent viruses. Therefore, this proved that the DaRV RDRP with GDN in motif C is functional. In the future it will be of interest to investigate the effects of transfecting fungal spheroplasts with DaRV with GDD sequence in motif C of its RDRP.

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Summary

Sphaeropsis sapinea and *Diaporthe ambigua* are important pathogens of forest and orchard tree species, respectively. Some isolates of *S. sapinea* are co-infected with two dsRNA viruses, SsRV1 and SsRV2. Isolates of *D. perjuncta* (formerly thought to be *D. ambigua*) are infected with a positive-stranded RNA virus known as DaRV. While *S. sapinea* is infected with a heterogeneous mixture of dsRNA elements of different sizes, *D. perjuncta* is infected with a single virus. This presents excellent opportunity for biocontrol of *Diaporthe*. The aim of this study was to assess these three viruses for possible application as biological control agents of *S. sapinea* and *D. ambigua*. This was done by transfecting these with *in vitro*-produced RNA from the cloned viral genomes and assessing the pathogenicity of the transfected isolates on apples and apple trees.

Attempts to transfect *S. sapinea* spheroplasts with SsRV1 and SsRV2 failed. Co-transfection of *S. sapinea* spheroplasts with both viruses also failed. Three isolates of *D. ambigua* and a single isolate of a *Phomopsis* sp. were successfully transfected with DaRV. Attempts to transfect the same fungi with a mutant of DaRV, bearing six codons for histidine immediately downsteam of an AUG thought to be a start codon for the translation of ORF1, failed.

DaRV was originally thought to be isolated from *D. ambigua*. The fungal isolates transfected with DaRV were thought to be *D. ambigua*. The transfectants did not resemble the naturally-infected isolate. The ITS regions from the ribosomal DNA operon of these isolates were amplified using ITS1 and ITS4 primer pair. The blast search revealed that the ITS sequence of the naturally-infected isolates are identical to *D. perjuncta*. One virus-free isolate was identified as a *Phomopsis* sp. while three other virus-free isolates were identified as *D. ambigua*. A PCR-based RFLP was developed to differentiate the naturally-infected *D. perjuncta* isolates from the virus-free *Phomopsis* sp. and *D. ambigua* isolates.

In the growth and pathogenicity studies, a DaRV-transfected, wild-type and negative control isolate of one *Phomopsis* and three *D. ambigua* isolates, were used. The

DaRV-transfected *Phomopsis* sp. had a higher growth rate than the wild-type isolate. This DaRV-transfected *Phomopsis* sp. was more virulent on apples than the wild-type isolate. The wild-type isolate was slightly more virulent than the DaRV-transfected *Phomopsis* sp. on apple trees.

There were no significant differences in growth rates between the DaRV-transfected and wild-type isolates of *D. ambigua* CMW5587 and *D. ambigua* CMW5287. There were no significant differences in virulence on apples between the DaRV-transfected and wild-type isolates of these fungi. The DaRV-transfected *D. ambigua* CMW5287 was more virulent than the wild-type isolate on apple trees. The DaRV-transfected *D. ambigua* CMW5587 had the same virulence as the wild-type isolate on both apples and apple trees. The DaRV-transfected *D. ambigua* CMW5288 had a slower growth rate than the wild-type isolate. There were no significant differences in virulence on apples between these isolates. The wild-type isolate of this isolate was significantly more virulent on apple trees than the DaRV-infected isolate.

Although transfection was successfully done, the effects of DaRV on the *Phomopsis* sp. and *D. ambigua* isolates are not conclusive. In order to obtain conclusive results, virus-free isolates of *D. perjuncta* must be transfected. During the course of this study, there were no available virus-free isolates of this fungus.

