Molecular and Biological Characterization of a Mitovirus in *Chalara elegans* (*Thielaviopsis basicola*)

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ABSTRACT

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A 2.8-kb double-stranded RNA (dsRNA) element in strain BK18 of *Chalara elegans* originally isolated from cotton soil in California was characterized by obtaining a full-length cDNA sequence (2,896 nucleotides in length) from a series of overlapping clones. Sequence analysis revealed the presence of one large open reading frame (ORF I) using the mitochondrial genetic code, with 20 to 34% amino acid identity to the ORF I of other previously reported fungal mitochondrial RNA viruses. The ORF I encoded a putative protein of 705 amino acids and contained the conserved motif characteristic of RNA-dependent RNA polymerases. Purification of mitochondria from strain BK18 confirmed the co-localization of this dsRNA, and northern blot hybridization with a strand-specific probe revealed the (+) single-stranded nature. This *Chalara elegans* mitovirus (CeMV) is designated as a new member of the genus *Mitovirus* of the family *Narnaviridae*. Using dsRNA-specific primers, the ORF I region (positions 427 to 2544) was obtained from an additional 2.8-kb dsRNA element in strain HA2 originating from carrot roots in the Netherlands. Both ORFs had 98% homology at the nucleotide and amino acid levels. CeMV was also found to be present in five additional strains of *C. elegans* from different geographic locations worldwide, and a 97 to

100% nucleotide sequence identity was observed within a 300-bp region of ORF I in these strains. To determine the biological effects of CeMV on *C. elegans*, attempts to cure strain BK18 of the dsRNA were made. Sequential transfers of mycelium at 35 to 37°C yielded a colony which lacked the 2.8-kb dsRNA when visualized on agarose gels and also in northern blot hybridization analysis. However, reverse transcription-polymerase chain reaction with specific primer sets revealed a band, indicating that dsRNA replication had been significantly repressed (latent). The wild type and latently infected strains were compared for colony morphology, growth rate, melanin production, various enzymatic assays (polyphenoloxidase, laccase, tyrosinase, and esterase), and virulence on carrot roots. Colony morphology on V8 agar was comparable between the two strains, while growth rate, melanin production, and virulence were enhanced in the latently infected strain. There were no detectable differences in enzymatic activity. Transmission electron microscopy of hyphae of the wild type and latently infected strains revealed differences in the number and size of the mitochondria, which were enhanced in the latently infected strain. Our results show that CeMV is a new member of the genus *Mitovirus* with some disruptive effects on its fungal host and is present in *C. elegans* strains from different locations worldwide.

Additional keywords: black root rot, fungal virus, mycovirus.

Double-stranded RNA (dsRNA) elements have been described in many filamentous fungi (19,36), and when associated with virus particles are termed mycoviruses (19). Since dsRNA elements do not have an extracellular phase during their life cycle, they are dependent on the fungal host for their replication. The elements can be transmitted between strains of the same fungal species through hyphal anastomosis (horizontal transmission) or via fungal propagules, e.g., conidia (vertical transmission) (12,33,36,40). Many of the dsRNA elements have been characterized at the molecular level and are classified into several virus families, including the *Hypoviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae* (14,20,22,58). These families are distinguished by differences in virion structure, genome organization, and expression strategy (19,21,22,58).

Previous studies have characterized the effects of some dsRNA elements on the fungal host phenotype (14,33). In some cases, dsRNA presence resulted in dramatic changes in culture morphology and physiology of the fungal host (3). For example, in the chestnut blight fungus, *Cryphonectria parasitica*, dsRNA presence reduced growth rate, sporulation, orange pigmentation, laccase and cutinase production, and virulence (induced hypovirulence) (2,14).

In other cases, such as *Nectria radicola*, the dsRNA element was associated with enhanced virulence of the host fungus (1). The relationship between the presence of dsRNAs and host fungi can be complex (1,10,42,48). Where multifragmented dsRNA populations occur, only certain dsRNA elements may induce symptoms in the fungal host (27,51). For example, in *Rhizoctonia solani*, a 6.4-kb dsRNA element was shown to enhance fungal virulence, while the presence of a 3.6-kb dsRNA element reduced the effects of the 6.4-kb element (27). By understanding the roles of dsRNAs in fungal hosts, their potential use as biological agents to control fungal pathogens through hypovirulence can be explored, and also the dsRNA–fungal host interactions can be better understood (14,33).

We are currently studying the extent of genetic diversity and molecular characteristics of dsRNA elements in *Chalara elegans* NagRaj and Kendrick (syn. *Thielaviopsis basicola* (Berk. & Br.) Ferr.). This filamentous fungus is a soilborne hyphomycete and a widespread facultative parasite on numerous plant species worldwide, causing the disease black root rot (43,59). Bozarth and Goenaga (6) first reported the presence of dsRNAs in *C. elegans* and Bottacin et al. (5) showed the diverse nature of dsRNA elements in this fungus. However, a molecular characterization of the dsRNA elements in *C. elegans* has not been achieved and their biological significance remains unknown.

The objectives of this study were to (i) obtain a cDNA sequence from a 2.8-kb dsRNA element in *C. elegans* and compare it to

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known virus groups; and (ii) attempt to cure wild-type strains of this dsRNA element and compare morphological characteristics, melanin production, virulence, and specific enzymatic activities (polyphenoloxidase, laccase, esterase, and tyrosinase) between wild-type and cured strains.

