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ANNOTATED SEQUENCE RECORD

Genome sequence of a novel endornavirus from the phytopathogenic fungus *Alternaria brassicicola*

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Abstract In an effort to discover new mycoviruses from phytopathogenic fungi, a dsRNA molecule of 10,290 nt, resembling those associated with the viruses belonging to the family *Endornaviridae*, was isolated from *Alternaria brassicicola*, one of the causal agents of rapeseed black spot disease. Genome analysis revealed the presence of a single open reading frame coding for a polyprotein of 3400 aa containing conserved viral methyltransferase (MTR), viral RNA helicase 1 (Hel-1), and RNA-dependent RNA polymerase (RdRp) domains. In addition, a cysteine-rich region (CRR) with conserved CXCC motifs, shared among several endornaviruses, was also identified between the MTR and Hel-1 domains. Phylogenetic analysis based on the RdRp sequence strongly suggested that the virus infecting *A. brassicicola* should be considered a representative of a novel endornavirus species, and this virus was designated as Alternaria brassicicola endornavirus 1 (AbEV1).

H.-H. Shang and J. Zhong contributed equally to this work.

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Introduction

The genus *Alternaria* includes a complex group of filamentous fungi, some of which cause major losses in a wide range of crops [1]. *Alternaria brassicicola*, *A. brassicae* and *A. raphanin* have been reported to be responsible for alternaria blackspot, an important disease of rapeseed. *Alternaria spp*. are known to harbor several viruses. For example, dsRNAs associated with virus-like particles were detected in *A. alternata* isolated from cotton seeds and Japanese pear trees [2–4]. Four of these dsRNAs make up the genome of a new mycovirus named Alternaria alternata virus 1 (AaV-1). This virus, which has a debilitating effect on the host, is distinct from most common mycoviruses (i.e., totiviruses, chrysoviruses, partitiviruses, reoviruses and hypoviruses) but related to Aspergillus mycovirus 341 (AsV341) [5]. Its genome was sequenced, and it showed a potential biocontrol effect [2]. Furthermore, dsRNAs present in the Japanese pear pathotype of *A. alternata* caused phenotypic changes in the host fungus through negative effects such as apoptosis-like cell death [6]. Also, two non-encapsidated dsRNAs of 8.3 and 5.5 kbp that were associated with spherical membrane vesicles in infected hosts were also detected in *A. alternata*; however, these molecules have not been sequenced yet [7]. A new mycovirus with a non-segmented dsRNA genome, related to the unassigned Curvularia thermal tolerance virus, has recently been reported from *A. longipes* [8]. However, no dsRNA virus has been reported from *A. brassicicola* yet. In this study, we sequenced the 10-kbp genome of a dsRNA virus from an isolate of *A. brassicicola*.

Endornaviridae is a recently approved family that includes dsRNA viruses infecting plants, fungi and oomycetes [9]. The viruses in this family have a non-encapsidated, single, linear, 9.8- to 17.6-kb, dsRNA

genome with a single open reading frame (ORF) encoding a unique polyprotein that is processed into functional proteins by virus-encoded proteases [10]. Four functional domains are often found in the polyproteins encoded by endornaviruses: viral methyltransferase (MTR), viral RNA helicase (Hel), glycosyltransferase (GT), and RNA-dependent RNA polymerase (RdRp). However, only the RdRp is universally present in all members of this family [11].

Here, we report the complete 10-kbp genome sequence of a novel dsRNA virus. We propose this virus as the prototype of a novel species in the family *Endornaviridae* and suggest the name "Alternaria brassicicola endornavirus 1" (AbEV1).

