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Molecular characterization of a novel endornavirus from the phytopathogenic fungus *Botrytis cinerea*

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Abstract The complete sequence of a novel endornavirus (Botrytis cinerea endornavirus 1, BcEV1) from the phytopathogenic fungus *Botrytis cinerea* strain HBtom-372 was determined. The BcEV1 coding strand is 11,557 nucleotides long, possessing an open reading frame (ORF) that codes for a polyprotein of 3,787 amino acid residues and lacks a site-specific nick. The polyprotein contains viral methyltransferase (MTR) domain, a cysteine-rich region (CRR), two putative viral helicase (DEXDc-like and Hel-1) domains, and an RNA-dependent RNA polymerase_2 (RdRp_2) domain. In phylogenetic analysis, BcEV1 clustered with several fungal endornaviruses, forming an independent clade, and it was detected in 4.2 % of *B. cinerea* strains collected from central China.

Botrytis cinerea Pers. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a widely distributed plant-pathogenic fungus, causing grey mold disease on more than 1,400 plant species, particularly on many economically important greenhouse-grown horticultural crops including strawberry, cucumber, table grape and tomato [1]. Mycoviruses are viruses that infect fungi and oomycetes [2]. In many cases, mycovirus infection has no visible effect on the host. However, infection by some mycoviruses in plant-pathogenic fungi can impair pathogenicity, which has potential for biocontrol of plant fungal diseases [3]. Mycoviruses are common in *Botrytis* spp., especially *B. cinerea* [4]. Most sequenced mycoviruses of *Botrytis* have been assigned to the viral families *Gammaflexiviridae*, *Alphaflexiviridae*, *Narnaviridae*, *Partitiviridae*, and *Totiviridae* [4], while some remain unclassified [5, 6]. Among the sequenced mycoviruses, Botrytis cinerea mitovirus 1 (BcMV1) [7, 8], Botrytis cinerea RNA virus 1 (BcRV1) [6], and Botrytis cinerea CCg378 virus 1 (Bc378V1) [9] impaired the virulence of *B. cinerea*. However, others, including Botrytis virus F (BVF) [10] and Botrytis virus X (BVX) [11], had no significant effects on host pathogenicity.

Endornaviruses are a group of viruses with double-stranded (ds) RNA genomes infecting plants, fungi and oomycetes without forming virions [12, 13]. Endornavirus genomes range in size from 9.8 to 17.6 kbp and possess a single large open reading frame (ORF) encoding a large polypeptide containing several conserved domains, of which only RNA-dependent RNA polymerase (RdRp) is universally present [14]. In most cases, endornavirus infections do not cause any visible symptoms on their hosts. Nevertheless, Helicobasidium mompa endornavirus 1 (HmEV1) and Vicia faba endornavirus (VfEV) reduce the virulence of the violet root rot fungus *H. mompa* [15] and confer cytoplasmic male sterility to *Vicia faba* plants [16], respectively. To date, endornaviruses have not been reported in *B. cinerea*. In this study, we have characterized a novel putative endornavirus, Botrytis cinerea endornavirus 1 (BcEV1), isolated from strain HBtom-372 of *B. cinerea*.

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B. cinerea strain HBtom-372 was originally isolated from a diseased tomato in Jingmen County, Hubei Province, China, and was stored as described previously [8]. Extraction and purification of dsRNA from HBtom-372 mycelium was performed as described previously [7]. DsRNA was fractionated by agarose gel (1 %, w/v) electrophoresis and visualized by staining with ethidium bromide (1.5 μg/L).

