MOLECULAR CHARACTERIZATION OF DOUBLE-STRANDED (DS) RNAS IN *CHALARA ELEGANS* AND BIOLOGICAL EFFECTS ON THE FUNGUS

by

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ABSTRACT

Molecular characterization of cDNA clones from double-stranded (ds) RNA in *Chalara elegans* was accomplished. The cDNA clones derived from a 5.3 kb dsRNA present in strains of *C. elegans* showed nucleotide sequence homology to viruses belonging to *Totiviridae*. The cDNA clones derived from a 2.8 kb dsRNA in strains BK18 and WASH revealed nucleotide sequence homology to the Mitovirus group. Two cDNA clones derived from a 12 kb dsRNA in strain NC1527 revealed no significant sequence homology to any known virus group. These results suggest the occurrence of at least three different virus groups in *C. elegans*. The genetic relatedness among dsRNAs in *C. elegans* strains was determined using Northern blot and cDNA sequence analysis. The clones derived from a 2.8 kb dsRNA cross-hybridized with 2.8 kb dsRNA occurring in *C. elegans* strains from diverse geographic regions worldwide. However, the cDNA clones derived from either a 5.3 kb or 12 kb dsRNA cross-hybridized only with the similar-sized dsRNAs, mainly in strains originating from same geographic regions. Sequence analysis of the full-length cDNA clone from the 2.8 kb showed the presence of one large open reading frame (ORF), when the mitochondrial genetic code was used. The ORF 1 contained RdRp conserved motifs present in other mitochondrial RNA viruses. This dsRNA copurified with mitochondria and named *Chalara elegans 18 virus* (Ce18V), a new mitovirus. A latently-infected strain (BK18C) which was obtained by high temperature incubation of 2.8 kb dsRNA-containing strain (BK18) for 2-3 months showed enhanced pathogenicity on carrots, but no differences in culture morphology or various enzymatic assays was detected. A full-length cDNA clone of the 5.3 kb dsRNA in strain CKP was also obtained. Sequence analysis revealed the presence of three ORFs,

which shared some homology either to the coat protein or RdRp regions of *Totiviridae*. Partial cDNA clones were obtained from additional dsRNA in strain CKP which shared some homology to the RdRp regions of Totivirus, suggesting coinfection by two distinctive totivirus-like dsRNAs in strain CKP of *C. elegans*. Results from this study demonstrate the presence of diverse novel dsRNA elements in *C. elegans* with differing biological effects.

DEDICATION

I dedicate this work to my husband and parents. Without their patience, understanding, support and most of all love, the completion of this work would not have been possible.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Origins of viruses

The origins of viruses are not clearly understood due to a lack of virus fossil records and the high rate of variation in their genome sequences. However, some new technologies, such as polymerase chain reaction (PCR) and other sensitive molecular techniques, have been used in many viral-related studies, resulting in enormous increases in our knowledge of viral genome sequences. Using the information from viral genomic sequences, comparative analysis has been done to show some similarities among viruses and between viral and cellular proteins, which give some idea as to how they possibly originated and evolved (Gorbalenya, 1995; McGeoch, 1995).

It has been postulated that the origins of life on planet Earth started with RNA as the genetic material, which later gave rise to cells with DNA genomes (Darnell and Doolittle, 1986). The nature of the earliest viruses can never be determined; however, it is likely that they arose very early during the evolution of life on Earth. It has been suggested that the elemental life form involving RNA replicons might be the origin of present RNA viruses (Becker, 2000; Holland and Domingo, 1998). Such primitive RNA molecules provide a replicate domain framework for the first true RNA viruses, which could have emerged when ancestral RNA replicons acquired genes for capsid, and possibly other proteins. These RNA viruses may have continued to evolve with the

evolution of the "DNA world" since the cells that had developed the DNA genomes still included in their genome the genes that coded for RNA molecules and RNA polymerases. This hypothesis was supported by comparative sequence analysis, which indicated that both RNA and DNA viruses have common archaic evolutionary roots in genome structural organization and in certain domains (Gorbalenya, 1995; McGeoch, 1995). However, it is also true that both DNA and RNA viruses could have emerged and evolved through a variety of mechanisms, such as mutation, recombination and reassortment of various genetic sources (Becker, 1998; Holland and Domingo, 1998). Indeed, it is quite unlikely that all viruses could have arisen from a single set of ancestral RNA molecules. It is more probable that viruses originated or evolved from many events by acquiring diverse genetic elements, such as other primitive replicons during the evolution of life.

1.2 Virus evolution

Virus evolution could have occurred *via* three major strategies, including recombination, mutation and reassortment (Holland and Domingo, 1998; Domingo et al., 1996). All of these forces may generate diversity in viral genomes under natural selection pressures.

1.2.1 Mutation

All RNA viruses have extremely high mutation rates because of lack of error-proof activity of viral RNA-dependent RNA polymerase (RdRp). Mutation rate can be defined as the actual rate of misincorporation of nucleotides, while the mutation

frequency can be defined as the real misincorporation of nucleotides that become established in the population. Several studies have shown that the averaged misincorporation rates are about 10^{-4} or one error per 10 kb genome (Domingo and Holland, 1994). This is a high rate compared to that of cellular DNA-dependent DNA polymerases (DdDps), which is about 10^{-9} (Rossinck, 1997). This high mutation rate can generate RNA viruses with great adaptability. In addition to the mutation rate, the mutation frequency can be one of the forces which generate diversity in viruses (Domingo and Holland, 1997; Rossinck, 1997). Similar mutation rates can be found in all RNA viruses, while the mutation frequency differs dramatically, depending on the virus (Aranda et al., 1993; Fraile et al., 1996).

1.2.2 Recombination and reassortment

Recombination has been considered to be one of the major mechanisms in the evolution of viruses. RNA recombination could occur either through homologous recombination between two nearly identical RNAs, or through nonhomologous recombination between two RNAs that have a short parallel stretch of complementarity (Simon and Bujarski, 1994). Phylogenetic analyses of many different RNA viruses, such as luteoviruses, nepoviruses and bromoviruses, have shown that recombination events could have occurred and played a role in virus evolution (Allison et al., 1989; Gibbs, 1995; LeGall et al., 1995). In addition to recombination, some RNA viruses have segmented genomes and reassortment among RNA segments could allow the evolution or generation of new viruses (Zaccomer et al., 1995).

2.1 Origins of plant viruses

A major debate in the field of plant viruses focuses on whether the origin of RNA viruses is monophyletic or polyphyletic (Gorbalenya, 1995; Rossinck, 1997; Rossinck, 2003). The monophyletic argument suggests that all RNA viruses were derived from an original set of domains with shuffling, which resulted in somewhat conserved domains with or without different genomic arrays (Gorbalenya, 1995; Dolja and Carrington, 1992). Genetic variation among viruses can occur during long-term mixed infections with different viruses, which eventually result in the loss or replacement of specific parts of the virus genome (Delmer et al., 1993). A well-known example can be found in *pea enation mosaic virus*. In this virus, both RNA1 and RNA2 contain conserved motifs; however, RNA1 is similar to a luteovirus, while RNA2 is similar to carmoviruses and tombusviruses (Delmer et al., 1993).

The argument for polyphyletic origin states that the genomes of viruses were derived from different origins, such as different portions of the host plant genome, and that they evolved independently (Gorbalenya, 1995). This theory is supported by evidence of sequence diversity present in specific viral regions, such as the movement protein gene. Many virus features are common for plant and animal viruses. However, plant viruses have to evolve specific mechanisms for successful transmission to the host plant, because plants have thick cell walls, which are absent in animal cells. Successful plant viruses must develop strategies to move both cell-to-cell and through the plant systemically. Sequence analysis of many movement protein genes has revealed that the sequence similarity is low among the viruses, which might indicate diverse origins (Goldbach, 1986; Goldbach et al., 1994).

Plant viruses may have genes of both monophyletic and polyphyletic origins and they use these two strategies together for adaptations in host plants. Some domains, such as RdRp regions, appear to be monophyletic, while other domains, such as movement genes, may have arisen from diverse origins, such as host genomes, which could provide better adaptations to specific host plants (Rossinck, 1997; Rossinck, 2003).

3.1 Origins of fungal viruses

Fungal viruses or mycoviruses are present in numerous fungi and typically contain double-stranded (ds) RNA genomes (Buck, 1986; Ghabrial, 1980; Ghabrial, 1998). The absence of an extracellular infection mode is one of the characteristics of these viruses and, therefore, they have evolved efficient horizontal transmission modes through either hyphal anastomosis between individual fungal isolates, or vertical transmission through fungal spores (Buck, 1986; Ghabrial, 1994). A large number of mycoviruses are known to be associated with cryptic symptoms in fungi, which is considered as strong evidence that these viruses have evolved in close association with their hosts (Bruenn, 1993; Lemke, 1979). The death of the fungal host caused by mycovirus pathogenesis would result in mycovirus elimination because of the apparent absence of an extracellular infection mode.

Several families of dsRNA viruses of fungi, including *Hypoviridae*, *Narnaviridae, Partitiviridae,* and *Totiviridae*, have been classified based on their fungal host, the number of genome segments and their capsid structure (Ghabrial et al., 1995A; Ghabrial et al., 1995B; Hillman et al., 1995; Hong et al., 1999; Tuomivirta and Hantula, 2004; Wickner et al., 2000). These dsRNA viruses are considered to be polyphyletic because of

the diversities in their genomes. Comparative genetic analysis of the amino acid sequences of RdRp regions, which are considered to be the most highly conserved genes among RNA viruses, revealed little similarity among dsRNA viruses (Koonin, 1991; Koonin, 1992). Phylogenetic analysis of RdRp regions among dsRNA viruses revealed a lack of conservation of primary sequence and size. This was interpreted as suggesting multiple evolution events occurring in different (+) strand RNA viruses (Koonin, 1992; Koonin and Dolja, 1993).

The mycoviruses are believed to have ancient origins (Bruenn, 1993; Ghabrial, 1998). Several genetic studies of dsRNA viruses have revealed that original dsRNA virus groups could infect cells before divergence of protozoa and fungi (Huang and Ghabrial, 1996; Ghabrial, 1998). This hypothesis is supported by the finding that the totivirus, Hv190SV, found in plant pathogenic fungi is more closely related to the Leishmaniaviruses (LRV1 and LRV2) found in protozoa than to the yeast viruses. This suggests that Hv190SV and LRVs existed prior to the divergence of protozoa and fungi, which is believed to have happened very early in evolution (Ghabrial, 1998).

4.1 DsRNAs in Cryphonectria parasitica

4.1.1 Introduction

In the 1960s, chestnut trees infected with the chestnut blight fungus, *Cryphonectria parasitica*, in North America were introduced to Europe, and the pathogen quickly started to infect European chestnuts and cause serious problems (Grente, 1965; Grente, 1969). Antonio Biraghi, an Italian pathologist, found that some of the originally infected trees survived, and consequently, Jean Grente, a French mycologist, isolated

non-lethal fungal strains from such infected trees (Biraghi, 1946; Anagnostakis, 1987, Grente, 1965). These strains had unique characteristics, such as lighter pigmentation and less virulence to host trees, so they were named "hypovirulent strains" (Grente and Sauret, 1969).

DsRNAs were found in these hypovirulent strains and these dsRNAs were considered a fungal virus (Choi and Nuss, 1992; VanAlfen et al., 1975). Infection by this virus could perturb various normal developmental processes of the host fungus, including reduction of sexual or asexual sporulation, reduction of virulence, and reduction of pigment production (Grente and Suaret, 1969; Griffin et al., 1983; Jaynes and Elliston, 1982). The transfer of these dsRNAs through hyphal anastomosis from hypovirulent to virulent strains resulted in the development of various hypovirulent characteristics in virulent strains, which gave rise to studies on developing the dsRNAs as potential biological control agents (Anagnostakis, 1982; VanAlfen et al., 1975).

The exact mechanisms of hypovirulence caused by dsRNAs are still not clearly understood, although numerous studies have been conducted (Elliston, 1982; Nuss, 1992; Nuss and Koltin, 1990, Dawe and Nuss, 2001). Molecular characterization of diverse dsRNA fragments, their effects on fungal hosts, and the potential use of dsRNAs as biological control agents will be discussed in this section.

4.1.2. Molecular characterization of dsRNA

4.1.2.1 Hypoviruses in C. parasitica

The best-studied hypovirus in *C. parasitica* is the *Cryphonectria* hypovirus 1-EP713 (CHV1-EP713) (Shapira et al., 1991). CHV1-EP713 was isolated from one of the

European hypovirulent isolates (EP713) of *C. parasitica*. In 1992, Choi and Nuss developed a full-length cDNA copy of this virus and transformed virus-free *C. parasitica* spheroplasts with this cDNA copy (Choi and Nuss, 1992; Chen et al., 1993). These transformants showed the hypovirulent characteristics, confirming that the dsRNA was the causal agent of hypovirulence in *C. parasitica* (Choi and Nuss, 1992; Chen et al., 1994). The development of full-length cDNA clones has been used in many different ways, including the functional analysis of CHV1-EP713 (Chen et al., 1993; Chen and Nuss, 1999; Chen et al., 2000).

The coding strand of CHV1-EP713 is 12,712 nucleotides long, excluding the poly(A) tail (Shapira et al., 1991). Two large open reading frames, ORF A and ORF B, were found in the genome. ORF A encods two polypeptides, p29 and p40, which are released from a polyprotein, p69, by autocatalytic activity of p69 (Choi et al., 1991a; Choi et al., 1991b). ORF B encods a large polyprotein and also releases a polyprotein (p48) by autocatalytic activity (Shapira and Nuss, 1991). The junction between ORF A and ORF B contains 5'-UAAUG-3', which may serve as both a termination codon (UAA) for ORF A and an initiation codon (AUG) for ORF B. The RdRp motifs were found in ORF B and shared sequence homology to that of potyviruses, which are one of the largest and most common plant virus groups (Choi et al., 1991; Koonin et al., 1991)

Research on *C. parasitica* field isolates revealed considerable variability in virulence and morphology, suggesting the possible presence of different hypoviruses in *C. parasitica* (Elliston, 1978; Elliston, 1985; Enebak et al., 1994b; MacDonald and Fulbright, 1991). The CHV1-Euro7 was selected to develop cDNA clones because its effects on the host fungus, *C. parasitica*, were quite different compared to that of CHV1-

EP713 (Chen and Nuss, 1999). Strain EP713 conferred highly debilitating symptoms, such as retarded growth rate, small cankers on chestnut trees, and reduced spore production. In contrast, *C. parasitica* infected with strain Euro7 showed faster growth, large canker expansion, and increased capacity for spore production compared to EP713 infected one. Full-length cDNA clones were developed for this second hypovirus in *C. parasitica,* and named CHV1-Euro7. Sequence comparison between these two hypoviruses, CHV1-EP713 and CHV1-Euro7, revealed high similarities at the level of both nucleotide and amino acid sequences (Chen and Nuss, 1999).

Two additional hypoviruses, CHV2 and CHV3, have been characterized in *C. parasitica*. CHV2 was first isolated in New Jersey from isolate NB58 of *C. parasitica* (Hillman et al., 1992). The genomic structure of CHV2 was quite similar to that of CHV1 (Hillman et al, 1992; Hillman et al., 1994). Both hypoviruses have two ORFs and poly A tails at the 3' end; however, CHV2 lacks the papain-like proteinase which is present in the ORFA of CHV1. The third hypovirus group (CHV3), first identified in a Grand Haven isolate, GH2, had a relatively smaller genome size (9 kb) and contained only one ORF rather than two ORFs as was found in CHV1 and CHV2 (Fulbright et al., 1983; Yuan and Hillman, 2001). However, the ORF in CHV3 contained a putative proteinase, RNA polymerase, and helicase domains that were similar to those identified in both ORFs in CHV1 and CHV2. An amino acid sequence comparison showed that CHV1 and CHV2 were more closely related to each other than to CHV3 (Smart et al., 2000).

4.1.2.2. Various dsRNAs in C. parasitica

In addition to the presence of hypoviruses in *C. parasitica*, the presence of other virus groups has been documented (Hillman et al., 1992; Hillman et al., 1994). In 1994, a small dsRNA from isolate NB631 of *C. parasitica* was reported (Polashock and Hillman, 1994). The culture morphology of this strain was similar to that of the dsRNA-free isolate, but virulence and laccase production were reduced. Full-length cDNA clones of the dsRNA were produced and revealed the complete nucleotide sequence of this dsRNA. The entire sequence of this dsRNA in *C. parasitica* was 2,728 bp long, which was much smaller than the other hypoviruses, which ranged from 9 kb to 12 kb. The deduced amino acid sequence analysis revealed that there were no large ORFs when the cytoplasmic genetic code was used. However, one large ORF was observed when the mitochondrial genetic code was used, suggesting that this virus might be present in the mitochondria. Isolation of mitochondria and nuclease assays confirmed the presence of this small dsRNA in mitochondria and it was named *Cryphonectria parasitica mitovirus* 1-NB631 (CpMV1-NB631) (Wickner et al., 2000).

In 1994, Enebak et al. reported the presence of multiple dsRNA fragments in a hypovirulent isolate of *C. parasitica*, and that these dsRNAs were genetically unique (Enebak et al., 1994a). Recently, Hillman et al. (2003) reported that virus particles were purified from a distinct hypovirulent isolate of *C. parasitica* that contained 11 dsRNA segments. These purified virus particles were reintroduced into a virus-free isolate of *C. parasitica*, resulting in morphology similar to that of dsRNA-containing isolates, suggesting that these dsRNAs were responsible for hypovirulence traits. Sequence

analysis of three large fragments among 11 revealed that these fragments were closely related to other known viruses belonging to *Reoviridae*.

In addition to the presence of different virus groups in *C. parasitica*, defective interfering (DI) and satellite dsRNAs were also observed (Hillman et al., 2000; Yuan and Hillman, 2001). In CHV3-GH2, an isolate which contained CHV3 hypovirus, an additional three small dsRNAs, [dsRNA2 (3.6 kb), dsRNA3 (1.9 kb) and dsRNA4 (0.9 kb)], were present. Sequence analysis of dsRNA2 revealed one ORF, which consisted of protease and helicase domains of hypovirus CHV3, indicating that this dsRNA could be a defective RNA of CHV3. However, the other two dsRNAs (dsRNA3 and dsRNA4) shared little sequence homology with dsRNA1 and dsRNA2 in CHV3-GH2, suggesting that these dsRNAs were satellite RNAs (Yuan and Hillman, 2001).

4.1.3. Functional analysis of hypoviruses

The full-length infectious clones of hypovirus in *C. parasitica* have been used in various aspects of research, including functional analysis of the hypovirus genome. A transformation study with ORF A of CHV1-EP713 into a dsRNA-free isolate showed the development of orange pigmentation, reduction of both asexual sporulation and laccase production, but no changes in virulence, suggesting that this ORFA region of CHV1-EP713 was responsible for other phenotypic changes but not virulence in the host fungus (Rigling et al., 1989; Rigling and Van Alfen, 1991; Nuss, 1996). Further investigations using various mutants of these hypoviruses showed that p29 regions could be the specific regions responsible for these phenotypic changes (Craven et al., 1993). For example, one deletion mutant, Δp29, which lost 88% of the p29 coding domain in ORF A, showed viral

replication and hypovirulence, but there was a restoration of phenotypic changes, such as reduction of orange pigment, increased conidiation and laccase production (Craven et al., 1993). Suzuki et al. (1999) further characterized the symptom determinant regions of p29 in CHV1-EP713. They found that the region from Phe-25 to Gln-73 of p29 could be essential for development of phenotypic changes and showed a moderate level of sequence similarity with the N-terminal region of the HC-pro papain-like protease in the potyvirus group. Four conserved cysteine residues, Cys-38, Cys-48, Cys-70, and Cys-72, were observed and more detailed studies using mutational analysis were conducted for these conserved regions. Mutation of both Cys-38 and Cys-48 revealed no changes, while mutation of Cys-70 resulted in the development of severe phenotypic traits, such as retarded growth rate and altered culture morphology. However, substitution of a glycine for Cys-72 showed a reduction of symptom expression (Craven et al., 1993; Suzuki et al., 1999).

The similarity between the fungal hypoviruses and plant potyviruses, especially functional and structural similarities for p29 and HC-pro, are particularly interesting (Koonin et al., 1991; Suzuki et al., 1999). It has been known that the HC-pro could be involved in various functions, such as aphid transmission, long distance movement, and promotion of genome amplification in plants (Atreya et al., 1992; Carrington et al., 1989; Cronin et al., 1995; Kasschau et al., 1997; Thornbury et al., 1985). Recently, HC-pro was considered to play an important role as a viral suppressor of post-transcriptional gene silencing (Kasschau and Carrington, 1998; Liave et al., 2000). In this regard, the relationships between the p29 and HC-pro could indicate that these two viral proteins may interact with ancestrally-related regulatory proteins in their respective hosts.

Full-length cDNA clones were developed from two isolates of *C. parasitica*, which contain different CHV1 viruses, CHV1-EP713 and CHV1-Euro7 (Chen and Nuss, 1999; Shapira et al., 1991). These isolates were selected because of the existence of significant phenotypic differences. Sequence analysis of these two hypoviruses revealed a high sequence identity, both at the nucleotide and amino acid levels, ranging from 87-93% and 90-98%, respectively. However, when these two hypoviruses were introduced independently into virus-free strains of *C. parasitica* to determine their effects on the host fungus, it revealed major differences in development of hypovirulence traits, which were similarly observed in the original dsRNA-containing field isolate. Therefore, this result could indicate that these two hypoviruses (CHV1-EP713 and CHV1-Euro7), are the major factors determining hypovirulence traits in *C. parasitica*, rather than host genetic background, and could be named as severe and mild hypoviruses, respectively. Because of the high nucleotide sequence similarity between CHV1-EP713 and CHV1-Euro7, recombinant chimeras were used to determine the viral determinants responsible for specific hypovirulent traits (Chen et al., 2000). A recombinant chimera containing CHV1-Euro7 ORF A and CHV1-EP713 ORF B showed severe hypovirulent traits, suggesting that the ORF B region of CHV1-EP713 could confer the severe hypovirulence traits. Further analyses using various recombinant chimeras have revealed that it could be possible to develop an engineered hypovirus which expressed different symptoms by mixing and matching specific domains of these two CHV1s (Chen et al. 2000).

4.1.4. Hypovirus effects on fungal gene expression

The changes in fungal virulence associated with infection of hypoviruses were considered as an important tool to understand the basis of fungal virulence. Comparative studies of extracellular enzyme activities showed some differences between virulent and hypovirulent isolates of *C. parasitica* and were extended to the genetic level (Powell and VanAlfen, 1987a; Powell and VanAlfen, 1987b; Zhang et al., 1993). Using sequences derived from known extracellular enzymes, several genes, such as cryparin, laccase, cutinase and cellulase, have been cloned from *C. parasitica* (Kazmierczak et al., 1996; McCabe and VanAlfen, 1999; Varley et al., 1992; Zhang et al., 1994; Zhang et al., 1998). Most enzymes could be down-regulated by infecting with CHV1; however, some of the enzymes could be up-regulated (Chen et al., 1996; Powell and VanAlfen, 1987b).

Laccase is a copper-containing phenol oxidase. It is not clearly understood what the function of this enzyme is in the development of virulence of *C. parasitica*. However, some studies have shown that laccases in other fungi could play important roles related to fungal development and virulence, including degradation of lignin, formation of fruiting bodies, and pigment production (Ander and Eriksson, 1976; Leatham and Stahmann, 1981; Leonard, 1971; Marbach et al., 1985). It has been speculated that the lack of canker penetration by hypovirulent isolates of *C. parasitica* could be related to the reduced expression level of laccase (Rigling et al., 1989; Rigling and VanAlfen, 1991). Genetic analysis of *C. parasitica* revealed the presence of three laccase genes, which are *lac 1*, *lac 2*, and *lac 3* (Kim et al., 1995; Rigling and VanAlfen, 1993). Expression levels of laccase genes could vary depending on the environment, such as nutrient medium, the age of the culture, and the amount of light available (Choi et al., 1992). CHV1 infection

could result in a 75% reduction in laccase enzyme activity (Rigling and VanAlfen, 1991; Choi and Nuss, 1992). Subsequent studies have revealed that laccase expression levels could be regulated at the level of transcription or stability of laccase mRNA (Choi et al., 1992; Rigling and VanAlfen, 1991). A similar result was observed in a hypovirulent isolate of another fungal species, *Diaporthe ambigua* (Smit et al., 1996).

Cutinase is another enzyme reduced by hypovirus infection. Cutinase is considered to be important in plant pathogenic fungi because it can degrade plant cuticles and help fungal penetration into host cells. Indeed, positive relationships between the amount of cutinase produced and virulence have been reported in some plant pathogenic fungi (Li et al., 2003; Rogers et al., 1994; Schafer, 1993). Varley et al. (1992) showed that a hypovirulent strain of *C. parasitica* produced significantly lower amounts of cutinase compared to that of a virulent strain, suggesting the dsRNA presence could have had some effect on cutinase gene expression. Several other hydrolytic enzymes, including polygalacturonases and cellulases, which are considered to play important roles in fungal peneration and pathogenesis, were also significantly reduced by infection of *C. parasitica* with a hypovirus (Gao et al., 1996; Gao and Shain, 1995).

Although a number of genes related to fungal pathogenesis have been characterized following infection by hypoviruses, many genes or proteins probably act in concert to cause hypovirulence traits, because fungal pathogenesis is a complex process. It has been proposed that hypovirus infection could perturb regulatory mechanisms in fungi, such as signal transduction pathways (Dawe and Nuss, 2001; Larson et al., 1992). The G proteins are known to be involved in signal transduction pathways and consist of three units (α, β, and γ). The Gα subunit cloned from *C. parasitica* and named *cpg-1*,

showed 98% homology to that of *Neurospora crassa* (Zhang et al., 1998; Gao and Nuss, 1996). Hypovirus infection resulted in the down-regulation of *cpg-1*, indicating an association between virus infection and signal transduction pathways in *C. parasitica*. The second G protein subunit gene, *cpg-2*, was also cloned from *C. parasitica* (Gao and Nuss, 1996). However, deletion of this gene from *C. parasitica* showed a mild phenotype compared to the deletion of *cpg-1*, suggesting that each G protein subunit had different effects on the development of hypovirulence traits. Although the detailed mechanisms by which hypoviruses interact with or regulate the signal transduction pathways of host fungi are not clearly understood, it is possible that the G proteins could be responsible for transmitting the virus signals to initiate complex hypovirulence phenotypic traits.

4.1.5. Hypovirus as potential biological control agent

Extensive studies on hypovirulence were first conducted to develop the potential of biological control agents. Several reports showed that the hypovirus-mediated hypovirulence could effectively contribute to control of chestnut blight disease in Europe (Anagnostakis, 1987). However, it has been observed that the efficacy of hypovirulence control strategies for chestnut blight disease in North America was much lower than in Europe (Dawe and Nuss, 2001). One of the possible reasons for this lower efficacy could be the presence of diverse vegetative compatibility groups (VCG)s in *C. parasitica* in North America (Anagnostakis, 1977; Anagnostakis and Day, 1979). Fungal isolates which belong to different VCGs cannot form hyphal anastomosis and virus transmission is restricted. Indeed, field isolates surveyed for their VCGs revealed that the VCGs in *C.*

parasitica populations in North America were more diverse relative to that found in Europe (Anagnostakis, 1988). Another way to spread hypovirus is through either asexual or sexual spores. However, hypovirus transmission through ascospores was restricted in *C. parasitica*. Also, the efficiency of hypovirus transmission through conidia was quite variable, ranging from 10 to 90%, depending on the fungal and hypovirus genome (Shain and Miller, 1992; Dawe and Nuss, 2001).

In this regard, the transgenic hypovirulent strains which contain the hypovirus genome in their chromosomes have some advantages in hypovirus transmission. Because the hypovirus genome has been integrated into the fungal chromosome, the viral genetic information could be inherited through ascospore progeny (Anagnostakis et al., 1998). Also, because of the allelic rearrangement of VCG loci, the progenies derived from hypovirus transgenic isolates could have diverse VCGs, which is beneficial for hypovirus transmission in nature. The possible use of hypoviruses as biological control agents was also examined in other fungal species, such as *Cryphonectria radicalis*, *Cryphonectria havenesis*, *Cryphonectria cubensis* and *Endothia gyrosa* (Chen et al., 1996a). Hypovirus infection using spheroplasts with synthetic transcripts resulted in various phenotypic changes, implying that the hypovirus could induce similar hypoviurlence traits in different fungal species. Further extension of hypovirus (CHV1-EP713) infection to other fungal species, such as *Valsa ceratosperma* and *Phomposis sp.*, were also reported, implying the hypovirus in *C. parasitica* could possibly be used as biological control agents of other plant pathogenic fungi (Sasaki et al., 2002).

5.1 Totivirus in Helminthosporium victoriae

5.1.1 Introduction

Helminthosporium victoriae was first described in 1946 and caused Victoria blight disease on oats (Meehan and Murphy, 1946). This fungus can produce a host-specific toxin called "victorin" and the amounts of victorin were correlated with fungal virulence (Wolpert et al., 1985). Isolates of *H. victoriae* with low virulence were found to produce low amounts of victorin (Ghabrial, 1986). The culture morphology of isolates with reduced virulence included retarded growth rate, and aerial mycelial collapse was also observed. These characteristics were transmitted through hyphal anastomosis. The presence of dsRNA in these isolates was observed and a correlation between dsRNA and diseased phenotype was reported (Ghabrial, 1980; Ghabrial, 1986).