Provenance of the virus material

A. brassicicola strain 817-14 was isolated from rapeseed leaves showing alternaria blackspot symptoms collected in 2013 from Hunan Province of China. The identity of the fungus has been verified by sequencing of its internal transcribed spacer (ITS) sequence (accession number KP174728). Viral dsRNA was extracted as described previously [12]. Contaminating single-stranded RNA (ssRNA) and DNA were eliminated by treatment with RNase-free DNase I (TaKaRa) and S1 nuclease (TAKARA). After being separated on an agarose gel, a fragment of approximately 10 kbp was excised, purified, and used for cDNA synthesis. A cDNA library was constructed using random hexadeoxynucleotide primers (TaKaRa) and sequenced. Based on the sequences obtained in the initial round of cloning and sequencing, sequence-specific primers were designed and used for RT-PCR to fill the gaps in the genome nucleotide sequence data. An adaptor was ligated to the 3' end of each strand, using T4 RNA ligase (Fermentas) [13, 14], to clone the termini of the dsRNAs. All of the amplified cDNA products were cloned into the pMD18-T vector (TaKaRa) and sequenced in both orientations in at least three independent experiments. Sequence alignments were performed using ClustalX [15]. Phylogenetic trees were constructed by the neighbor-joining method using MEGA 4. Bootstrap tests were carried out with 1000 re-samplings using Poisson correction, and the "complete deletion" function was used for missing data [16].

Sequence properties

The complete genome sequence of AbEV1 was determined and found to be 10,290 nt in length, with a G+C content of 48.5 % (accession number KP239989). A single open reading frame (ORF) was found starting at nt 53 and terminating at nt 10,255, preceded and followed by untranslated regions (UTRs) of 52 and 35 nt at the 5' and 3' ends, respectively (Fig. 1a). The 3' end of the genome was characterized by the presence of 10 cytosines and the absence of a poly(A) tail. The single ORF potentially encodes a 383.7-kDa polyprotein (3400 aa). A specialized BLAST CD search with default search parameters detected three conserved domains in this putative polypeptide, i.e., a viral MTR (pfam01660), a Hel-1 (pfam01443) and an RdRp (pfam00978) (Fig. 1). A comparison of all protein-coding sequence regions of AbEV1 to those of other viruses was performed, and the results are shown in Table 1.

The MTR region (255 aa) was found at aa positions 425-679 and was predicted to code for a 28.2-kDa protein, sharing the highest degree of identity of 32 % (E-value, 0; query cover, 99 %) with that of Tuber aestivum endornavirus (TaEV). Four conserved motifs of the 'Sindbis-like' supergroup of ssRNA viruses [17] were also detected (Fig. S1a). A 26.3-kDa Hel-1 domain was identified between aa residues 1784 and 2020. It showed the highest degree of identity (identity, 24 %; E-value, 7e-07; query cover, 99 %) with the Hel-1 of Gremmeniella abietina type B RNA virus XL1 (GaBRV-XL1) and contained the motifs (I to VI) (Fig. S1b) [18] belonging to superfamily 1. The putative RdRp domain, from aa position 2984 to 3238, was located at the C-terminal end of the polyprotein and contained conserved motifs I-VIII [18] that has similarity (E value: 7.21e-13) to those of the subfamily RdRP_2 (cl03049) (Fig. S1c). Moderate aa sequence identities of this RdRp domain to those of other known endornaviruses were found, with Rhizoctonia cerealis endornavirus 1

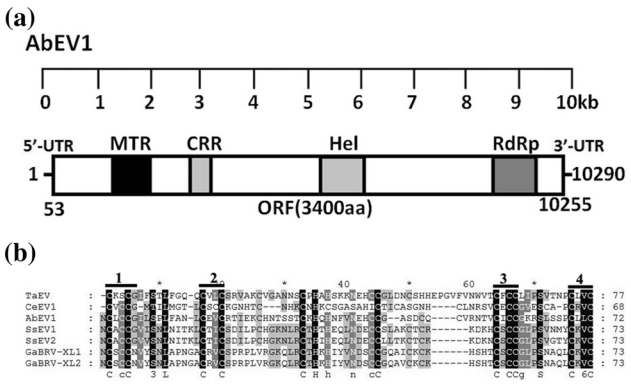

Fig. 1 (a) Diagrammatic representation of the genome organization of AbEV1. The longer box represents the large ORF, and the conserved domains are represented by smaller shaded boxes. MTR, viral methyltransferase; CRR, cysteine-rich region; Hel-1, viral helicase superfamily 1; RdRp, RNA-dependent RNA polymerase. (b) Multiple alignment of the cysteine-rich regions (CRRs) of AbEV1 and other endornavirus. The CXCC motifs are indicated by solid lines and the numbers 1-4

