Characterization of a novel dsRNA endornavirus in the plant pathogenic fungus *Thielaviopsis basicola*

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Double-stranded (ds) RNA elements are commonly present in strains of the plant pathogenic fungus *Thielaviopsis basicola* which infects a wide range of plant species. To characterize a novel 12 kb dsRNA in strain NC1527 of this fungus, reverse transcription-polymerase chain reaction (RT-PCR) was used to obtain an 11,602 bp cDNA sequence from overlapping cDNA clones. Northern blot analysis confirmed that 15 individual cDNA clones that covered the entire length of the cDNA sequence all hybridized to the 12 kb dsRNA. An open reading frame (ORF) search revealed that the 5′non-coding region of this sequence spans 27 nucleotides, followed by one large ORF of 11,575 bp nucleotides, which potentially encodes a large putative polyprotein of 3858 amino acid residues. Specialized Basic Local Alignment Search Tool (BLAST) searches of conserved domains indicated that the putative polyprotein contained a viral RNA helicase1 (Hel), glycosyl transferase (GT) and RNA-dependent RNA polymerase (RdRp) domain regions. BLASTp searches in the protein database using translated nucleotides showed that the cloned dsRNA had homology to endornaviruses, which are present in a few fungi as well as some plant species. The amino acid homologies ranged from 28% to 34% in the RdRp domain region, 23–32% in the helicase domain region and 29–30% in GT region. We designate this dsRNA element as a new endornavirus, *Chalara elegans endornavirus* 1 (CeEV1). Phylogenetic comparison of the sequence of RdRP with other endornavirus indicated that CeEV1 was relatively distant and may be an ancestral form.

**Keywords:** Endornavirus; double-stranded RNA; mycovirus; RNA-dependent RNA polymerases

**Introduction**

Double-stranded (ds) RNA elements are known to occur in a wide range of fungi and are presently classified into six families. Examples of dsRNA groups include the *Hypoviridae* found in the chestnut blight fungus *Cryphonectria parasitica* (Anagnostakis 1982; Nuss 1992; Hillman et al. 1994; Duwe and Nuss 2001), *Partitiviridae* in *Discota destructiva* (Rong et al. 2001), *Totiviridae* in *Helminthosporium victoriae* and *Thielaviopsis basicola* (Ghabrial et al. 1987; Park et al. 2005; Park et al. 2006a), *Reoviridae* in *Rosellinia necatrix* (Osaki et al. 2002), *Narnaviridae* in *Ophiostoma novo-ulmi* (Hong et al. 1998, 1999) and *T. basicola* (Park et al. 2006b) and *Endornaviridae* in *Helicobasidium mompa*, the violet root rot fungus (Osaki et al. 2006).

*Chalara elegans* (*synanam. T. basicola*) is a soilborne hylomycete and a widespread facultative parasite on numerous plant species. It has been previously reported that dsRNA elements and virus-like particles (VLPs) were present in the cytoplasm of one strain of *C. elegans* (Bozarth and Goenaga 1977). Additional studies on these dsRNAs and their effects on growth and virulence of *C. elegans*, and on the diversity, complexity and transmission of dsRNA elements, have been carried out (Park et al. 2005, 2006a, 2006b). Thirty-six out of forty-three wild-type isolates (83.7%) were found to contain dsRNA elements, which were assigned to nine groups based on the similarity of banding patterns of the different molecular weight sizes (Bottacin et al. 1994). Studies on the diversity of the dsRNA elements in *C. elegans* have indicated that a high degree of genetic dissimilarity exists among different molecular size dsRNA elements, even within a single strain. This genetic diversity may have resulted from geographic separation of strains carrying unique dsRNA elements (Park et al. 2006a). Multiple dsRNA elements (from 2.0 to 12 kb in size) can occur in a single strain of *C. elegans*. At present, at least two groups of dsRNAs have been identified in *C. elegans*, including a 5.3 kb totivirus in *Totiviridae* (Park et al. 2005) and a 2.8 kb mitovirus in *Narnaviridae* family (Park et al. 2006b).