The presence of dsRNAs in *H. victoriae* is particularly interesting not only because of the hypovirulence caused by these dsRNAs, but also because of the presence of two viruses in infected isolates. Based on their sedimentation values, these two viruses were named *Helminthosporium victoriae* 190S virus (Hv190SV) and Hv145S virus (Sanderlin and Ghabrial, 1978). Hypovirulence appeared to be correlated with the concentration of Hv145S virus, rather than of Hv190S virus. However, no fungal isolates which contained only Hv145S virus alone were found, suggesting that the Hv145S virus depended on Hv190S virus for replication and/or encapsidation (Sanderlin and Ghabrial, 1978). Soldevila et al. (2000) reported that the Hv145S encoded its own RdRp gene, indicating it can replicate by itself without the help of the Hv190S virus. However, further studies related to encapsidation with or without Hv190S are needed.

5.1.2. Helminthosporium victoriae 190S (Hv190S) virus

Helminthosporium victoriae 190S (Hv190S) has isometric virus particles, 40 nm in diameter, and the total nucleotide sequence is 5,178 bp in size (Huang and Ghabrial, 1996). The complete nucleotide sequence has been determined and the virus is classified in the family *Totiviridae*, which can infect either fungi or parasitic protozoa (Ghabrial, 1998). Two large overlapping open reading frames (ORF 1 and ORF 2) were found and they encoded a coat protein (CP) and an RNA-dependent RNA polymerase (RdRp) protein, respectively. The junction between these two open reading frames has a 5'-AUGA-3' sequence which overlaps with the stop codon (UGA) for the upstream CP ORF and the translational start codon (AUG) of downstream RdRp ORF (Huang and Ghabrial, 1996; Huang et al., 1997).

Genome expression strategies of the Hv190S virus appeared to be quite different from that in other totiviruses, such as ScV-L-A in yeast and LRV1 in protozoa. Other totiviruses expressed their RDRP protein as a fusion protein (CP-RdRp) by fusing ORF1 and 2 via a ribosomal translational frameshifting (Icho and Wickner, 1989). However, Hv190S virus seemed to translate both CP and RdRp proteins by using an internal initiation mechanism (Soldevila and Ghabrial, 2000). Similar expression strategies of Hv190S virus were predicted in two other totiviruses (SsRV1 and SsRV2), which can infect the filamentous fungus *Sphaeropsis sapinea* (Preisig et al., 1998). The observation that totiviruses infecting filamentous fungi could be different in terms of expression of RdRps compared to the totivirus infecting either yeast or parasitic protozoa could have important implications in understanding the interactions between totiviruses and fungal hosts during their evolution.

5.1.3 Helminthosporium victoriae 145S (Hv145S) virus

Four dsRNAs were associated with Hv145S virus and their sizes were 2.7, 2.9, 3.1 and 3.6 kbp, respectively (Sanderlin and Ghabrial, 1978). Northern blot hybridization using cDNA clones of Hv145S virus revealed that there was no detectable homology between Hv145S and Hv190S viruses (Soldevila et al., 2000; Soldevila and Ghabrial, 2000). In addition, the four dsRNA fragments in Hv145S virus each contained unique nucleotide sequences. Highly conserved sequences at their 5'- and 3'-ends were observed, implying that all four dsRNAs may utilize the same RdRp protein for their replication (Soldevila et al., 2000).

Sequence analyses of these Hv145S viruses revealed some sequence homology to other chrysoviruses, which belong to the family *Partitiviridae*, and all of them contained only one large ORF. The amino acid sequence of dsRNA 1 showed the highly conserved motifs of RdRps of RNA viruses, while the remaining dsRNAs 2, 3, and 4 did not show any statistically significant sequence similarity to known viruses. However, dsRNA 2 contained a coding region for a potential coat protein, which is a characteristic of the family *Partitiviridae* (Ghabrial et al., 1995).

5.1.4 Interactions between dsRNA and host fungi

Because of their limited genome size, it is not surprising that viruses use host fungal proteins for their own use. Recently, one cellular protein called HV-p68 was found to copurify with the viral dsRNAs in *H. victoriae* (Soldevila et al., 2000). Sucrose density gradient centrifugation with purified virus preparations from diseased isolates of *H. victoriae* revealed several differently sedimenting species, including virion

components (HV190S-1 and HV190S-2), and host protein HV-p68. Agarose gel electrophoresis of each sediment revealed that the HV-p68 fraction contained mainly 145S dsRNAs. The complete sequence of HV-p68 revealed 70% identity to the alcohol oxidase protein in yeasts. Expression of the HV-p68 gene at the transcriptional level was elevated (10-20 fold) in diseased isolates and the protein product was also found to be overproduced when Western blot analysis was performed. Further experiments revealed that HV-p68 could bind both viral RNAs (190S and 145S dsRNAs), suggesting the presence of RNA-binding activity in HV-68 protein (Soldevila et al., 1998; Soldevila and Ghabrial, 2001).

It has been speculated that HV-p68 in *H. victoriae* could have functions similar to that of alcohol oxidase in yeast. In yeast, alcohol oxidase can convert alcohol to aldehyde, which is toxic to both yeasts and filamentous fungi. Accumulation of such toxic intermediates by overexpression of HV-p68 could lead to the various diseased phenotypes seen in *H. victoriae*. Also, it has been suggested that the HV-p68 in *H. victoriae* could involve the signal transduction pathways similar to that observed in *C. parasitica*; however, more detailed studies are required (Ghabrial et al., 2002).

6.1. Mitoviruses in Ophiostoma novo-ulmi

6.1.1 Introduction

The plant pathogenic fungus *Ophiostoma ulmi* is an ascomycete and can cause Dutch elm disease (Laut et al., 1973; Karnosky, 1979). This fungus can be transmitted by elm bark beetles, infect the xylem of elm trees and produce wilt toxins called cerato-ulmins (CU), which result in the blockage of the xylem and death of trees (Lea and

Brasier, 1983). The first serious loss of elm trees caused by this fungus occurred around 1910 in Northern Europe, and the pathogen spread rapidly world-wide to regions including North America and part of Asia (Brasier, 1991; Karnesky, 1979). A new more aggressive species called *O. novo-ulmi*, which can cause almost 100% mortality in mature elms, was discovered during the 1940s. Two races of *O. novo-ulmi*, called the Eurasian (EAN) and North American (NAN) races, have been distinguished and appear to have different geographic origins, such as Ukraine or Romania for EAN race and North America for NAN race (Brasier, 1991).

The presence of dsRNAs in *Ophiostoma* was first observed in 1980 (Brasier, 1983). Both *O. ulmi* and *O. novo-ulmi* appeared to contain multiple dsRNA fragments. However, the frequency of dsRNAs was much higher in *O. ulmi* than in *O. novo-ulmi* (Brasier, 1986). At first, the correlation between the presence of dsRNAs and their effects on the fungal host was undetermined, because of the multiplicity of dsRNA fragments and large differences in fungal genetic backgrounds. However, more detailed studies on the dsRNAs revealed that some specific dsRNA fragments present in both *O. ulmi* and *O. novo-ulmi* could cause diseased phenotypic traits in the fungal host (Brasier, 1986).

The diseased phenotypic trait was first described in an *O. novo-ulmi* NAN race isolate from France and named as a disease-factor (d-factor) in 1983 (Brasier, 1983; Sutherland and Brasier, 1995). Many *Ophiostoma* isolates infected with the d-factor were obtained from natural diseased trees and showed quite variable phenotypes. Based on the order of discovery, different d-factors have been numbered, such as d^1-, d^2-, d^3- and d^{12}. Hyphal anastomosis between diseased and healthy isolates resulted in the

development of "d-reaction" in the healthy isolate, which showed slower growth rate and distinct culture morphology (Brasier, 1984; Brasier, 1986). Experiments with isolates containing marker genes revealed that the d-factor could be caused by extranuclear elements (Brasier, 1984).

6.1.2. d^2-factors associated with dsRNA fragments

Examination of d^2-factor-containing isolates revealed the presence of 10 dsRNA fragments by polyacrylamide gel electrophoresis (Rogers et al., 1986). The fragments were various sizes, ranging from 0.33 kb (RNA-10) to 3.49 kb (RNA-1) (Rogers et al., 1986). However, minor differences both in the sizes and numbers of dsRNA fragments were observed among d^2 factor isolates of *O. novo-ulmi*. For example, L13d^2 isolate contained all 10 dsRNA fragments except RNA-7. Also, further analysis using a high resolution PAGE system revealed two more dsRNA fragments, RNA-1b and RNA-3b, in L8d^2 isolate for a total of 12 dsRNA fragments in one isolate (Cole et al., 1998).

Electron microscopy did not detect any virus-like particles in d^2 factor isolates of *O. novo-ulmi*. However, the dsRNA fragments were co-purified with the mitochondria fraction, suggesting that these dsRNA fragments could localize in the mitochondria (Buck, 1986). Also, it has been shown that the diseased isolates of *O. novo-ulmi* had reduced cytochrome oxidase levels compared to the healthy isolates, indicating the d factors could affect mitochondrial function (Rogers et al., 1988).

Transmission studies showed that all dsRNA fragments could be transferred into dsRNA-free isolates by hyphal anastomosis if both isolates belonged to the same vegetative compatibility group (Brasier, 1984; Rogers et al., 1986). Transfer rate of

dsRNA fragments through conidia occurred at a high frequency, up to 100%; however, some of the progeny derived from single conidia showed the loss of several dsRNA fragments (Rogers et al., 1986). A few isolates derived from single conidia also showed reversion from a diseased phenotype to a healthy phenotype and these changes were correlated with the loss of some dsRNA fragments, such as RNA-4, RNA-7 and RNA-10, implying that these dsRNAs could possibly be responsible for the diseased phenotype in *O. novo-ulmi* (Cole et al., 1998; Rogers et al., 1986).

Sequence analysis of dsRNA fragments following the development of cDNA clones showed that RNA-4 consisted of 2,599 nucleotides and an ORF, which may encode a 783 amino acid protein (Hong et al., 1999). This ORF had 11 UGA codons, which do not act as stop codons but encode for tryptophan in the mitochondria of most fungi (Hong et al., 1998; Hong et al., 1999). Also, the dsRNA sequence contained high ratios of A and U residues, which is a characteristics of mitochondrial DNA genomes. The putative proteins in ORF1 showed the conserved motifs present in the RdRp of RNA viruses (Hong et al., 1998; Hong et al., 1999).

Sequence analysis of RNA-7 showed it to be 1,057 nucleotides long and could be derived from RNA-4 with three internal deletions (Hong et al., 1999). Because of large deletions in the RdRp regions of RNA-7, RNA-7 replication could be dependent on RNA-4. Both 5' and 3'- ends of RNA-7 showed sequence identity to those regions of RNA-4, indicating that these regions could act as recognition sites for the RdRp encoded RNA-4. Other dsRNA fragments, such as RNA-3a (2,617 bp), RNA-5 (2,474 bp) and RNA-6 (2,343 bp), were also characterized by developing cDNA clones (Hong et al., 1998; Hong et al., 1999). Each of these dsRNAs contained one large ORF, which could

encode RdRp proteins of 718 amino acids, 729 amino acids and 695 amino acids, respectively. These ORFs also showed mitochondrial genome characteristics, similar to those found in RNA-4.

Comparison of nucleotide sequences among the dsRNAs revealed a range of identities both at the nucleotide and amino acids levels. Nucleotide identities among RNAs 3a, 4, 5, and 6 ranged from 42.9% to 54.8% and amino acid identities of ORFs among them ranged from 20.3% to 33.3% (Hong et al., 1998; Hong et al., 1999). Because of these large differences in RdRp regions among dsRNA fragments, it is more likely that each dsRNA fragment replicated independently in one isolate of *O. novo-ulmi*. Also, a dsRNA transfer study through conidia or ascospores showed that dsRNA 1a, 1b, 2 and 3a could replicate independently, because it was found that some progenies, derived either from single conidia or ascospores, contained only one dsRNA fragment such as RNA-2 (Rogers et al., 1986). Similar single conidia progenies which had only one dsRNA fragment or had lost a specific dsRNA fragment were observed, suggesting these large dsRNAs 1a, 1b, 2 and 3a could replicate independently (Cole et al., 1998). In contrast to the independent replication of the large dsRNA fragments, small dsRNAs such as RNA-7 to 10, could be derived from the large dsRNA fragments and be too small to encode their own RdRps, suggesting that they may need the large dsRNA fragment for their replication. Indeed, a dsRNA transfer study through single conidia showed that the failure of the transfer of dsRNA-1b *via* single conidia resulted in the loss of the smaller dsRNA-8, suggesting that the dsRNA-8 needed the dsRNA-1b for replication (Rogers et al., 1986). Although it is not clearly known how these independent dsRNA fragments could be present together in one mitochondrion, the dsRNA transfer study through

conidia showed that these dsRNAs could be present in one cell because a high number of progeny derived from single conidia contained all these dsRNAs (Rogers et al., 1986; Cole et al., 1998).

6.1.3. Problems in use of dsRNA fragments as biological control agents

The relationship between the presence of dsRNAs and various d-factors and symptoms in *O. novo-ulmi* led to interest in the use of these dsRNAs as potential biological control agents for Dutch elm disease. However, there are several problems to be overcome. In field experiments, the number of conidia required for the development of successful infection of elm trees is quite different between d-factors in infected and healthy isolates (Webber, 1987; Webber 1993). Infection of the highly susceptible tree host, *U. procera*, required 50,000 conidia of a d^2-infected isolate, but only 500 to 1,000 conidia of a healthy isolate. Therefore, inoculation of sufficient numbers of conidia of a diseased fungal isolate to compete with a healthy isolate in nature could not be easily obtained. In addition to this problem, other factors, such as the relative resistance of the tree host, various dsRNA effects on the fungus, and complex environmental conditions, need to be considered during evaluation of dsRNAs as a biological control agent for this pathogen (Sutherland and Brasier, 1997; Webber, 1993).

These dsRNAs would be better biological control agents if d-factor associated dsRNAs could be easily transferred from diseased to healthy isolates and infect a much larger population of *O. novo-ulmi*. However, it has been suggested that vegetative compatibility reactions could greatly restrict dsRNA transfers among the *O. novo-ulmi* population, depending on the severity of the incompatibility reaction (Webber, 1993).

High transmission of dsRNA fragments through conidia has been observed; however, a proportion of the conidia showed a loss of some dsRNA fragments (Rogers et al., 1986). Moreover, dsRNA transmission through ascospores of *O. novo-ulmi* is quite restricted. Therefore, more detailed work related to the d-factor associated dsRNAs and host fungus is needed before the use of these dsRNA fragments as biological control agents of Dutch elm disease is feasible.

6.1.4. Presence of OnuMV3a in other fungi-Sclerotinia homoeocarpa

The presence of dsRNA elements in *Sclerotinia homoeocarpa*, which is the causal agent of dollar spot disease of turfgrass, was observed and could cause hypovirulence traits such as retarded growth rate and abnormal culture morphology (Zhou and Boland, 1997; Zhou and Boland, 1998). Two genetically distinct dsRNA fragments were detected in *S. homoeocarpa*; however, only the L-dsRNA (2.6 kb) seemed to be correlated with the hypovirulence traits (Deng and Boland, 2001). Molecular characterization of the L-dsRNA was recently reported by developing full-length cDNA clones (Deng and Boland, 2003).

The L-dsRNA was 2,632 bp in size and showed one large open reading frame (ORF) when the mitochondrial genetic code was used. This ORF might encode a protein of 720 amino acids containing conserved motifs of RdRp regions of other RNA viruses. Ultrastructural analysis with TEM showed the presence of degenerated mitochondria in a hypovirulent isolate of *S. homoeocarpa*, and the L-dsRNA was co-purified with mitochondria, indicating that the L-dsRNA has similar characteristics to other known mitoviruses (Boland, 1992).

Sequence analysis of L-dsRNA revealed interesting results, which were the high sequence identities at both the nucleotide (92.4%) and amino acid (95.1%) levels to the *O. novo-ulmi mitovirus* 3a-Ld (OnuMV3a) in *O. novo-ulmi* (Deng and Boland, 2003). Due to the high sequence identities, these two dsRNAs were considered to be conspecific, and the L-dsRNA in strain Sh12B of *S. homoeocarpa* was named *O. novo-ulmi mitovirus* 3a-Sh12B (OnuMV3a-Sh12B). It is not clearly understood how these similar dsRNAs could be present in two taxonomically distinct fungi; however, because of the presence of high sequence identities between these two dsRNA fragments, it has been proposed that horizontal transmission of the dsRNA between these two different fungal groups may have occurred (Deng and Boland, 2003).

7.1. Overview of the plant pathogenic fungus, Chalara elegans

Chalara elegans NagRaj & Kendrick (syn. *Thielaviopsis basicola* (Berk. &Br.) Ferr.) is a plant pathogenic fungus which causes black root rot disease on more than 100 host plants (Nag Raj and Kendrick, 1975; Yarwood, 1981). This fungus has been widely distributed in many regions worldwide, including agricultural and non-cultivated soils. Although the optimum conditions for *C. elegans* vary depending on the host plant species (Lloyd and Lockwood, 1963; Mauk and Hine, 1988), this fungus generally grows best at temperatures between 20°C to 25°C and pHs from 5.5 to 6.5 (Punja, 1992).

The pathogen initially infects the host plant roots through a wound site and results in the development of above-ground symptoms, including stunting, wilting and chlorosis of the foliage. In the early stages of disease, the infected roots are normally white; however, the root systems become black and water-soaked as the disease progresses.

Two types of asexual spores have been found on infected roots and are produced in culture. One type is the phialospores (endoconidia), are hyaline, cylindrical in shape and are produced first in large numbers. The other type, chlamydospores, are darkly pigmented, thick-walled and responsible for the long term survival of *C. elegans*.

Various strategies have been attempted to control the black root rot disease. In some cases, such as *C. elegans* on groundnut, resistant cultivars have been successfully developed and used for controlling the disease (Jones and Van Der Merwe, 1986; Cilliers, 2001). However, because little is known about the cultural practices which may reduce or limit the development of black root rot disease, in most cases preventative strategies, such as sanitation and avoidance of wounding have been used to control black root rot disease due to *C. elegans* (Daughtrey et al., 1995; Punja et al., 1992).

A large number of field isolates of *C. elegans* were shown to contain dsRNAs, which were quite variable both in number and size (Bozarth and Goenaga, 1977; Bottacin et al., 1994). In some strains, only one dsRNA fragment near 2.8 kb in size was observed, while other strains contained multiple dsRNA fragments ranging from 2.5 kb to 12 kb in size, suggesting the possible presence of diverse mycovirus groups in *C. elegans* (Bottacin et al., 1994). Previous experiments revealed that certain dsRNA fragments could have some effect on the host fungus; however, the relationship between the dsRNA and *C. elegans* is not clearly understood (Bottacin et al., 1994; Punja, 1995). Research related to dsRNA characterization, including the development of cDNA clones, could help to elucidate the roles of dsRNAs in *C. elegans*. Sequence information and molecular characterization of dsRNAs in *C. elegans* should provide insight into the biology of

dsRNA and its function in *C. elegans,* as has been demonstrated for other plant pathogenic fungi.

8.1 Research objectives

The objectives of this study were to: 1) develop cDNA clones from diverse dsRNA fragments in *C. elegans* isolates and determine the genetic relatedness among dsRNA fragments by Northern blot analysis; 2) compare cDNA sequence information to known virus groups to determine how many different dsRNA groups are present in *C. elegans*; 3) develop full-length cDNA clones of specific dsRNA fragments to characterize them at the molecular level; and 4) attempt to eliminate dsRNA fragments in *C. elegans* strains using various methods and to use these dsRNA-cured strains to understand the relationship between the specific dsRNA fragment and the biology of *C. elegans*.

CHAPTER 2

OCCURRENCE OF GENETICALLY DIVERSE DOUBLE-STRANDED RNA ELEMENTS IN *CHALARA ELEGANS* (*THIELAVIOPSIS BASICOLA*) [a]

2.1. Introduction

Chalara elegans Nag Raj and Kendrick (syn. *Thielaviopsis basicola* (Berk. and Br.) Ferr.) is a soilborne dematiaceous hyphomycete that causes black root rot disease on numerous plant species, which include several economically important crops (Nag Raj and Kendrick, 1975; Punja et al., 1992; Yarwood, 1981). A high proportion (up to 80%) of field isolates of *C. elegans* were reported to contain dsRNA fragments ranging in size from 2.6 kb to 12 kb, and some isolates contained eight or more fragments (Bottacin et al., 1994; Bozarth and Goenaga, 1977). The extent of genetic diversity among these dsRNA elements is unknown. Such information would enhance the understanding of how these elements may have originated and been disseminated in the fungal population as well as providing insights into their potential effects on the biology and pathogenicity of *C. elegans*. Specific dsRNA fragments were previously shown to alter pathogenicity, spore production, growth rate, pigmentation, and survival of some strains of *C. elegans* when they were eliminated from the specific strain (Bottacin et al., 1994; Punja 1995).

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The degree of genetic diversity among dsRNA fragments in fungi is generally determined by a comparison of molecular weight sizes on agarose gels, Northern blot hybridization, and sequence characterization (Ahn and Lee, 2001; Buck, 1986; Dawe and Nuss, 2001). Currently, there are at least four described virus families in fungi, including *Partitiviridae*, *Narnaviridae*, *Hypoviridae* and *Totiviridae* (Ghabrial et al., 1995A; Ghabrial et al., 1995B; Hillman et al., 1995; Hong et al., 1999; Wickner et al., 2000). In *Cryphonectria parasitica* and *Rhizoctonia solani*, a wide range of nucleotide sequence diversity was reported among dsRNA fragments within isolates (Anagnostakis, 1982; Bharathan and Tavantzis, 1990; Chen et al., 1996). The presence of multiple infections with several related or unrelated dsRNA fragments in one isolate can also lead to complex relationships among dsRNA fragments (Nuss and Koltin, 1990; Rong et al., 2001). For example, up to 12 unencapsidated dsRNAs, ranging in size from 0.33 to 3.5 kb (all belonging to *Narnaviridae*), were observed in one isolate of the Dutch elm disease fungus, *Ophiostoma novo-ulmi* (Hong et al., 1999). Co-infection with two distinct dsRNAs (belonging to *Totiviridae*) was observed in one isolate of *Sphaeropsis sapinea* (Preisig et al., 1998). The presence of defective dsRNA and/or satellite dsRNA, which can induce significant changes in fungal phenotype, was reported in *C. parasitica* (belonging to *Hypoviridae*) and *Saccharomyces cerevisiae* (belonging to *Totiviridae*) (Hillman et al., 2000; Wickner, 1996). All these reports indicate that complex dsRNA elements can occur within a single fungal species.

The objective of this study was to determine the extent of genetic diversity among dsRNA fragments in *C. elegans*. We developed cDNA clones from selected dsRNA fragments in six representative strains of *C. elegans* using random and specific primers,

and assessed the genetic relatedness among dsRNA fragments in 21 strains through Northern blot hybridization. Lastly, we obtained sequence information for all cDNA clones and compared this to previously published virus groups.

2.2. Materials and Methods

2.2.1. Fungal isolates.

The strains of *C. elegans* included in this study are described in Table 1. All cultures were maintained on V8 agar (V8A, containing V8 juice, 150 ml; Bacto agar, 15 g; distilled water, 850 ml; ampicillin, 100 mg) at ambient room temperature (21-23°C). Actively-growing cultures were initiated by transferring an 8-mm diameter mycelial plug to a fresh V8A plate and incubating at room temperature for 4-5 days prior to conducting the analysis described below.

2.2.2. Extraction of dsRNA

Each strain was transferred to 100 ml of potato dextrose broth (PDB, Difco Laboratories, Detroit, MI), and incubated at room temperature for 10-14 days. Mycelium was harvested through Miracloth® using a vacuum, blotted dry between paper towels, and then ground with a mortar and pestle in 2X STE (0.1 M Tris-HCl, 0.2 M NaCl and 2 mM EDTA, pH 6.8) and transferred to a 15 ml Falcon tube. The volume was adjusted to 5 ml with 2X STE and 500 µl of 10% SDS was added. Each sample was incubated at 37°C for 30 min with occasional hand-mixing. An equal volume of phenol: chloroform: isoamylalcohol (25: 24: 1) was added, the mixture was incubated at room temperature for 5 min and centrifuged at 8,000 g for 10 min. The supernatant was transferred to a new

TABLE 1. Strains of *Chalara elegans* included in this study.

Strain designation	dsRNA fragment sizes present (kb)[a]	Host or substrate of origin	Geographic origin	Year isolated	Source
AK4N1	3.0, 3.6, 5.3	Cotton	Clarkdale, Arkansas	1989	C. Rothrock
AK89-2	3.6, 5.3	Cotton	Clarkdale, Arkansas	1989	C. Rothrock
AK89-4	2.7, 3.0, 3.6, 5.3	Cotton	Clarkdale, Arkansas	1989	C. Rothrock
AK89-7	2.7, 3.6, 5.3	Cotton	Hope, Arkansas	1989	C. Rothrock
AK308	2.7, 3.6, 5.3	Cotton	Hope, Arkansas	1989	C. Rothrock
BK18	2.8	Cotton soil	Kings Co., California	1991	B. Holtz
BK25	2.8	Cotton soil	Kings Co., California	1991	B. Holtz
BK28	2.7, 5.3	Cotton soil	Kings Co., California	1991	B. Holtz
BC92CO	2.8	Carrot	Cloverdale, British Columbia	1992	Z. Punja
CKP	2.7, 3.0, 3.6, 5.3	Chickpea	Pullman, Washington	1993	W. Kaiser
F374	2.7, 5.3	Cotton	Kern Co., California	1989	J. Marlow
HA	2.8	Carrot	Amsterdam, Holland	1990	Z. Punja
HB93-2	2.8	Motherwort	Cloverdale, British Columbia	1993	L. MacDonald
NC1	2.7, 3.0, 3.6, 5.3, 6.3, 6.8, 12	Tobacco	Allegheny, North Carolina	1988	D. Shew
NC2	2.7, 3.0, 3.6, 5.3, 6.3, 12	Tobacco	Watauga, North Carolina	1988	D. Shew
NC1526	2.7, 3.0, 5.0, 5.3, 6.8, 12	Tobacco	Mitchell, North Carolina	1987	D. Shew
NC1527	2.7, 3.0, 3.6, 5.3, 6.3, 12	Tobacco	Madison, North Carolina	1987	D. Shew
OR1	2.0, 2.7, 3.6, 5.3	Petunia	Clackomas Co., Oregon	1991	J. Marlow
OR2	2.0, 2.5, 3.6, 5.3, 6.8	Vinca	Texas	1989	J. Marlow
TX	2.7, 3.0, 3.6, 5.3	Cotton	West Texas	1991	K. Arthur
WASH	2.8	Carrot	Bellingham, Washington	1989	Z. Punja

[a] Sizes were determined following electrophoresis on a 1% agarose gel at 60V for 3 hr. Banding patterns are shown in Fig.1.

Falcon tube, an equal volume of chloroform: isoamylalcohol (24: 1) was added and mixture incubated at room temperature for 5 min, then centrifuged at 8,000 g for 10 min. The aqueous phase was transferred to a new 15 ml Falcon tube and precipitated with an equal volume of isopropanol followed by centrifugation at 8,000 g for 15 min. The resulting pellet was washed with 70% ethanol and air-dried by inverting the tubes. The pellet was resuspended in 100 µl TE buffer, treated with RQ1 DNase (Promega, Madison, WI) by incubating at 37°C for 30 min, followed by RNase A treatment by adding 30 µl of 5 M NaCl and 1 µl of 5 mg/ml RNase A (USB, Cleveland, OH) at 37°C for 10 min. Total nucleic acids were further treated with phenol/ chloroform purification, precipitated with two volumes of 100% ethanol and 0.1 volume of 3 M NaOAc (pH 6.8) by incubating at -20°C for 1 hour, and washed with 70% ethanol (Sambrook et al., 1989). The pellet was resuspended in 4.25 ml of 1X STE and 750 µl of 100% ethanol, and applied to a CF-11 cellulose column prepared in a 5 ml syringe with glass wool plug. The column was washed with 15 ml of STE: EtOH (85%: 15%) mixture and the dsRNA sample was eluted by adding 4 ml of 1X STE (Morris and Dodds, 1979). The dsRNA was precipitated with ethanol, resuspended with 30 µl of diethylpyrocarbonate (DEPC) treated water, and used for low-melting agarose gel purification.

2.2.3. Low-melting agarose gel purification of dsRNA fragments

The dsRNA samples prepared by CF-11 column chromatography were subjected to electrophoresis at 80 V for 30 min in a 1% low melting agarose gel and the dsRNA fragments were examined under UV light. The predominant dsRNA bands (2.8, 5.3 and 12 kb in size, see Fig. 1) were cut out with a scalpel and the gel-blocks containing the

described dsRNA bands were resuspended in 450 µl of 1X STE and incubated at 65°C until the gel was completely melted. The sample was treated twice with 500 µl of phenol with vortexing for 1 min and centrifuged at 10,000 rpm for 10 min at room temperature. The aqueous layer was treated with 500 µl of chloroform: isoamylalcohol (24:1), centrifuged at 18,000 g for 10 min, precipitated with ethanol by incubating at -80°C for 1 hr, centrifuged at 18,000 g for 30 min, and washed with 70% ethanol. The dsRNA pellet was dried under speed vacuum for 10 min, resuspended in 20 µl DEPC-treated water and used for cDNA production and cloning.