A dsRNA band of high molecular weight was excised and purified (Fig. 1a) using an AxyPrep™ DNA Gel Extraction Kit (Axygen Scientific, Inc. Union City, USA). A cDNA library was produced using a random-primer-mediated PCR amplification protocol [6], cloning and sequencing as described previously [5]. The terminal sequences of the dsRNA were cloned using a standard RLM-RACE procedure [5, 8] performed on three separate occasions (Fig. S1). Two gaps between the cDNA contigs were amplified by RT-PCR with virus-specific primers EV2-F/EV2-R and EV3-F/EV3-R, and the amplicons were subsequently sequenced. All amplicons were separated by agarose gel electrophoresis, cloned into *E. coli* DH5α, and sequenced [8]. All partial cDNA sequences were assembled to obtain the full-length cDNA sequence of the target dsRNA. Sequence analysis, including ORF finding, homology searching with the BlastN and BlastP programs, and multiple sequence alignments, was performed as described previously [5]. Phylogenetic trees based on the sequences of MTR, Hel and RdRp domains were constructed using the neighbor-joining (NJ) method and tested with a bootstrap of 1,000 replicates in MEGA 5.2 [17].

Northern hybridization was performed to confirm the authenticity of the cDNA sequences generated from BcEV1 and the potential presence of a nick at the 5′ terminus of the BcEV1 dsRNA genome. Two DNA probes (nt positions 58-818 for probe 1, nt positions 5305-6019 for probe 2) were designed based of the full-length BcEV1