In this study, we characterized an approximately 12 kb dsRNA element, the largest dsRNA element that has been described in *C. elegans*. Based on the molecular characteristics and phylogenetic analysis of an 11,602 bp cDNA sequence, we designate this dsRNA element as *Chalara elegans endornavirus* 1 (CeEV1), a new member of the *Endornaviridae*. This is the first report that an...
endornavirus exists in *C. elegans* and also coexists with a totivirus within a single isolate of *C. elegans*.

**Materials and methods**

**Fungal strains**

Strain NC1527 of *C. elegans* has been reported to contain 6–8 dsRNA fragments ranging in size from about 2.8 to 12 kb (Park et al. 2006a). This strain was originally isolated from tobacco (*Nicotiana tabacum L.*) in Madison County of North Carolina in 1987 by D. Shew. The isolate was maintained on V8A medium (V8 juice, 150 ml; Bacto agar, 15 g; distilled water, 850 ml; streptomycin, 200 mg/L) at room temperature (21°C–23°C), or at 4°C for long-term storage.

**Reverse transcription-polymerase chain reaction (RT-PCR) and cDNA cloning**

Total dsRNA extraction and purification was conducted according to the procedures described by Bottacin et al. (1994) and Park et al. (2005). The 12 kb dsRNA fragment was collected from a 0.7% low-melting agarose gel using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA). Previously, Park et al. (2006a) had derived a 1.9 kb region of partial cDNA sequence from the 12 kb dsRNA element in NC1527 which included two overlapping clones (AY556457, 1458 bp) and (AY556458, 734 bp). From this 1.9 kb sequence, several forward and reverse primers were designed, combined with random primer, and RT-PCR was performed to extend the cDNA sequence. A series of primers from each new extended sequence was then designed (data not shown) and used in a subsequent RT-PCR (Park et al. 2005). The cDNA fragments from RT-PCR were collected using the MinElute gel extraction kit (Qiagen, Valencia, CA). The fragments were cloned into TOPO vector (Invitrogen, Carlsbad, CA) or pGEM Easy vector (Promega, Madison, WI, USA) following the manufacturer’s procedures. Single white colonies were transferred into 3 ml LB with 100 mg/L ampicillin or 50 mg/L kanamycin and incubated at 37°C overnight with vigorous shaking. The plasmid DNA was extracted using Wizard Plus Midiprep’s kit (Promega, Madison, WI, USA) and digested with *EcoR*I to confirm the insertion. Then 10 µl of the 100 ng/µl plasmid with appropriate-size insertions was sent to UBC NAPS Unit (University of BC, Vancouver, BC, Canada) for sequencing with standard M13 forward and reverse primers or sent to Macrogen Inc. (Seoul, South Korea) for sequencing using M13 forward and reverse primers for TOPO vector and T7 and SP6 primers for pGEM vector.

For the 5′-end and 3′-end sequence, rapid amplification of cDNA ends (RACE) was carried out using SMARTer RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA). GSP1 and GSP2 were designed as 5′-endR1: CCAGAGTGTCTACTAAGTCCG CGAAACA, 3′-endF1: CAAGACAGACAAACCTCCTG GGACAA. The nested primers were designed as 5′-endR2: GTACCTCTAATCGTTGTGTGCACTGTCG, 3′-endF2: GATGGATGACCCCCTCCTGAGATCG. The first strand cDNA reaction was conducted following the protocol of the RACE kit manual. The 5′-RACE and 3′-RACE PCR reaction was the same as described above but 2.5 µl of 5′- or 3′-cDNA was added instead of 10 µl.

**Northern blot analysis and sequence analysis**

To confirm that cDNA clones from RT-PCR were derived from the 12 kb dsRNA element, Northern blots were carried out. Fifteen clones covering the entire cDNA sequence were selected and the DNA fragments of insertions collected from plasmids were used as probes. At least 3–4 clones derived from each RT-PCR fragment were sequenced in both directions. The sequences were aligned and a consensus sequence was obtained using the BioEdit software (Hall 1999). The whole cDNA consensus sequence was aligned manually in BioEdit. Then the consensus sequence was used for nucleotide and protein similarity searches by Basic Local Alignment Search Tool (BLAST)n and BLASTp. The open reading frames (ORFs) were found using NCBI ORF Finder. The specialized BLAST search of conserved domains (CDs) from conserved domains database (CDD) was performed with default search parameters to find the CDs from deduced amino acid sequences.