2.2.4. cDNA production and cloning

A 2.5 µl sample of dsRNA was mixed with 1 µl of random hexanucleotides (3 µg/µl) (Invitrogen, Carlsbad, CA), boiled for 10 min and immersed in ice. First-strand cDNA was synthesized using 3.5 µl of heat-denatured dsRNA with random primer mixture, 1 µl of 100 mM DTT (Gibco BRL, Grand Island, NY), 2 µl of 5X first strand synthesis buffer, 0.5 µl of each 10 mM dNTP (Amersham, NJ), 0.25 µl of RNase inhibitor (10 unit/µl) (Invitrogen, Carlsbad, CA), 0.5 µl of Superscript II reverse transcriptase (200 u/µl) (Gibco BRL, Grand Island, NY) and 2.25 µl of DEPC-treated water. The mixture was incubated at 42°C for 45 min and denatured at 99°C for 5 min. The second-strand cDNA was synthesized in PCR reactions containing the following components: 10 µl of first-strand cDNA synthesis, 1 µl of each 10 mM dNTPs, 1.5 µl of 50 mM MgCl, 1 µl of random primer (3 µg/µl), 0.5 µl of Taq polymerase (5 unit/µl), 0.5 µl of Taq extender (5 unit/µl), 5 µl of 10X Taq extender buffer, and 30.5 µl of DEPC-treated water. Amplification conditions were one cycle at 94°C for 5 min, 35 cycles each

at 94°C for 1 min, 55°C for 45 sec, 72°C for 2 min, and one cycle at 72°C for 10 min for final extension. Amplified RT-PCR products were subjected to electrophoresis at 60 V for 1 hr in a 1% agarose gel and visualized under UV light. The distinct DNA bands were purified using Qiaquick gel extraction kit (Qiagen, GmbH, Germany), and cloned using the TOPO vector kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

2.2.5. RT-PCR with dsRNA specific primers

Specific primers were designed based on sequence information derived from cDNA clones of dsRNA fragments in strains BK18, CKP, and NC1527 (Table 2). As a positive control, RT-PCR was performed with total nucleic acids extracted from strain CKP using various combinations of these forward and reverse primer sets. Also, these dsRNA-specific primer sets were used to either extend or generate more cDNA fragments from dsRNAs in several *C. elegans* strains with or without combinations of random primer.

2.2.6. DNA sequencing

Plasmids containing inserts were selected and sent to the Nucleic Acids Protein Service (NAPS) unit at the University of British Columbia (Vancouver, BC) for bi-directional sequencing using M13 reverse and forward primers. Sequences of cDNA inserts obtained were then compared to known fungal virus groups using the BLAST program (http://www.ncbi.nlm.nih.gov/ BLAST/) and also used to align partial cDNA fragments with each other.

2.2.7. Northern blot hybridization

DsRNA was extracted from the 21 strains listed in Table 1, purified using CF 11 as described above, loaded on a 0.7% agarose gel, and electrophoresed at 60 V for 3 hr in 0.5 X TBE buffer. The gel was treated with 0.2 N HCl for 10 min, denatured with 50 mM NaOH and 1.5 M NaCl for 20 min, and neutralized with 1 M Tris and 1.5 M NaCl for 20 min. The dsRNA was transferred by capillary action to a nylon membrane (Roche Molecular Biochemicals, Switzerland) with 10X SSC (Sodium Chloride/Sodium Citrate) overnight and cross-linked to the membrane by UV irradiation (Stratagene Crosslinker). Hybridization with dsRNA-specific cDNA probes was performed using the non-radioactive DIG system according to the manufacturer's protocol (Roche Molecular Biochemicals, Switzerland).

2.3. Results

2.3.1. dsRNA banding patterns

The dsRNA banding patterns in 15 strains of *C. elegans* are shown in Fig. 1 and the approximate molecular weight sizes are summarized in Table 1. All strains shown in Fig. 1 contain multiple fragments, ranging in size from 2.0 kb to 12 kb. The remaining six strains not shown in Fig. 1 contained only a single 2.8 kb dsRNA fragment (Table 1).

2.3.2. Development of cDNA clones

Specific dsRNA fragments (2.8 and 5.3 kb) with high intensity fluorescence on agarose gels were selected from strains WASH and CKP, respectively, of *C. elegans* to represent

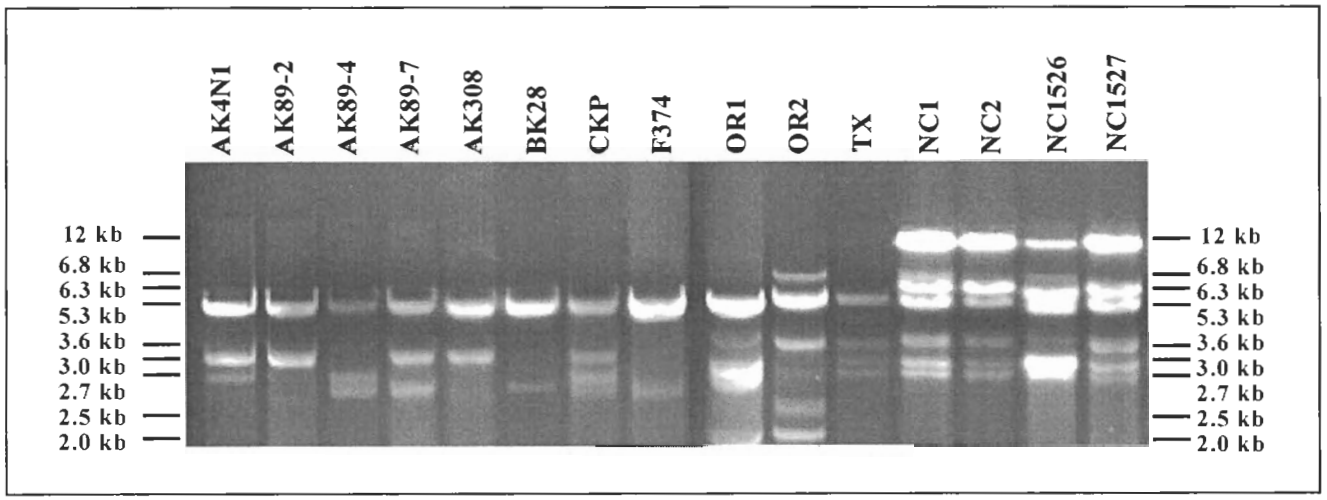

Fig. 1. Banding patterns and sizes of dsRNA extracted from 15 *C. elegans* strains. DsRNA was electrophoresed through a 0.7% agarose gel and stained with ethidium bromide. Strains are described in Table 1.

TABLE 2. Primer sequences used to develop cDNA clones from dsRNAs in *C. elegans*.

Strain	Target dsRNA fragment	Primer	Sequence (5' to 3')	Length (nt)
CKP	5.3 kb dsRNA	C1CF1	CTG CGT CTG ACT CGA TTT TT	20
		C1CR1	TGA ACG AAC CCT CGG GGT AT	20
		C1CF3	TGG TTG TGG TGT GTT AAT GGT	21
		C1CR3	GAC AAT AAT TCA CCT AAA CGG	21
BK18	2.8 kb dsRNA	BK18F1	CCA AAA CAA TGG CCT AGT GG	20
		BK18R2	CAG TTC TCT CCA AAA CTA GGA TCT G	25
NC1527	12 kb dsRNA	NC-F1	ATC TTT GAG GCG AGA ATC GC	20
		NC-F2	TGA CAT GTT GTT GAA CAA CAC T	22

the two predominant sizes present in most strains. RT-PCR amplification using random primers generated multiple bands when viewed on agarose gels, varying in size from 100 bp to almost 1 kb. Several large and distinct RT-PCR bands were selected, purified and cloned into TOPO vector, yielding cDNA clones with inserts of various sizes ranging from 450 bp to 1 kb. To confirm that these clones were originally derived from the dsRNA fragments, Northern blot hybridization was performed prior to sequence analysis. One cDNA clone derived from strain CKP showed a strong signal with the original 5.3 kb dsRNA fragment, but none of the cDNA clones derived from strain WASH showed any hybridization signals, suggesting these latter clones did not originate from the 2.8 kb dsRNA fragment (data not shown).

RT-PCR with total nucleic acids extracted from strain CKP using dsRNA-derived primers designed from the partial 5.3 kb cDNA clone of this strain was successful. All primer sets amplified a single band and sizes were as predicted, indicating these primers were dsRNA-specific and RT-PCR could be used to generate or extend more cDNA fragments from dsRNAs in *C. elegans* (Fig. 2). However, all RT-PCR trials using dsRNA extracted from strains of *C. elegans* other than CKP were unsuccessful using these primers, indicating the potential existence of genetic dissimilarity between dsRNA fragments in these different *C. elegans* strains (data not shown). Additional RT-PCR reactions using either random primers only, or a mixture of random primers and dsRNA-derived primers, were performed with dsRNA extracted from additional *C. elegans* strains, including AK89-2, BK18, OR1, NC1527, and WASH. These reactions generated more cDNA clones, which are described in Table 3. RT-PCR using primers BK18F1 and

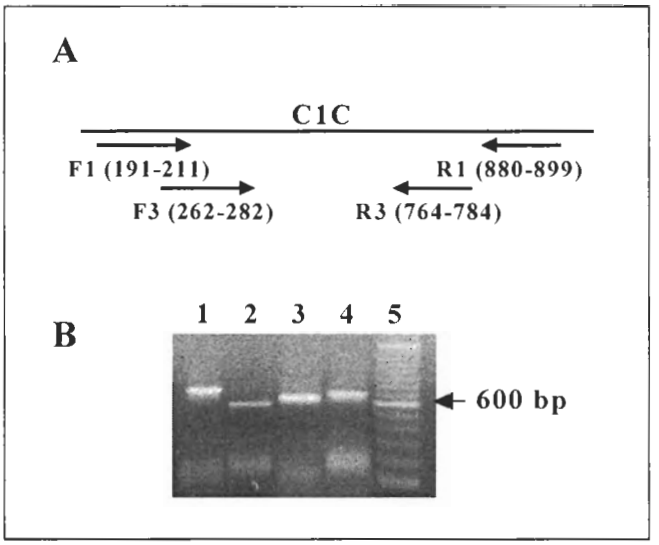

Fig. 2. Reverse transcription polymerase chain reaction (RT-PCR) using dsRNA specific primers derived from clone C1C from a 5.3 kb dsRNA fragment in strain CKP. **A,** Position of primers. Four primers were designated as F1, F3, R1, and R3. Numbers and arrows indicate the position and orientation of each primer relative to clone C1C. **B,** RT-PCR amplification products on a 1% agarose gel. Lane 1=F1 and R1; lane 2=F3 and R3; lane 3=F1 and R3; lane 4=F3 and R1; lane 5=100 bp ladder.

BK18R2, derived a partial cDNA clone of the 2.8 kb dsRNA fragment in strain BK18, generated one more cDNA clone (WASH-3) from the 2.8 kb dsRNA fragment in WASH strain (data not shown). The size of the WASH-3 clone was about 300 nucleotides long, corresponding to the predicted size based on sequence information.

2.3.3. Northern blot hybridizations

To estimate the relatedness among dsRNA fragments within a single strain of *C. elegans*, Northern blot hybridizations using all cDNA probes were first performed with the original six *C. elegans* strains from which the cDNA fragments were derived. Most of these strains (AK89-2, CKP, OR1, and NC1527) contained multiple dsRNA fragments, ranging in size from 2.5 kb to 12 kb (Fig. 1); however, two strains (BK18 and WASH) contained only one dsRNA fragment about 2.8 kb in size (Table 1). All cDNA clones hybridized to one specific dsRNA fragment in each strain without exception, indicating a lack of homology among dsRNA fragments within a single strain of *C. elegans*. This sequence diversity among dsRNA fragments within a single strain was also confirmed by analysis of two distinct clones (NC1527A1 and NC1527B1) derived from one strain, NC1527 (containing six fragments) (Fig. 3). These clones hybridized to a 5.3 kb and 12 kb dsRNA fragment, respectively, with no cross hybridization observed to any of the other four dsRNA fragments present in this strain (Fig. 3).

To examine the genetic relatedness among dsRNA fragments in additional *C. elegans* strains, dsRNAs were extracted from 15 strains and probed with all cDNA clones. The AK89-2C1 clone derived from the 5.3 kb dsRNA fragment in strain AK89-2 hybridized to a similar-sized dsRNA fragment in all Arkansas strains, suggesting the

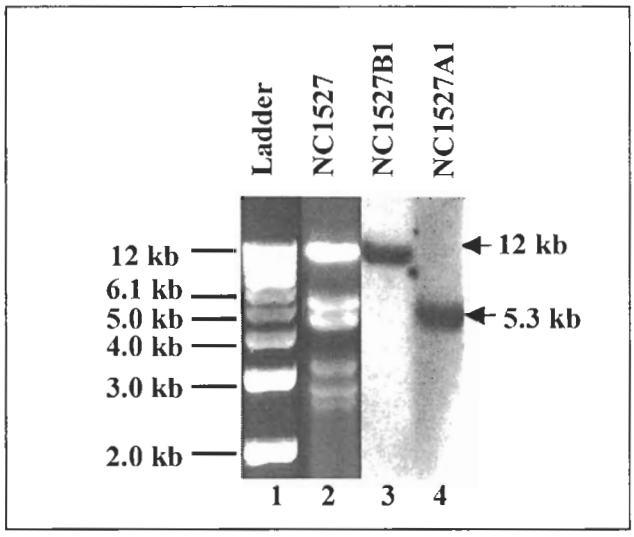

Fig. 3. Hybridization of double-stranded (ds) RNAs in strain NC1527 of *C. elegans* with cDNA probe NC1527A1 (from a 5.3 kb fragment) and NC1527B1 (from a 12 kb fragment). Lane 1=1 kb DNA ladder. Lane 2=dsRNA banding pattern in strain NC1527 on a 0.7% agarose gel stained with ethidium bromide. Lanes 3 and 4=autoradiographs of Northern blots of dsRNAs shown in lane 2, probed with NC1527B1 (lane 3) and NC1527A1 (lane 4), respectively.

presence of sequence similarity among these fragments. No cross-hybridization was observed with any of the other lower molecular weight dsRNA fragments (Fig. 4). A similar result was observed with clones NC1527A1 and NC1527B1 derived from North Carolina strain NC1527. These clones hybridized to either the 5.3 kb or 12 kb dsRNAs in other North Carolina strains, respectively, but not to any of the other dsRNA fragments present (Fig. 4).

Clone C1C, derived from the 5.3 kb dsRNA fragment in strain CKP, hybridized to only the original 5.3 kb dsRNA fragment in this strain and not to any other dsRNAs (including a 5.3 kb fragment) in other *C. elegans* strains, indicating that this dsRNA fragment was unique (Fig. 4). In contrast, cDNA clone OR1(11C1), derived from the 5.3 kb fragment in Oregon strain OR1, cross-hybridized with a 5.3 kb dsRNA fragment in North Carolina *C. elegans* strains. All 5.3 kb dsRNA fragments in North Carolina strains showed a signal with the OR1(11C1) probe, indicating the presence of some sequence homology among these dsRNA fragments (Fig. 4). However, no cross-hybridization was observed using this probe to other different-sized dsRNA fragments in additional *C. elegans* strains (Fig. 4).

Northern blot hybridization using cDNA clones BK18C3 and WASH-3 derived from a 2.8 kb fragment showed strong cross-hybridizations with all *C. elegans* strains which contained a single 2.8 kb dsRNA fragment, regardless of their geographic origin, suggesting that these similar-sized dsRNA fragments were closely related (Fig. 5). No cross-hybridization was observed with any of the other multiple dsRNA fragments in other *C. elegans* strains.

Fig. 4. Hybridization of cDNA probes to double-stranded (ds) RNAs in *Chalara elegans* strains. **A,** Schematic drawing of dsRNA banding patterns in *C. elegans* strains from Fig. 1. **B-E,** Autoradiographs of Northern blots of dsRNAs shown in panel **A**, probed with AK89-2C1, (panel B) C1C, (panel C) OR1(11C1), or (panel D) NC1527B1 cDNA's, respectively (see Table 3). Numbers indicate the size of the dsRNA fragment detected by a given probe.

A
12 kb
6.8 kb
6.3 kb
5.3 kb
3.6 kb
3.0 kb
2.7 kb
2.5 kb
2.0 kb
AK4N1
AK89-2
AK89-4
AK89-7
AK308
BK28
CKP
F374
OR1
OR2
TX
NC1
NC2
NC1526
NC1527
B
5.3 kb
Probe AK89-2C1
C
5.3 kb
Probe C1C
D
Probe OR1(11C1)
E
Probe NC1527B1
5.3 kb
12 kb

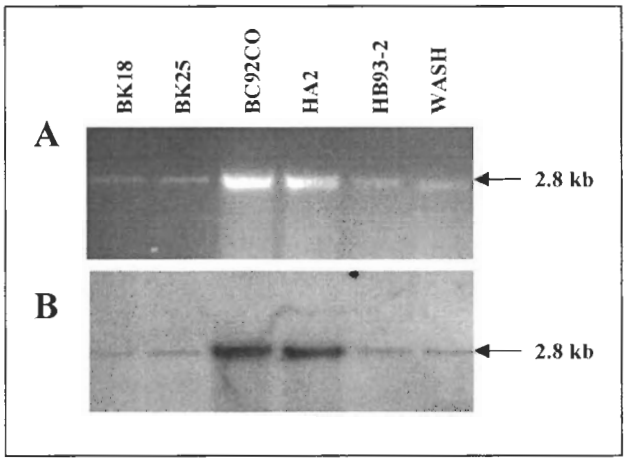

Fig. 5. Hybridization of BK18C3 probe to 2.8 kb double stranded (ds) RNAs in *C. elegans* strains. **A)** DsRNA banding patterns of six *C. elegans* strains on a 0.7% agarose gel stained with ethidium bromide. **B)** Autoradiographs of Northern blots of dsRNAs shown in panel A probed with BK18C3.

2.3.4. Sequence analysis of cDNA clones

Sequence analysis was performed on the eight dsRNA-derived cDNA clones recovered in this study. The sequence information was used to align overlapping partial cDNA clones and to compare levels of sequence similarity with previously published virus groups using BLAST. The sequences of these clones have been deposited into GenBank, with accession numbers as follows: BK18C3 (AY556460), WASH-3 (AY556453), AK89-2C1 (AY556454), C1C (AY556459), OR1(11C1) (AY556455), NC1527A1 (AY556456), NC1527B1 (AY556457) and NC1-1 (AY556458). The cDNA clones BK18C3 and WASH-3, derived from the 2.8 kb dsRNA fragment in strains BK18 or WASH, respectively, had 98% sequence identity at the nucleotide level, confirming the close genetic similarity predicted from the results of Northern hybridization analysis. BLAST searches with these two clones revealed some sequence similarity to the RdRp regions of viruses in the Family *Narnaviridae* (Table 3). The highest identity was 57% to the RdRp region of *Ophiostoma novo-ulmi mitovirus 4-Ld.* Sequence analysis of four more cDNA clones [AK89-2C1, C1C, OR1(11C1) and NC1527A1], which all hybridized to 5.3 kb dsRNA fragments in Northern blots, showed homology to viruses in the family *Totiviridae*, indicating they likely belonged to this group (Table 3). However, depending on the cDNA clone, identities ranged from 23% to 43% to either the coat protein or RdRp regions of *Totiviridae*. Clones NC1527B1 and NC1-1 derived from a 12 kb dsRNA fragment in strain NC1527 overlapped with each other and established a continuous 2.5 kb nucleotide sequence (data not shown). A BLAST search with this extended partial cDNA clone did not reveal significant homology to any of the previously described virus groups, indicating it might be a new virus.

Table 3. Characterization of cDNA clones derived from specific dsRNAs in *C. elegans* based on Northern blot hybridization and sequencing.

Strain designation	Clone	Size of clone	Hybridization pattern	Highest identities to[a] previously-published viruses	Proposed virus group
BK18	BK18C3	418 bp	To 2.8 kb dsRNA fragments in strains from California	RdRp region of OnuMV-4 (57%)	*Narnaviridae*
WASH	WASH-3	305 bp	Washington, British Columbia and Holland (n=6)	RdRp region of OnuMV-4 (55%)	*Narnaviridae*
AK89-2	AK89-2C1	1,520 bp	To 5.3 kb dsRNA fragment in all Arkansas strains (n=5)	Coat protein region of SsRV1 (35%)	*Totiviridae*
CKP	C1C	669 bp	To 5.3 kb dsRNA fragment in strain CKP only (n=1)	RdRp region of Hv190SV (44%)	*Totiviridae*
OR1	OR1(11C1)	954 bp	To 5.3 kb dsRNA fragment in OR1 and all North Carolina strains (n=5)	RdRp region of SsRV1 (23%)	*Totiviridae*
NC1527	NC1527A1	1,764 bp	To 5.3 kb dsRNA fragment in all North Carolina strains (n=4)	Coat protein region of SsRV1 (35%)	*Totiviridae*
NC1527	NC1527B1	1,458 bp	To 12 kb dsRNA fragment	None	Unknown
	NC1-1	734 bp	in all North Carolina strains (n=4)	None	Unknown

[a]RdRp=RNA-dependent RNA polymerase; OnuMV-4=*Ophiostoma novo-ulmi* Mitovirus-4; SsRV1=*Sphaeropsis sapinea* RNA virus 1; and Hv190SV=*Helminthosporium victoriae* 190S virus.

2.4. Discussion

In fungi, most viruses are known to be present as multiple fragments and the genetic relationships among them, even within a single strain, are quite variable (Buck, 1984; Chu et al., 2002; Bharathan and Tavantzis, 1990; Kanematsu et al., 2004). In *C. parasitica*, Tartaglia et al. (1986) reported that three dsRNA species were found in strain GH2. Further studies revealed that two of the dsRNA fragments shared some sequence homology at both 5' and 3' ends, but not with the third fragment (Tartaglia et al., 1986). Additional cloning and sequence analysis of these dsRNA fragments revealed that one dsRNA fragment was a satellite RNA, while another was a defective RNA derived from one dsRNA fragment by an internal deletion (Hillman et al., 2000). In addition to these defective interfering dsRNAs and satellite RNAs, mixed infections with two or more related or unrelated viruses have also been reported (Ghabrial, 1998; Nuss and Koltin, 1990; Preisig et al., 1998). The presence of two unrelated viruses was observed in *Helminthosporium victoriae*, and these two viruses were classified as either *Totiviridae* or *Chrysoviridae* (Soldevila et al., 2000; Huang and Ghabrial, 1996). Also, Preisig et al. (1998) reported the occurrence of two distinct Totiviruses in an isolate of *S. sapinea*; the identities between these two viruses at the coat protein and RdRp levels were only 38% and 36%, respectively.

The genetic relatedness among dsRNA fragments in *C. elegans* strains was first determined in this study by Northern blot hybridizations using several dsRNA-derived cDNA clones. Most of the clones were found to hybridize to similar-sized dsRNA fragments in other *C. elegans* strains only if they originated from similar geographic regions. This high degree of relatedness among similar-sized dsRNA fragments was not

unexpected, since identical dsRNA banding patterns were observed on agarose gels in most *C. elegans* strains which originated from the same geographic region. In addition, Punja and Sun (1999) previously demonstrated using random amplified polymorphic DNA (RAPD) analysis that strains of *C. elegans* from a similar geographic region were more closely related genetically than those strains which were derived from different geographic regions. Several studies have shown that the distribution of dsRNA hybridization groups was correlated with the geographic distribution of host fungi (Enebak et al., 1994; Peever et al., 1997). In *C. parasitica*, clones of CHV3-GH2 dsRNA derived from Michigan isolates hybridized to several other Michigan dsRNAs, but not to other dsRNAs found in isolates from Tennessee and West Virginia (Paul and Fulbright, 1988). Also, clones of CHV1 dsRNA in a *C. parasitica* strain derived from Europe hybridized only to dsRNAs from European isolates and not to any other North American isolates, except CHV2-NB58 (Enebak et al., 1994a; Enebak et al., 1994b; Hillman et al., 1992). Although the genetic relatedness among dsRNAs in natural field populations of *R. solani* was considerably large, a higher genetic relatedness among dsRNAs was also observed in isolates from a common geographic region when compared to isolates of distant geographic origin (Bharathan and Tavantzis, 1990).

There are some examples, however, which demonstrate that the genetic relatedness among dsRNA fragments is not limited to isolates of similar geographic origin. Two small dsRNA fragments (1.5-2.5 kb) present in *Discula destructiva*, the dogwood anthracnose fungus, shared sequence homology regardless of the geographic origin of isolates (Rong et al., 2001). Similar results were also found in *C. parasitica* and *R. solani*. The cDNA clones from SR2 dsRNA type of *C. parasitica* hybridized widely to

those dsRNAs present in eastern North American isolates (Enebak et al., 1994b), while dsRNAs from a Japanese isolate of *R. solani* AG 3 hybridized to dsRNAs of some North American isolates (Bharathan and Tavantzis, 1990; Bharathan and Tavantizis, 1991). In our study, cross-hybridization of cDNA probes among strains of *C. elegans* from different geographic regions was rare and was only observed with the 2.8 kb dsRNA fragment. Clone BK18C3 hybridized to the 2.8 kb dsRNA fragment in many *C. elegans* strains, regardless of the geographic origin of the strains, which included North America and Europe. However, no cross-hybridization was observed to other dsRNA fragments of different sizes, indicating that the 2.8 kb dsRNA fragment was unrelated to additional dsRNA fragments present in other isolates. Interestingly, this fragment was never found in association with any other dsRNA elements. With regard to the 5.3 kb dsRNA fragment, cross-hybridization was observed to a similar-sized dsRNA fragment present in North Carolina and Oregon strains, indicating some cross-homology between these dsRNA fragments despite their disparate geographic origins. No cross-hybridization was observed with any other different-sized dsRNA elements.

Sequence analysis of the cDNA clones from the 2.8 kb and 5.3 kb dsRNA fragments obtained in this study suggested the presence of at least two virus groups in *C. elegans*. Most cDNA clones which hybridized to the 5.3 kb dsRNA fragment shared sequence homology to viruses in the *Totiviridae*, while the cDNA clones which hybridized to a 2.8 kb dsRNA fragment shared sequence homology to *Narnaviridae*. The occurrence of a Totivirus in *C. elegans* was not surprising since it is the one of the largest groups found in fungi (Nomura et al., 2003; Cheng et al., 2003; Ghabrial, 1998; Wickner et al., 2000). However, the presence of dsRNA with sequence similarity to the

Narnaviridae family was unexpected because its occurrence is more restricted. Only a few fungi, including *O. novo-ulmi*, *C. parasitica*, and *S. cerevisiae*, contain a mycovirus belonging to the family *Narnaviridae* (Hong et al., 1999; Polashock and Hillman, 1994; Wickner, et al., 2000). In addition to these virus groups, it is likely that additional related and/or unrelated viruses are present in *C. elegans*. Two cDNA clones (NC1527B1 and NC1-1) derived from a North Carolina strain had no significant sequence homology to any of the previously published virus groups. These clones hybridized to only one dsRNA fragment (approximately 12 kb in size), which is larger in size compared with other viruses (Mitovirus and Totivirus) present in *C. elegans,* but similar in size to CHV1 (Hypovirus) in *C. parasitica*. Sequence analysis of these clones showed a 19% identity at the amino acid level to the polyprotein regions of fava bean dsRNAs, which shared some homology to *Potyviridae* (data not shown).

The extent of genetic variation within *C. elegans* populations was previously determined using RAPD analysis (Punja and Sun, 1999). It was proposed that host plant species might have an important role in exerting selection pressure on *C. elegans* populations. This may result in the evolution of distinct subpopulations, since many isolates derived from the same hosts were more similar to each other based on RAPD analysis, when compared with isolates derived from different hosts (Punja and Sun, 1999). Such genetic differentiation of subpopulations in fungi is considered to be an important factor leading to genetic diversity of dsRNA fragments, since dsRNA transmission is dependent on the fungal host (Rogers et al., 1986; McCabe et al., 1999; Ihrmark et al., 2000; Cortesi et al., 2001). In this study, we observed that the extent of variation among partial cDNA clones from a 5.3 kb dsRNA fragment with sequence

homology to the *Totiviridae* group was greater compared with the cDNA clones from a 2.8 kb dsRNA fragment with sequence homology to the *Narnaviridae* family. Furthermore, the fungal strains containing the Totivirus dsRNA fragments were genetically more diverse compared to those isolates that harbored the *Narnaviridae* dsRNA fragments, based on RAPD marker analysis (Punja and Sun, 1999). Therefore, in light of the previously demonstrated high degree of genetic diversity among strains of *C. elegans* (Punja and Sun, 1999), a high degree of diversity among dsRNA fragments would be a predicted outcome, as was demonstrated in this study. In the absence of demonstrable hyphal anastomosis and exchange of cytoplasm among strains of *C. elegans* (author, unpublished data), the dsRNA elements in this pathogen should continue to remain distinct. Geographic isolation of strains may also preserve the uniqueness of dsRNA sequences. In contrast, however, the widespread occurrence and high genetic similarity among the 2.8 kb dsRNA fragments in *C. elegans* strains suggests that either horizontal transmission of this fragment may somehow have occurred or that this strain was widely disseminated. A high transmission rate of dsRNA through conidia (close to 100%) can be demonstrated (author, unpublished data). These conidia (phialospores) are an important inoculum source to initiate disease and can survive in soil for extended periods and can potentially carry dsRNAs to new sites (Punja et al., 1992; Punja, 1995).

The biological effects of the dsRNA elements in *C. elegans* appear to differ depending on the strain and specific fragment present (Bottacin et al., 1994; Punja, 1995). A distinct hypovirulence phenotype reported in fungi such as *C. parasitica* and *D. ambigua* (Dawe and Nuss, 2001; Rong et al., 2001) is absent in *C. elegans*. The loss of specific dsRNA fragments from *C. elegans* strains can result in enhanced growth but

reduced sporulation, pigmentation and survival of the fungus (Punja 1995). The Northern hybridization data and the partial sequence information from this study provide evidence for considerable genetic diversity among dsRNA elements in *C. elegans*. Additional information, including the development of full-length cDNA clones of dsRNA elements, has confirmed the uniqueness of specific dsRNA elements in *C. elegans* (see chapters 3 and 4).