Table 1 Percent aa sequence identity between AbEV1 and other endornaviruses determined by multiple alignments of the complete polyprotein sequence and other conserved domains

Virus	Acronym	Accession no.	Host	Genome length (aa)	Full sequence	MTR	Hel	RdRp
Tuber aestivum endornavirus	TaEV	YP_004123950.1	F	9,760	14.7	30.2	-	29.0
Sclerotinia sclerotiorum endornavirus 1	SsEV1	YP_008169851.1	F	10,770	13.9	29.0	20.9	24.5
Sclerotinia sclerotiorum endornavirus 2	SsEV2	YP_009022070.1	F	10,513	14.4	28.6	20.0	24.3
Gremmeniella abietina type B RNA virus XL2	GaBR-XL2	ABD73306.1	F	10,374	14.3	25.9	20.5	26.4
Gremmeniella abietina type B RNA virus XL1	GaBR-XL1	YP_529670.1	F	10,375	14.1	25.5	20.9	26.3
Rhizoctonia cerealis endornavirus 1	RcEV1	YP_008719905.1	F	17,486	10.6	14.5	17.3	29.3
Rhizoctonia solani endornavirus - RS002	RsEV-RS002	AHL25280.1	F	14,694	-	12.4	9.3	-
Yerba mate endornavirus	YmEV	YP_009046830.1	P	13,954	9.8	–	14.8	28.0
Persea americana endornavirus	PaEV	YP_005086952.1	p	13,459	9.7	-	8.9	25.6
Oryza rufipogon endornavirus	OrEV	YP_438202.1	P	17,635	9.1	-	9.3	27.9
Bell pepper endornavirus	BPEV	YP_004765011.1	P	14,728	11.7	16.9	12.7	25.9
Oryza sativa endornavirus	OsEV	YP_438200.1	P	13,952	10.5	-	9.1	28.0
Vicia faba endornavirus	VfEV	YP_438201.1	P	17,635	10.6	-	10.1	26.3
Phytophthora endornavirus 1	PeV1	YP_241110.1	O	13,883	9.8	-	16.0	27.3
Lagenaria siceraria endornavirus-California	LsEV-CA	YP_009010973.1	P	15,088	10.8	-	-	28.0
Phaseolus vulgaris endornavirus 1	PvEV1	YP_009011062.1	P	13,908	9.9	-	12.7	27.4
Phaseolus vulgaris endornavirus 2	PvEV2	BAM68540.1	P	14,820	10.6	18.8	14.8	24.1
Helicobasidium mompa endornavirus 1	HmEV1	YP_003280846.1	F	16,614	8.8	-	15.6	26.9
Grapevine endophyte endornavirus	GEEV	YP_007003829.1	P	12,154	8.5	-	22.4	19.8
Chalara endornavirus CeEV1	CeEV1	ADN43901.1	F	11,602	9.1	-	13.9	21.0

"-" indicates that this domain was not present in the virus isolate. MTR, methyltransferase; Hel, viral RNA helicase; RdRp, RNA-dependent RNA polymerase; P, plant; F, fungus; O, oomycete. Because the full-length aa sequence of RsEV-RS002 had not be determined, it has been excluded from the RdRp domain comparison

(RcEV1) being the best match (identity, 30 %; E-value, 4e-35; query cover, 97 %). Using the SMART web server, an additional putative cysteine-rich region (CRR) (61aa) was also identified at aa positions 971-1031, sharing maximum sequence identity of 38 % (E-value, 7e-07; query cover, 99 %) with that of Sclerotinia sclerotiorum endornavirus 1 (SsEV1). Multiple alignment analysis of the potential CRRs of AbEV1 and other selected endornaviruses showed that AbEV1 and SsEV1 contained only two highly conserved CXCC signatures, whereas other endornaviruses have three or four of these conserved motifs (Fig. 1b).