Several previously published endornavirus full-length amino acid sequences and some other dsRNA or ssRNA virus RdRp, RNA helicase and glycosyl transferase (GT) partial sequences were retrieved from GenBank. The RdRp, helicase and GT region sequence alignments were conducted using ClustalW Multiple Alignment in BioEdit. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). The alignments were converted into MAGA format and phylogenetic analysis was conducted using neighbor joining algorithm with 1000 replicates of bootstrap. Complete-deletion option was used for gaps and missing data.

**Results**

**cDNA cloning and Northern blot analysis**

An 11,602 bp cDNA contiguous consensus sequence was obtained from more than 100 overlapping clones generated from a 12 kb dsRNA element in strain NC1527 of *Chalara elegans*. The clones containing the large cDNA fragments used to conduct Northern blot analysis are shown in Figures 1 and 2. Each region of the consensus
sequence was derived from at least three clones. Some overlapping regions were derived from more than 6–8 clones. The Northern blot results showed that all these clones hybridized only to the 12 kb dsRNA of NC1527 which confirmed that the clones obtained from RT-PCR fragments were derived from the 12 kb dsRNA element and had not arisen from contamination by other dsRNA fragment in NC1527 (Figure 1).

Sequence analysis
The nucleotide BLAST search of the 11,602 bp sequence showed that there was no significant homology to any other nucleotide sequences in GenBank except to the previously deposited sequences derived from the same 12 kb dsRNA element in C. elegans (Park et al. 2006a). However, the translated nucleotide BLAST search showed 26–34% amino acid identity in the RdRp region to several endornaviruses, 23–32% identity in RNA helicase region, 29–30% in UGT and 22–45% identity in an unknown region. Therefore, we designated the 12 kb dsRNA element of C. elegans as a new member of the Endornavirus genus, CeEV1. The almost full-length 11,602 bp nucleotide sequence has been deposited in GenBank under accession number GQ494150. At the time of our submission, six endornaviruses were completely sequenced, including those from cultivated rice (Oryza sativa endornavirus) (OSV) (Moriyama et al. 1995), wild rice (Oryza rufipogon endornavirus) (ORV) (Moriyama et al. 1999), broad bean (Vicia faba endornavirus) (VFV) (Pfeiffer 1998) and the fungus H. mompa (HmEV1-670) (Osaki et al. 2006). The latter is the first report of a fungal endornavirus whose complete nucleotide sequence is 16,614 bp in size. Another complete fungal endornavirus sequence is Gremmeniella abietina type B RNA virus XL2 (GaBRV-XL2) (Accession: DQ399290). An endornavirus has also been found in a protist group species, Phytophthora endornavirus (PEV1), of size 13,883 bp (Hacker et al. 2005). There is one additional newly reported grapevine endophyte endornavirus GEEV (Espach et al. 2012) described after our CeEV1 sequence was deposited in GenBank (4 October 2010). This dsRNA sequence from grapevine aligned to our CeEV1 at 29% identity with E-value 3e-168.

Functional domains
The ORF search of the 11,602 bp cDNA sequence showed that the 5’ non-coding region spans 27 nucleotides, an AUG start codon was initiated at position 28 and was followed by one large ORF of 11,575 bp nucleotides which potentially encoded a long putative polyprotein of 3858 amino acids with a predicted molecular weight of 426,627.96 Daltons. Specialized BLAST CDs search with default search parameters showed that the large putative polyprotein contained several conserved functional domains. These domains were: Viral_helicase1 (Hel) located near the N-terminal at 1200–1500 aa; RecD producing ATP-dependent exoDNAse (exonuclease V) located at 1400–1500 aa which overlapped with Hel domain; GT1_Gtf_like domain and GTs overlapping domains located at 2600–2800 aa region; and RdRp domain region (3550–3750 aa). The box with question mark is the unknown sequence region (465–772 aa).
RNA_dep_RNAP and RdRp 2 both located at 3500–3858 aa region (Figure 2). There are two more domains described recently in endornaviruses, one is AAA_30 (pfam13604) located at 1200–1400 aa. This family of domains contains a P-loop motif that is characteristic of the AAA superfamily. Many of the proteins in this family are conjugative transfer proteins. There is a Walker A and Walker B present. A second domain is UvrD_C_2 (pfam13538) located at 1400–1500 aa. This domain is found at the C-terminus of a wide variety of helicase enzymes and has a AAA-like structural fold.