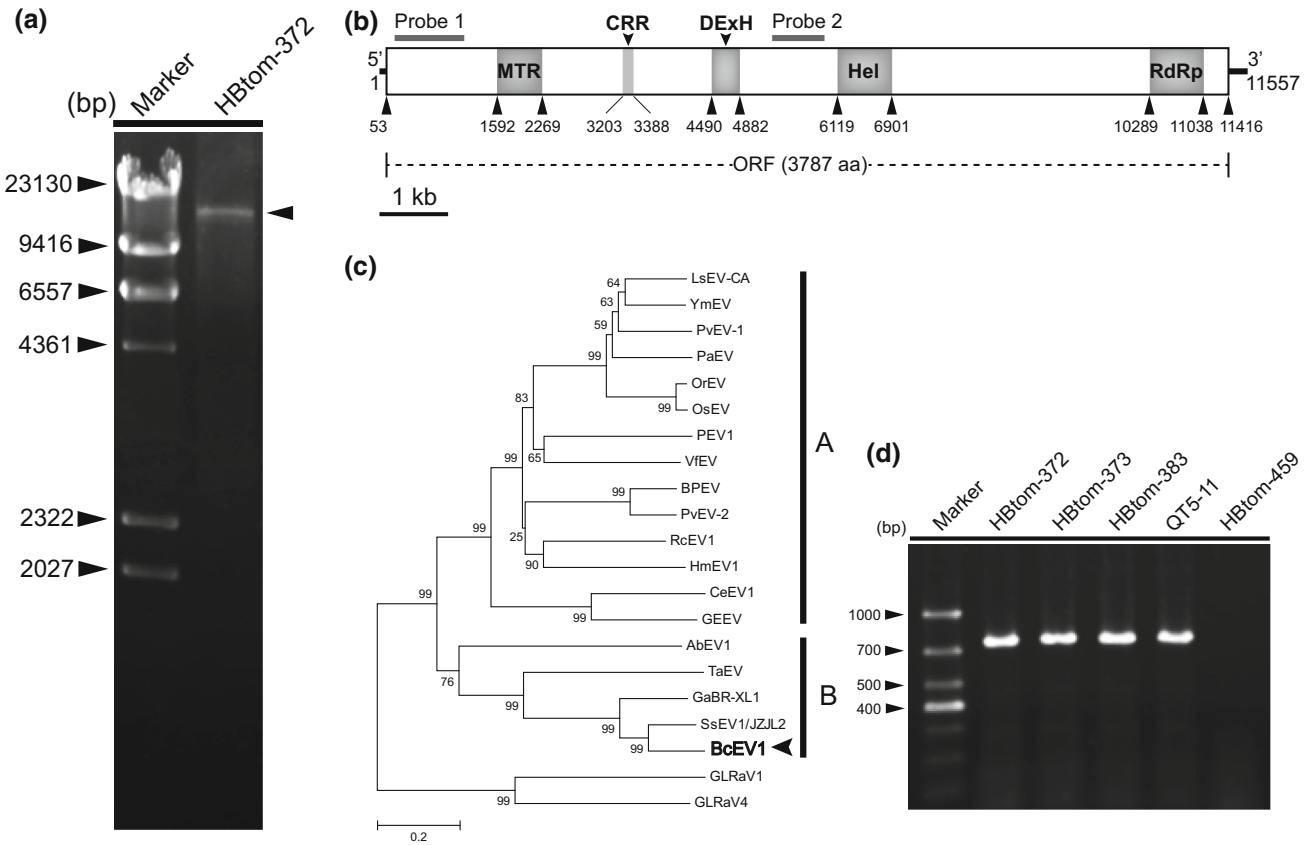

Fig. 1 Molecular characteristics and RT-PCR detection of Botrytis cinerea endornavirus 1 (BcEV1). (a) Agarose gel electrophoresis of gel-purified BcEV1 dsRNA extracted from the mycelium of *Botrytis cinerea* strain HBtom-372. Marker, λ-*Hin*d III digest DNA marker. (b) Schematic diagram of the genome organization of BcEV1. BcEV1 is 11,557 bp long and contains a large ORF encoding a polyprotein of 3,787 aa. Grey boxes are conserved domains: MTR, viral methyltransferase; CRR, cysteine-rich region; DExH box; Hel-1, viral helicase superfamily 1; RdRp, RNA-dependent RNA polymerase. (c) Phylogenetic analysis of BcEV1 and other endornaviruses presented in the NJ trees inferred from the RdRp sequences. For extended names of viruses used for constructing the phylogenetic tree, please refer to Table 1. Grapevine leafroll-associated virus 1 (GLRaV1, GenBank accession no. EF103901) and grapevine leafroll-associated virus 4 (GLRaV4, GenBank accession no. KP313764) were used as outgroups. (d) RT-PCR detection of BcEV1 in four *B. cinerea* strains

Table 1 Percentage of sequence identities between BcEV1 and other endornaviruses according to the multiple alignments of the full-length nucleotide (nt) sequence, polyprotein sequence and the amino acid (aa) residue sequence of different domains

Virus	Acronym	Host[1]	Genome length (nt)	aa sequence identity				Accession no.
				Full sequence	MTR	Hel	RdRp	
Sclerotinia sclerotiorum endornavirus 1	SsEV1/JZJL2	F	10,770	39.1	61.95	35.88	77.2	NC_021706
Gremmeniella abietina type B RNA virus XL1	GaBR-XL1	F	10,375	30.28	48.68	32.57	67.6	NC_007920
Alternaria brassicicola endornavirus 1	AbEV1	F	10290	16.61	28.33	21.19	30.16	NC_026136
Tuber aestivum endornavirus	TaEV	F	9,760	15.63	29	-	41.73	NC_014904
Chalara endornavirus 1	CeEV1	F	11,602	11.14	-	18.73	28.52	GQ494150
Rhizoctonia solani endornavirus - RS002	RsEV-RS002	F	14,694	9.89	15.42	16.3	-	KC792590
Rhizoctonia cerealis endornavirus 1	RcEV1	F	17,486	8.81	16.81	13.79	30.98	NC_022619
Helicobasidium mompa endornavirus 1	HmEV1	F	16,614	8.43	-	13.7	27.91	AB218287
Grapevine endophyte endornavirus	GEEV	P	12,154	12.44	-	14.45	25.88	NC_019493
Oryza sativa endornavirus	OsEV	P	13,952	11.51	-	16.73	25.49	D32136
Persea americana endornavirus	PaEV	P	13,459	11.63	-	17.8	27.06	NC_016648
Yerba mate endornavirus	YmEV	P	13,954	11.49	-	14.39	27.73	NC_024455
Oryza rufipogon endornavirus	OrEV	P	17,635	11.43	-	18.63	25.1	NC_007649
Phaseolus vulgaris endornavirus 1	PvEV-1	P	13,908	10.92	-	18.63	25.88	AB719397
Phaseolus vulgaris endornavirus 2	PvEV-2	P	14,820	10.72	15.49	17.49	26.27	AB719398
Bell pepper endornavirus	BPEV	P	14,728	10.43	13.27	16.73	29.8	NC_015781
Lagenaria siceraria endornavirus-California	LsEV-CA	P	15,088	9.73	-	-	26.27	NC_023641
Vicia faba endornavirus	VfEV	P	17,635	8.86	-	15.85	25.2	AJ000929
Phytophthora endornavirus 1	PEV1	O	13,883	11.1	-	11.49	25.39	AJ877914