MATERIALS AND METHODS

Fungal growth and dsRNA extraction. The strains of *C. elegans* used in this study are listed in Table 1. All strains originated from diverse geographic regions and host plants and each contained a 2.8-kb dsRNA, except for strain AK208, which contained no detectable dsRNA element (5). Growth conditions and extraction of total nucleic acids were conducted as described by Park et al. (38).

cDNA development and sequence analysis. The development of cDNA and sequence analysis was performed as described by Park et al. (38). Briefly, the dsRNA was extracted through a CF11 column, as described by Morris and Dodds (35), and purified using low-melting agarose gel. Based on the sequence information from a partial cDNA clone (BK18C3), which was previously developed by using random hexanucleotide primers (38), several specific primers were designed and used in reverse transcription-polymerase chain reaction (RT-PCR) to develop a full-length cDNA as described by Park et al. (38). Amplified RT-PCR products were analyzed by running the samples in a 1% agarose gel, purified using Qiaquick gel extraction kit (Qiagen, GmbH, Germany), ligated into the TOPO cloning vector, and transformed into *Escherichia coli* strain TOP10 provided by the TOPO one-shot kit (Invitrogen, Carlsbad, CA). Several recombinants having an insert of the expected size were selected and sent to the sequencing lab 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 at both ends of the 2.8-kb dsRNA element, the rapid amplification of cDNA end (RACE) kit (Roche, Indianapolis, IN) was used according to the manufacturer's protocol. Combinations of oligo(dT) primers and dsRNA-specific terminal primers were used to generate RT-PCR products. On each end, three specific primers were used for strain BK18. The forward and reverse primers were BK18 3'-3 (5'-TCCAACAA-TTACTCTACCTTCAGC-3'), BK18 3'-2 (5'-TGCTGGAAA-AGTCTCAACCC-3'), BK18 3'-1 (5'-GCTCCGTTACTCATATG-GATTG-3'), BK18 5'-3 (5'-TGGGTTTAAATCCACGAGGT-3'), BK18 5'-2 (5'-GGTCATAGGTTGGTTTCTCCA-3'), and BK18 5'-1 (5'-GTGCCATAAGACAATGCCA-3'). For comparison, cDNA clones were also generated from a 2.8-kb dsRNA element in strain HA2 because this strain represented the most distant geographic origin (Europe). For the 5' and 3' ends, three specific primers were used with oligo(dT), as follows: HA 5'-3 (5'-GAGACCCCTCCTTGGAATTT-3'), HA 5'-2 (5'-GAAACCATTAT-TTCTTCATAATTTGG-3'), HA 5'-1 (5'-TCCACTTTTATCTAC-TGAAACACCTG-3'), HA 3'-3 (5'-CCATACCTCCCGCTTAA-AAA-3'), HA 3'-2 (5'-TCTTCGGGAAGCTGGAATTA-3'), and HA 3'-1 (5'-CCTGGACTTGGTGCCTTAGA-3'). RT-PCR products of 5'/3' RACE were ligated into TOPO vector and sequenced as described above. Both terminal sequences were examined for

their potential secondary structures using the MFOLD program (32,60). Based on their sequence information, the full-length sequence of the 2.8-kb dsRNA element in strains BK18 and HA2 was obtained by aligning partial cDNA clones using the computer programs of BioEdit (Ibis Therapeutics, Carlsbad, CA). RT-PCR using the RACE kit was repeated three times for each end to ensure the accuracy of the sequences and the completeness of full length of cDNA clones. Multiple sequence alignments with known virus groups were also performed with ClustalW Multiple Alignment (53) in the BioEdit program. The phylogenetic analysis was conducted using the program MEGA version 3.0 (Sudhir Kumar, The Biodesign Institute at Arizona State University, Tempe, AZ). Bootstrap with 150 replicates was applied in MEGA analysis. Maximum parsimony trees were inferred by using the CNI (level 1) search option with initial tree by random addition (10 reps). Complete-deletion option was used for gaps and missing data. PAUP version 4.0 (Sinauer Associates, Sunderland, MA) was also used to conduct the phylogenetic analysis with 150 bootstrap replicates.

Northern blot hybridization with strand-specific probe of 2.8-kb dsRNA. Total nucleic acids were extracted from strain BK18 using Trizol reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD) and electrophoresed on a 1% agarose gel. Total nucleic acids were transferred to a nylon membrane by capillary reaction in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight. Northern blot hybridization was performed according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Strand-specific DNA probes using Digoxigen (DIG) (Roche Molecular Biochemicals, Switzerland) were prepared using the PCR procedure described by Finckh et al. (17). The primers, BK18-3F1 (5'-CCAAAACAATGGCCTAGTGG-3') and BK18-3R2 (5'-CAGTTCTCTCCAAAAGTAGGATCTG-3'), were used for (+) and (-)-strand DNA probes, respectively. Hybridization was performed overnight at 42°C with 20 ng of each probe per milliliter hybridization buffer with gentle shaking. Detection was done according to the manufacturer's protocol (Boehringer Mannheim, Germany).

Genetic diversity in 2.8-kb dsRNA of *C. elegans*. Northern blot hybridizations with the 2.8-kb dsRNA-specific cDNA probe was performed to determine the genetic relatedness among 2.8-kb dsRNA elements present in six strains of *C. elegans* (Table 1). The dsRNA was extracted using CF11 column as described above and electrophoresed on a 1% agarose gel and used for northern hybridization using the nonradioactive DIG system according to the manufacturer's protocol (Roche Molecular Biochemicals).

RT-PCR using both BK18-3F1 and BK18-3R2 primers was also attempted in all *C. elegans* strains in which a 2.8-kb dsRNA element was found (Table 1). As a positive control, RT-PCR was performed with total nucleic acids extracted from strain BK18 using these forward and reverse primer sets. All RT-PCR amplicons were gel-purified and sequenced as described above and compared to one another.