A phylogenetic tree based on viral RdRp domain sequences showed that the clade containing endornaviruses was divided into two main clusters: I and II (Fig. 2). Cluster I included endornaviruses with larger genomes that infect members of the Oomycetes and Basidiomycetes as well as plants, and cluster II included viruses with smaller genomes that infect members of the Ascomycetes. AbEV1 was grouped with endornaviruses in cluster II but separated from other endornaviruses, such as TaEV and GaBRV-XL1. The genome organization of AbEV1 was similar to that of endornaviruses in cluster II, which are characterized by smaller genomes and the lack of a UGT domain, as is the case for the endornaviruses GaBRV-XL1 [19] and SsEV1 [20]. Phylogenetic trees based on the full-length aa sequence of the polyprotein and the MTR and Hel domains showed topologies similar to that of the RdRp-based tree (Fig. S2), further supporting the phylogenetic status of AbEV1. In conclusion, we propose that this virus AbEV1 be considered a novel member of the family *Endornaviridae*.

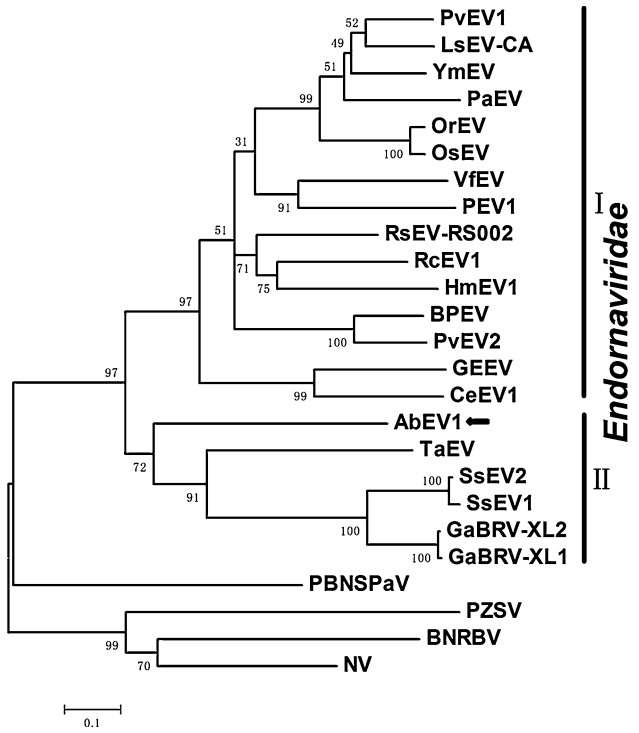

Fig. 2 Phylogenic tree constructed using the viral RdRp sequences of endornaviruses and related viruses, by the neighbor-joining method with 1,000 bootstrap replicates. Values near the branches indicate the percentage of bootstrap replicates supporting the branch. The respective virus names and GenBank accession numbers are listed in Table 1, except for the following: PZSV, pelargonium zonate spot virus (NP_619771.1) (overall aa sequence identity: 24.2 %); BNRBV, blueberry necrotic ring blotch virus (YP_0 04901701.1) (overall aa sequence identity: 22.8 %); NV, Ngewotan virus (AFY98072.1) (overall aa sequence identity: 22.5 %); PBNSPaV, plum bark necrosis stem pitting-associated virus (CDM63857.1) (overall aa sequence identity: 19.8 %)

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