[1] F, fungus; P, plant; O, oomycete

cDNA sequence. BcEV1 dsRNA was separated on a 1 % (w/v) agarose gel containing 2 % formaldehyde in 1× MOPS buffer [18], and the denatured RNAs were transferred to Immobilon-Ny membranes (Millipore, Bedford, MA, USA) by capillary transfer [19]. The probes were prelabeled with enzyme as described by the manufacturer (GE Healthcare) for hybridization with the denatured dsRNA blotted on the membrane. The chemiluminescent signals of the probe-RNA hybrids were detected using a CDP-Star kit (GE Healthcare).

In order to investigate the distribution of BcEV1 in China, 94 *B. cinerea* strains from Shaanxi, Shandong and Hubei provinces (15 counties, Table S2) were tested for the presence of BcEV1 using RT-PCR with the primer pair E-RT-F and E-RT-R (Table S1) designed to amplify a specific band of 740 bp in size.

Sequencing data revealed that the complete genome sequence of the dsRNA was 11,557 bp long, with a GC content of 38.1 % which contained a single large ORF (GenBank accession no. KU923747) and two short untranslated regions (UTRs), 52 nt and 141 nt in length, at the 5′ and the 3′ termini, respectively. The 3′-UTR terminated with seven cytosine residues. Northern hybridization analysis showed that only one dsRNA element was detected by both probe 1 and probe 2 (Fig. S2a), suggesting no site-specific nicks at the 5′-terminus of the BcEV1 coding strand.

The large ORF was predicted to encode a putative polypeptide of 3,787 aa residues (Fig. 1b). The results of BlastP search showed that this polypeptide is most closely related to endornavirus-encoded polypeptides, particularly those of Sclerotinia sclerotiorum endornavirus 1 (SsEV1/JZJL2, 39.1 % aa identity) and Gremmeniella abietina type B RNA virus XL1 (GaBRV-XL1, 30.28 % aa identity) (Table 1). CDD database searches and multiple sequence alignment analysis revealed that the polypeptide contained a viral MTR domain, a DEXDc domain (DExH box), a viral Hel superfamily 1 domain, and an RdRp_2 superfamily domain (Fig. 1b, Fig. S3). Furthermore, a cysteine-rich region (CRR) with a cysteine content of 22.9 % from nucleotide 3203-3388 was detected. Two conserved signatures sequences "CxCCG" were discovered following multiple alignment analysis of the CRRs in BcEV1 and other endornaviruses (Fig. S2b). The CRR with CxCCG was hypothesized to possibly have an enzymatic role during polyprotein processing [20].

Three phylogenetic trees with similar topologies were generated based on the polypeptide sequences of the RdRp (Fig. 1c), viral Hel and MTR domains (Fig. S4a, b). Two subclades, A and B, were detected in all three phylogenetic

trees, with BcEV1 clustering in subclade B, together with other endornaviruses with genome size below 12.5 kbp. This result is consistent with previous phylogenetic investigations of endornaviruses [18] and the proposed establishment of two genera within the family *Endornaviridae*.

In this study, the complete sequence of an endornavirus from the *B. cinerea* strain HBtom-372 is described. Based upon currently valid species demarcation criteria for the family *Endornaviridae* (i.e., less than 75 % sequence identity), BcEV1 should be considered a novel endornavirus. BcEV1 infection was detected in only four of the 94 tested *B. cinerea* strains (Fig. 1d) collected in several provinces of central China. These results indicate that BcEV1 might be distributed in other regions where *B. cinerea* occurs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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