Mitochondrial association of the 2.8-kb dsRNA element. Extraction of mitochondria was performed using the method described by Rogers et al. (46). Briefly, mycelium was mixed with 15% sucrose in 20 ml of Tris-EDTA (TE) buffer and homogenized

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

Strain	Geographic origin	Substrate and year of isolation	Source	dsRNA
AK208	Hope, AK	Cotton, 1989	C. Rothrock	—
BC92CO	Cloverdale, BC	Carrot, 1992	Z. Punja	2.8 kb
BK18	Kings County, CA	Cotton soil, 1991	B. Holtz	2.8 kb
BK25	Kings County, CA	Cotton soil, 1991	B. Holtz	2.8 kb
HA2	Amsterdam, Holland	Carrot, 1990	Z. Punja	2.8 kb
HB 93-2	Cloverdale, BC	Motherworth, 1993	L. MacDonald	2.8 kb
WASH	Bellingham, WA	Carrot, 1989	Z. Punja	2.8 kb

with 20 g of glass beads (Sigma, St. Louis, MO) for 2 min. The homogenate was mixed with an equal volume of 1% (wt/vol) sucrose in TE buffer and centrifuged at $1,300 \times g$ for 10 min at 4°C, and the supernatant and pellet (fraction A) were collected in separate tubes. The supernatant was centrifuged at $14,000 \times g$ 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 of 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 milliliter of mitochondrial fraction was loaded at the top of a sucrose gradient and centrifuged at $64,000 \times g$ 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 CF11 column as described above. The presence of dsRNA in each subcellular fraction, including mitochondria fraction (fraction M), was examined on a 1% agarose gel.

Curing of dsRNA. Mycelial plugs of strain BK18 were placed on fresh V8 agar plates (100 × 15 mm) (38) amended with either cycloheximide (5 and 10 µg/ml) or ribavirin (10 or 20 mg/ml) and incubated at 25°C. These concentrations were chosen because they inhibited mycelial growth but did not prevent it entirely. 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 to 4 days and then hyphal-tipped and grown in 50 ml of potato dextrose broth (PDB) (Difco Laboratories, Detroit) at 25°C for 2 weeks. The mycelium was used for further analysis of dsRNA presence as described previously.

In another trial to eliminate the 2.8-kb dsRNA element from strain BK18, high incubation temperatures were used. Colonies were grown on V8 agar plates at temperatures of 30, 35 to 37, or 45°C until two-thirds of the plate was covered with fungal mycelium. Two replicate plates were used at each temperature. Because of a 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 putatively cured strains, and the concentration was adjusted to 20 ng/µl using a spectrophotometer and used for RT-PCR amplification. RT-PCR was performed with a 2.8-kb dsRNA-specific primer set (BK18F1 and BK18R2) and electrophoresed on a 1% agarose gel to determine if the 2.8-kb element was present.

Colony characteristics of a putatively dsRNA-cured strain. A dsRNA-cured strain (BK18C) was compared with its wild-type dsRNA-containing strain (BK18) with regard to colony morphology and growth rate. Both strains were grown on V8 agar plates at room temperature for 2 weeks and examined for any 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 conducted twice and the data averaged.

Virulence assessment. The virulence of strains BK18C and BK18 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 5.25% NaOCl) for 5 min, and rinsed with sterilized 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 to 8 days. Degree of colonization was determined by measuring both the width and the length of the developing fungal colony and averaging the two values. Three replicates were included for each strain and the experiment was conducted three times.

Comparison of melanin production. The extent of melanin production in strains BK18 and BK18C was compared. The strains were grown in PDB at 21 to 23°C for 3 weeks and mycelium and melanized chlamydospores (57) were harvested using Miracloth and vacuum suction. The mycelium was dried overnight at 37°C and weighed, and 10 mg of mycelium was transferred to a 1.5-ml microcentrifuge tube. One milliliter of 1 N NaOH and 10% DMSO was added to each tube, incubated at 80°C for 1 h, and centrifuged at $12,000 \times g$ for 10 min. The supernatant was retained and used to estimate the melanin pigment by absorption at A585 on a spectrophotometer (Nicolet Evolution 300; Thermo Electron). Three replicates were prepared for each strain and the experiment was repeated twice.

Enzyme activity assays. The activities of polyphenoloxidase, 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 conducted twice. The polyphenoloxidase activity was assessed on Bavendamm's medium containing either 0.5% tannic acid or gallic acid (Sigma, St. Louis, MO) (4). 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) as a substrate. An 8-mm agar plug from the margin of a colony grown on Bavendamm's medium for 7 days was transferred to a 1.7-ml microcentrifuge tube. One milliliter of autoclaved double-distilled water was added and incubated at 4°C for 30 min, vortexed briefly, and centrifuged at 14,000 rpm for 10 min. Two hundred microliters of supernatant was 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 per 5 min 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) was used as a substrate and absorbance at 475 nm was used. Esterase activity was measured using p-nitrophenyl butyrate as a substrate as described by Kunoh et al. (28).

Transmission electron microscopy. Transmission electron microscopy (TEM) was used to compare mitochondrial ultrastructure between strains BK18 and BK18C. Both strains were grown on V8 agar plates for 10 days and then 1 mm³ pieces of agar containing mycelium were cut either from the margin of the colony (young) or from the center of the colony (old), fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, rinsed in buffer, and transferred to 2% osmium tetroxide in 0.05 M phosphate buffer for 2 h. Samples were dehydrated in an ethanol series, transferred to 100% acetone, exposed to propylene oxide, and then infiltrated and embedded in epon/araldite. Each sample was sectioned, stained with 0.4% lead citrate and 2% uranyl acetate, and viewed with a Hitachi H7600 TEM at 80 kV. Approximately 200 images were viewed to identify representative sections of each strain. TEM images were digitally captured and analyzed for mitochon-

drial size using image analysis software (Adobe Photoshop, Adobe Systems Incorporated, San Jose, CA). After optimizing for contrast, mitochondria were masked and an aggregate mitochondrial area (number of pixels) was calculated using the image histogram information provided for the selection. This area was then compared with the total area of the cell in each image, thus providing a mitochondrial/cell area ratio. All images were captured at the same magnification ($\times 7,200$) to ensure accurate comparison between samples. The numbers of mitochondria in each cell were also recorded. Data from 10 replicate samples from each of the two strains and culture ages were compared using one-way analysis of variance to test for significant differences at $P < 0.05$.