CHAPTER 3

MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF A MITOVIRUS IN *CHALARA ELEGANS (THIELAVIOPSIS BASICOLA)*[a]

3.1. Introduction

The occurrence of double-stranded (ds) RNA elements has been described in many filamentous fungi (McCabe et al., 1999; Ghabrial, 1998); these dsRNAs can be associated with virus particles, and hence are termed mycoviruses (Ghabrial, 1998). These dsRNA elements do not have an extracellular phase during their life cycle and are dependent on the fungal host for their replication. Mycoviruses can be transmitted between strains of the same fungal species either through hyphal anastomosis (horizontal transmission) or via fungal propagules, e.g. conidia (vertical transmission) (Buck, 1986; Cortesi et al., 2001). Increasingly, dsRNA fragments in fungi are being sequenced and they have been classified into several families, including the *Hypoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae* (Ghabrial et al., 1995a; Ghabrial et al., 1995b; Hillman et al., 1995; Hillman et al., 2003). These families are distinguished by differences in virion structure, genome information and expression strategy (Ghabrial et al., 1995a; Ghabrial et al., 1995b; Hillman et al., 1995; Hillman et al., 2003).

[a] Prepared as a manuscript submitted to *Phytopathology*.

A number of previous studies have characterized the effects of dsRNA elements on the phenotype of the fungal host (Buck, 1984; Brasier, 1986; McCabe et al., 1999). In some cases, dsRNA presence resulted in dramatic changes to culture morphology and physiology of the fungal host (Anagnostakis and Day, 1979). In the chestnut blight fungus, *Cryphonectria parasitica*, dsRNA presence reduced growth rate and sporulation, enhanced yellow pigmentation, reduced laccase and cutinase production, and reduced virulence (induced hypovirulence) (Anagnostakis, 1982; Dawe and Nuss, 2000). In other cases, the dsRNA element was associated with enhanced virulence of the host fungus, as in *Nectria radicicola* (Ahn and Lee, 2001). Research has shown that the relationship between the presence of dsRNAs and host fungi is complex (Ahn and Lee, 2001; Chu et al., 2002; Ghabrial, 1980). In multi-fragmented dsRNA populations, only certain dsRNA fragments may induce symptoms in the fungal host (Jian et al., 1998; Soldevila and Ghabrial, 2001). In *Rhizoctonia solani*, the presence of a 6.4 kb dsRNA fragment was shown to enhance fungal virulence, while the addition of a 3.6 kb dsRNA fragment reduced the effects of the 6.4 kb fragment (Jian et al., 1998). Understanding the role of dsRNAs in fungal hosts is important not only for their potential use as biological agents to control fungal pathogens through hypovirulence, but also to enhance the fundamental understanding of dsRNA-fungal host interactions (McCabe et al., 1999; Tavantzis, 2001).

Chalara elegans NagRaj and Kendrick (syn. *Thielaviopsis basicola* (Berk. & Br.) Ferr.), a filamentous fungus that causes black root rot disease on higher plants, is a soil-borne hyphomycete and a widespread facultative parasite on numerous plant species worldwide (Punja, 1995; Yarwood, 1981). Bozarth and Goenaga (1977) first reported the presence of dsRNAs in *C. elegans* and some studies have further characterized these

dsRNA elements (Bottacin et al., 1994; Punja, 1995). Recently, Park et al. (2004) reported a high degree of genetic diversity among dsRNA elements in a number of *C. elegans* strains. However, a detailed molecular characterization of the dsRNA elements in *C. elegans* has not been achieved.

The objectives of this study were to: 1) develop and characterize a full-length cDNA clone from a 2.8 kb dsRNA fragment in *C. elegans* and compare its sequence to known virus groups; 2) attempt to cure wild-type strains of this dsRNA element and compare the cultural characteristics, virulence, and enzymatic activities (polyphenoloxidase, laccase, esterase, and tyrosinase) between wild-type and cured strains.

3.2. Materials and Methods

3.2.1. Fungal growth and dsRNA extraction

Strain BK18 of *C. elegans* was originally isolated from cotton soil in King's County, CA (provided by B. Holtz) and was used in this study. This strain harbored a 2.8 kb dsRNA fragment (Bottacin et al., 1994). Growth conditions and extraction of total nucleic acids were as described in Chapter 2.

3.2.2. cDNA development and sequence analysis

The dsRNA was extracted through a CF-11 column as described by Morris and Dodds (1979), treated with RNase A (USB, Cleveland, OH) in 0.8 M NaCl at 37°C for 10 min and RQ1, RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min and further purified using a low-melting agarose gel. Based on the sequence information

from a partial cDNA clone (BK18C3), which was previously developed by using random primers (Park et al., 2003), several specific primers were designed and used in RT-PCR to develop a full-length cDNA. The gel-purified dsRNA fragment was denatured at 99°C for 10 min and reverse transcribed using 1 μl of random primers (Invitrogen, Carlsbad, CA) with Superscript II reverse transcriptase (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. PCR was performed with the first-strand cDNA using Taq DNA polymerase (Invitrogen, Carlsbad, CA) and Taq extender (Stratagene, La Jolla, CA). PCR amplification was carried out using 35 cycles for 1 min at 94°C, 45 sec at 55°C and 2 min at 72°C, and terminated by a 10 min elongation at 72°C. Amplified RT-PCR products were analyzed by running the samples in a 1% agarose gel, and purification of the specific fragments was done using the gel extraction kit from Qiagen (GmbH, Germany) according to their protocol. The cDNA fragments were ligated into the TOPO cloning vector, and transformed using the TOPO one-shot kit (Invitrogen, Carlsbad, CA). Several recombinants having an insert of the expected size were selected by *Eco*RI restriction enzyme analyses and purified with the QIAgen plasmid kit (GmbH, Germany). Sequencing was conducted at the University of British Columbia, NAPS unit. Nucleotide sequences of the insert in recombinant plasmids were determined using the M13 forward and –21M13 primers set.

To obtain cDNA clones encompassing both ends of the 2.8 kb dsRNA fragment, the rapid amplification of cDNA ends (RACE) kit (Roche, Indianapolis, IN) was used according to the manufacturer's protocol. Several dsRNA specific primers for both 5'- and 3'- ends were designed and used for RT-PCR (Table 4). RT-PCR products of 5'/3'

TABLE 4. Primer sequences used to develop cDNA of 2.8 kb dsRNA in *C. elegans*

Primer	Sequence (5' to 3')	Length (nt)
BK18F1	CCAAAACAATGGCCTAGTGG	20
BK18F2	TGCGTGAGAAGAGAATAAGGAA	22
BK18R1	CTGGGAATCTATCAGTGGCTG	21
BK18R2	CAGTTCTCTCCAAAACTAGGATCTG	25
5'-1	GTGCCCATAAGACAATGCCA	20
5'-2	GGTCATAGGTTGGTTTCTCCA	21
5'-3	TGGGTTTTAATCCACGAGGT	20
3'-1	GCTCCGTTACTCATATGGATTG	22
3'-2	TGCTGGAAAAGTCTCAACCC	20
3'-3	TCCAACAATTTACTCTACCTTCAGC	25

RACE were electrophoresed through a 1% agarose gel, purified using a gel-extraction kit (Qiagen, Chatsworth, CA), ligated into TA vector and sequenced as described above. Based on their sequence information, the full-length sequence of the 2.8 kb dsRNA fragment was obtained by aligning partial cDNA clones using the computer programs of the BLAST and Bioedit. Multiple sequence alignments with known virus groups were also performed with CLUSTAL W (Thompson et al., 1994).

3.2.3. Northern blot hybridization with strand-specific probes from the 2.8 kb dsRNA

Total nucleic acids were extracted using Trizol according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD) and electrophoresed on a 1% agarose gel. The dsRNA was further purified using the CF-11 method as described above. DsRNA and total nucleic acids were electrophoresed on a 1% agarose gel. The gel was soaked in 50 mM NaOH/150 mM NaCl for 15 min for denaturation and in 10X SSC (Sodium Chloride/Sodium Citrate) for 10 min for neutralization. Total nucleic acids were transferred to a nylon membrane by capillary action in 10X SSC overnight. Prehybridization was performed in prehybridization buffer (5X SSC, 0.1% sodium-lauroylsarcosine, 0.02% SDS and 1% blocking reagent) for 4-6 hr at 42°C according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Strand-specific DNA probes using Digoxigen (Roche Molecular Biochemicals) were prepared using the PCR procedure described by Finckh et al. (1991). The primers, BK18-3F1 (5'-CCAAAACAATGGCCTAGTGG-3') and BK18-3R2 (5'-CAGTTCTCTCCAAAACTAGGATCTG-3'), were used for (+) and (-)-strand DNA probes, respectively. Hybridization was performed overnight at 42°C with 20 ng of each

probe/ml hybridization buffer with gentle shaking. Blots were washed twice in 2X washing solution (2X SSC and 0.1% SDS) for 15 min each at room temperature, then twice in 0.5X washing solution (0.5X SSC and 0.1% SDS) at 68°C for 15 min. Detection was done according to the manufacturer's protocol (Boehringer Mannheim, Germany).

3.2.4. Mitochondrial association of the 2.8 kb dsRNA element

Extraction of mitochondria was performed using the method described by Rogers et al. (1987). Briefly, mycelium were grown in 1 liter of PDB for 3 weeks, harvested, mixed with 15% sucrose in 20 ml TE buffer and homogenized with 20 g of glass beads (Sigma, St. Louis, MO) for 2 min. The homogenate was mixed with an equal volume of 1% (w/v) sucrose in TE buffer, centrifuged at 1,300 *g* for 10 min at 4°C, and the supernatant and pellet (fraction A) collected in separate tubes. The supernatant was centrifuged at 14,000 *g* for 30 min at 4°C and the supernatant (fraction B) and pellet were collected in separate tubes. The pellet was resuspended with 20% sucrose in 4 ml TE buffer and used for further purification of mitochondria. Both 35% and 60% sucrose in TE buffer were used for preparation of a sucrose gradient. One ml of mitochondrial fraction was loaded at the top of sucrose gradient and centrifuged at 64,000 *g* for 90 min at 4°C in a Beckman SW 60 rotor. Each fraction, 20% (fraction C), 35% (fraction D) and 60% (fraction E), the band (fraction M) between 35% and 60%, and pellet (fraction F) in the bottom of 60% sucrose were collected in separate tubes and used for further purification of dsRNA using CF-11 column as described above. The presence of dsRNA in each sub-cellular fraction, including the mitochondrial fraction (fraction M), was examined on a 1% agarose gel.

3.2.5. Transmission of dsRNA through conidia

Colonies were grown on V8 agar plates (see Chapter 2) at 25°C for 5 days and conidia harvested by placing several drops of double distilled water onto the colony surface, collecting the suspension with a pipette, and transferring to a 1.7 ml microcentrifuge tube. The conidial suspension was diluted up to 1,000-fold with double-distilled water and used to inoculate a V8 agar plate. After 3-4 days of incubation at 25°C, hyphal tip transfers from individual developing colonies were made onto 1.5% water agar plates and incubated at 25°C for 5 days. Each colony derived from 50 single conidia transfers was examined for the presence of dsRNA following the method described above.

3.2.6. Curing of dsRNA

Mycelial plugs of strain BK18 were placed on fresh V8 agar plates (100 x 15 mm) amended with concentrations of either cycloheximide (5 μg and 10 μg/ml) or ribavirin (10 mg or 20 mg/ml), and incubated at 25°C. Two replicate plates each were included for each treatment. After two-thirds of the plate was covered with fungal mycelium, agar plugs from the margin of each colony were transferred to fresh plates containing the same concentration of either cycloheximide or ribavirin. Consecutive transfers were repeated for up to four generations. Control cultures were serially transferred onto V8 agar without any chemicals. All cultures were subsequently transferred to fresh V8 agar plates and grown for 3-4 days, then hyphal-tipped and grown in 50 ml of potato dextrose broth (PDB) (Difco, MI) at 25°C for 2 weeks. Mycelium was used for further analysis of dsRNA presence as described above.

In another trial to eliminate the 2.8 kb dsRNA fragment from strain BK18, high incubation temperatures were used. Colonies were grown on V8 agar plates at temperatures of 30°C, 35-37°C, or 45°C until two-thirds of the plate was covered with fungal mycelium. Two replicate plates were used at each temperature. Because of significant reduction of fungal growth above 35°C, all plates incubated above 35°C were kept in plastic bags to maintain moisture and enhance mycelial growth. Control colonies were maintained at 25°C with regular transfers. After several serial transfers on V8 agar plates at these incubation temperatures over 2 months, each colony was transferred to 50 ml of PDB and incubated at 25°C for 2 weeks and used for analysis of dsRNA banding patterns as described above. Total nucleic acids were extracted from any potentially cured strains, the concentration was adjusted to 20 ng/µl and 10 µl of sample used for RT-PCR amplification. RT-PCR was performed with the 2.8 kb dsRNA specific primer set (BK18F1 and BK18R2-Table 4) and electrophoresed through a 1% agarose gel to confirm the presence of 2.8 kb fragment.

3.2.7. Cultural characterization of a putatively dsRNA-cured strain

One dsRNA-cured strain (BK18C) was compared to its wild-type dsRNA-containing strain (BK18) for cultural characteristics including colony morphology and growth rate. Both strains were grown on V8 agar plates at room temperature for 2 weeks and examined for any visible morphological differences. To compare mycelial growth rates, an 8-mm agar plug from the actively growing margin of each colony was placed on a V8 agar plate and incubated at 25°C. The diameter of the fungal colony was measured

daily until the mycelium had covered the entire plate. Three replicate plates were used for each strain and the experiment was repeated.

3.2.8. Virulence assessment

The virulence of strains BK18C and BK18 strains on carrot roots (*Daucus carota* L.) was determined. Mature carrot roots were purchased from a retail store, cut into 10-cm long sections, surface-sterilized with 10% Javex® (containing 4.5% of Sodium hypochlorite) for 5 min and rinsed with autoclaved double distilled water for 5 min. Each carrot segment was placed in a sterile plastic box lined with moistened filter paper. An 8-mm diameter mycelial plug from the actively growing margin of each strain was placed in the center of each carrot root. The boxes were sealed with parafilm and incubated at 25°C for 5-8 days. Degree of colonization was determined by measuring both the widest and the longest length of the developing fungal colony and averaging the values. Three replicates were included for each strain and the experiment was repeated.

3.2.9. Enzyme activity assays

The activities of polyphenol oxidase, laccase, esterase and tyrosinase were compared between strains BK18 and BK18C. For each enzyme assay, three replicates were used for each strain and the experiment was repeated. The polyphenoloxidase activity was assessed on Bavendamm's medium containing either 0.5% tannic acid (Sigma, St. Louis, MO) or gallic acid (Sigma, St. Louis, MO) (Bavendamm, 1928). Actively growing agar plugs from each strain were inoculated onto both media and incubated at 25°C in the dark for 10 days. Development of dark brownish pigmentation

on the media indicated the presence of phenoloxidase activity. The intensity and zone of pigmentation was compared visually for all strains.

The laccase activity of each strain was measured using 2,6-dimethoxyphenol (DMOP) (Sigma, St. Louis, MO) as a substrate. An 8-mm agar plug from the margin of a colony grown on Bavendamn's medium for 7 days was transferred to a 1.7 ml microcentrifuge tube. One ml of autoclaved double distilled water was added then incubated at 4°C for 30 min, vortexed briefly, and centrifuged at 14,000 rpm for 10 min. Two hundred µl of supernatant were mixed with 800 µl of 2.5 mM DMOP in 100 mM sodium buffer at pH 6.0. The absorbance at 468 nm was measured at 25°C at 5 min interval using a spectrophotometer and the relative enzymatic activity was calculated from the changes in absorbance per 5 min. Tyrosinase activity of each strain was measured following the same procedure as for measuring laccase activity, except that 3,4-dihydroxy-L-phenylalanine (L-DOPA) (Sigma, St. Louis, MO) was used as a substrate and absorbance at 475 nm was mentioned. Esterase activity was measured using p-nitrophenyl butyrate as a substrate as described by Kunoh et al. (1990).

3.2.10. Electron microscopy

Transmission electron microscopy was used to compare mitochondrial ultrastructure between strains BK18 and BK18C. Both strains were grown on V8 agar plates for 10 days and then processed at the Bio-Imaging lab at the University of British Columbia (Vancouver, Canada). Briefly, mycelial fragments of both strains (BK18 and BK18C) were scraped from the margin of the colony, fixed using glutaraldehyde and frozen in a Baltec HPM10 high pressure freezing machine for 5 days. Samples were

further processed for resin infiltration and embedding following a standard protocol (http://www.emlab.ubc.ca/protocol.hltm) with transfer baskets. Each sample was sectioned, stained with 2% uranyl acetate (12 min.), followed by lead citrate (6 min.) and viewed at 80 kV with a Hitach H7600 TEM. Approximately 200 sections were viewed to identify representative images of each strain.

3.3. Results

3.3.1. cDNA development and sequence analysis

RT-PCR reactions with dsRNA specific primers generated four fragments ranging in size from 800 bp to 1.2 kb; these were aligned with each other based on their overlapping sequence (Fig. 6A). For 5'- and 3'-ends, single RT-PCR products were generated using a combination of oligo(dT) primers and dsRNA-specific terminal primers. These RT-PCR terminal products were cloned, sequenced and used to obtain the full-length sequence for the 2.8 kb dsRNA in *C. elegans* (Fig. 6).

Sequence analysis of the complete cDNA showed that the dsRNA was 2,896 nucleotides long. The nucleotide composition was 32.4% A, 16.8% C, 15.7% G and 35.0% U. No long open reading frame (ORF) was identified on either strand when the standard cytoplasmic genetic code was applied. However, a large putative ORF, designated ORF I, was identified when the mitochondrial genetic code of fungi was used (Fig. 6B). The mitochondrial genetic codes uses UGA as a codon to encode tryptophan rather than as a translation terminator. Translation of the ORF may be initiated at the AUG codon at nucleotide position 427 and terminated at the UAG stop codon at

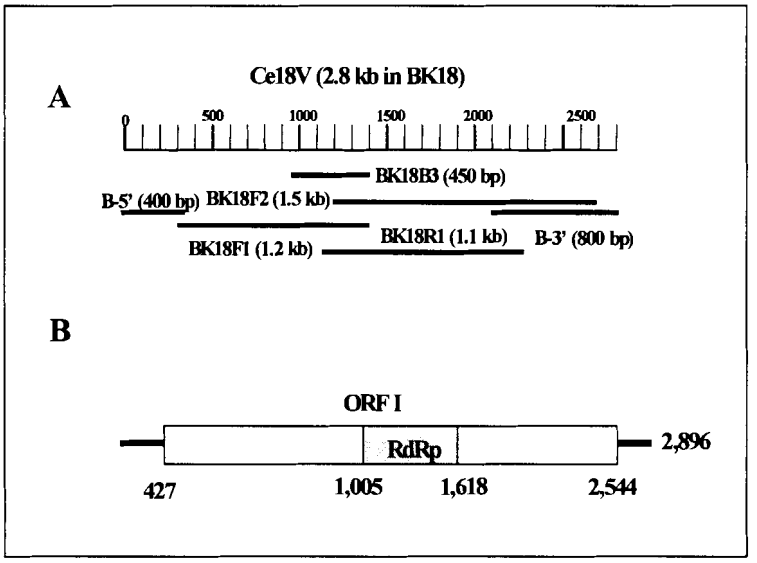

Fig. 6. Schematic representation of alignment of partial cDNA clones derived from the 2.8 kb dsRNA in the BK18 strain **(A)** and its putative genomic organization **(B)**. **(A)** Eight partial cDNA clones were obtained from the 2.8 kb dsRNA fragment and aligned with each other to obtain the full-length sequence. **(B)** Putative diagrammatic representation of the genome organization of the 2.8 kb dsRNA. One large potential open reading frame (ORF I) was found and predicted to encode a putative RNA-dependent RNA polymerase (RdRp). The region containing the conserved motifs associated with RdRps is represented in the gray box.

nucleotide position 2,544 (Fig. 6B). The putative ORF I is predicted to encode 705 amino acids with a molecular weight of 81.45 kDa. ORF I showed a codon preference of either A or U in the third position, which is a characteristic of mitochondrial codons. Analysis of this protein revealed the typical amino acid sequence motifs (I-VI) of RdRp including the highly conserved GDD motif (Fig. 7) (Poch et al., 1989). No other ORF of significant length was observed in the negative strand.

Using a BLAST search, the ORF I in the 2.8 kb dsRNA showed homology at the amino acid level to the RdRp regions of other mitoviruses, such as *O. novo-ulmi* OnuMV3a-Ld, OnuMV4-Ld, OnuMV5-Ld and OnuMV6-Ld, *Cryphonectria mitovirus* 1 (Hong et al., 1999), mitovirus-like dsRNA from *R. solani* (Lakshman et al., 1998), and L-dsRNA from *S. homoeocarpa* (Deng et al., 2003) (Table 5). The highest amino acid sequence homology of the putative ORF I region in the 2.8 kb dsRNA was to the RdRp of OnuMV4-Ld, with 34% identity when the mitochondrial genetic code was used. Comparative sequence analysis revealed that the 2.8 kb dsRNA was related to other mitochondrial dsRNAs, with sequence identities in the range of 44-53% (Table 6). Sequence comparisons at the amino acid level among known mitoviruses revealed homologies of 20% to 34% for the entire RdRp-like proteins and 46% to 71% for only the RdRp I-VI motifs only (Table 5). The 2.8 kb dsRNA in *C. elegans* has been designated as *Chalara elegans 18 virus* (Ce18V) and its complete sequence has been deposited in Genbank under accession no. AY563138.

The 5'- and 3'-UTR of Ce18V are 426 nt and 352 nt long, respectively (Fig. 6). Both terminal sequences were examined for their potential secondary structures using the

Fig. 7. Comparison of conserved motifs (I-VI) of the RdRp of *C. elegans* Ce18V to that of other Mitoviruses. * indicates identical amino acids residues; : and . indicate higher and lower number of chemically similar residues, as defined in the CLUSTAL W program. Ce18V=*Chalara elegans* 18 Virus; CpMV1-NB631=*Cryphonectria parasitica* mitovirus 1-NB631; GaMV-S1=*Gremmeniella abietina* mitochondrail RNA virus; L-dsRNA=*Sclerotinia homoeocarpa* mitovirus; OnuMV3a-Ld=*Ophiostoma novo-ulmi* mitovirus 3a-Ld; OnuMV4-Ld=*Ophiostoma novo-ulmi* mitovirus 4; OnuMV5-Ld= *Ophiostoma novo-ulmi* mitovirus 5; OnuMV6-Ld= *Ophiostoma novo-ulmi* mitovirus 6; RsM2-1A1=*Rhizoctonia solani* 1A1 M2 dsRNA.

```
                                        I
                   ________________________________________________
Ce18V         -SGKLGIVKDPEGKRRIIAMVDYHSQLVLRSIHDGLLNKLRNLPQDRTYNQDP  244
CpMV1-NB631   MGKLSVVYDQAGKARIVAITNSWIQTAFYSLHLHVFKLLKNIDQDGTFDQER  376
GaMV-S1       --SLSLIYDPECKVRIVAMLDYTTQLFLRPIHNDLFKLLKKLPQDRTFTQNP  283
L-dsRNA       LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKGLPMDGTFNQQA  312
OnuMV3a-Ld    LGKLAIKEEAAGKARVFAMADSITQSVMAPLNSWVFSKLKDLPMDGTFNQQA  311
OnuMV4-Ld     -LGKLSIVHDPELKERVIAMVDYTTQFALRPIHNILLNNLSKLPCDRTFTQDP  274
OnuMV5-Ld     FGKISIVKDPELKMRVIAMVDYHSQFVLKKIHNSLFNKLKLIKSDRTFTQDP  289
OnuMV6-Ld     IRRLSIVHDPECKERVIAIFDYGSQMVLKPIADVLFDLLRNIPSDRTFTQSP  286
RsM2-1A1      LSQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFAILRNIPNDGTFDQEA  384
               ::.:  :    * *:.*: :    * .:  :    ::  *  :   * *: *.

                              II
                         ____________
Ce18V         -NNAWEENKECFHSLDLSSATDRFPVKLQSRLLTEMYSDPSFGENWMNLLLNR  296
CpMV1-NB631   LNEP---TQKFYGFDLTAATDRLPIDLQVDILNIIFKN-SPGSSWRSLLRIK  434
GaMV-S1       LNDWEDNEHSFWSIDLTAATDRFPISLQRRLLLYIYSDPEIANSWQNLLVHR  335
L-dsRNA       YKEGLLHDVEFYSYDLSSATDRLPMAFQKQIISVLFGS-DFADDWATLLVGR  371
OnuMV3a-Ld    YQDGLLHDVEFYSYDLSSATDRLPMAFQKQIISVLFGS-KFAKDWATLLVGR  370
OnuMV4-Ld     HKWNDDHKERYHSLDLSAATDRFPIFLQQKLISLIFNDYEFGKNWRNLLVDR  330
OnuMV5-Ld     IFTTPTMGHRFWSMDLSAATDRFPIDLQERLLSYLYGS-EISSAWKQLLIDR  343
OnuMV6-Ld     THTDLDNKSKFWSIDLSSATDRFPIVFQKRVLQKILGK-QMTDSWERIMIGS  342
RsM2-1A1      SQEKAVTAGKAFSYDLTAATDRLPVILTAFILSTIVGIRTFGGLWRSILVKR  442
                        . **::****:*: :    :: :           *  ::

                                  III
                      _______________________________    ______
Ce18V         EEGLSGERLRYAVGQPMGAYSSWAAFTLSHHLVVAWCTYKSK-KVIRSSQYII  353
CpMV1-NB631   YKSPQG-FLTYAVGQPMGAYSSFAMLALTHHVIVQVAALNSG-FTTRFTDYCI  485
GaMV-S1       -NGLN--PIKYSVGQPMGAYSSWPAFTLSHHLVVHWCAHLCN-INKFK-DYII  387
L-dsRNA       WYLKDI-PYRYSVGQPMGALSSWAMLALSHHVIVQIAAMRVG--KLSFTNYAL  422
OnuMV3a-Ld    WYLKDI-PYRYSVGQPMGALSSWAMLALSHHVIVQIAAMRVG--KLPFTNYAL  421
OnuMV4-Ld     NYDYQGISYRYSVGQPMGAYTSWAAFTLTHHLVVHWAAELAG--LKNFKDYII  381
OnuMV5-Ld     YKTPEGDELHYKVGQPMGAYSSWAAFTLTHHLVVFYSARMAG--IKDFTNYIL  395
OnuMV6-Ld     FLAPDGDTVSYNCGQPMGAQSSWPMFTLAHHVIVRVAANRCG--LSNFDKYII  394
RsM2-1A1      LKVSDG-PYFYEVGQPMGALSSWPGLALTHHWIVQVAAFRVTNSKSWNTEYEI  505
                 .     *  ****** :*:. ::*:** :*    :           .* :

                    IV                  V             VI
              ______________      __________     _________
Ce18V         LGDDIVIKDNDIARKYIGQMSKLG-VAISMQKTHVSKD--TYEFAKRWMHKG  402
CpMV1-NB631   LGDDIVIAHDTVASEYLKLMETLG-LSISSGKSVISSEFTEFAKKLKGRNNF  535
GaMV-S1       LGDDIVIHNDNIAKKYIEIMG-KLGVGLSNSKTHVSKD--TYEFAKRWIHKG  436
L-dsRNA       LGDDIVIADKAVATSYHMIMTQILGVEINLSKSLVSSN--SFELAKRLVTMD  472
OnuMV3a-Ld    LGDDIVIADKAVATSYHMIMTQILGVEINLSKSLVSNN--SFEFAKRLVTMD  471
OnuMV4-Ld     LGDDIVIKNNKVAQIYINLMTKWG-VDISLSKTHVSYD--TYEFAKRWIKNK  430
OnuMV5-Ld     LGDDIVINNDKVAKYYIRTMKRLG-VELSMNKTHVSKN--TYEFAKRWFKNK  444
OnuMV6-Ld     LGDDIVINNDNVALKYMEIMNDFK-VEISRNKTHVSND--TYEFAKRWIKNK  443
RsM2-1A1      LGDDIVIFNELIAQEYLNIMAVIG-CEINLNKSISSRCRPVFEFAKRTCWGF  557
              ******* ..  :*  *   *         :.  *:  *      :    :
```

TABLE 5. Sequence identity comparison (%) of entire amino acids (upper triangle) and conserved motifs only (lower triangle) of RdRp regions between *C. elegans* Ce18V and known viruses.