The CD search for five complete nucleotide sequences of endornaviruses revealed domain RdRP_2 (pfam00978) is present in all 5 endornaviruses, the domain helicase is present in VFV and PEV but not in ORV and OSV. GT1 is present in PEV, OSV and HmEV1. GT domain was not present in VFV and HmEV1. Domain RNA_dep_RNAP (cd01699) was only present in CeEV1, Glyco_transf_28 was only present in HmEV1, and domain of mannosyltransferases OCH1 and a capsular polysaccharide synthesis (CPS) protein domain (pfam05704) were present only in OSV and ORV (data not shown). The newly described GEEV did not contain a GT domain, but contained a Tnp_zf-ribbon_2 (pfam13842) located at 800 aa which is close to the unknown domain region in our ORF (Figure 2). This zinc-ribbon domain is frequently found at the C-terminal of proteins derived from transposable elements.

**Phylogenetic analysis of CeEV1 and other endornaviruses**

Phylogenetic neighbor joining analysis of endornavirus RdRp region (491 aa of alignments) from 15 sequences was conducted (Figure 3). The phylogeny of 15 RdRp showed several clades with high Bootstrap values. The endornaviruses from dicotyledonous plants (bottle gourd, melon and kidney bean) grouped together with 86% value, and from rice were clustered at 100%; they both grouped together with 99% value separating them from the others. The VFV from broad bean grouped with PEV1 from a protist group with 57% value, and with fungus HmEV1 and other two monocotyledonous plants – barely and seagrass – grouped together with 68% value. The GaBX2, CeEV1 and PmV1 and PmV2 which are two ssRNA virus, grouped together with less than 50% bootstrap value. CeEV1 was distant from all others. Single-strand RNA virus from tomato was chosen as an out-group (Figure 3).

**Discussion**

This study is the first to show the presence of an endornavirus in strain NC1527 of *C. elegans*, designated CeEV1. A totivirus was also previously reported to occur in this strain (Park et al. 2006a). One large ORF was found in the 11,602 bp cDNA sequence of CeEV1 (almost complete) and the large putative polyprotein of 3858 amino acid was found to have homology to endornaviruses. Members of the genus *Endornavirus* classified in *Endoviridae* family (Gibbs et al. 2000; Fukuhara et al. 2006) are widely distributed in various plants species, such as rice, broad bean, kidney bean, barley and bell pepper. They are also reported to occur in fungi such as *H. mompa* and *G. abietina* (GaBXL2, ABD73306.1), and *Z. marina* (Zostera marina (seagrass), GaBX2 = Gremmeniella abietina type B RNA virus XL2, PmV1 = *Pineapple mealybug* w/associated virus-1, PmV2 = *Pineapple mealybug* w/associated virus-2, Tom = Tomato (*Solanium lycopersicum*), Accession numbers and sequence region of RdRp are shown in the trees. Figures 3 and 4).
Endornavirus dsRNAs share a common ancestor with the alpha-like supergroup of single-stranded RNA (ssRNA) viruses as proposed by Gibbs et al. (2000). The monophyletic origin of Endornaviridae has been proposed by Fukuhara et al. (2006) based on 350 aa and 99 aa analysis of RdRp regions. Fukuhara et al. (2006) concluded that the origin of the endornaviruses and the origin of host may not be consistent. The existence of genome heterogeneity of endornaviruses was suggested by Osaki et al. (2006). The CeEV1 endornavirus was found to contain more domains than in any of the other endornaviruses described to date. Endornaviruses containing different domains suggests the existence of heterogeneity among members of this group (Osaki et al. 2006) and endornaviruses with similar domains may be closely related by sharing similar evolutionary mechanisms. We predicted that at least six functional domains are encoded in the large ORF on the linear dsRNA element in CeEV1. An unknown sequence similarity region was present at the N-terminal region of CeEV1 as well as in 5 other endornaviruses (data not shown). This might be a transcription factor NF-X1 which contains NFX-type Zn$^{2+}$-binding and R3H domains as shown in GEEV (Espach et al. 2012).