RESULTS

cDNA development and sequence analysis. RT-PCR with dsRNA-specific primers generated four fragments ranging in size from 450 bp to 1.2 kb; these were aligned with each other based on their overlapping sequence (Fig. 1A). For the 5' and 3' ends, RT-PCR products were generated using a combination of oligo(dT) primers and dsRNA-specific terminal primers, using three specific primers for each end. At least three amplicons for the 5'-terminal region and 3'-terminal region were obtained from strain BK18. These RACE amplicons were cloned and multiple colonies from each amplicon were obtained. Several colonies were checked for the insertion and then one of them from each amplicon was sequenced using M13 forward and reverse primers. To confirm the sequences of 5' and 3' regions obtained from RACE, RT-PCR was performed using the 20-bp sequences from the ends of these regions as forward (F-5end) and reverse (R-3end) primers, respectively, combined with primer R1007 (from 1,007 to 987 bp in the CeMV sequence) and primer F2517 (from 2,517 to 2,536 bp in CeMV sequence). Two fragments (1 kb and 0.8 kb) at the 5' region and one fragment (0.4 kb) at the 3' region were obtained. The 5' fragments were sequenced using the F-5end, R1007, and BK18 5'-3 primers. The R-3end and F2517 primers were used for sequencing the 3' fragment. The sequences of these fragments showed 99% identity with the sequences obtained from RACE. In total, two to three clones from different amplicons for each of the end regions were sequenced twice and two to four clones for each middle region were also sequenced twice. The consensus sequences from all clones for all regions were used to obtain the full-length sequence for the 2.8-kb dsRNA in strain BK18 of *C. elegans* (Fig. 1).

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. 1B). Mitochondrial genetic codes use UGA as a codon to encode tryptophan rather than 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 nucleotide position 2544 (Fig. 1B). The putative ORF I is predicted to encode 705 amino acids with a molecular weight of 81.45 kDa. The 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 RNA-dependent RNA polymerases (RdRp), including the highly conserved GDD motif (Fig. 2) (39). No other ORF of significant length was observed in the negative strand.

Using Blast search, the ORF I in the 2.8-kb dsRNA showed some identity at the amino acid level to the RdRp regions of other mitoviruses, such as *Ophiostoma novo-ulmi* OnuMV3a-Ld, OnuMV4-Ld, OnuMV5-Ld, and OnuMV6-Ld, *Cryphonectria* mitovirus 1 (24), mitovirus-like dsRNA from *R. solani* (29), and

L-dsRNA from *Sclerotinia homoeocarpa* (16) (data not shown). The highest amino acid sequence identity of the putative ORF I region in the 2.8-kb dsRNA was to the RdRp-like protein of OnuMV4-Ld, with 34% identity when the mitochondrial genetic code was used. Nucleotide comparison analysis revealed that the 2.8-kb dsRNA was related to other mitochondrial dsRNAs, with sequence identities in the range of 44 to 53% (data not shown). 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 the RdRp I-VI motifs only (data not shown, Fig. 2). The 2.8-kb dsRNA in *C. elegans* strain BK18 has been designated as *Chalara elegans* mitovirus (CeMV) and its complete sequence has been deposited as GenBank accession no. AY563138. A culture of *C. elegans* BK18 has been deposited at ATCC (Rockville, MD).

The 5'- and 3'-untranslated region (UTR) of CeMV are 426 and 352 bp long, respectively (Fig. 1). Both 5'-UTR (nucleotides 1 to 27) and 3'-UTR regions (nucleotides 2868 to 2896) could be potentially folded into the stable stem-loop structure (Fig. 3). Also, a potential panhandle structure between 5'- and 3'-UTR regions has been predicted in CeMV (Fig. 3).

Genetic diversity of 2.8-kb dsRNA in *C. elegans* strains. The partial cDNA probe (BK18C3) derived from the 2.8-kb dsRNA in strain BK18 hybridized to all 2.8-kb dsRNA elements in six different strains of *C. elegans* (BK18, BK25, BC92CO, HA2, HB93-2, and WASH) (Fig. 4). RT-PCR amplification using a specific primer set (BK18-3F1 and BK18-3R2) was also performed with total nucleic acids extracted from these *C. elegans* strains. The RT-PCR of all 2.8-kb dsRNA-containing strains successfully generated one band near 300 bp in size (data not shown). These RT-PCR amplicons were gel-purified, cloned, and sequenced to confirm that they were derived from the 2.8-kb dsRNA element. The clones shared high sequence identities (97 to 100%) to each other at the nucleotide level (data not shown), indicating a high degree of homology. The sequences of these clones have been deposited as GenBank accession nos. DQ168141 (BC92CO), DQ168142 (BK25), DQ168143 (HB93-2), and AY556453 (WASH).

In strain HA2, more than seven cDNA clones were obtained and sequenced. For the 5' and 3' ends, several amplicons each were also obtained from RACE and four colonies from four amplicons were sequenced forwards and backwards. When all the

Fig. 1. Schematic representation of alignment of a series of partial cDNA clones derived from the 2.8-kb double-stranded RNA (dsRNA) in strain BK18 of *Chalara elegans* and its putative genomic organization. **A**, Ten partial cDNA clones were obtained from the 2.8-kb dsRNA and aligned with each other to obtain the full-length sequence. **B**, Putative diagrammatic representation of the genome organization of 2.8-kb dsRNA. One large potential open reading frame (ORF I) was predicted and encodes a putative RNA-dependent RNA polymerase (RdRp). The shaded box represents the region of conserved motifs of RdRp.

sequences were aligned with each other, it resulted in a sequence 2,813 bp in length, which showed 98% nucleotide identity with CeMV. This was not a full-length sequence, with about 50 bp missing downstream of nucleotide 2632 and about 27 bp missing at the 3' end. To fill in these missing regions, the specific primer

F2517 and R-3end were used to conduct RT-PCR for strain HA2. A 0.4-kb fragment was obtained and sequenced with F2517 and R-3end. The sequence from nucleotide 2583 to 3' end was 99% identical with CeMV. The 50-bp gap and 27-bp 3' end missing part were 100% identical with CeMV. In total, two clones for the