	Ce18V	CpMV1-NB631	GaMV-S1	L-dsRNA	OnuMV3a-Ld	OnuMV4-Ld	OnuMV5-Ld	OnuMV6-Ld	RsM2-1A1
Ce18V		24	32	23	21	34	33	27	20
CpMV1-NB631	48		22	24	24	23	23	25	23
GaMV-S1	66	51		23	23	34	33	31	20
L-dsRNA	51	57	50		95	24	24	25	23
OnuMV3a-Ld	52	57	51	99		24	23	26	27
OnuMV4-Ld	71	50	72	53	54		35	29	21
OnuMV5-Ld	68	50	67	53	54	73		35	21
OnuMV6-Ld	61	51	66	52	53	63	67		23
RsM2-1A1	46	55	48	64	64	47	46	51	

Ce18V=*Chalara elegans 18 virus*; CpMV1-NB631=*Cryphonectria parasitica mitovirus 1-NB631*; GaMV-S1=*Gremmeniella abietina mitochondrial RNA virus S1*; L-dsRNA=*Sclerotinia homoeocarpa mitovirus*; OnuMV3a-Ld=*Ophiostoma mitovirus 3a*; OnuMV4-Ld=*Ophiostoma novo-ulmi mitovirus 4-Ld*; OnuMV5-Ld=*Ophiostoma novo-ulmi mitovirus 5-Ld*; OnuMV6-Ld=*Ophiostoma novo-ulmi mitovirus 6-Ld*

TABLE 6. Comparison of nucleotide sequence identities (%) of *C. elegans* Ce18V with related fungal viruses.*

	CpMV1-NB631	GaMV-S1	L-dsRNA	OnuMV3a-Ld	OnuMV4-Ld	OnuMV5-Ld	OnuMV6-Ld	RsM2-1A1
Ce18V	48	51	48	46	53	50	47	44
CpMV1-NB631		48	48	48	48	49	48	43
GaMV-S1			48	48	54	53	52	43
L-dsRNA				92	49	48	48	43
OnuMV3a-Ld					49	49	48	42
OnuMV4-Ld						55	52	43
OnuMV5-Ld							54	41
OnuMV6-Ld								40

Ce18V=*Chalara elegans 18 virus*; CpMV1-NB631=*Cryphonectria parasitica mitovirus 1-NB631*; GaMV-S1=*Gremmeniella abietina mitochondrial RNA virus S1*; L-dsRNA=*Sclerotinia homoeocarpa mitovirus*; OnuMV3a-Ld=*Ophiostoma mitovirus 3a*; OnuMV4-Ld=*Ophiostoma novo-ulmi mitovirus 4-Ld*; OnuMV5-Ld=*Ophiostoma novo-ulmi mitovirus 5-Ld*; OnuMV6-Ld=*Ophiostoma novo-ulmi mitovirus 6-Ld*

MFOLD program (Mathews et al., 1999; Zuker et al., 1999). Both 5'-UTR (nucleotides 1-27) and 3'-UTR regions (nucleotide 2,868-2,896) could be potentially folded into the stable stem-loop structure (Fig. 8). Also, a potential panhandle structure between the 5'- and 3'-UTR regions has been predicted in Ce18V (data not shown).

3.3.2. Detection of viral ssRNA form of Ce18V

Hybridization with both (+) and (-) strand probes resulted in the detection of a strong signal with the dsRNA band, confirming that it was double-stranded in nature (Fig. 9). However, another additional smeared band, which migrated faster than the full-length dsRNA, was detected only when the (+) strand probe was used (Fig. 9). With the negative strand probe, there was no additional signal produced, except with the dsRNA band (Fig. 9).

3.3.3. Mitochondrial association of Ce18V

Each cellular fraction obtained from the mitochondria extraction was retained, purified using a CF-11 column for dsRNA and electrophoresed on a 1% agarose gel to determine whether Ce18V was present. A sharp dsRNA band of approximately 2.8 kb in size was observed in cellular fractions A, B and mitochondrial fraction M, but not any other fractions, confirming the association between Ce18V and mitochondria (Fig. 10).

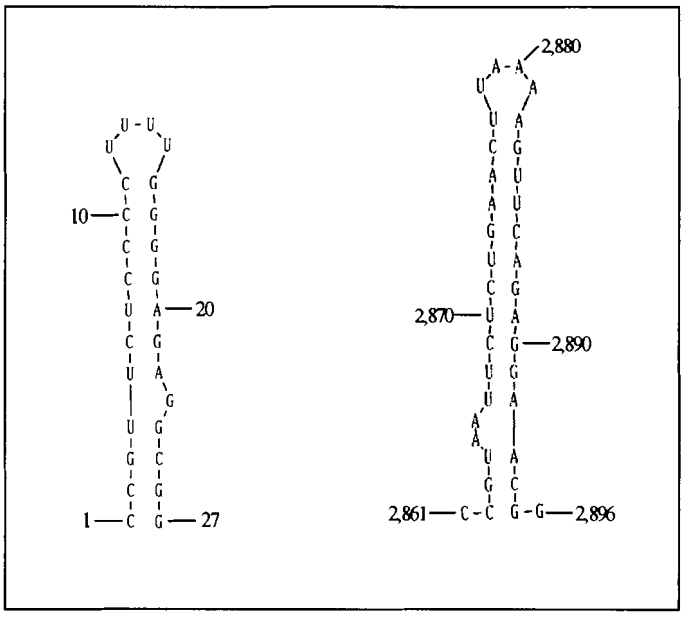

Fig. 8. Potential secondary structures of the 5'- and 3'- ends of the (+) strand of Ce18V. in strain BK18 of *C. elegans*. Both ends [1-27 (5'-end) and 2,861-2,896 (3'-end)] were folded using the MFOLD program (Zuker et al., 1999).

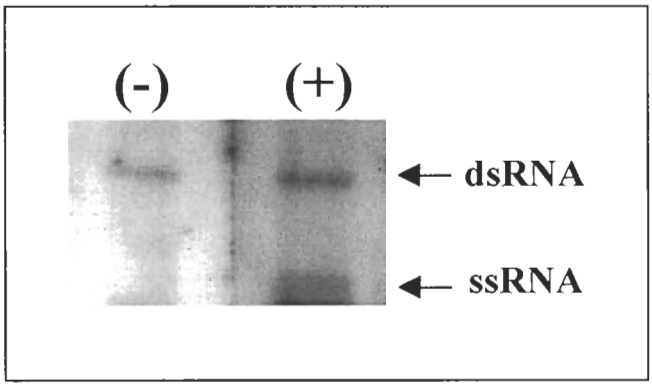

Fig. 9. Northern blot hybridization using a strand-specific probe of Ce18V. Total nucleic acids from *C. elegans* were extracted and probed with either (-) or (+) strand specific probes of Ce18V. The positions of dsRNA and ssRNA species are indicated.

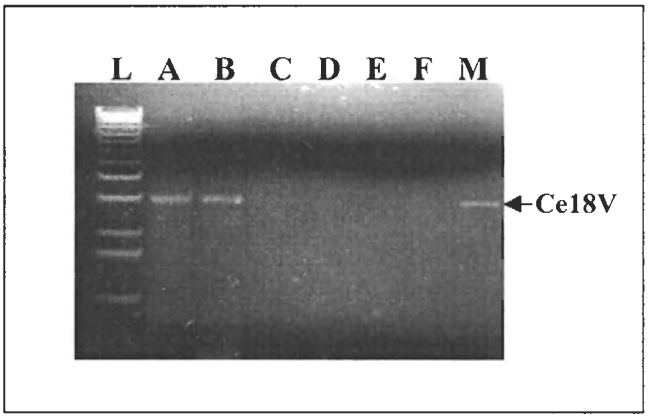

Fig. 10. Mitochondrial purification from strain BK18. The presence of Ce18V was observed in the purified mitochondria fraction (lane M), as well as in fractions A (the pellet after centrifugation at 1,300 *g* for 10 min) and B (the supernatant after centrifugation at 14,000 *g* for 30 min).

3.3.4. Phylogenetic relationships

Genetic relatedness was analyzed by comparing amino acid sequences of the conserved motifs of RdRp of Ce18V to known viruses (Fig. 11). Based on multiple alignments of RdRp regions, a phylogenetic tree was drawn (Fig. 11). There was a close relationship between Ce18V and viruses belonging to the genus *Mitovirus*, including OnuMV3a-Ld, OnuMV4-Ld, and OnuMV5-Ld, and OnuMV6-Ld (Fig. 11).

3.3.5. Transmission of dsRNA through conidia

All of the progeny (total of 50) obtained by transferring hyphal tips from single conidial colonies of strain BK18 showed the presence of dsRNA. There was no change in dsRNA banding patterns in any of the progeny when compared to the parental strain (Fig. 12).

3.3.6. Curing of dsRNA

When grown on V8 agar plates amended with various concentrations of either cycloheximide or ribavirin, all colonies showed significant growth retardation. However, following extended periods of growth and repeated transfers, colonies were recovered from plates treated with these two chemicals. The dsRNA banding pattern in all colonies was found to be identical to that of the wild-type dsRNA-containing strain (data not shown). Also, hyphal-tipping was attempted either combined with chemical treatments or without any treatments. No dsRNA-cured strains were obtained, indicating that the hyphal-tipping and chemical treatments had not eliminated the 2.8 kb dsRNA from strain BK18 of *C. elegans*.

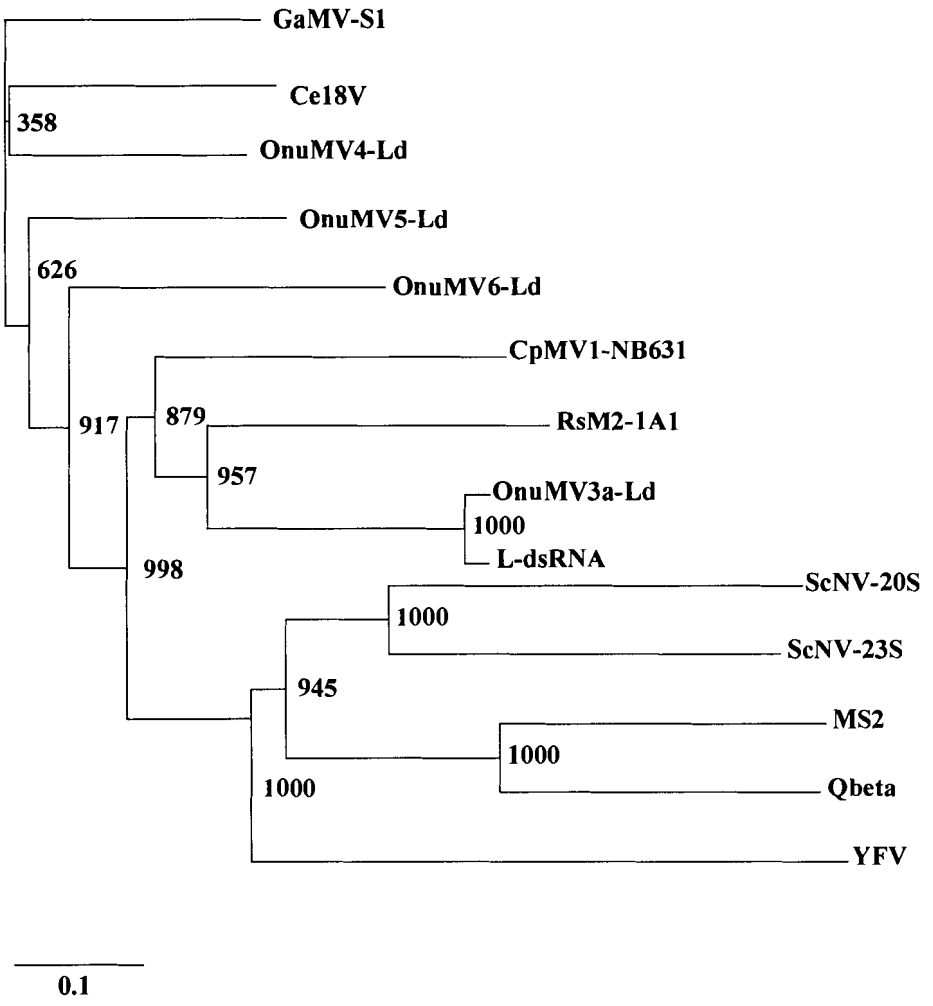

Fig. 11. Phylogenetic relationships among the conserved motifs of RdRps of Ce18V and other known Mitoviruses. Analysis was done using Clustal X program (Thompson et al., 1997) and the tree was drawn with TREEVIEW program. Bootstrap values are shown on the branches of the tree. Virus notations are as in Fig. 7. ScNV-20S (*Saccharomyces cerevisiae* 20S narnavirus), ScNV-23S (*Saccharomyces cerevisiae* 23S narnavirus), Q beta (Qbeta Bacteriophage), MS2 (MS2 Bacteriophage), and YFV (Yellow fever virus) were used as outgroups.

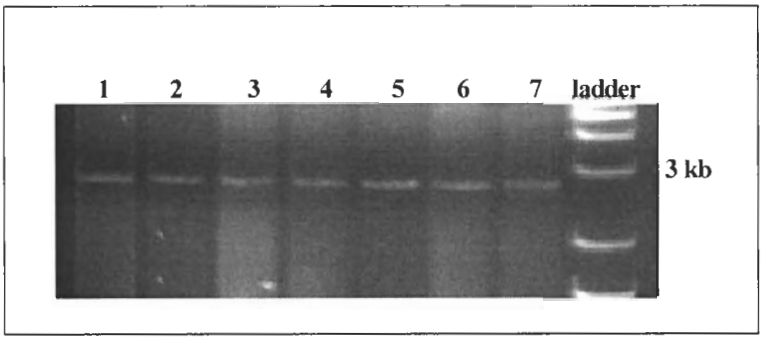

Fig. 12. Agarose gel electrophoresis of dsRNA derived from colonies obtained from single conidia of strain BK18. All progenies (lane 1 to 6) showed the same dsRNA-banding pattern compared to that of parental strain (lane 7), indicating the 2.8 kb dsRNA fragment was transmitted through all conidia.

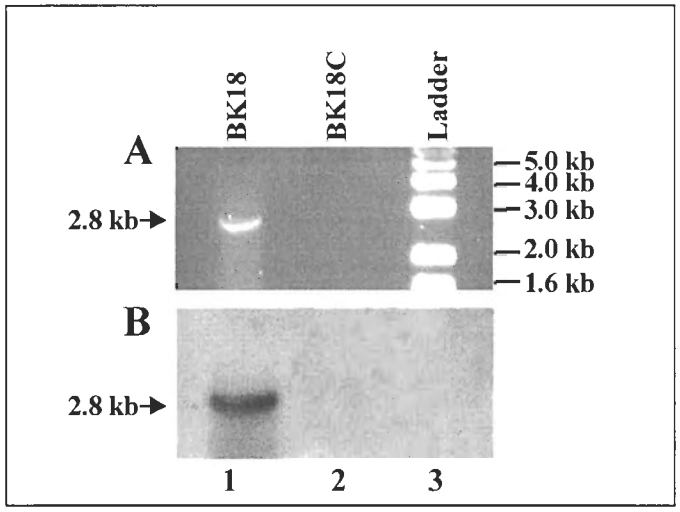

Fig. 13. Change in dsRNA banding pattern in strain BK18 after high temperature (35-37 C) incubation. **(A)** Ethidium bromide stained agarose gel of dsRNA extracted from BK18 (lane 1) and BK18C (lane 2). Lane 3 contains the 1 kb DNA ladder. **(B)** Autoradiograph of Northern blot of dsRNA shown in (A) probed with BK18C3.

During subculturing at 35-37°C incubation temperatures over an extended period (about 2 months), loss of the 2.8 kb dsRNA fragment was observed in one colony and resulted in the recovery of a putatively-cured strain, referred as BK18C (Fig. 13A). Northern blot hybridization with a partial cDNA probe (BK18C3) from the 2.8 kb dsRNA fragment did not reveal a signal in this strain (Fig. 13B).

RT-PCR amplification using a specific primer set (BK18F1 and BK18R2) was performed with total nucleic acids extracted from several *C. elegans* strains, including the 2.8 kb dsRNA-containing strains BK18 and WASH, putatively dsRNA-cured strain BK18C, and a dsRNA-free wild type strain AK208. The RT-PCR of strain BK18 generated one expected band near 300 bp in size (Fig. 14). However, RT-PCR amplification of strains BK18C and AK208 also generated a band identical in size to that of the RT-PCR amplification product of strain BK18 (Fig. 14). These RT-PCR products were gel-purified, cloned and sequenced to confirm that they were derived from the 2.8 kb dsRNA fragment. The clones shared high sequence identities (up to 98%) at the nucleotide level (Fig. 15), suggesting a latent infection with the 2.8 kb dsRNA fragment in both putatively dsRNA-cured (BK18C) and dsRNA-free wild type (AK208) strains. This latent-infected strain (BK18C) was subjected to further studies of culture morphology, pathogenicity, various enzyme assays and electron microscopy.

3.3.7. Characterization of a putatively-cured strain

3.3.7.1. Culture morphology and virulence

The colony morphology of strain BK18C resembled that of its wild-type strain (Fig. 16A). The growth rate of BK18C was slightly greater compared to that of BK18

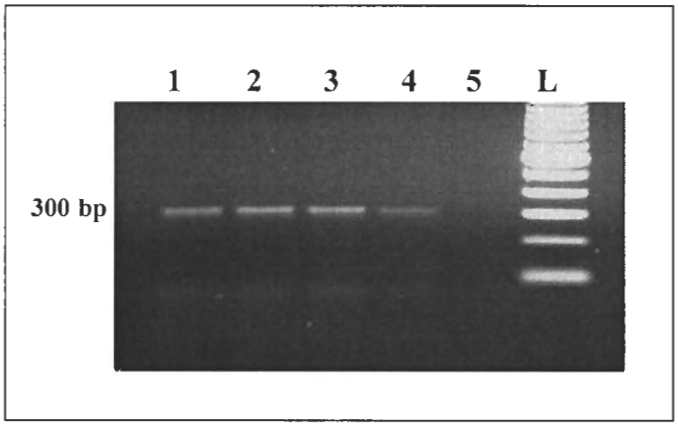

Fig. 14. Gel electrophoresis of RT-PCR amplification products with Ce18V specific primer sets. All trials with several strains, including BK18 (lane 1), BK18C (lane 2) WASH (lane 3) and AK208 (lane 4), successfully generated one RT-PCR fragment near 300 bp, indicating the Ce18V was present in these strains. AK208 is a wild-type strain with no detectable dsRNAs on polyacrylamide gels. Control RT-PCR products using water (lane 5).

Fig. 15. Comparison of nucleotide sequences of two cDNA clones (BK18B3 and WASH-3) derived from the 2.8 kb dsRNA fragment of BK18 and WASH strain, respectively. * indicates identical nucleotides and arrows represent the locations of primers used for RT-PCR (BK18F1 and BK18R2).

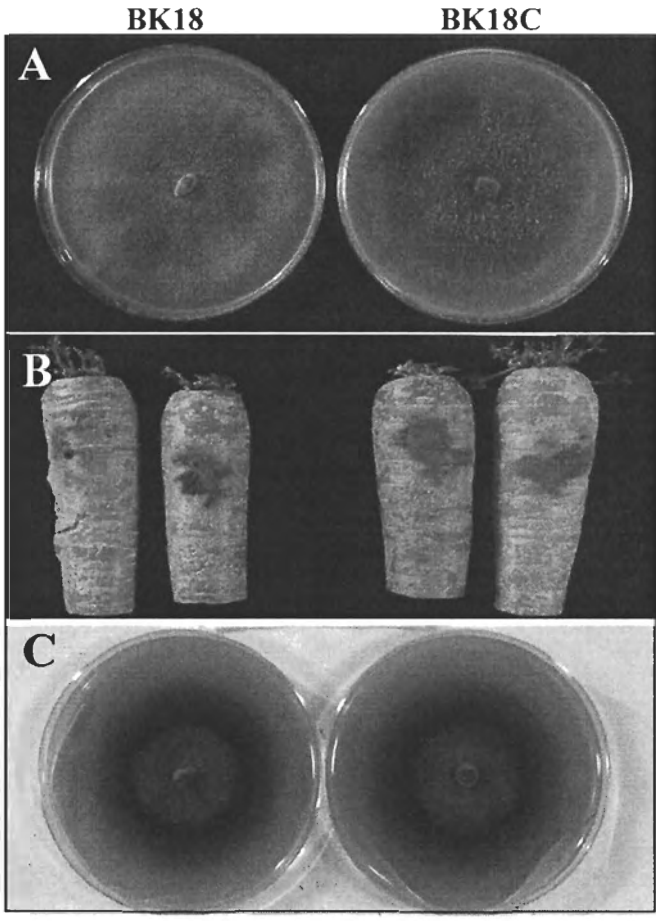

Fig. 16. Comparison of phenotypic characteristics of dsRNA-containing (BK18) and latently-infected (BK18C) strains. **(A)** Similar colony morphologies on V8 agar plates. **(B)** Virulence test on carrot roots. Carrot roots inoculated with strain BK18C showed greater colonization compared to its original dsRNA-containing (BK18) strain. **(C)** Bavendamm's reactions after 10 days incubation at 25°C in the dark, showing phenoloxidase color.

(Fig. 17A), and the virulence on carrot roots was slightly enhanced in BK18C compared to BK18 (Fig. 16B,17B).

3.3.7.2. Enzyme activity assays

Several enzyme activities were compared between BK18C and BK18. All colonies growing on Bavendamm's media containing either 0.5% tannic acid or 0.5% gallic acid produced a dark brown color reaction, indicating the presence of polyphenoloxidase in each culture (Fig. 16C). Similar enzyme activities, including laccase, esterase and tyrosinase, were observed between two strains (Table 7).

3.3.8. TEM pictures

Comparison of mitochondrial ultrastructure in strains BK18 and BK18C using TEM showed differences in size of mitochondria between the strains (Fig. 18), with strain BK18 containing smaller mitochondria compared to strain BK18C.

3.4. Discussion

The full-length cDNA clone from the 2.8 kb dsRNA fragment (Ce18V) in *C. elegans* strain BK18 was obtained by RT-PCR using a combination of random and dsRNA-specific primers and was characterized as a *Mitovirus* belonging to the family *Narnaviridae*. The sequence was 2,896 bp in length and using the mitochondrial genetic code contained a large open reading frame (ORF) that shown sequence similarity with several motifs, which are conserved among viral RdRps. The putative Ce18V RdRps showed the greatest similarity (34%) to that of an *Ophiostoma* mitovirus (OnuMV4-Ld).

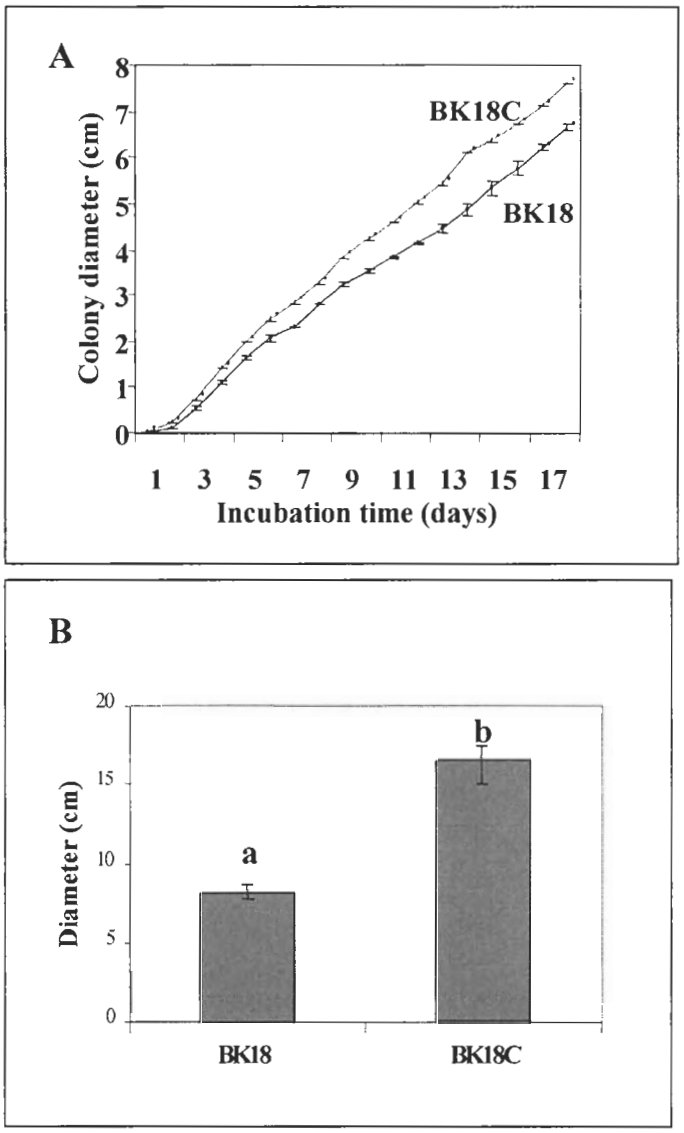

Fig. 17. (A) Comparison of growth rates on V8 agar between dsRNA-containing (BK18) and latently-infected (BK18C) strains. Strain BK18C grew better than strain BK18. Vertical bars represent standard errors of the mean. **(B)** Virulence test using carrot roots inoculated with latently-infected (BK18C) and dsRNA-containing (BK18) strains. Letters denote significant differences according to the difference of least squares means ($P<0.05$).

Table 7.	Comparison of various enzymatic activities between BK18 and BK18C strains.			
Strain	Trial	Enzyme Activity (absorbance change/5 min)		
		Laccase	Tyrosinase	Esterase
BK18	1st	0.0028	0.0049	0.0110
	2nd	0.0036	0.0051	0.0143
BK18C	1st	0.0036	0.0038	0.0117
	2nd	0.0039	0.0042	0.0123

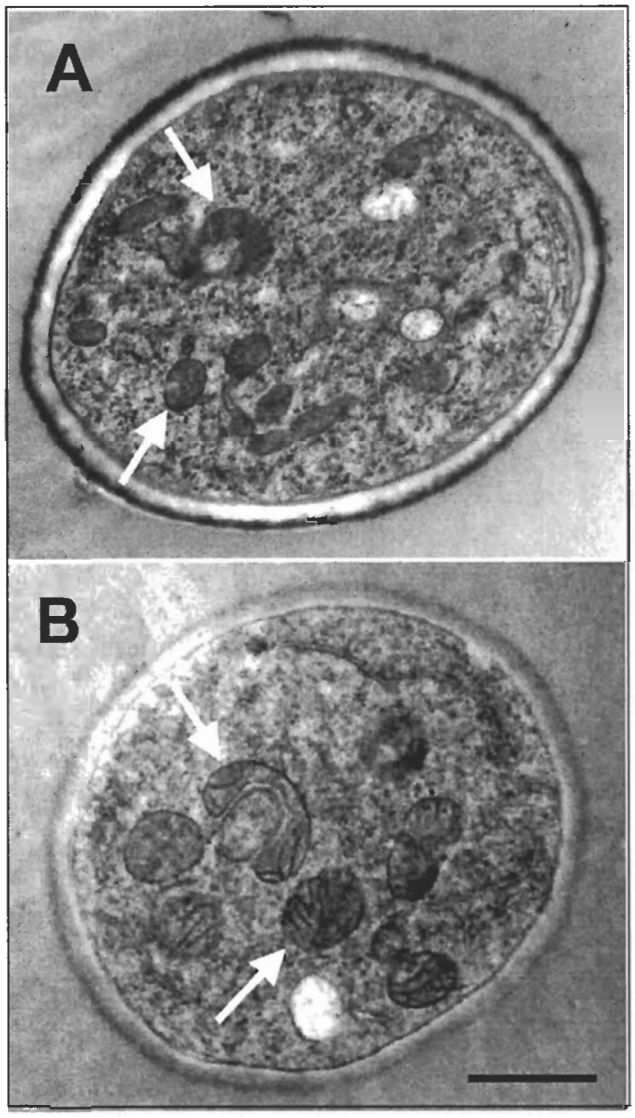

Fig. 18. Comparison of mitochondrial structures of dsRNA-containing (BK18) **(A)** and latently-infected (BK18C) strains **(B)** using transmission electron microscopy (TEM). Locations of mitochondria in the cell of each strain are indicated by arrows. Note smaller mitochondria in strain BK18. Bar is representing 500 nm.

In general, it has been suggested that two viruses are different when the sequence identity at the protein level is lower than 50% (Shukla et al., 1994). In this regard, Ce18V is likely to be a new member of the mitovirus group, since its highest sequence identity at the protein level to all previously described mitoviruses was 39% to OnuMV4-Ld.

Both nucleotide and amino acid sequences of Ce18V further supported the close relationships between Ce18V and other mitoviruses. Ce18V showed an A-U rich nature (67.4%), which is considered to be characteristic of mitoviruses as well as fungal and plant mitochondrial genomes (Paquin et al., 1997). The codon preference of either A or U in the third position, which is characteristic of mitochondrial codons (Cummings et al., 1990; Paquin et al., 1997; Unseld et al., 1997), was also observed in the ORF of Ce18V. In addition to the similar genome size to mitoviruses (2.3 kb to 3.2 kb), the predicted molecular mass (81.45 kDa) of the putative RdRp protein of Ce18V is within the size range of RdRp proteins of other mitoviruses (92.2 kDa in OnuMV4-Ld, 86.1 kDa in OnuMV5-Ld, 80.0 kDa in OnuMV6-Ld of *O. novo-ulmi*). Analysis of the amino acid sequence of Ce18V showed the typical RdRp motifs (II-VI) including conserved GDD motifs found in other RNA virus genomes (Bruenn, 1993; Poch et al., 1989). For the mitochondrial dsRNAs only, Hong et al. (1998) proposed an additional conserved motif among RdRp proteins and designated this region as motif I. Within motif I of RdRps in mitoviruses, there are nine positions showing identical amino acids and an additional 17 positions having chemically similar amino acids. The presence of motif I in Ce18V was found to be similar, confirming that the Ce18V is closely related to previously described mitoviruses.