Opsomming

Sphaeropsis sapinea en *Diaporthe ambigua* is belangrike patogene van onderskeidelik denne- en vrugteboomspesies. Sommige isolate van *S. sapinea* word gekoïnfekteer met twee dsRNS virusse, naamlik SsRV1 en SsRV2. Isolate van *D. perjuncta* (voorheen behandel as *D. ambigua*) word geïnfekteer met 'n positief gestringde RNS virus bekend as DaRV. *Sphaeropsis sapinea* word dus geïnfekteer met 'n heterogene mengsel van dsRNS elemente van verskillende groottes, terwyl *D. perjuncta* met 'n enkele virus geïnfekteer word. Hierdie eienskap bied dus 'n uitstekende geleentheid vir die biologiese beheer van *Diaporthe*. Die doel van hierdie studie was om die moontlikheid te ondersoek dat die bogenoemde drie virusse gebruik kan word as biologiese beheeragente van *S. sapinea* en *D. ambigua*. Dit is gedoen deur transfeksie van die fungi met *in vitro*-geproduseerde RNS afkomstig van die gekloonde virale genome, asook patogenisiteitstudies met die getransfekteerde isolate op appels en appelbome.

Pogings om *S. sapinea* sferoplaste met SsRV1 en SsRV2 te transfekteer, was onsuksesvol. Ko-transfeksie van *S. sapinea* sferoplaste met beide virusse het ook misluk. Drie isolate van *D. ambigua* en 'n enkele isolaat van 'n *Phomopsis* spesie is suksesvol gestransfekteer met DaRV. Pogings om dieselfde fungi te transfekteer met 'n mutant van DaRV, met ses kodons vir histidien direk stroomaf van 'n AUG, wat beskou word as 'n beginkodon vir die translasie van ORF1, was ook onsuksesvol.

Daar is aanvanklik gemeen dat DaRV van *D. ambigua* geïsoleer is. Die isolate gestransfekteer met DaRV is ook beskou as *D. ambigua*. Die transfektante het egter morfologies verskil van die natuurlik geïnfekteerde isolate. Die ITS gebiede van die ribosomale operon van hierdie isolate is dus geamplifiseer met primers ITS1 en ITS4. 'n BLAST-soektog het gewys dat die ITS basispaarvolgorde van die natuurlik-geïfekteerde isolate identies was aan *D. perjuncta*. Een virusvrye isolaat is geïdentifiseer as 'n *Phomopsis* spesie, terwyl drie ander isolate as *D. ambigua* geïdentifiseer is. 'n PCR-gebaseerde RFLP is ontwikkel om tussen die natuurlik geïnfekteerde *D. perjuncta* isolate, die virusvrye *Phomopsis* spesie, en *D. ambigua* isolate te onderskei.

In die groei- en patogenisiteitstudies is 'n DaRV-getransfekteerde, 'n wilde tipe, en 'n negatiewe kontrole elk van een *Phomopsis* en drie *D. ambigua* isolate gebruik. Die DaRV-getransfekteerde *Phomopsis* isolaat het vinniger gegroei, en was meer virulent op appels as die wilde tipe. Die wilde tipe het egter groter letsels op die appelbome gemaak as die DaRV-getransfekteerde isolaat.

Daar was geen betekenisvolle verskille tussen die groeitempo van die DaRV-getransfekteerde *D. ambigua* CMW5287 en die virusvrye *D. ambigua* CMW5588 isolate nie. Daar was ook geen betekenisvolle verskille in virulensie op appels tussen die DaRV-getransfekteerde en wilde tipes nie. Op appelbome het die DaRV-getransfekteerde *D. ambigua* CMW5287 egter groter letsels veroorsaak as die wilde tipe. Die DaRV-getransfekteerde *D. ambigua* CMW5587 het dieselfde virulensie getoon op beide appels en appelbome as die wilde tipe. Die DaRV-getransfekteerde *D. ambigua* CMW5288 het stadiger gegroei as die wilde tipe, maar daar was nie betekenisvolle verskille tussen die twee isolate op appels nie. Die wilde tipe was betekenisvol meer virulent op appelbome as die DaRV-geïnfekteede isolaat.

Alhoewel transfeksie suksesvol was, kon die effek van DaRV op die *Phomopsis* spesie en *D. ambigua* isolate nie uitgeklaar word nie. Om die effek van DaRV op sy gashere te bepaal, is dit nodig dat virus-vrye isolate van *D. perjuncta* getransfekteer word. Tydens die verloop van hierdie studie was daar egter geen virus-vrye isolate van hierdie fungus beskikbaar nie.