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CeMV	-SGKLGIVKDPEGKRRIIAMVDYHSQVLVRSIHDGLLNKLRLNPQDRTYNQDP	244
CpMV1-NB631	MGKLSVVDQAGKARIVAITNSWIQTAFYSLHLHVFKLLKNIDQDGTDFQER	376
GaMV-S1	--SLSLIYDPECKVRIVAMLDYTTQLFLRPIHNDLFKLLKKLPQDRFTQNP	283
L-dsRNA	LGKLAIKEEAAGKARVFAMADSIQSVMAPLNSWVFSKLGKLPMDGTFTNQQA	312
OnuMV3a-Ld	LGKLAIKEEAAGKARVFAMADSIQSVMAPLNSWVFSKLGKDLPMDDGTFTNQQA	311
OnuMV4-Ld	-LGKLSIVHDPELKERVIAIMVDYTTQFALRPIHNILLNNLSKLPDRTFTQDP	274
OnuMV5-Ld	FGKISIVKDPELKMRVIAIMVDYHSQFVLKKIHNSLFNKLKLIKSDRTFTQDP	289
OnuMV6-Ld	IRRLSIVHDPECKERVIAIFDYGSQMVLPKIADVLFDDLNRIPSDRTFTQSP	286
RsM2-1A1	LSQFALKEEAAGKIRLFALMDSITQSVMSPLHDYMFALRNIPNDGTDFDQEA	384
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II		
CeMV	-NNAWEENKECFHSLDLSSATDRFPVKLQSRLLTEMYSDPSFGENWMNLLLNR	296
CpMV1-NB631	LNEP---TQKFYGFDLTAATDRLPIDLQVDILNIIIFKN-SPGSSWSRSLRIK	434
GaMV-S1	LNDWEDNEHSFWSIDLTAATDRFPIQLRRLLLYIYSDPEIANSWQNLVHR	335
L-dsRNA	YKEGLLDHVEFYSDYDLSSATDRLPMAFQKQIISVLFSG-DFADDWATLLVGR	371
OnuMV3a-Ld	YQDGLLDHVEFYSDYDLSSATDRLPMAFQKQIISVLFSG-KFAKDWTLLVGR	370
OnuMV4-Ld	HKWNDDHKERYHSLDLAATDRFPIFLQKQLISLIFNDYEFGNWRNLLVDR	330
OnuMV5-Ld	IFTTPTMGHRFWSMDLSAATDRFPIQLERLLSYLYGS-EISSAWKQLLIDR	343
OnuMV6-Ld	THTDLNKSFKWSIDLSSATDRFPIVQKRVLQKILGK-QMTDSWERIMIGS	342
RsM2-1A1	SQEKAVTAGKAFSYDLTAATDRLPVILTAFILSTIVGIRTFGGLWRSILVKR	442
	. **::*****::: : : : *	
III		
CeMV	EEGLSGERLRYAVGQPMGAYSSWAAFTLSHHLVVAWCTYKSK-KVIRSSQYII	353
CpMV1-NB631	YKSPQG-FLTYAVGQPMGAYSSFAMALATHHVIVQVAALNSG-FTTRFTDYCI	485
GaMV-S1	-NGLN--PIKYSVGQPMGAYSSWPAFTLSHHLVHVHCAHLN-INKFK-DYII	387
L-dsRNA	WYLKDI-PYRYSVGQPMGALSSWAMLALSHHVIVQIAAMRVG--KLSFTNYAL	422
OnuMV3a-Ld	WYLKDI-PYRYSVGQPMGALSSWAMLALSHHVIVQIAAMRVG--KLPFTNYAL	421
OnuMV4-Ld	NYDYQGISYRYSVGQPMGAYTSWAAFTLTTHHLVHVHAAELAG--LKNFKDYII	381
OnuMV5-Ld	YKTPEGDELHYKVGQPMGAYSSWAAFTLTTHHLVVFYSARMAG--IKDFTNYIL	395
OnuMV6-Ld	FLAPDGDVSYNCGQPMGAQSSWPMFTLAHHVIVRVAANRCG--LSNFDKYII	394
RsM2-1A1	LKVSDG-PYFYEVGQPMGALSSWPGALATHHWIVQVAAFRVNTSKSWNTEYEI	505
	. * ***** :*: :*: * : : *	
IV		
V		
VI		
CeMV	LGDDIVIKNDIARKYIGQMSKLG-VAISMQKTHVSKD--TYEFAKRWMHKG	402
CpMV1-NB631	LGDDIVIAHDTVASEYLKLMETLG-LSISSGKSVISSEFTEFAKKLKGRNNF	535
GaMV-S1	LGDDIVIHNDNIARKYIEIMG-KLGVGLSNSKTHVSKD--TYEFAKRWIHKG	436
L-dsRNA	LGDDIVIAADKAVATSYHMIMTQILGVEINLSKSLVSSN--SFELAKRLVTMD	472
OnuMV3a-Ld	LGDDIVIAADKAVATSYHMIMTQILGVEINLSKSLVSNN--SFELAKRLVTMD	471
OnuMV4-Ld	LGDDIVIKNNKVAQIYINLMTKWG-VDISLSKTHVSYD--TYEFAKRWIKNK	430
OnuMV5-Ld	LGDDIVINNDKVAKYYIRTMKRLG-VELSMNKTHVSKN--TYEFAKRWFKNK	444
OnuMV6-Ld	LGDDIVINNDNVALKYMEIMNDFK-VEISRKNTHVSN--TYEFAKRWIKNK	443
RsM2-1A1	LGDDIVIFNELIAQEYLNIMAVIG-CEINLNKSISSRCRPVFEFAKRTCWGF	557
	***** .. : * * * :. * : : :	

Fig. 2. Comparison of conserved motifs (I-VI, 208 residues) in RNA-dependent RNA polymerase (RdRp) regions of *Chalara elegans* mitovirus (CeMV) to other mitoviruses. * Indicates identical amino acids residues; : and . indicate higher and lower chemically similar residues, respectively, as defined in the CLUSTAL W program. CeMV(AY563138) = *C. elegans* mitovirus; CpMV1-NB631(NP_660174) = *Cryphonectria parasitica* mitovirus 1-NB631; GaMV-S1(AAN05635) = *Gremmeniella abietina* mitochondria RNA virus; L-dsRN(AAA021337) = *Sclerotinia homoeocarpa* mitovirus; OnuMV3a-Ld(NP_660176) = *Ophiostoma novo-ulmi* mitovirus 3a-Ld; OnuMV4-Ld(NP_660179) = *Ophiostoma novo-ulmi* mitovirus 4; OnuMV5-Ld(NP_660180) = *Ophiostoma novo-ulmi* mitovirus 5; OnuMV6-Ld(NP_660181) = *Ophiostoma novo-ulmi* mitovirus 6; and RsM2-1A1(AAD17381) = *Rhizoctonia solani* M2-1A1 dsRNA.