It has been suggested that the presence of stem-loop structures at both 5'- and 3'-ends may be a characteristic of mitoviruses. Hong et al. (1998) showed that both the 5'- and 3'- end terminal sequences of *O. novo-ulmi* mitoviruses (OnuMV3a-Ld, OnuMV4-Ld, OnuMV5-Ld and OnuMV6-Ld) could be folded into stem-loop structures. Also, they suggested that the 5'-ends of both *R. solani* M2 RNA and *C. parasitica* mitochondrial RNA could form the possible stem-loop structure. Recently, similar structures were found at both 5'- and 3'-ends of L and S-dsRNA in *S. homoeocarpa* (Deng et al., 2003). The predictions of secondary structure for both 5'- and 3'-ends of Ce18V using the MFOLD program resulted in the possible formation of stem-loop structure. Both 5'- (nucleotides 1-27) and 3'- (nucleotide 2,868-2,896) ends can be folded into potentially stable stem-loop structure, providing additional evidence that Ce18V is closely related to other mitoviruses. Also, the presence of panhandle structure was predicted in Ce18V. It has been suggested that the presence of panhandle structures in some mitoviruses, such as OnuMV4 and OnuMV6, may play an important role in replication and transcription (Hong et al., 1998; Hong et al., 1999). Therefore, these secondary structures predicted in Ce18V may play similar functions that have been suggested for other mitoviruses.

Compared to other fungal RNA viruses, mitoviruses are unusual not only because of their rarity in fungi but also because of their association with mitochondria. Most mitoviruses, including *Ophiostoma* mitoviruses (Hong et al., 1999), *C. parasitica* mitovirus 1-NB631 (Polashock and Hillman, 1994) and *S. homoeocarpa* L-dsRNA (Deng et al., 2003), are known to have several UGA codons, which have been speculated to follow the mitochondrial genetic code to encode tryptophan rather than using a stop codon in the universal code (Paquin et al., 1997). Several reports have shown that

mitoviruses can be co-purified with mitochondrial extraction, indicating their presence in mitochondria for at least part of their life cycle (Polashock and Hillman, 1994; Deng et al., 2003). Cole et al. (2000) also reported that RdRp protein activity could be detected in mitochondria for mitovirus OnuMV6-Ld in *Ophiostoma* strain, but not from uninfected strains, suggesting that mitovirus RNA could be translated in the mitochondria. In this regard, our results showing the presence of UGA codons in the ORF of Ce18V and the co-purification of dsRNA with mitochondria are consistent with the results for other mitoviruses. However, in our study, the relative amounts of dsRNA observed in the mitochondria and in other subcellular fractions were similar. In contrast, in *Ophiostoma* and *Sclerotinia*, a strong mitovirus band was observed in the mitochondria fraction compared to other sub-cellular fractions, indicating that most mitoviruses were present in the mitochondria rather than the cytoplasm (Deng et al., 2003; Hong et al., 1998). While it is possible that Ce18V is present in both mitochondria and cytoplasm, this needs to be resolved through more experimentation, since sub-cellular extracts can be contaminated with mitochondria during purification and this may have occurred in our study.

Mycoviruses can be spread between strains either by vertical transmission through conidia or horizontal transmission via hyphal anastomosis (Ihrmark et al., 2002; McCabe et al., 1999). A high transmission rate of Ce18V through conidia of *C. elegans* was observed in this study. In contrast, horizontal transmission of dsRNA via hyphal anastomosis was not demonstrated in *C. elegans* (author unpublished data). The efficiency of virus transmission through either conidia or hyphal anastomosis differs depending on the fungal species (Tavantzis, 2001). Although the transmission rate of dsRNA through conidia is usually close to 100% in most fungal species, there are a few

reports which show variable transmission rates, ranging from 0 to 100% (Chen et al., 1996; Rogers et al., 1986; Shain and Miller, 1992; Tavantzis, 2001). The horizontal transmission rate between fungal strains via hyphal anastomosis is much more variable and is generally lower due to vegetative incompatibility (Nuss and Koltin, 1990; Milgroom and Cortesi, 1999). In *O. ulmi*, horizontal transmission of dsRNA was restricted by vegetative incompatibility (Brasier, 1984). There are no previous studies on vegetative incompatibility groups in *C. elegans*. The high degree of genetic diversity among dsRNA elements reported by Park et al. (2003), suggests that movement of dsRNA elements between strains is likely to be rare and that hyphal anastomosis may be infrequent.

To elucidate the role of dsRNAs in *C. elegans*, we attempted to eliminate Ce18V from strain BK18 using different methods, including treatment with cycloheximide or ribavirin, hyphal tip transfer, and high incubation temperatures. These treatments have been reported in other studies for elimination of dsRNAs (Fink and Styles, 1972; James, 2001; Vazguez, 1978). Most of the experiments were unsuccessful, indicating a high degree of stability of the dsRNA. In a previous study, Bottacin et al. (1994) reported that dsRNA-cured cultures of *C. elegans* were obtained from spontaneous sectors displaying altered colony morphology. In the present study, a change in dsRNA banding patterns was not observed in fungal sectors with unusual colony morphology during long-term subculturing, suggesting that spontaneous changes in dsRNA banding patterns may be infrequent in *C. elegans*.

During subsequent serial transfers at 35-37°C, a colony was obtained in which Ce18V was not seen in agarose gels and this strain was designated BK18C. Northern

blot hybridization confirmed the absence of Ce18V in BK18C. However, RT-PCR with total nucleic acids from putatively dsRNA-cured BK18C and a dsRNA-free wild type strain of *C. elegans* amplified one band, which was the same size as that in dsRNA-containing strain BK18, indicating a possible latent infection with the 2.8 kb dsRNA fragment. Sequencing of these RT-PCR products showed that these fragments were derived from the dsRNA and shared high sequence identities (up to 98%).

Numerous reports have demonstrated that the presence of dsRNA in fungi can affect specific biochemical and physiological pathways in the fungal host (Liu et al., 2003a; Liu et al., 2003b; Dawe and Nuss, 2000; Jian et al., 1997). In *Diaporthe ambigua*, the causal agent of Diaporthe canker disease, dsRNA-containing strains showed lower activities of phenoloxidase, gallic acid oxidation and oxalic acid accumulation (Smit et al., 1996). The transfer of dsRNA from a dsRNA-containing strain to a dsRNA-free strain through hyphal anastomosis resulted in changes in all enzymatic activities, indicating that the dsRNA altered physiological processes in the fungal host (Smit et al., 1996). However, it has also been reported that the presence of dsRNA in some fungi could not be correlated to any specific symptoms and such viruses have been called latent viruses (Ghabrial, 1998). In some cases, the presence of latent infection with certain virus could only be detected by RT-PCR. For example, mitovirus OnuMV3a dsRNA in *S. homoeocarpa* was detected by RT-PCR but not by other methods, such as agarose gel electrophoresis and Northern blot analysis (Deng et al., 2003). A similar observation was made for one of the dsRNAs present in *R. solani* (Lakshman and Tavantzis, 1994). These results are consistent with our observation regarding the concentration of dsRNA present in *C. elegans* BK18C. However, the effects of latently-infected dsRNA on host fungi are

quite different. In *S. homoeocarpa*, a change of OnuMV3a concentration in some isolates was observed after storage at 4°C and resulted in the development of hypovirulence traits such as reduced growth and virulence (Deng, 2003). The development of latent infection of Ce18V in *C. elegans* affected mitochondria size, fungal mycelium growth and virulence only slightly. It is noteworthy that the presence of Ce18V in *C. elegans* does not appear to induce remarkable hypovirulence as has been reported for other mitoviruses in fungi (Deng et al., 2003; Rogers et al., 1988). For example, mitovirus OnuMV-4Ld, which shared the highest amino acid sequence identity to Ce18V, caused hypovirulence in *O. novo-ulmi* (Hong et al., 1999). Reports of dsRNA in fungi are biased towards viruses that display a distinct phenotype, such as hypovirulence, and as a result, these dsRNAs have gained interest as biological control agents for plant pathogenic fungi (Anagnostakis and Day, 1979; Nuss and Koltin, 1990). However, latent infections can play an important role in mycovirus evolution because lower selection pressure compared to that of the highly expressive fungal viruses may give more advantages for survival and spread of latent viruses among host fungal populations (Ghabrial, 1998). The Ce18 virus in *C. elegans* is widely distributed in many geographic regions, which could be indirect evidence showing the successful adaptation of this virus in the host fungus, *C. elegans*, while causing minimally disruptive effects on its host.

CHAPTER 4

COINFECTION OF TWO DISTINCT TOTIVIRUS-LIKE DOUBLE-STRANDED (DS) RNA ELEMENTS IN *CHALARA ELEGANS (THIELAVIOPSIS BASICOLA)*[a]

4.1. Introduction

Double-stranded (ds) RNA elements have been reported in a large number of fungi (Ghabrial, 1998). Although many of these dsRNAs occur in plant pathogenic fungi, their effects on the host fungi are not always clearly understood (Ghabrial, 1980; Nuss and Koltin, 1990). Some dsRNAs, such as in the chestnut blight fungus *Cryphonectria parasitica*, can induce severely debilitating phenotypes, resulting in hypovirulence (Anagnostakis, 1982) and the dsRNAs associated with hypovirulence in *C. parasitica* have been extensively studied. The transmission of dsRNAs through hyphal anastomosis from dsRNA-containing to dsRNA-free strains resulted in the development of hypovirulence traits in the dsRNA-recipient strains (Anagnostakis and Day, 1979).

The genetic information contained in dsRNA fragments can be ascertained by developing complementary DNA (cDNA). Based on this sequence information, dsRNAs in fungi have been classified into various virus groups, including the hypoviruses in *C. parasitica* (Hillman et al., 1995), totiviruses in *Helminthosporium victoriae* (Ghabrial et

[a] Prepared as a manuscript submitted to *Virus Research.*

al., 1995), mitoviruses in *Ophiostoma novo-ulmi* (Wickner et al., 1995), partitiviruses in *Discula destructiva* (Rong et al., 2001), and unclassified viruses in *Diaporthe ambigua* (Presig et al., 2000). Furthermore, the development of cDNA clones from specific dsRNA fragments has been used to study genetic diversity to better understand the population structures of both dsRNAs and host fungi (Peever et al., 1997; Bharathan and Tavantzis, 1990). The development of full-length cDNAs from dsRNA fragments can also result in the construction of infectious cDNA clones, such as CHV1 in *C. parasitica*, which has great potential to be used for biological control studies (Moleleki et al., 2003; Dawe and Nuss, 2001).

Chalara elegans is a soil-borne plant pathogen that causes black root rot disease on more than 100 plant species worldwide, including a wide range of vegetables, field crops and ornamental plants (Punja et al., 1992; Yarwood, 1981). Natural populations of *C. elegans* contain multiple dsRNA fragments, which vary in size from 2.5 kb to 12 kb (Bottacin et al., 1994). Partial cDNA clones from these dsRNAs, including from a 2.8 kb, 5.3 kb and 12 kb fragment, were developed and shown to have some sequence homology to viruses in the *Narnaviridae* and *Totiviridae* (Park and Punja, 2003; Park et al., 2003). This suggests that at least two or three virus groups occur in *C. elegans*. Genetic diversity studies have also been conducted using these partial cDNA clones (Park et al., 2004). The genetic relatedness among the 5.3 kb dsRNA fragment, the most common size of dsRNA fragment present in *C. elegans,* revealed a high degree of genetic diversity compared to the 2.8 kb and 12 kb dsRNA fragments (Park et al., 2004).

In this study, we constructed a full-length cDNA clone from the 5.3 kb dsRNA fragment in *C. elegans*. Sequence analysis revealed three putative open reading frames

(ORFs), which encoded either a coat protein or RdRp, and these ORFs showed some homology to that of viruses belonging to *Totiviridae*. Partial cDNA clones were coincidentally obtained from an additional dsRNA fragment in strain CKP and sequence analysis of these also showed some homology to the RdRp region of viruses in the family *Totiviridae*. The two fragments (Ce-dsRNA1 and Ce-dsRNA2) had 56% and 50% sequence identities at the level of nucleotide and amino acid sequences, respectively, in conserved motifs of the RdRp regions. This implies coinfection by two distinctive totivirus-like dsRNA fragments occurring in one strain of *C. elegans*. Sequence data from both dsRNA fragments were analyzed for phylogenetic relationships to other known viruses. The presence of virus-like particles in the mycelium of strain CKP of *C. elegans* was also observed using transmission electron microscopy (TEM).

4.2. Materials and Methods

4.2.1. Fungal strain

A dsRNA-containing strain (CKP) of *C. elegans*, originally isolated from chickpea (*Cicer arietinum*) near Pullman, Washington, U. S. A. in 1993 by W. Kaiser, was used in this study. It contained multiple dsRNA fragments ranging from 2.5 kb to 6.8 kb in size (Bottacin et al., 1994). This isolate was maintained on V8 agar [V8A; V8 juice, 150 ml; Bacto agar, 15 g; distilled water, 850 ml; ampicillin, 100 mg) either at room temperature (21-23°C) or at 4°C for long term storage. Actively growing cultures were obtained by transferring fungal mycelium onto fresh V8A dishes or into 100 ml of potato dextrose broth (PDB, Difco, Detroit, MI).

4.2.2. dsRNA isolation and purification

Extraction and purification of dsRNA was conducted as previously described (Park and Punja, 2003). Briefly, fungal mycelium of strain CKP grown in PDB for 2 wk was harvested through Miracloth® using vacuum filtration and homogenized with a mortar and pestle in 2X STE buffer. After extraction with phenol, dsRNA was further purified by chromatography on CF11 (Morris and Dodds, 1979) and low-melting agarose gel electrophoresis. The purified dsRNA was dissolved in DEPC-treated water and used for the production of cDNA clones.

4.2.3 Development of full-length cDNA

A partial cDNA clone from a 5.3 kb dsRNA fragment in strain CKP was obtained previously by RT-PCR using random primers (Park et al., 2003). Several dsRNA-specific primers were designed based on the sequence of the partial cDNA clone and used for RT-PCR with random primers to extend the partial cDNA fragment. The primers used for this study are listed in Table 8. RT-PCR was performed according to the method previously described by Park et al. (2004). RT-PCR products of high molecular mass were purified from a 1% agarose gel using the Qiagen gel-extraction kit (Qiagen, Chatsworth, CA), and cloned into pCR2.1-TOPO vector using the TA cloning kit (Invitrogen) according to the manufacturer's protocol. To obtain cDNA clones for the terminal regions of the 5.3 kb dsRNA fragment, the rapid amplification of cDNA ends (RACE) kit (Roche, Indianapolis, IN) was used according to the manufacturer's protocol.

TABLE 8. Primer sequences used to develop cDNA clones from a 5.3 kb dsRNA in strain CKP of *C. elegans*

Primer	Sequence (5' to 3')	Length (nt)
6A1-F	TTCAGTGTCCATTTGTGGGA	20
6A1-R	GAGGTGCCGTAGACAGCGTA	20
C2D-GapF1	TTATCACCGGGCTGAGTTTT	20
C2D-GapR1	GCGTGCAGCTTCTTCATAAAC	21
CV1-F1	GCATTCATACGTTAGGCCGT	20
CV1-R1	GCACTGACGTTAGAGCACCA	20
5'-1	TACCTGCGGAAAGCAGAGTC	20
5'-2	GCGATGACACCAGACAAAAA	20
5'-3	CCGTCGAGAACAGAATGTGA	20
3'-1	TCACCCGTAGAGGTTATGGC	20
3'-2	CAACGGAGAGCTCATGACAA	20
3'-3	GACAAACTGAAAAGGGCGTC	20

RT-PCR products for both 5' and 3' terminal ends were run on a 1% agarose gel, gel-purified and cloned into TOPO vector as described above.

4.2.4. Northern blotting

Northern blot hybridization analysis was performed as described by Park et al. (2004). The dsRNA fragments in a 1% agarose gel were treated with 0.2 N HCl for depurination, denatured with 50 mM NaOH and 1.5 M NaCl, and neutralized with 1 M Tris, pH 7.4, and 1.5 M NaCl. DsRNA was transferred to a nylon membrane by overnight capillary action in 10X SSC and the DIG DNA labeling kit (Boehringer Manheim, Germany) was used for detection following the manufacturer's protocol.

4.2.5. Sequence analysis

Twelve dsRNA-derived cDNA clones were sent to the sequencing lab NAPS unit at the University of British Columbia, Vancouver, BC and sequenced with either M13 or dsRNA-derived primer sets. Based on their sequences, the partial cDNA clones were aligned using the Bioedit program to generate a full-length sequence of the 5.3 kb dsRNA fragment. Sequence comparison were performed with the BLAST program (http://www3.ncbi.nlm.gov/BLAST). In addition, multiple sequence alignments and phylogenetic analysis were performed using CLUSTAL W (Thompson et al., 1997) and dendogram were drawn with the TREE View program.

4.2.6. Transmission electron microscopy (TEM)

Strain CKP was inoculated onto V8A and incubated at room temperature for 5 days. Several samples of agar blocks, that included the actively growing margin of colonies, were selected and prepared for transmission electron microscopy in the Bio-imaging lab at the University of British Columbia (Vancouver, Canada). Briefly, each sample was fixed using glutaraldehyde and frozen in a Baltec HPM10 high pressure freezing machine for 5 days. High pressure frozen samples were further processed for infiltration and embedding with resin using transfer baskets. Each sample was ultra thin-sectioned with a Leica Ultracut ultramicrotome, stained with 2% uranyl acetate (12 min.) followed by lead citrate (6 min.) and viewed with a Hitachi H7600 TEM at 80 kV.

4.3. Results

4.3.1. Development and sequence analysis of cDNA from 5.3 kb dsRNA

RT-PCR with random primers amplified several cDNA clones, ranging in size from 500 bp to 1.2 kb of the 5.3 kb dsRNA fragment in strain CKP. These cDNA clones (12 in total) were initially characterized by Northern blot hybridization analysis to confirm that they were derived from dsRNA in strain CKP (Fig. 19). Based on the sequence information from the 12 clones which hybridized to the 5.3 kb dsRNA fragment, several dsRNA-derived primers were designed (Table 8). These primers were used to amplify additional regions which were not amplified by RT-PCR using random primers.

Up to five cDNA clones (1A, 6A, C1C, C2D, C7) were obtained to cover most of the region of the 5.3 kb dsRNA fragment in strain CKP and were aligned with each other based on their sequence overlap (Fig. 20A). RT-PCR following the manufacturer's

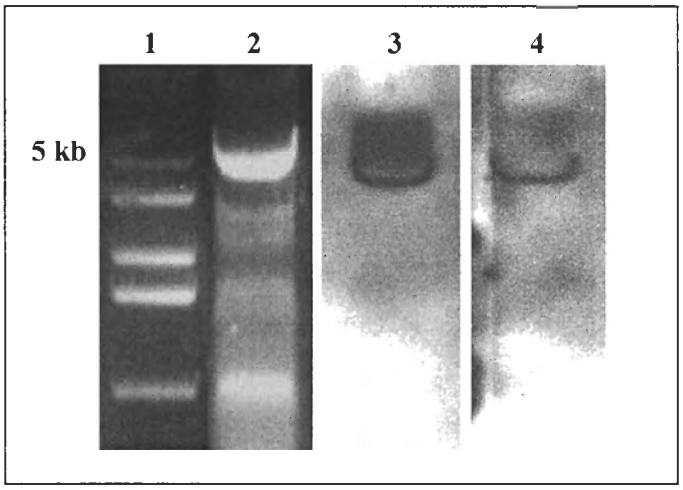

Fig. 19. Gel electrophoresis and Northern blot analysis of the 5.3 kb dsRNA in strain CKP of *C. elegans*. The dsRNAs were electrophoresed on a 1% agarose gel (lane 2) and used for Northern blot analysis with either C2D (lane 3) or C4B (lane 4) probes, derived from two dsRNA fragments, Ce-dsRNA1 and Ce-dsRNA2, respectively, in strain CKP.

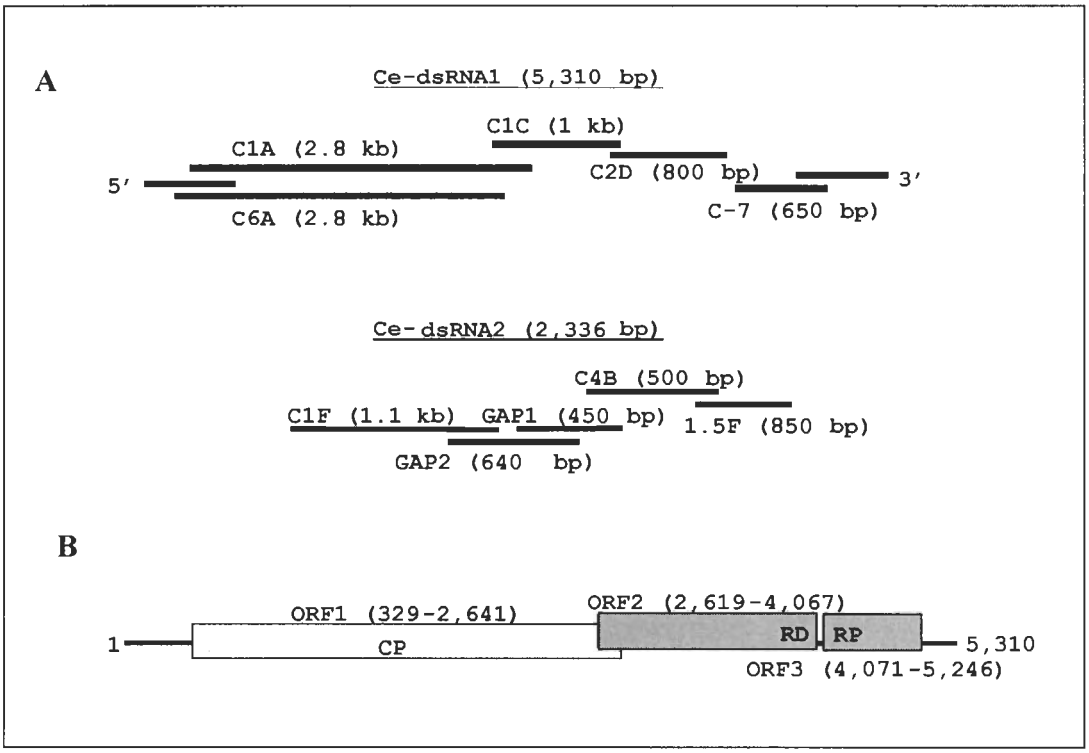

Fig. 20. Schematic representation of alignments of partial cDNA clones (**A**) derived from two dsRNA fragments (Ce-dsRNA1 and Ce-dsRNA2) in strain CKP of *C. elegans* and potential genomic organization of Ce-dsRNA1 (**B**). CP=coat protein and RDRP=RNA-dependent RNA polymerase.

Fig. 21. The complete cDNA sequence of Ce-dsRNA1. The shaded sequences show the conserved motifs (I-VIII) of RNA-dependent RNA polymerases (RdRp) of dsRNA viruses.

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AACCGCTGCTTCCATGGAGGCTACTTCTCTTAACCATTTTTTGTCTGGTGTCATCGCCGCCCCTAAAGGTGGACGTTTGG
              M  E  A  T  S  L  N  H  F  L  S  G  V  I  A  A  P  K  G  G  R  L
              ├──▶ ORF1
CCGAAGACTCTGCTTTCCGCAGGTACCGTGCCCACGTCCGCACCACCGCCACTATTGGCGGTAACACGGACGCCCGTAA
A  E  D  S  A  F  R  R  Y  R  A  H  V  R  T  T  A  T  I  G  G  N  T  D  A  R  N

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  A  F  I  R  Y  E  V  G  R  S  S  G  K  R  G  Q  L  L  A  A  P  P  D  D  S  R

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 R  I  E  A  S  Y  P  T  N  A  V  L  A  E  D  F  L  G  L  A  K  K  Y  S  N  F

CTGCCCAGTTCCAGTTTTCGTCTCTGGCCGCAGTCGCTGAGCGTCTAGCTAAAGGCCTCGCTGTCCATGCAACCGTTGC
S  A  Q  F  Q  F  S  S  L  A  A  V  A  E  R  L  A  K  G  L  A  V  H  A  T  V  A

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  D  V  D  S  V  A  L  R  G  G  A  P  L  I  V  A  G  L  G  T  Y  D  G  P  I  N

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 S  L  I  S  S  V  F  I  P  R  L  V  N  N  V  L  T  G  D  V  F  S  V  L  A  N

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  V  D  G  D  G  F  A  K  A  A  T  E  A  L  R  I  V  G  A  N  M  A  Q  S  D  Q

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  K  T  K  L  A  W  E  D  I  E  L  A  G  D  R  D  T  T  F  S  A  Y  R  A  K  F

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  P I S D Y L W D R G Q S P F C A P G E L L N L G S T

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 I G F L V R H V T F D D D G I P T T E H V P T S R E

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  A P T N E G R N D V *
           M R A E T M S R Y E E F G H L G K Y L S D L L
          ORF2
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                                I
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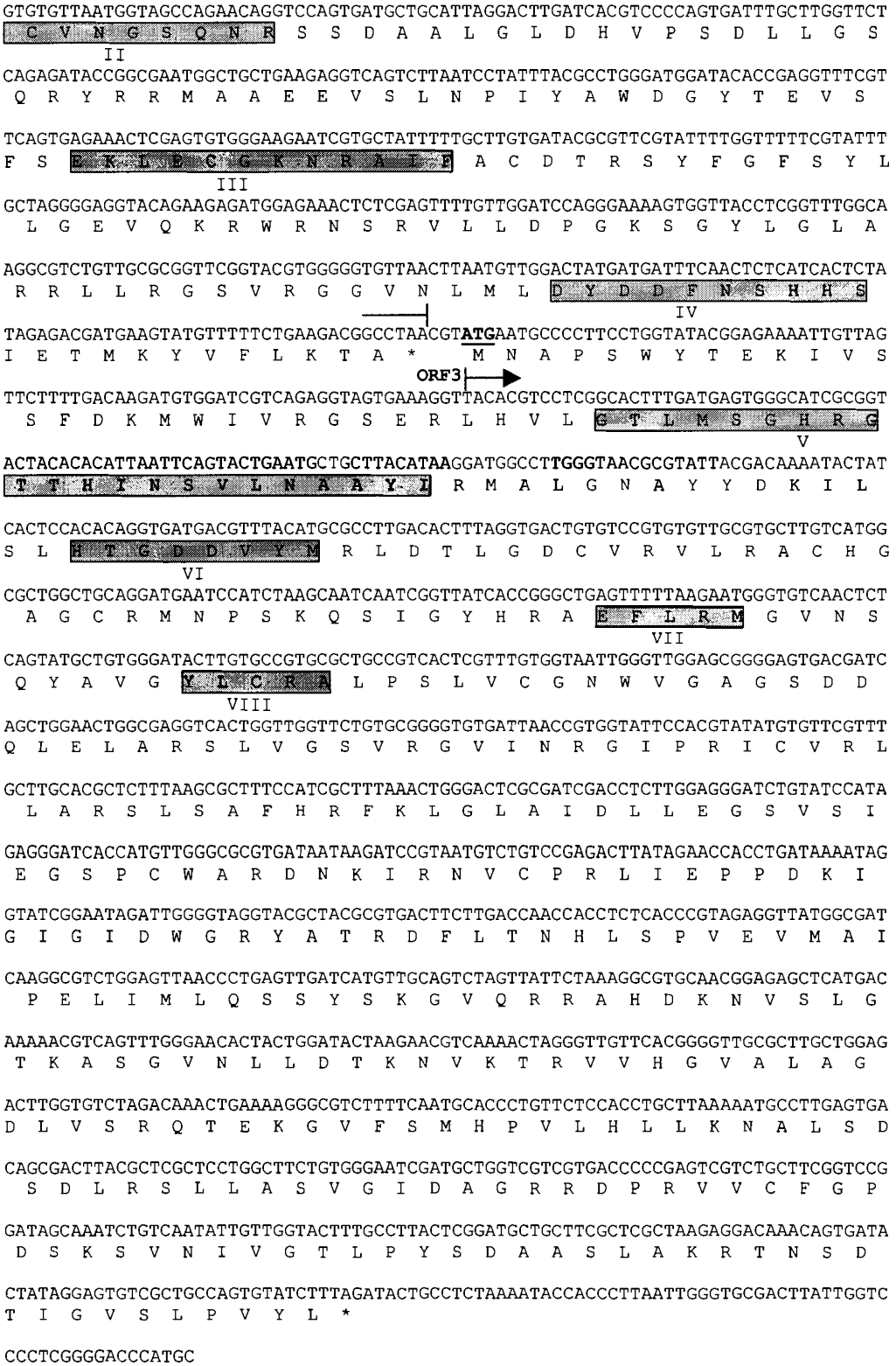
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II
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Q R Y R R M A A E E V S L N P I Y A W D G Y T E V S
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F S E K L E C G K N R A L E A C D T R S Y F G F S Y L
III
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L G E V Q K R W R N S R V L L D P G K S G Y L G L A
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R R L L R G S V R G G V N L M L D Y D D F N S H H S
IV
TAGAGACGATGAAGTATGTTTTTCTGAAGACGGCCTAACGTATGAATGCCCCTTCCTGGTATACGGAGAAAATTGTTAG
I E T M K Y V F L K T A * M N A P S W Y T E K I V S
ORF3
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V
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T T H I N S V L N A A Y I R M A L G N A Y Y D K I L
CACTCCACACAGGTGATGACGTTTACATGCGCCTTGACACTTTAGGTGACTGTGTCCGTGTGTTGCGTGCTTGTCATGG
S L H T G D D V Y M R L D T L G D C V R V L R A C H G
VI
CGCTGGCTGCAGGATGAATCCATCTAAGCAATCAATCGGTTATCACCGGGCTGAGTTTTTAAGAATGGGTGTCAACTCT
A G C R M N P S K Q S I G Y H R A E F L R M G V N S
VII
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Q Y A V G Y L C R A L P S L V C G N W V G A G S D D
VIII
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L A R S L S A F H R F K L G L A I D L L E G S V S I
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E G S P C W A R D N K I R N V C P R L I E P P D K I
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D L V S R Q T E K G V F S M H P V L H L L K N A L S D
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S D L R S L L A S V G I D A G R R D P R V V C F G P
GATAGCAAATCTGTCAATATTGTTGGTACTTTGCCTTACTCGGATGCTGCTTCGCTCGCTAAGAGGACAAACAGTGATA
D S K S V N I V G T L P Y S D A A S L A K R T N S D
CTATAGGAGTGTCGCTGCCAGTGTATCTTTAGATACTGCCTCTAAAATACCACCCTTAATTGGGTGCGACTTATTGGTC
T I G V S L P V Y L *
CCCTCGGGGACCCATGC

protocol using RACE kits with several dsRNA-derived primers generated four clones to cover the 5'- and 3'- end sequences. These additional clones were sequenced and used to construct the full-length cDNA sequence of the 5.3 kb dsRNA. The alignments of partial cDNA clones are represented in Fig. 20 and the full-length clone is designated as *Chalara elegans dsRNA 1* (Ce-dsRNA1).

