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Genome sequence of a novel victorivirus identified in the phytopathogenic fungus *Alternaria arborescens*

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Abstract Strains of the phytopathogenic fungus *Alternaria* spp. have been found to contain a variety of double-stranded RNA (dsRNA) elements indicative of mycovirus infection. Here, we report the molecular characterization of a novel dsRNA mycovirus, Alternaria arborescens victorivirus 1 (AaVV1), from *A. arborescens*, the tomato pathotype of *A. alternata*. Using next-generation sequencing of dsRNA purified from an *A. arborescens* strain from the United States of America, we found that the AaVV1 genome is 5203 bp in length and contains two open reading frames (ORF1 and 2) that overlap at the tetranucleotide AUGA. Proteins encoded by ORF1 and ORF2 showed significant similarities to the coat protein (CP) and the RNA-dependent RNA polymerase (RdRp), respectively, of dsRNA mycoviruses of the genus *Victorivirus*. Pairwise comparisons and phylogenetic analysis of the deduced amino acid sequences of both CP and RdRp indicated that AaVV1 is a member of a distinct species of the genus *Victorivirus* in the family *Totiviridae*.

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Introduction

Mycoviruses are widespread in a variety of fungal species [7]. An increasing number of mycoviruses have been reported in recent years, which have single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) or single-stranded DNA (ssDNA) genomes. Of these, dsRNA genomes are the most prevalent. Mycoviruses with dsRNA genomes are classified into the seven families: *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Reoviridae*, *Endornaviridae*, *Megabirnaviridae* and *Quadriviridae* [7]. Also, mycoviruses with linear positive-sense ssRNA genomes are currently classified into the five families: *Alphaflexiviridae*, *Barnaviridae*, *Gammaflexiviridae*, *Hypoviridae* and *Narnaviridae*. In order to screen for mycoviruses with different types of genomes, we can commonly employ dsRNA purification and subsequent electrophoresis, which can be applied to mycoviruses that possess a dsRNA genome as well as to those having a ssRNA genome, which produce a dsRNA intermediate during replication [15]. Although most mycoviruses cause no apparent symptoms on their host, some of them reduce the virulence of host-phytopathogenic fungi, which highlights the potential of mycoviruses as biological control agents against plant fungal pathogens [7].

Alternaria alternata is a ubiquitous fungus found in a variety of plants, causing leaf spot or other diseases. Many

A. alternata strains have been found to harbor dsRNA elements of different sizes, which are indicative of mycovirus infections [9, 19]. Indeed, we previously found a mycovirus with four dsRNA segments, Alternaria alternata virus 1 (AaV1), from the non-pathogenic *A. alternata* strain EGS 35-193, which is closely related to Aspergillus mycovirus 341 and Aspergillus foetidus virus-fast (AfV-F) [2, 11]. Moreover, we have also found that a Japanese pear pathotype of *A. alternata* contains dsRNA elements responsible for phenotypic morphological changes in the host [6]. However, in most of these *A. alternata* strains, the nucleotide sequences and effects on host fungi of these dsRNAs remain unknown. Recently, mycoviruses have also been found in other members of the genus *Alternaria*. One example is Alternaria longipes dsRNA virus 1, found in *A. longipes*, which causes brown spot disease on tobacco plants. This virus contains a single dsRNA genome with two open reading frames (ORFs) and belongs to a new family of dsRNA mycoviurses [13]. Also, Alternaria brassicicola endornavirus 1 was found in *A. brassicicola*, which causes black spot disease in rapeseed [18].

Here, we report the complete genome sequence of a dsRNA virus, Alternaria arborescens victorivirus 1 (AaVV1), from *A. arborescens* (synonyms: *A. alternata* tomato pathotype, *A. alternata* f. sp. *lycopersici*) [1, 8, 16]. A single dsRNA segment of approximately 5.2 kbp has two ORFs putatively coding for the coat protein (CP) and RNA-dependent RNA polymerase (RdRp). Phylogenetic analysis of the RdRp indicated that AaVV1 is a member of a distinct virus species of the genus *Victorivirus*.

Provenance of the virus material

The *A. arborescens* strain used in this study was EGS 39-128 [6]. Fungi were initially grown on dextrose agar plates at 25 °C, and fungal mycelia from the plate were cultured in YG broth medium (0.5 % (w/v) yeast extract and 2 % glucose) with reciprocal shaking at 25 °C for 7 days. Total nucleic acid extraction and purification of dsRNA were performed as described previously [15]. The purity and size of the dsRNA were confirmed by electrophoresis in 1.0 % (w/v) agarose gels, which indicated that the strain EGS 39-128 contained an approximately 5.2-kbp dsRNA as described previously [6] (Fig. S1). Using an input of 27.5 ng of this dsRNA, library preparation for deep sequencing was carried out using an NEBNext® Ultra RNA Library Prep Kit for Illumina Version 2.0 (New England Biolabs, Ipswich, MA). Following measurement of the library quality and quantity on a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad CA), sequencing was performed on a MiSeq benchtop sequencer using a MiSeq Reagent Kit v2 (50 cycles) (Illumina, San Diego, CA) with 51 single-end reads. The raw sequence reads were formatted to FASTQ data using MiSeq Reporter v2.3 (Illumina), followed by read trimming and *de novo* assembly of contigs using CLC Genomics Workbench 6.5.1 (CLC bio, Aarhus, Denmark). Assembled sequences were compared to those in the GenBank database using BLASTP in the National Center for Biotechnology Information (NCBI). Multiple alignments and phylogenetic analysis of the amino acid sequences were perfomed using MEGA 6.0 [20].