5' region and three clones for the 3' region from different amplicons, as well as five clones for other regions (overlapping), were sequenced twice. The full-length 2.8-kb cDNA sequence from strain HA2 has been deposited as GenBank accession no. DQ173015. One large ORF was found in the 2.8-kb dsRNA element in strain HA2 when the mitochondrial genetic code was used. Comparison of the ORF region between strains HA2 and BK18 revealed high sequence identity (98%) at both the nucleotide and amino acids levels. In addition, the conserved motifs found in the RdRp region in both of these dsRNAs showed 100% amino acid sequence identity, indicating these viruses were the same.

Detection of viral single-stranded RNA form of CeMV. Hybridization with both (+) and (−) strand-specific probes resulted in the detection of a strong signal with the dsRNA band, confirming that it was double-stranded in nature (Fig. 5). However, another additional smeared band, which migrated faster than the full-length dsRNA, was detected only when the (+) strand probe was used (Fig. 5). With the negative strand probe, there was no additional signal produced, except with the dsRNA band, suggesting CeMV has a (+) single-stranded nature (Fig. 5).

Mitochondrial association of CeMV. Each fraction obtained from the mitochondria extraction was retained, purified with CF11 column for dsRNA, and electrophoresed on a 1% agarose gel to determine whether CeMV was present in the mitochondria fraction. A distinct dsRNA band of 2.8 kb was observed in cellular fraction A and mitochondrial fraction M, but not in other fractions, confirming the association between CeMV and mitochondria (Fig. 6).

Phylogenetic relationships. Genetic relatedness was analyzed by comparing amino acid sequences of RdRp of CeMV to known

viruses (Fig. 7). Based on multiple alignments of the RdRp regions (717 amino acid residues), an unrooted maximum parsimony tree was reconstructed with 150 bootstrap replicates using MEGA3 program (Fig. 7). The data matrix file has been deposited in TreeBASE (accession no. S1396 and matrix accession no. M2512). This analysis indicated that the nearest relationship between CeMV and other viruses belonging to the genus *Mitovirus* was with OnuMV4-Ld and OnuMV5-Ld. We also conducted the parsimony analysis for the RdRp conserved motif region (208 amino acid residues). The parsimony tree (data not shown) showed that the CeMV was more closely related to GaMV-S1 than to OnuMV4-LD and OnuMV5-Ld, but this was not supported by bootstrap analysis. The PAUP 4.0 program was also used to analyze the RdRp region and RdRp motif region. The relationships revealed from the parsimony trees by PAUP (data not shown) were consistent with that from the trees by MEGA3.

Curing of dsRNA. When grown on V8 agar plates amended with various concentrations of either cycloheximide or ribavirin, all colonies showed significant growth inhibition. 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 BK18 (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*.

During subculturing at 35 to 37°C incubation temperatures over an extended period (about 2 months), loss of the 2.8-kb dsRNA

Fig. 3. A, Potential secondary structures of 5' end (from 1 to 27 nucleotides) and 3' end (from 2,861-2,896 nucleotides) of *Chalara elegans* mitovirus (CeMV) in strain BK18 of *C. elegans*. B, Panhandle structure of the (+) strand of the CeMV. Using the MFOLD program, (+) strand of the CeMV was folded and ΔG values (kcal/mole) were calculated.

Fig. 4. Hybridization of BK18C3 probe to 2.8-kb double-stranded RNAs (dsRNAs) in six *Chalara elegans* strains. A, dsRNA banding pattern in the six strains on 1% agarose gel stained with ethidium bromide. B, Autoradiograph of northern blots of dsRNAs shown in panel A probed with BK18C3.

Fig. 5. Northern blot hybridization using a strand-specific probe of *Chalara elegans* mitovirus (CeMV). Total nucleic acids were extracted and probed with either (−) or (+) strand-specific probes of CeMV. Double-stranded RNA (dsRNA) was detected with both probes; however, single-stranded RNA (ssRNA) was detected only when (+) strand-specific probe was used, appearing as a smear on the gel.

element was observed in one colony to result in the recovery of a putatively cured strain, referred to as BK18C (Fig. 8A). Northern blot hybridization with a partial cDNA probe (BK18C3) developed from the 2.8-kb dsRNA element did not detect a signal in this strain (Fig. 8).

RT-PCR amplification using a specific primer set (BK18F1 and BK18R2) was performed with total nucleic acids extracted from putatively dsRNA-cured strain BK18C and a dsRNA-free wild-type strain AK208 (Table 1). RT-PCR amplification generated a band identical in size to that of the RT-PCR amplification product of strain BK18 (data not shown). These RT-PCR products were cloned, sequenced, and confirmed to have been derived from the 2.8-kb dsRNA element (data not shown). The latently infected strain (BK18C) was subjected to further studies of culture morphology, pathogenicity, various enzyme assays, and electron microscopy.

Characterization of a putatively-cured strain. Culture morphology and virulence. The colony morphology of strain BK18C resembled that of its wild-type strain (Fig. 9A). The growth rate of BK18C was slightly greater compared with that of BK18 (Fig. 10A), and the virulence on carrot roots was enhanced in BK18C compared with that in BK18 (Figs. 9D and 10B).