Our similarity search and CD search did not reveal a virion coat protein sequence and domain, indicating that CeEV1 is not associated with particles, which is one of the characteristics of endornaviruses.

The phylogenetic analyses indicated that CeEV1 was relatively distant from the previously described endornaviruses. The phylogenies conducted using 15 RdRp sequences confirmed that the endornaviruses from monocotyledonous and dicotyledonous plants did not co-evolve with their host (Fukuhara et al. 2006).

A UGT region was found in endornaviruses PEV1, OSV, ORV (Hacker et al. 2005) and Bp22 in bell pepper (Valverde and Gutierrez 2007) but not in VFV from Vicia faba. It was suggested that the UGT gene in PEV1 was not acquired recently from the Phytophthora genome and may have been acquired by a progenitor endornavirus of an ancient host that predates the separation of bacteria, fungi and plants. The VFV possibly originated from the same UGT-containing ancestral virus as PEV1, ORV and OSV and was lost (Hacker et al. 2005). The presence of UGT in CeEV1 may support this suggestion and share an ancestor with them or may be the ancestor of them. This needs more data to support.

The functions of the dsRNA endornaviruses in plants are unclear (Moriyama et al. 1995; Fukuhara et al. 2006). These endornaviruses are not associated with disease symptoms in their hosts, except for VFV associated with cytoplasmic male sterility (CMS) because it was only found in CMS plants and was not present in fertile lines (Pfeiffer et al. 1993; Pfeiffer 1998). In the fungus H. mompa, HmEV1-670 was found as the hypovirulence factor which reduced virulence of the host (Ikeda et al. 2003). In this study, two RdRp domains were present in CeEV1. One of them is the RNA-dep-RNAP domain (cd01699) which belongs to RT-like superfamily. In this superfamily, one group called poly (A)-type retrotransposons contain fungal mitochondrial introns and transposable elements that lack long terminal repeats. Whether CeEV1 is a retrotransposon remains to be determined. Plant endornaviruses were considered as a kind of RNA plasmid and might be related to hypoviruses, which also lack particles and are transmitted vertically (Fukuhara et al. 1993). They were proposed to be plasmid-like replicons since endornaviruses can replicate independently of their host genome (Lefebvre et al. 1990; Moriyama et al. 1995; Horiuchi et al. 2001). Their replication is regulated to produce a constant low concentration in all tissues except pollen (Moriyama et al. 1996, 1999). It was also suggested that these replicons evolved from a defective alpha-like single-stranded RNA virus (Gibbs et al. 2000). Osaki et al. (2006) suggested the genome heterogeneity found in endornaviruses supported the hypothesis that RNA recombination had occurred possibly through horizontal transmission between plants and parasitic organisms due to the evidence of the horizontal transmission of HmEV1-670. They also proposed that fungi might have acquired their ancestral viruses from plants or vice versa during host infection (Osaki et al. 2006). The wide distribution and genome heterogeneity of endornaviruses (Fukuhara et al. 2006; Osaki et al. 2006) suggests that horizontal transfer of the ancestral endornaviruses from fungi to plants could have occurred although there is no evidence yet to support this. It is possible that fungal endornaviruses may have co-evolved with their hosts and RNA recombination with the host genome may have taken place or genome interactions may have occurred between fungal endornavirus and the host plant especially if they possess retrotransposon properties. Since CeEV1 is an ancestral form, it is possible that broad bean might have acquired its ancestral virus from the fungus, since the plant pathogen C. elegans also infects a wide range of plants, including beans. However, this hypothesis remains to be proven.

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