The complete nucleotide sequence of Ce-dsRNA1 was 5,310 bp long and is deposited in GenBank under accession number AY561500. The nucleotide composition of Ce-dsRNA1 was 20.2% A, 25.0% C, 27.5% G, and 27.1% U. Sequence analysis revealed the presence of three large putative open reading frames (ORFs) on the full-length positive sense strand of Ce-dsRNA1 (Fig. 20, 21). The putative ORF 1 was initiated at the AUG codon at nucleotide position 329 (underlined) and was terminated at the UAG codon at nucleotide position 2,641. ORF1 can encode a protein of 770 amino acids with a predicted molecular weight of 80.71 kDa. Homology search with the amino acid sequence revealed that the putative protein encoded by ORF1 has significant homology to the coat protein region of other mycoviruses belonging to the family *Totiviridae* (Fig. 22). The highest homology of ORF1 was to the coat protein of *Spharopsis sapinea RNA virus 1* (SsRV1), with 45% identity. Amino acid identities between ORF1 of Ce-dsRNA1 and other selected viruses in the family *Totiviridae* were compared and are listed in Table 9. Amino acid identities ranged from 33% to 45%, indicating that Ce-dsRNA1 in *C. elegans* is related to viruses in the *Totiviridae*.

The overlapping region between ORF1 and the putative ORF 2 was 22 nucleotides long and ORF 2 was in the +1 frame relative to ORF1 (Fig. 20, 21). ORF 2 started at the AUG codon at nucleotide position 2,619 and terminated at the UAA

TABLE 9. Sequence identity comparison (%) of the entire coat protein and RNA-dependent RNA polymerase of Ce-dsRNA1 to other dsRNAs in *Totiviridae*.

	Capsid protein	RdRp
CmRV	33	31
GaRVL1	34	19
Hv190SV	45	37
SsRV1	41	39
SsRV2	35	32

CmRV=*Coniothyrium minitans RNA virus*; GaRVL1=*Gremmeniella abietina RNA virus* L1; Hv190SV=*Helminthosporium victoriae 190S virus*; SsRV1=*Sphaeropsis sapinea RNA virus* 1; SsRV2=*Sphaeropsis sapinea RNA virus* 2.

Fig. 22. Comparison of deduced amino acid sequences of the coat protein region of Ce-dsRNA1 to known dsRNA viruses in *Totiviridae*. CmRV=*Coniothyrium minitans RNA virus*, Hv190SV=*Helminthosporium victoriae 190S virus*, SsRV1=*Sphaeropsis sapinia RNA virus1*.

```
Ce-dsRNA1    MEATSLNHFL SGVIAAPKGG RLAEDSAFRR YRAHVRTTAT ---------- --IGGNTDAR
CmRV         RFLASAASPL TGTVAGISAG TIGQDNQYRR YRAGLTIGVH ---------- --EHGSYTQA
HV190SV      MSHTTITNFL AGVIARPQEE TLLAT---KR SDVIGQLCAP ---------Q PLSAGNEDSR
SsRV1        MEHALRNAFL AAVIASPRGG TLLTAPTKKG KEKKAPLATY RRYRANIRTE STIAGNVDAR
                :     * :..:*       :      :          .                 •.

Ce-dsRNA1    NAFIRYEVGR SSGKRGQLLA APPDDSRRIE ASYPTNAVLA EDFLGLAKKY SNFSAQFQFS
CmRV         RRSIFYEVGR RYGRLTDALG PHGAEAVPID ASVRINAAEA ANFEGFARKF SNFSPQWLTM
HV190SV      TTSIFHEIGR AVNTKGKALA VAGMEAPLVE ASYPTNAVLV EDFIGLAKKY TNFSATFEYS
SsRV1        VAGIQYEIGA RYERVGEAFA PVPEEQVLFE AAYPSSAALA EDFVGFARKY SNFSATFAHS
                * :*:*        . :.     :   .: *:   .*. .  :* *:*:*: :***. :

Ce-dsRNA1    SLAAVAERLA KGLAVHATVA D--VDSVALR GGAPLIVAGL GTYDGPINSL ISSVFIPRLV
CmRV         DLCGIAERLA KGVAAQSVYG G--VNIVNLR GHLPVRVVAL GTLDSPQTAS NNSVFIPRTV
HV190SV      SLAGVVERLA RGLALVASLK TSRVP--ICW GNNPLAVHAL ATYDGPVNSL TSAVFIPRLV
SsRV1        SLAGLVERVA RTLGALTVFP SGTFDQDAIR GGRPLMIAAL GTLDGPVNSL AGSVFIPRLV
             .*..:.**:* : :.  :       .       *  *: : .* .* *.* .:   .:***** *

Ce-dsRNA1    NNVLTGDVFS VLANAIAGEG ASIATDIAEI DPSTRQPVIP EVDGDGFAKA ATEALRIVGA
CmRV         DTVGNDHVFA VLAAAANGEG AAVSTDVLRL DANTNEPVIP AVSGPSLASA CIEALRIVGA
HV190SV      NNALTGDVFA VLCNCVAGEG GTVVTDTIEL DANTRQPIVP EVGPLGVPGA IVDALRLLGS
SsRV1        DSVISPDVFT ILINAAAGEG SRVITDVLEL DATTRRPIVP TLRDSSMLLP CVEALRILGA
             :.. . .**: :*  .  *** . : **  .: *..*..*::*  :   ..  .   :***::*:

Ce-dsRNA1    NMAQSDQGLL FSLAVTRGIH SVVSWLPTAM KAALLG-ICC VWTFQCPFVG FITGSKLHG-
CmRV         NMEASGAGDL FAYAVTRGIH AVVSVVAHTD EGGYMRALLR HGRFRVPYGG INQALRDYPA
HV190SV      NMIASDQGPL FALALTRGIH RVLSVVGHTD EGGIVRDLLR CGGFGLPFGG IHYGLEEYSG
SsRV1        NMAACDQGPL FAFALVRGLN AVLSLVGHTD EAGVTRDIFR VSGFDIPFGG IHFGLEPYAG
             **  .. * * *: *:.**::  *:* :  :  :..    :      *  *: * :  . . :

Ce-dsRNA1    -PPSLLSTVS RSVLLCRCHC TDPAAAVAPS DPGENYNGEW FPTFYSGTKG GDPTVRPGGD
CmRV         LPAAGALATH VISSWVDAIA LKTAAVVAHS DPCVIASGGL YPTVFTSSQG DITPPGTDEG
HV190SV      LPALQFNSAA ATAAYVDGIA LVTAAVVAHA DPGERYNGEW FPTFFDGTTH ADTMRRSGDS
SsRV1        LPAVASNAGA DACCYVDALL MTSGALVAHC DPGQEYGGRW FPTVLQGTGP DTAEVRPGQS
              *.    :               ..* ** . **    .*   :**.  .:     .   .. .

Ce-dsRNA1    CPGNSETSGR IRSQLLADCE KFFRNYIPAL GRIFGLTGSP NLAVTVAVGM SRFLHAD--P
CmRV         DSPTDADARA IGRQIAGDLG RFAPTFMAGL LRIFGLQTSS QVAEAHFCTV GGMYLSENVD
HV190SV      TEGTAAMADR NRATVACPQQ LFWRPYITAL GACFSTAGDI SVAERFQCAA SHSLGAD--P
SsRV1        QEGTADMANR NRGLLASSMP RFAEHYARGL GRLFAIEGDA RIVVNILSAS ARLLPDN--C
                .   :       :       *   :  .*    *.   .   :.        .     :

Ce-dsRNA1    RHLRYASVAP WYWIEPTSLL PSDFLGSVAE MNGSGSFGGK DSTKTKLAWE DIELAG-DRD
CmRV         RHLRHKTVAP YFWVEPTSLI EVGFLGSTAE TAGFGSLVTP GDQAMIPTFE RVREMDRGRN
HV190SV      RHLRLPSVAP YFWIEPTGLI PHDFLGSVAE EEGFASYCWR DTTRTRPAWD SIVLSG-PRD
SsRV1        RHLRYPSVSP YFWVEPTSLL PPDFLGTAAE LNGCGSLAMR GTSRTRQAWE DIERVG-DED
             ****  :*:* ::*:***.*:   .***:.**   * .*     .      :::  :   .  .:
```

```
Ce-dsRNA1    TTFSAYRAKF LSPRRAWFMA HWNGHPDNGL GCIRLRQADP NGFIHPG-RG TSGADLRDRL
CmRV         ANFSTIAFKM RTARTSGLVC AHAAAPTP-L AGLKLYQFDQ DSLILAGDQG PTNGDVPTKH
HV190SV      TTFSAYHIRM KGARTAWFLA HWLGHPENGL GATRVRQLDP NAVLHPG-PC EGNEQVRDRV
SsRV1        VTFTGYNVAM PFARSSWFLI HWLNHPANGL GALRVRQLDP NGVIHPG-PC ADNPDVRDRV
             ..*:     :   .* : ::       *   * .  :: * *  :..: .*      . ::  :

Ce-dsRNA1    EDDAPISDYL WDRGQSPFCA PGELLNLGST IGFLVRHVTF DDDGIPTTEH VPTSREFLDT
CmRV         VAADPLSSYL WVRGQSAIPA PAEMINTQAS YAAKYKNITW DDDFEGTVSD LPKAWELEHD
HV190SV      EADLPLTDYL WLRGQSPFPA AGELLNLTSE WGILFRHVTF TDDGDLNPEH LPAAHEMADT
SsRV1        EASLPITDYL WVRGQSPFPA PGEFLNLTGT LGLLARHLTM DDDGIPTEEH LPTGREFRDT
                 *::.** * ****.: * ..*::*  .   .   :::*   **   . .. :* . *: .

Ce-dsRNA1    TVTIEVGRPL SISIGKSNCP DSKARRARTR ATIELGAASR RARAFGSAAV AEMPTLSTAP
CmRV         TMWRVTVPTA FSVTGPSNYL DREARRARSR AAISLAQAAL RARAYGEANS PVIDVSNVPP
HV190SV      TVTMTVGRPI GIAPGRYNAG DNQARRARTR ASVELSAASR RARVFGRPDV GEMPTLTSAP
SsRV1        AVTVVAGRPV GLPNGAHNAY DSQARRARTR ATRELAASAA RARLYGRATV AEMPILTSAP
             ::   .  .      *  *   * :*****:* *: .*. ::  *** :* .     :   . .*

Ce-dsRNA1    RALTFAPSRL MVKDT-GGGA GSSRHGADSG GSGADDAARK ADGVPVRATA HNQPVRFPTL
CmRV         TWDDERAPVV VFSDEIHTSD PGTAQGR--G IGNPPDVTAP VTGAPLVPIP HHQPLRGPPF
HV190SV      APIPASPAYD GNRGGEAGGV TGRGNNRSAA PGHASWSERQ ADGVPVNVTP HHNALRAPPF
SsRV1        VLRAAPPRPE AVNPMEGGGP GHDLARHSGM GRHTYPPDRE QRGDPAVPVP QHQALRAPQM
                   .           .                .         * *    . :::.:* * :

Ce-dsRNA1    ANPTGPTRSS AAPAAAARDQ SAGGPSVDIV RSGGDAASEV GSDVAPAPAP GVESGVVSSS
CmRV         PRGAGVMGGG VAPPPPAGPG GPPAGPGPNP PPPPPPGGDG GDDAAAAAAV AGGDVHGAAG
HV190SV      PRQQGAL-GG GGNVPLPPAP GAAPPPPPGP PNGPPAGPPP SDDGSSNPAA PVPTAIHAPP
SsRV1        PRPG------ ----PNPQGG GVIPPPPPLP PSGGGDGSGP SSGGSCAQED TNPAPPVAP-
             ..             . .    .    .          .    ... :            :.

Ce-dsRNA1    VAAPTNEGRN DV
CmRV         ALPAPQV--- --
HV190SV      AAAQADRAEG Q-
SsRV1        GPAQPDGPAN E-
               . .:
```

codon at nucleotide position 4,067. No overlapping nucleotides were observed between ORF 2 and the ORF 3. Only three nucleotides after the termination of ORF2, ORF 3 was initiated at an AUG codon at nucleotide position 4,071 and was terminated at the UAG codon at nucleotide position 5,246. ORF2 and ORF 3 encoded two putative proteins, one of which was 482 amino acids in length with a molecular weight of 53.66 kDa, and the other was 391 amino acids in length with a molecular weight of 42.7 kDa, respectively. Homology search of these two proteins revealed that both ORF2 and ORF3 had significant homology to the RdRp of other viruses belonging to the family *Totiviridae*. The highest homology of both ORF2 and ORF3 was to the RdRp of *SsRV1* with 41% amino acid identity. Amino acid sequence analysis of both ORF2 and ORF3 revealed conserved motifs, which are found in RdRps in other dsRNA viruses of simple eukaryotes (Fig. 23). Both nucleotide and amino acid identities of ORF2 and ORF 3 of Ce-dsRNA1 were compared to RdRps of selected totiviruses and are shown in Table 9.

The 5' UTR and 3' UTR regions were 329 bp and 66 bp long, respectively (Fig. 20, 21). One short ORF was found upstream of ORF1 and potentially encoded for a polypeptide with 47 amino acid residues. However, analysis of secondary structure for this region revealed that it was highly structured (data not shown). By comparison of the optimal consensus sequence found in eukaryotic start codons of $GCC^{A}/_{G}CCAUGG$, the context near AUG codon in ORF 1 (UCCAUGG) was slightly better than that of upstream ORF in 5'-UTR (GUUAUGC) (Fig. 21) (Kozak, 1991). Therefore, the small ORF in 5'-UTR of Ce-dsRNA1 may not be translated. For both 5'- and 3'-UTR regions, secondary structure analysis predicted the presence of a stem-loop structure, which has been observed in several other Totiviruses (Fig. 24).

```
                I          II            III           IV
Ce-dsRNA1       LQGRG      WCVNGSQNR     EKLECGKNRAIF  DYDDFNSHHS
Ce-dsRNA2       LQGRA      WCVNGSHTP     LKLEHGKSRAIF  DYDDFNSHHS
CmRV            LLGRD      WAVNGAHSG     PKLEHGKTRAIF  DYDDFNSHHT
EbRV1           LQGRG      WCVGGAHNL     EKVEQGKGRAIY  DYSDFNSQHS
GaRVL1          LLGRA      WAVNGAHSG     PKLEAGKTRAIF  DYDDFNSHHS
GLV             LLGKV      WGTTGSGYI     EKPELTKVRAVI  DQSNFDRQPD
Hv190SV         LQGRY      WCVNGSQNA     VKLENGKDRAIF  DYDNFNSQHS
LRV1-1          LLGRG      WAANGSHSR     AKLEHGKTRLLL  DYDDFNSQHT
LRV2-1          LRGRG      WAANGGHSR     QKLEHGKSRLLL  DFEDFNSQHS
ScV-L1          LMNRG      WVPGGSVHS     TKYEWGKQRAIY  DYDDFNSQHS
ScV-La          LENGV      IMPGGSVHS     TKYEWGKVRALY  DFDDFNSQHS
SsRV1           LLGRA      WCVNGSQND     PKLEHGKTRAIF  DFDDFNSHHS
SsRV2           LQGRA      WAVNGSQSG     PKLEHGKTRAIF  DYDDFNSHHS
TVV             LLGRG      WSRSGSHYY     RKLEHGKERFIY  DYTDFNSQHT
TVVII           LQGRG      WVKKGAHHH     PKLEHGKTRFIY  DYTDFNSQHS
                * .          *.          * *  * * :    *  :*: :

                          V                    VI        VII   VIII
Ce-dsRNA1       TLMSGHRGTTHINSVLNAAYI     HTGDDVYM     EFLRM  YLCRA
Ce-dsRNA2       TLMSGHRGITFVNSVLNAAYL     HTGDDVYI     EFLRM  YLPRA
CmRV            TLMSGHRLTTYINSVCNEAYL     HVGDDVYL     EFLRV  YLARA
EbRV1           SLMSGHRATTYWNSVLNAAYV     HVGDDVLI     EFLRV  YVARS
GaRVL1          TLMSGHRGTTFINSVLNKAYL     HVGDDVYF     EFLRN  YFARA
GLV             GLPSGWKWTALLGALINVTQL     VQGDDIAL     EFLRR  YPARM
Hv190SV         TLMSGHRATTFTNSVLNAAYI     HAGDDVYL     EFLRL  YLCRA
LRV1-1          TLMSGHRATSFINSVLNRAYI     HVGDDILM     EFLRV  YLARV
LRV2-1          TLMSGHRATTFINTILNTAYL     HVGDDVIF     EFLRV  YVARA
ScV-L1          TLLSGWRLTTFMNTVLNWAYM     HNGDDVMI     EFLRV  YLSRS
ScV-La          TLFSGWRLTTFFNTALNYCYL     HNGDDVFA     EFLRV  YLTRG
SsRV1           TLPSGHRGTTIVNSVLNAAYI     HTGDDVYI     EFLRM  YLARS
SsRV2           TLMSGRRGTTYISSVLNEVYL     HVGDDVYL     EFLRL  YLARA
TVV             TLPSGHRATTFINTVLNWCYT     CAGDDVIL     EFLRK  YPCRA
TVVII           TLPSGHRATTFINSVLNRAYL     HTGDDVLL     EFLRL  YPARA
                 * ** :  :       *          ***:      ** *   *  *
```

Fig. 23. Alignment of the deduced amino acids of the eight conserved motifs of RdRp of both Ce-dsRNA1 and -dsRNA2 to known totiviruses. CmRV=*Coniothyrium minitans RNA virus*, EbRV1=*Eimeria brunetti RNA virus 1*, GaRVL1= *Gremmeniella abietina RNA virus L1*, GLV=*Giardia lamblia virus*, Hv190SV=*Helminthosporium victoriae 190S virus*, LRV1-1=*Leishmania RNA virus 1-1*, LRV2-1=*Leishmania RNA virus 2-1*, ScV-L1=*Saccharomyces cerevisiae virus L1*, ScV-La=*Saccharomyces cerevisiae virus La*, SsRV1=*Sphaeropsis sapinea RNA virus 1*, SsRV2= *Sphaeropsis sapinea RNA virus 2*, TVV=*Trichomonas vaginalis virus*, TVVII= *Trichomonas vaginalis virus II.*

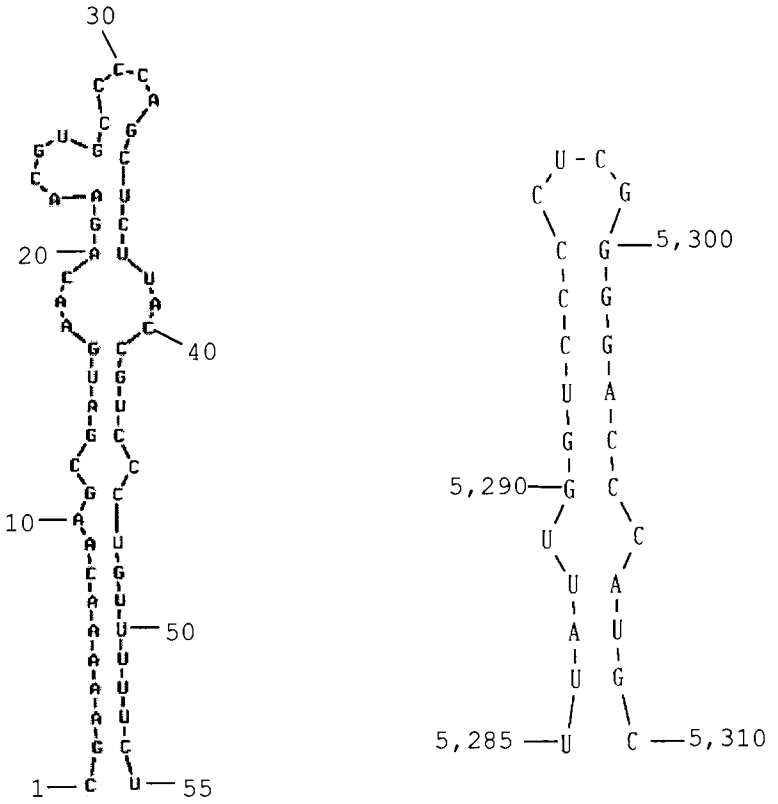

Fig. 24. Potential secondary structures of both 5'- and 3'- ends of (+) strand of the Ce-dsRNA1 in strain CKP of *C. elegans*. The putative folded structures of the 5'- and 3'-ends were obtained from the MFOLD program (Zuker et al., 1999).

4.3.2. Partial cDNA clones of an additional dsRNA fragment in strain CKP

During the initial RT-PCR with random primers, one additional cDNA clone (C4B), which was 500 bp in size, was coincidentally obtained during cDNA development from the dsRNA in strain CKP. Sequence analysis of the C4B clone revealed that it could not align with any part of Ce-dsRNA1, suggesting that this clone was not derived from Ce-dsRNA1. Northern blot hybridization analysis showed that the C4B clone could hybridize to the 5.3 kb dsRNA fragment, but not to any other dsRNA fragments present in strain CKP (Fig. 19). This suggested that this partial cDNA clone could have been derived from an additional dsRNA fragment which was very close in size to Ce-dsRNA1 (Fig. 19). Based on the sequence information of the C4B clone, several dsRNA-specific primers were designed and used to extend this partial cDNA in both directions (Table 8). Four more cDNA clones (C1F, GAP1, GAP2 and 1.5F) were obtained, aligned with each other according to their sequences, and this clone was designated as *Chalara elegans dsRNA 2* (Ce-dsRNA2) (Fig. 20).

The partial nucleotide sequence of Ce-dsRNA2 was 2,336 bp long and is deposited in GenBank under accession number AY556461. Homology search with the deduced amino acid sequence showed a significant homology to the RdRp regions of Totiviruses. The highest sequence homology was to the RdRp region of SsRV1, with 45% amino acid identity. All conserved motifs which are present in the RdRp regions of other RNA viruses were also found in the deduced amino acid sequence of Ce-dsRNA2 (Fig. 23). Both nucleotide and amino acid sequences of conserved motifs in the RdRp regions between Ce-dsRNA1 and Ce-dsRNA2 revealed an identity of 56% and 50%, respectively (Fig. 25).

Fig. 25. Comparison of the nucleotide (**A**) and deduced amino acid sequences (**B**) of conserved motifs of RdRp regions of Ce-dsRNA1 and Ce-dsRNA2.

A.

```
Ce-dsRNA1       -TACAAGGACGTGGCTGTAAACCAGTTGACCTGGCAGATGAGCTATACAAGCGGACTGAT
Ce-dsRNA2       TTACAAGGCCGCGCTACTGCATCTGTCGATCTCAAAGAGGAGATTCGATATCGTTGTGAC
                 ******* ** *    *  * * ** ** **   *** *** *     * **   ***

Ce-dsRNA1       CCAGTGGGTGTTCAGGAGCAAGTGCT-CCCGTTGA--GTGATGATTTGCGGCGAGCGATT
Ce-dsRNA2       GCTGAGTCCGTCTCTAAACAGGTTCTGAATGTTGATCCTGAATCATTGAGACCTCATGTT
                 * * *   **     * ** ** **    *****   ***    *** * *      **

Ce-dsRNA1       GATGCGACGATAGAGCATGAACTGCCTGTGGGCGAGTTGCCTGATATGGAGGAGTGGTGG
Ce-dsRNA2       CGCGCGATTATTGAGTCTGAGGTCCAGTCTTGCAGCCTACCCCCTCTGGATAGTTTCTGG
                   ****  ** ***  ***  * *      **    * **   * ****    *  ***

Ce-dsRNA1       TCATCGAGGTGGTTGTGGTGTGTTAATGGTAGCCAGAACAGGTCCAGTGATGCTGCATTA
Ce-dsRNA2       TCTTCTCGCTGGTTATGGTGTGTTAACGGTTCGCATACCCCCGCTGCTTCGCAAGGTCTA
                ** **  * ***** ********** ***   ** * *    *   *      *   **

Ce-dsRNA1       GGACTTGATCACGTCCCCAGTGATTTGCTTGGTTCTCAGAGATACCGGCGAATGGCTGCT
Ce-dsRNA2       GGCATTGATCACGATGTGTTTTCTTCAACACACACTCGTGTGTATCGCCGCATGGCTGCA
                **  *********       *  **         ***     ** ** ** ********

Ce-dsRNA1       GAAG-AGGTCAGTCTTAATCCTATTTACGCCTGGGA-TGG-ATACACCGAGGTTTCGTTC
Ce-dsRNA2       GAAGTAAGGGAGTCAGAA-CCATTGTCTGATTGGAATTGTCGTGTCTCTGTGTCTCCTTC
                **** * *  **** ** **  * *  *  *** * **   *    *   ** ** ***

Ce-dsRNA1       AGTGAGAAACTCGAGTGTGGGAAGAATCGTGCTATTTTTGCTTGTGATACGCGTTCGTAT
Ce-dsRNA2       -GCT-CAAGTTGGAGCATGGTAAGTCTCGCGCCATTTTCTCTTGTGATACGCGATCTTAT
                 *    **  * ***  *** ***  *** ** *****  ************ ** ***

Ce-dsRNA1       TTTGGTTTTTCGTATTTGCTAGGGGAGGTACAGAAGAGATGGAGAAACTCTCGAGTTTTG
Ce-dsRNA2       TTTGCCTTTGAATGGTTACTGGGTACTCTCCAGAAAAATTGGGCCAATCGCCGCGTGCTT
                ****  ***   *  ** ** **     * ***** *  ***   **    ** **  *

Ce-dsRNA1       TTGGATCCAGGGAAAAGTGGTTACCTCGGTTTGGCAAGGCGTCTGTTGCGCGGTTCGGTA
Ce-dsRNA2       TTGGATCCAGGTTTTGGTGGACACACAGGGATCATAGGTAGGAT-TAGGTCGTTCTCTAA
                **********     ****  **   **  *   * *  *  * * *  ** *     *

Ce-dsRNA1       -CGTGGGGGTGTTAACTTAATGTTGGACTATGATGATTTCAACTCTCATCACTCTATAGA
Ce-dsRNA2       GAGAGGTGGCGTCAATTTGATGCTAGACTATGACGATTTTAATTCTCACCATTCGCTCCC
                  * ** ** ** ** ** *** * ******** ***** ** ***** ** **  *

Ce-dsRNA1       GACGATGAAGTATGTTTTTCTGAAG-A-CGGCCTAACGTATGAATGCCCCTTCCTGGTAT
Ce-dsRNA2       GGTTATG-GCTATG-GTTTATGAAGAAGCTGCAAAGGGCACCGACGCTCCAAAGTGGTAC
                *   ***   ****  *** ***** * * **  *  * *   * ** **    *****

Ce-dsRNA1       ACGGAGAAAATTGTTAGTTCTTTTGACAAGATGTGGATCGTCAGAGGTAGTGAAAGGTTA
Ce-dsRNA2       GTTGATAAACTCGTTGCTTCGGTTTATTCTTCTTTTATTAAAGATGGTAATAGACAACGA
                   ** *** * ***  ***  ** *       *  **       **** *  *     *

Ce-dsRNA1       CACGTCCTCGGCACTTTGATGAGTGGGCATCGCGGTACTACACACATTAATTCAGTACTG
Ce-dsRNA2       CGCGTATTAGGTACTCTAATGAGCGGTCACCGCGGTATAACCTTTGTAAACTCCGTTCTC
                * ***  * ** *** * ***** ** ** *******  **     * ** ** ** **
```

```
Ce-dsRNA1       AATGCTGCTTACATAAGGATGGCCTTGGGTAACGCGTATTACGACAAAATACTATCACTC
Ce-dsRNA2       AATGCGGCTTATCTCCGGCATTCCCTCGGTCCCTCGTTATTCGACTCGCTTTCGTCACTT
                ***** *****  *  **    ** * ***  * ***  * ****    *    *****

Ce-dsRNA1       CACACAGGTGATGACGTTTACATGCGCCTTGACACTTTAGGTGACTG-TGTCCGTGTGTT
Ce-dsRNA2       CATACAGGTGATGATGTCTATATTAACGCTGGTACACTTGATGAGTGCGGTTCGATTCTT
                ** ********** ** ** **   *  **  **  * * *** **  ** **  * **

Ce-dsRNA1       GCGTGCTTGTCATGGCGCTGGCTGCAGGATGAATCCATCTAAGCAATCAATCGGTTATCA
Ce-dsRNA2       G-ACGCTGCTCGTTCTTACGGGTGTCGGATGAATCCTACCAAACAATCGATCGGTTATGT
                *   ***  ** *      ** **  **********  * ** ***** *********

Ce-dsRNA1       CCGGGCTGAGTTTTTAAGAATGGGTGTCAACTCTCAGTATGCTGTGGGATACTTGTGCCG
Ce-dsRNA2       CGGTGCTGAGTTTTTGAGGATGGGTGTCTCTACAAAGTGTGCCTACGGATATTTACCTCG
                * * ********** ** *********    *  *** ***    ***** **    **

Ce-dsRNA1       TGCG
Ce-dsRNA2       AGCT
                 **
```

B.

```
Ce-dsRNA1       LQGRGCKPVDLADELYKRTDPVGVQEQVLPLS-DDLRRAIDATIEHELPVGELPDMEEWW
Ce-dsRNA2       LQGRATASVDLKEEIRYRCDAESVSKQVLNVDPESLRPHVRAIIESEVQSCSLPPLDSFW
                ****.  .*** :*:  * *. .*.:*** :. :.**  : * ** *:   .** ::.:*

Ce-dsRNA1       SSRWLWCVNGSQNRSSDAALGLDHVPSDLLGSQRYRRMAAEEVSLNPIYAWDGYTEVSFS
Ce-dsRNA2       SSRWLWCVNGSHTPAASQGLGIDHDVFSSTHTRVYRRMAAEVRESEPLSDWNCRVSVSPS
                ***********:. ::. .**:**   .   :: *******  . :*:  *:  ..** *

Ce-dsRNA1       EKLECGKNRAIFACDTRSYFGFSYLLGEVQKRWRNSRVLLDPGKSGYLGLARRLLRGSVR
Ce-dsRNA2       LKLEHGKSRAIFSCDTRSYFAFEWLLGTLQKNWANRRVLLDPGFGGHTGIIGRIRSFSKR
                 *** **.****:*******.*.:*** :**.* * ******* .*: *:  *:   * *

Ce-dsRNA1       GGVNLMLDYDDFNSHHSIETMKYVFLKTAMN--APSWYTEKIVSSFDKMWIVRGSERLHV
Ce-dsRNA2       GGVNLMLDYDDFNSHHSLPVMAMVYEEAAKGTDAPKWYVDKLVASVYSSFIKDGNRQRRV
                *****************: .*  *: ::* .  **.**.:*:*:*. . :*  *..: :*

Ce-dsRNA1       LGTLMSGHRGTTHINSVLNAAYIRMALGNAYYDKILSLHTGDDVYMRLDTLGDCVRVLRA
Ce-dsRNA2       LGTLMSGHRGITFVNSVLNAAYLRHSLGPSLFDSLSSLHTGDDVYINAGTLDECGSILDA
                ********** *.:********:* :** : :*.: *********:. .**.:*  :* *

Ce-dsRNA1       CHGAGCRMNPSKQSIGYHRAEFLRMGVNSQYAVGYLCRALPSLVCGNWVGAGSDD
Ce-dsRNA2       ARSYGCRMNPTKQSIGYVGAEFLRMGVSTKCAYGYLPRA----------------
                .:. ******:******  ********.:: * *** **
```

4.3.3. TEM analysis

Putative virus particles were observed in strain CKP of *C. elegans*, which were not seen in mycelial tissue of another strain BK18, which does not contain Ce-dsRNA1 or Ce-dsRNA2 (Fig. 26). Clusters of isometric particles were observed in the cytoplasm of actively growing hyphae of strain CKP (Fig. 26). The approximate size range of each particle was 35-45 nm in diameter, which fits into the size range of known Totivirus particles (Caston et al., 1997; Ghabrial, 1994).