Comparison of melanin production. Melanin production was compared between strains BK18 and BK18C. Colonies of BK18C appeared darker in color and there was a significant difference in melanin production between these two strains (Fig. 9B). The spectrophotometric absorbance values for melanin content for strains BK18 and BK18C were 0.49 and 0.62, respectively ($P < 0.01$).

Enzyme activity assays. Several enzyme activities were compared between strains 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. 9C). There were no significant differences among the various enzyme activities, including laccase, esterase, and tyrosinase, between the two strains (data not shown).

TEM. Comparison of mitochondrial ultrastructure between strains BK18 and BK18C using TEM showed differences in the number and size of mitochondria (Fig. 11). Strain BK18 contained significantly fewer and smaller mitochondria (lower mitochondrial area in the cell) compared with strain BK18C, especially in the young mycelium (Table 2). In the older hyphae from the center of the colony, there were still fewer mitochondria in strain BK18 compared with BK18C and the overall area of the cell occupied by mitochondria was lower in older hyphae than in young hyphae (Fig. 11; Table 2).

DISCUSSION

The full-length cDNA sequence obtained from a series of clones from the 2.8-kb dsRNA element (CeMV) in *C. elegans* strain BK18 was characterized as a member of the genus *Mitovirus* belonging to the family *Narnaviridae* (55). The sequence was 2,896 bp in length and contained a large ORF that predicted a protein with several motifs, which are conserved among viral RdRps. The putative CeMV RdRp showed the greatest identity (34%) to that of an *Ophiostoma* mitovirus (OnuMV4-Ld) when the mitochondrial genetic code was used. In general, it has been suggested that two viruses are different when the sequence identity at the protein level is lower than 50% (49). In this regard, CeMV is considered a new member of the mitovirus group, since its highest sequence identity at the protein level of entire RdRp to all previously described mitoviruses was 34% to OnuMV4-Ld, 33% to OnuMV5-Ld, and 32% to GaMV-S1.

Both nucleotide and amino acid sequences of CeMV further supported the close relationships between CeMV and other

Fig. 7. Unrooted maximum parsimony tree reconstructed using MEGA3, based on the RNA-dependent RNA polymerase region (717 amino acid residues) of *Chalara elegans* mitovirus (CeMV) with the other known mitoviruses. Bootstrap values (with 150 replicates) over 60% are shown on the branches of the tree. Virus notations are as in Figure 2. ScNV-20S(NP_660178) = *Saccharomyces cerevisiae* 20S narnavirus; ScNV-23S(NP_660177) = *S. cerevisiae* 23S narnavirus; Qbeta(NP_046752) = bacteriophage Q beta; MS2(RRBPBM) = bacteriophage MS2; and YFV(P19901) = Yellow fever virus.

Mitochondria purification

Fig. 6. Mitochondria purification from strain BK18. The presence of *Chalara elegans* mitovirus was observed in purified mitochondria fraction (lane M), as well as in cellular fraction A (the pellet after centrifugation at $1,300 \times g$ for 10 min).

mitoviruses. CeMV showed an A-U rich nature (67.4%), which is considered to be characteristic of mitoviruses as well as fungal and plant mitochondrial genomes (37). The codon preference of either A or U in the third position, which is characteristic of mitochondrial codons (13,37,54), was also observed in the ORF of CeMV. In addition to the similar genome size to mitoviruses (2.3 to 3.2 kb), the predicted molecular mass (81.45 kDa) of the putative RdRp protein of CeMV is within the size range of RdRp proteins of other mitoviruses (92.2 kDa in OnuMV4-Ld, 86.1 kDa in OnuMV5-Ld, and 80.0 kDa in OnuMV6-Ld of *O. novo-ulmi*) (24). Analysis of the amino acid sequence of CeMV showed the typical RdRp motifs (II-VI) including conserved GDD motifs found in other RNA virus genomes (8,39). For the mitochondrial dsRNAs only, Hong et al. (23) 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. A similar motif I was present in CeMV, confirming that CeMV 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. (23) 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* (16). The predictions of secondary structure for both 5' and 3' ends of CeMV using the MFOLD program resulted in the possible formation of stem-loop structure. Both 5' (nucleotides 1 to 27) and 3' (nucleotide 2868 to 2896) ends can be folded into potentially stable stem-loop structures, providing additional evidence that CeMV is related to other mitoviruses. Also, the presence of panhandle structure was predicted in CeMV. 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 (23,24). Therefore, these secondary structures predicted in CeMV may play similar functions that have been suggested for other mitoviruses.

Compared with other fungal RNA viruses, mitoviruses are unusual not only because of their infrequent occurrence in fungi but also because of their association with mitochondria. Most mitovi-

uses, including *Ophiostoma* mitoviruses (24), *C. parasitica* mitovirus 1-NB631 (41), and *Sclerotinia* L-dsRNA (16), 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 (37). Several reports have shown that mitoviruses can be co-purified with mitochondrial extraction, indicating their presence in mitochondria for at least a part of their life cycle (16,40). Cole et al. (11) also reported that RdRp protein activity could be detected in mitochondria for mitovirus OnuMV6-Ld in an *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 four UGA codons in the ORF of CeMV and the co-purification of dsRNA with mitochondria are consistent with the results for other mitoviruses.

Sequencing of the RT-PCR products from six strains of *C. elegans* showed that these fragments were derived from the 2.8-kb dsRNA element and shared high sequence identities (97 to 100%)

Fig. 8. Change in double-stranded RNA (dsRNA) banding pattern in strain BK18 after high temperature (35 to 37°C) incubation. **A**, dsRNA extracted from BK18 (lane 1) and BK18C (lane 2). The 2.8-kb element was absent in BK18C. Lane 3 shows the 1-kb DNA marker. **B**, Autoradiograph of northern blot of dsRNA shown in **A** probed with BK18C3. There was no signal in lane 2 (BK18C), indicating the 2.8-kb dsRNA element was not detected.