4.3.4. Phylogenetic relationships of Ce-dsRNA1 and dsRNA2 using the conserved regions of RdRp

Genetic relatedness was analyzed by comparison of amino acid sequences of the conserved motifs in the RdRp regions of both dsRNA fragments in *C. elegans* (Ce-dsRNA1 and Ce-dsRNA2) with other Totiviruses (Fig. 23, 27). High amino acid sequence similarities among these viruses, including the two dsRNA fragments in *C. elegans*, were found in the conserved regions (Fig. 23). A phylogenetic tree was produced based on multiple alignments of conserved motifs of RdRps. This showed that both dsRNA fragments (Ce-dsRNA1 and Ce-dsRNA2) in *C. elegans* were more closely related to those Totiviruses which can infect filamentous fungi, such as SsRV1, SsRV2, and Hv190SV, than to other Totiviruses, such as ScV-L1, UmVH1 and GLV, which are present in yeast, the smut fungi and protozoa (Fig. 27).

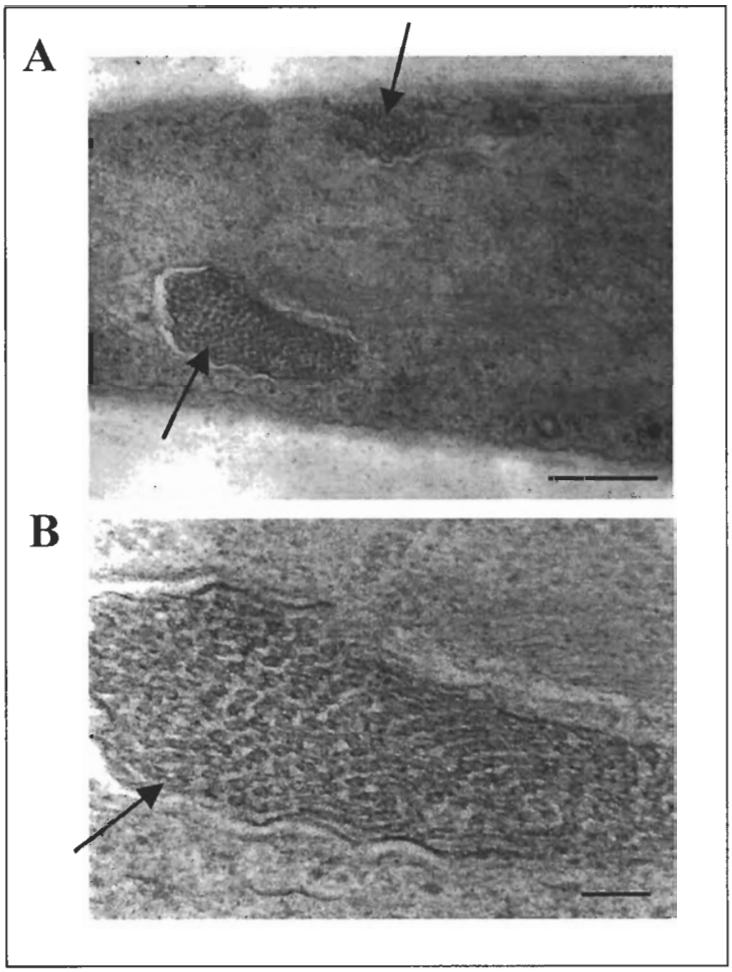

Fig. 26. The presence of virus-like particles in strain CKP using transmission electron microscope (TEM). **(A)** Location of virus-like particles (arrow) in the cytoplasm of mycelium of strain CKP. **(B)** An enlarged view of virus-like particles. Each scale bar represents 500 nm in **(A)** and 100 nm in **(B)**, respectively.

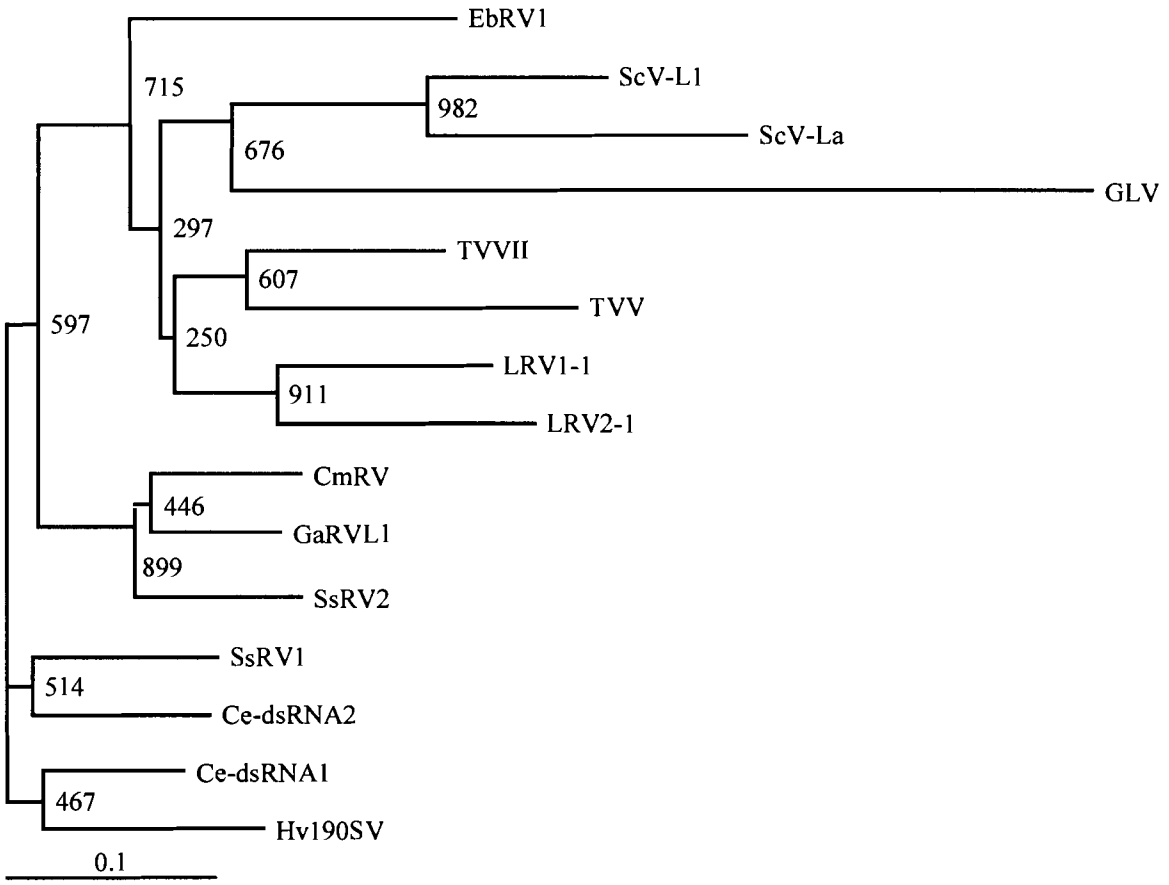

Fig. 27. Phylogenetic relationships among the conserved motifs of RdRps of both Ce-dsRNAs (dsRNA1 and dsRNA2) and other known totiviruses. Values for bootstrap replicates (out of 1000) are indicated. CmRV=*Coniothyrium minitans RNA virus*, EbRV1=*Eimeria brunetti RNA virus 1*, GaRVL1= *Gremmeniella abietina RNA virus L1*, GLV=*Giardia lamblia virus*, Hv190SV=*Helminthosporium victoriae 190S virus*, LRV1-1=*Leishmania RNA virus 1-1*, LRV2-1=*Leishmania RNA virus 2-1*, ScV-L1=*Saccharomyces cerevisiae virus L1*, ScV-La=*Saccharomyces cerevisiae virus La*, SsRV1=*Sphaeropsis sapinea RNA virus 1*, SsRV2= *Sphaeropsis sapinea RNA virus 2*, TVV=*Trichomonas vaginalis virus*, TVVII= *Trichomonas vaginalis virus II.*

4.4. Discussion

Isometric dsRNA mycoviruses (35-45 nm in size) are currently classified into two families, *Totiviridae* and *Partitiviridae* (Ghabrial et al., 1995). *Partitiviridae* viruses have two genome segments, ranging in size from 1.4 to 3.0 kb, and a single coat protein subunit, ranging from 42 to 73 kDa in size. *Totiviridae* viruses have a single genome segment ranging in size from 4.6 to 6.7 kb, and a single coat protein subunit ranging from 73 to 88 kDa in size. In this regard, both the genome fragment size (5.3 kb) and molecular weight of the coat protein (80.71 kDa) of Ce-dsRNA1 in *C. elegans* are similar to those of *Totiviridae*, indicating that Ce-dsRNA1 may belong to this group.

The size of the overlapping region between ORF1 and ORF2 in Totivirus can be quite variable. Some Totiviruses, such as ScV-L-A (130 nt), LRV1-1 and LRV 1-4 (71 nt each) and GLV (122 nt) have relatively long overlapping regions; while other Totiviruses such as SsRV1, SsRV2 (2 nt), CmRV (4 nt) and Hv190SV (overlap directly), have short or direct overlapping regions, (Cheng et al., 2003; Huang and Ghabrial, 1996; Icho and Wickner, 1989; Preisig et al., 1998; Wang et al., 1993). Genome analysis of Ce-dsRNA1 revealed that the overlapping region was relatively short (22 nt), which is similar to that found in SsRV1 and Hv190SV, implying a close relationship to these viruses. Interestingly, at 40 codons upstream to the proposed start codon of ORF2 in Ce-dsRNA1, the presence of a stop codon was observed in the same reading frame, which is similar to that found in both SsRV1 (8 codons upstream) and SsRV2 (12 codons upstream) (Preisig et al., 1998). The genome organization is considered to be important for the translation mechanism because this stop codon may severely restrict the region for any frameshift event (Kozak, 1991).

Two different strategies for the expression of ORF2 have been suggested in *Totiviridae* (Huang and Ghabrial, 1996; Wang et al., 1993). One could be a fusion protein between the coat protein and the RdRp regions by ribosomal frameshifting, which has been suggested in ScV-L-A and GLV. It is known that -1 or +1 ribosomal frameshifting can successfully occur when both the slippery site and pseudoknot structure are found either within the overlapping region of ORF1 and ORF2 or near that region, especially for the pseudoknot structure (Icho and Wickner, 1989; Wang et al., 1993). Although a stem-loop structure within the overlapping region between ORF1 and ORF2 of Ce-dsRNA1 was predicted (data not shown), neither a pseudoknot nor a slippery site was observed, indicating that the expression strategy of ORF2 in Ce-dsRNA1 could be different. Another strategy which has been proposed to translate ORF2 in *Totiviridae* is through internal initiation (Huang and Ghabrial, 1996; Preisig et al., 1998). It has been suggested that Hv190SV can express the RdRp as a separate non-fused polypeptide via a coupled termination-reinitiation mechanism (Huang and Ghabrial, 1996). Recently, Soldevila and Ghabrial (2000) demonstrated that the RdRp in Hv190SV could be expressed independent of a coat protein as a non-fused polypeptide. A similar mechanism has been proposed for the Totiviruses, including SsRVs and CmRV, which are considered to be more closely related to Ce-dsRNA1 (Cheng et al., 2003; Preisig et al., 1998). Therefore, it is possible that the expression of ORF2 in Ce-dsRNA1 could employ the internal initiation strategy.

Sequence analysis of Ce-dsRNA1 in *C. elegans* revealed the presence of a third open reading frame (ORF3), which has not been observed previously in other Totiviruses. However, a homology search of both ORF2 and ORF3 revealed a significant homology

to known RdRps of Totiviruses. Also, the eight conserved motifs of RdRp domains were equally distributed in both ORFs (with 4 conserved motifs present in each ORF), indicating that these ORFs may produce a fusion protein. Many RNA viruses are considered to use the ribosomal readthrough strategy during translation to make a fusion protein (Herr et al., 2000). A readthrough of UAA at the 4,055 position of ORF2 could be supported by the fact that both ORF2 and ORF3 are in the same frame, which is essential for a readthrough translation. Also, the predicted molecular mass of this fusion protein was 96.36 kDa, which is similar to the size of other RdRps in Totiviruses. For example, the RdRps encoded by SsRV1 and SsRV2 were predicted to be 92.2 kDa and 90.8 kDa, respectively (Preisig et al., 1998). Therefore, it is possible that starting translation at AUG in 2,619 position (ORF2) may extend until the UAA at 5,244 position (the end of ORF3) to produce a fusion RdRp protein. However, this needs to be further clarified experimentally.

Transmission electron microscopy revealed the presence of virus-like particles (VLPs) in the cytoplasm of fungal mycelium of strain CKP of *C. elegans*. These VLPs were ~35-45 nm in size and isometric in shape, similar to VLPs found in the *Totiviridae* group, further supporting the close relationship of these two dsRNAs (Ce-dsRNA1 and -dsRNA2) to the totivirus group (Caston et al., 1997; Ghabrial, 1994). Viruses in the family *Totiviridae* are unique because the host ranges of these viruses are limited to fungal and protozoan cells (Ghabrial, 1998). Three genera, including *Totivirus*, *Giardiavirus* and *Leishmaniavirus* have been placed in the family *Totiviridae* (Ghabrial et al., 1995). The *Totivirus* genus is different from other genera because, to date, it has been found only in fungi, rather than in parasitic protozoa that are the host of viruses

belonging to the genera *Giardiavirus* and *Leishmaniavirus* (Ghabrial, 1998). Based on the information derived from additional virus genomes in the genus *Totivirus*, it has been suggested that a new genus is needed (Huang and Ghabrial, 1996; Ghabrial, 1998; Preisig et al., 1998; Cheng et al., 2003). Totiviruses such as SsRVs, Hv190SV and CmRV infect filamentous fungi, while other Totiviruses infect yeast and smut fungi. The viruses infecting filamentous fungi are more closely related in several ways, by having a similar genomic organization, internal initiation translation mode (rather than frameshift mechanism), a proline-rich region near the C-terminus of coat protein, and a relatively high level of amino acid identity (Cheng et al., 2003; Ghabrial, 1998). Similar features were found in Ce-dsRNA1, suggesting that this virus is more closely related to the viruses infecting filamentous fungi. Phylogenetic analysis also confirmed these close relationships. However, the taxonomic classification of Ce-dsRNA1 is unclear because of the difference in the number of putative ORFs in Ce-dsRNA1 (three instead of two).

Partial cDNA clones of an additional dsRNA fragment (Ce-dsRNA2) present in strain CKP of *C. elegans* were coincidentally obtained during the development of the full-length cDNA clones of Ce-dsRNA1. Although a full-length cDNA clone was not developed for this dsRNA fragment, Northern blot hybridization suggested that the size of the full-length of Ce-dsRNA2 may be similar to that of Ce-dsRNA1, since the dsRNA fragments, which could hybridize to cDNA probes derived from either Ce-dsRNA1 or Ce-dsRNA2 fragment, were indistinguishable. In addition to the size similarity, the amino acid sequence homology to known virus groups using the partial sequence of Ce-dsRNA2 revealed a close relationship to the totivirus group, which is the same group to which Ce-dsRNA1 was assigned, suggesting that these two dsRNAs in strain CKP of *C.*

elegans are genetically related to one other. However, attempts to align the partial cDNA clones derived from Ce-dsRNA2 with Ce-dsRNA1 failed, suggesting the nucleotide sequences of these two dsRNA fragments are indeed distinct.

Coinfection by two distinct Totiviruses has been observed in one filamentous fungus, *S. sapinea* (Preisig et al., 1998). Sequence and genomic organization analysis showed that these two viruses were similar in size and both were classified as Totiviruses. In addition to the report of coinfection by two viruses in both *C. elegans* and *S. sapinea*, it was interesting that the closest phylogenetic relationship of both *C. elegans* dsRNAs (Ce-dsRNA1 and -dsRNA2) was to SsRV1, which is one of the dsRNA fragments present in *S. sapinea*. It has been proposed that dsRNA viruses in the family *Totiviridae* may have been present in a single ancient cell before divergence between fungi and protozoa (Bruenn, 1993; Ghabrial, 1998). Therefore, it is possible that certain dsRNA fragments could have infected one fungal cell before divergence of *C. elegans* and *S. sapinea*, and then coevolved with its respective host, resulting in two distinct viruses with some degree of homology, such as that observed between Ce-dsRNA1 and SsRV1. Another possible explanation for the origin of these viruses could be the transmission of dsRNA between those two fungal species. The latter is unlikely since the most prevalent dsRNA fragment in *C. elegans* is the 5-6 kb dsRNA fragment, which has some homology to *Totiviridae* with various ranges (23%-44%) (Park et al., 2003; Park et al., 2004). Northern blot analysis indicated that genetic diversity between these fragments in *C. elegans* was high (Park et al., 2004), implying that this 5.3 kb dsRNA fragment must have been present in *C. elegans* for a long time. Therefore, if dsRNA transmission

occurred from other fungi, that event must have occurred even earlier to generate the genetic diversity seen among the 5.3 kb dsRNA fragments in *C. elegans*.

The transmission of dsRNAs through conidia in strain CKP is close to 100% (data not shown) and most wild-type isolates of *C. elegans* contain dsRNA elements (Bottacin et al., 1994). Therefore, these dsRNAs have been well maintained in the fungal populations. Most dsRNAs in the family *Totiviridae* do not seem to have any obvious phenotypic effects on their host (Ghabrial, 1998). However, multiple dsRNA fragments can be found in any given strain of a fungal species and complex interactions between these fragments have been reported. Two different virus groups, namely the totivirus Hv190SV and chrysovirus-like virus Hv145SV, were present in *H. victoriae* (Ghabrial, 1998). It has been reported that the lytic/diseased phenotype observed in virus-infected *H. victoriae* isolates was due to the over expression of Hv-p68 which is associated with chrysovirus-like virus Hv145SV, rather than totivirus Hv190SV (Soldevila et al., 2000). Similarly, three additional dsRNA fragments about 3-4 kb in size are also present in strain CKP containing both Ce-dsRNA1 and Ce-dsRNA2 (Park et al., 2003; Park et al., 2004). Additional studies are required to elucidate the potential interactions among these dsRNA fragments and their physiological effects on *C. elegans*.

CHAPTER 5

GENERAL DISCUSSION

5.1. General discussion

It has been previously demonstrated that a large number of field isolates of *C. elegans* contain a range of dsRNA elements, which were different in size and intensity on 1% agarose gels (Bottacin et al., 1994). Because of the widespread occurrence of dsRNAs *C. elegans*, it has been suggested that these dsRNAs could have some effects on the host fungus; however, sequence information to elucidate relationships among these dsRNAs *C. elegans* and to viruses in other fungi were lacking.

In this study, attempts to develop cDNA clones from the dsRNA elements in *C. elegans* were successful. Although cDNA clones for dsRNAs that were faint in intensity on agarose gel were not obtained, cloning of intensely staining dsRNA fragments was successful, suggesting this procedure could be used to generate more cDNA clones from other dsRNA fragments in the future. Several partial cDNA clones were derived from three different-sized dsRNA elements (2.8 kb, 5.3 kb and 12 kb) in six different *C. elegans* strains (AK89-2, BK18, CKP, OR1, NC1527 and WASH). These strains were selected because they originated from diverse geographic regions and showed a range of dsRNA banding patterns, which represented the major dsRNA banding patterns found previously in *C. elegans* strains (Bottacin et al., 1994).

It is common that multiple bands of dsRNA in fungi are evident when viewed on agarose gels (Ghabrial et al., 1998; Nuss and Koltin, 1990). The genetic relationships between dsRNA fragments in one isolate are variable, depending on the isolates and host fungi (Bharathan and Tavantzis, 1991; Enebak et al., 1994a). In *C. parasitica*, four different-sized dsRNA fragments were observed in one isolate (CHV3-GH2) and cross-hybridization occurred only between dsRNA1 and dsRNA2, but not with other dsRNA fragments (Hillman et al., 2000; Yuan and Hillman, 2001). Similar results were observed in other fungi, such as *R. solani* (Bharathan and Tavantzis, 1990; Bharathan and Tavantzis, 1991). In this regard, our data are different from that found in other fungi, since none of the cDNA clones cross-hybridized to other dsRNA fragments present in the same strain of *C. elegans*, suggesting that genetically distinct dsRNA fragments could be present in one strain. However, it could be an indication of the detection limit of Northern blot analysis using partial cDNA clones. Further work is needed to understand the genetic relationships between dsRNA fragments within a strain of *C. elegans*.

Mixed infections, with more than one dsRNA virus group, are common in fungi (Ghabrial, 1998). The presence of three dsRNA virus groups, such as *Hypoviridae*, *Narnaviridae* and *Partitiviridae*, has been observed in *C. parastica* (Hillman et al., 2003; Nuss and Koltin, 1990; Polashock and Hillman, 1994). Based on Northern blot and sequence analysis of cDNA clones, we revealed that at least three dsRNA groups, including *Narnaviridae*, *Totiviridae*, and *Potyviridae*, could be present in *C. elegans*. It was not surprising to find the presence of totivirus-like dsRNAs in *C. elegans* because *Totiviridae* is the one of the major virus families infecting fungi (Ghabrial, 1998). The presence of dsRNA belonging to *Narnaviridae* in *C. elegans* was not expected because of

its rare presence in fungi. Also, it would be interesting to demonstrate if the12 kb dsRNA fragment in *C. elegans* is genetically related to the best known *C. parasitica* hypovirus, which shared some sequence homology with viruses belong to *Potyviridae.* Sequence analysis using the information derived from partial cDNA clones of the 12 kb dsRNA fragment in strain NC1527 showed a relatively low amino acids sequence identity to *Potyviridae*.

The genetic relatedness study using Northern blot and sequence analysis showed how many different dsRNA groups are present in *C. elegans*, and also indicated how these dsRNA elements could be transferred among *C. elegans* populations. Mycoviruses lack on extracellular route of infection and can only be transferred by using horizontal transmission through hyphal anastomosis between fungal strains, or vertical transmission via asexual or sexual spores (Ghabrial, 1998; Nuss and Koltin, 1990). All progenies derived from single conidia of strains BK18 and CKP contained the parental dsRNAs, indicating that vertical dsRNA transmission via conidia is highly efficient in *C. elegans*. However, an attempt to demonstrate efficiency horizontal transmission of dsRNAs using RAPD markers was unsuccessful. No dsRNA-containing progenies, which showed the RAPD patterns of dsRNA-free parental strain, were obtained, indicating the possible presence of blockage mechanisms for horizontal dsRNA transmission among *C. elegans* populations. However, it has also been suggested that horizontal dsRNA transfer should occur between *C. elegans* strains, since similar dsRNA banding patterns were observed in strains derived from close geographic regions (Bottacin et al., 1994). In this regard, Northern blot and sequence analysis using cDNA clones revealed that similar-sized dsRNA elements could be genetically related, supporting horizontal dsRNA transmission

among *C. elegans* strains or spread of specific strains over a specific geographic region. The role of vegetative compatibility groups, which are considered to be the one of the important factors to limit horizontal dsRNA transmission in fungi, needs to be studied in *C. elegans*.

The development of full-length cDNA clones of both the 2.8 kb and 5.3 kb dsRNA fragments in *C. elegans* suggests that if a transformation protocol is established, the development of genetically engineered *C. elegans* strains using various genetic sources derived from dsRNA fragments could lead to interesting studies to understand the interaction between dsRNA and host fungi. It has been suggested that dsRNA infection could alter expression of many fungal genes (Dawe and Nuss, 2000; Liu et al., 2003a). In *C. parasitica*, cDNA differential display experiments revealed a surprisingly large number of differences in PCR amplicons between dsRNA-free and dsRNA-containing strains (Chen et al., 1996). Transformation studies with specific fungal genes such as CPG-1 confirmed that virus infection could perturb CPG-1 signaling pathways in *C. parasitica* (Zhang et al., 1998; Gao and Nuss, 1996). Fungal genetic studies using transformation techniques and cDNA differential display experiments could give interesting information related to the interactions between the dsRNA and the biology of *C. elegans*.

Various attempts have been tried to eliminate the dsRNA in *C. elegans*, however, most trials failed, suggesting that the presence of dsRNA is quite stable in the fungal hosts. Only two strains (BK18 and CKP), which were incubated at high temperature (35°C) for more than 2 months, revealed the change of dsRNA banding patterns on agarose gel analysis. However, RT-PCR with the 2.8 kb dsRNA specific primer sets in

BK18 successfully amplified the partial cDNA indicating the high incubation temperature could lead to the repression of dsRNA quantity, rather than complete elimination. The change of 2.8 kb dsRNA quantity in BK18 strain appeared to have no detectable effects on *C. elegans* suggesting that BK18 strain could be latently infected with 2.8 kb dsRNA. Latent infection of viruses is widespread phenomenon in fungi (Buck 1986, Ghabrial 1980). For the plant pathologist view, the latent infection could lead to little interest because these infections have no overt effects on host fungi. However, this latency has been considered to be one of the important strategies for virus spread among host population. It has been suggested that selection pressure against to latent viruses could be relatively lower compared to those virulent viruses. In addition to the importance for understanding the virus transmission among host fungal populations, more precise studies related to latent infections are required because there are some possibilities that the latent infections could convert to overt infections, which can develop various effects on host fungi. The isolates of *S. homoeocarpa* latently infected by OnuMV3a displayed conversion to the hypovirulent phenotype after lower temperature incubation (Deng 2003). The lower temperature incubation treatment has been attempted to lead the conversion to hypovirulent isolate of *C. elegans*, however, no conversion was observed (data not shown). It could be a interesting study to find out 1) if the latent infections could convert to overt infections and 2) what kinds of environmental factors could cause the conversion to overt infection of dsRNA in *C. elegans*.

Finally, many studies related to dsRNA fragments in plant pathogenic fungi have been focused on the development of biological control agents, because dsRNAs could cause the hypovirulence phenotype in host fungi (Anagnostakis, 1980). In this study,

attempts to elucidate the relationship between the specific dsRNA fragments and pathogenicity of *C. elegans* were unsuccessful. However, the loss of a small dsRNA fragment in strain CKP of *C. elegans* showed some changes in culture morphology, growth rate, and virulence on carrots roots suggesting that dsRNA fragments could affect the host fungus *C. elegans*. It would be interesting to study how different relationships, such as hypoviruence, hypervirulence or no overt effects, are present between specific dsRNA fragments and *C. elegans*, and what the major factors are to determine these various interactions and how they could be used to control black root rot diseases caused by *C. elegans*.

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