Fig. 9. Comparison of phenotypic characteristics between double-stranded RNA (dsRNA)-containing (BK18) and latently infected (BK18C) strains of *Chalara elegans*. **A**, Similar colony morphologies on V8 agar plates. **B**, Extraction of melanin pigment from the two strains. Note greater pigmentation in strain BK18C. **C**, Bavendamm's reactions after 10 days incubation at 25°C in the dark, showing phenoloxidase color. **D**, Virulence test on carrot roots. Carrot roots inoculated with strain BK18C showed greater colonization compared with its original dsRNA-containing (BK18) strain.

Fig. 10. A, Comparison of growth rates on V8 agar between double-stranded RNA (dsRNA)-containing (BK18) and latently infected (BK18C) strains of *Chalara elegans*. Strain BK18C grew better than strain BK18. Vertical bars represent standard error of the mean. B, Virulence test using carrot roots inoculated with latently infected (BK18C) and dsRNA-containing (BK18) strains. Letters denote significant differences in lesion diameter according to the difference of least squares means ($P < 0.05$).

to CeMV. It was interesting that the strains originated from diverse geographic regions and hosts, and were genetically distinct based on random amplified polymorphism DNA patterns (44), but harbored the same CeMV.

Mycoviruses can be spread between strains either by vertical transmission through conidia or horizontal transmission via hyphal anastomosis (25,33). 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% (9,25,33,45,52). The horizontal transmission rate between fungal strains via hyphal anastomosis is much more variable and is generally lower due to vegetative incompatibility (12,34,36). A high transmission rate (100%) of CeMV through conidia of *C. elegans* has been observed. In contrast, horizontal transmission of dsRNA via hyphal anastomosis could not be demonstrated in *C. elegans* (Y. Park and Z. Punja, unpublished data). The efficiency of virus transmission through either conidia or hyphal anastomosis differs depending on the fungal species (51). In *O. ulmi*, horizontal transmission of dsRNA was restricted by vegetative incompatibility (7). There are no previous studies on vegetative incompatibility groups in *C. elegans*; therefore, the extent to which dsRNA elements may be transmitted between strains is unknown.

To elucidate the role of the 2.8-kb dsRNA in *C. elegans*, we attempted to eliminate CeMV 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 to eliminate dsRNAs (18,26,56). During subsequent serial transfers at 35 to 37°C, a colony was obtained in which CeMV was not seen in agarose gels and this strain was designated BK18C. Northern blot hybridization confirmed the absence of CeMV 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 possible suppressed replication (latent infection) with the 2.8-kb dsRNA element. Therefore, no strains completely free of CeMV were recovered in this study. The high incubation temperature may have suppressed viral replication or eliminated the virus from a subset of the mitochondria.

Numerous reports have demonstrated that the presence of dsRNA in fungi can affect specific biochemical and physiological pathways in the fungal host (14,27,31). 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 (50). 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 had altered physiological processes in the fungal host (50). 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 (19). In some cases, the presence of latent infection with certain viruses could only be detected by RT-PCR, implying their replication was suppressed. 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 (16). A similar observation was made for one of the dsRNAs present in *R. solani* (30). These results are consistent with our observation, implying a reduced concentration of CeMV in *C. elegans* strain BK18C, either through suppressed replication or elimination of the virus from a portion of the mitochondria by temperature treatment. The effects of latently infected dsRNA on host fungi are quite different. In *S. homoeocarpa*, an increase in 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 (15). Continuous subculture of BK18C over a 12 month period, however, or incubation of cultures at 4°C for several months, did not alter the latency of CeMV (Y. Park and Z. Punja, unpublished data). An additional wild-type strain of *C. elegans* (AK208) was also found to contain the 2.8-kb dsRNA at subliminal levels detectable only by RT-PCR, suggesting that latency can occur among strains in natural populations. The conversion from detectable to latent infection by CeMV in strain BK18C enhanced mitochondria number and size, fungal mycelium growth, melanin production, and virulence. While these differences were significant, it is noteworthy that the presence of CeMV in *C. elegans* did not appear to induce hypovirulence as has been reported for other mitoviruses in fungi (16,47). For example, mitovirus OnuMV4-Ld, which is most closely related to CeMV, caused hypovirulence in *O. novo-ulmi* (24). Reports of dsRNA in fungi are biased toward 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 (3,36). Similarly, latent infections by mycoviruses are reported infrequently. However, latent infections can play an important role in mycovirus evolution because lower selection pressure compared with that of the highly expressive fungal viruses may provide more advantages for survival and spread of latent viruses among host fungal populations (19). The CeMV is widely distributed among strains of

Fig. 11. Comparison of mitochondrial structures in double-stranded RNA-containing (BK18) (**A and C**) and latently infected (BK18C) strains (**B and D**) of *Chalara elegans* using transmission electron microscopy. Top panel corresponds to hyphae from the actively growing margin of colonies; lower panels are older hyphae from the center of colonies. Locations of mitochondria in the cell of each strain are indicated by arrows. Note smaller and fewer mitochondria in strain BK18, as well as fewer mitochondria in older hyphae compared with young. Scale bar = 2 μ m.

TABLE 2. Comparison of mitochondrial number and size in young and old mycelium of strains BK18 and BK18C of *Chalara elegans*

Measurement ^a	Young mycelium		Old mycelium		ANOVA test ^b	<i>P</i> value ^c	
	Strain BK18	Strain BK18C	Strain BK18	Strain BK18C		Number	Size
Number of mitochondria per cell	3.8	6.2	2.9	3.7	BK18 young $\times$ BK18 old	0.2944	0.1220
Mitochondrial size (area as a percentage of total area of cell)	5.44	7.17	3.90	4.09	BK18C young $\times$ BK18C old	0.0052	0.00087
					BK18 young $\times$ BK18C young	0.0093	0.03140
					BK18 old $\times$ BK18C old	0.3281	0.85110

^a Measurements were made from digitized images of TEM section of the two strains. To estimate mitochondrial size, the area in the cell occupied by mitochondria was expressed as a percentage of the area of the entire cell.

^b One-way analysis of variance (ANOVA) was conducted between the specific strain combinations at *P* = 0.05.

^c *P* values in bold are significantly different.

C. elegans from different 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.

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