Sequence properties

The 2,270,543 raw reads obtained by MiSeq sequencing were assembled into 2255 contigs, 28 of which had an average coverage of more than 100. One of these contigs, with an average coverage of 198, was 5206 bp in length, which corresponds well to the length of the dsRNA purified from EGS 39-128. A BLAST search of the 5206-bp contig revealed significant similarity to the complete genome sequence of Coniothyrium minitans RNA virus (CmRV), a dsRNA mycovirus that belongs to the genus *Victorivirus* (*E*-value, 0; greatest hit length, 2447) [4]. A whole-genome comparison between the 5206-bp contig and CmRV showed 66 % identity. The nucleotide sequence of this putative mycovirus, which we called "Alternaria arborescens victorivirus 1" (AaVV1), has been deposited in the GenBank/EMBL/DDBJ databases under accession number LC086813.

The 5'- and 3'-terminal genomic sequences of AaVV1 were determined using a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA). As a result, we obtained almost identical terminal sequences by RACE, which indicated that the AaVV1 genome is 5203 bp in length with a GC content of 58.0 %. The GC content of the AaVV1 genome is similar to that of other victorivirus genomes, including CmRV (59.2 %), Magnaporthe oryzae virus 2 (MoV2) (61.9 %) and Epichloe festucae virus 1 (EfV1) (60.3 %). An ORF search revealed that the AaVV1 genome contains two ORFs in the same strand (Fig. 1a). ORF1 begins at an AUG codon (nt 327-329) and terminates with a UGA codon (nt 2655-2657). ORF2 begins at an AUG codon (nt 2654-2656) and terminates with a UAA codon (nt 5141-5143). ORF2 is in the -1 frame of ORF1, with the AUG codon of the ORF2 overlapping the UGA codon of ORF1 at the tetranucleotide AUGA at nt 2653-2656, which is a characteristic feature of many victoriviruses [10, 12, 14]. The 5' and 3' untranslated regions (UTR) were found to be 326 and 60 bases long, respectively. In the 5'-UTR, the octanucleotide sequence 5'-AGGGUUCC-3', which is conserved in several victoriviruses, including CmRV and EfV1, was found at nt

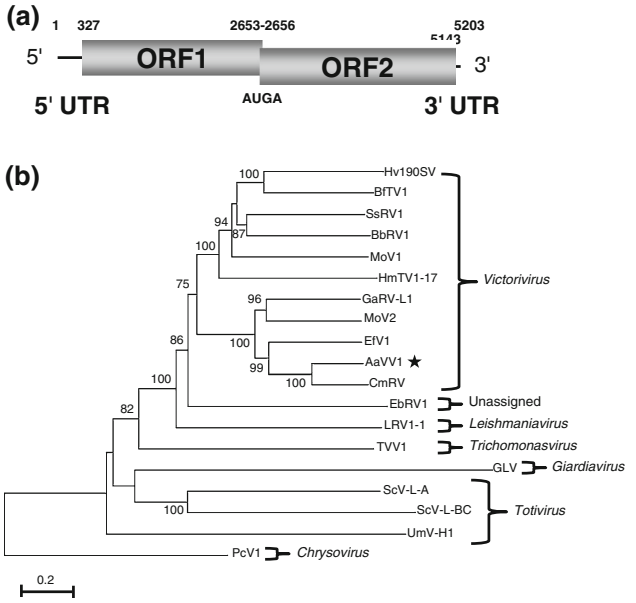

Fig. 1 Genome organization of Alternaria arborescens victorivirus 1 (AaVV1) and its phylogenetic relationships to selected viruses of the family *Totiviridae*. (a) Genome organization of AaVV1. (b) Phylogenetic tree of AaVV1 and other selected dsRNA viruses based on RdRp amino acid sequences, generated by the neighbor-joining method. A star indicates the position of AaVV1. Brackets indicate genera. Numbers proximal to nodes represent bootstrap support values for 1,000 replicates (only the bootstrap scores over 70 % are shown). The scale bar below the tree represents the phylogenetic distance. The following RdRp amino acid sequences were used to construct the tree, with their abbreviated names and accession numbers in parentheses: Epichloe festucae virus 1 (EfV1, AM261427), Coniothyrium minitans RNA virus (CmRV, AF527633), Saccharomyces cerevisiae virus L-A (ScV-L-A, J04692), Saccharomyces cerevisiae virus L-BC (ScV-L-BC, U01060), Gremmeniella abientina RNA virus L1 (GaRV-L1, AF337175), Leishmania RNA virus 1-1 (LRV1-1, M92355), Helicobasidium mompa totivirus 1-17 (HmTV1-17, AB085814), Helminthosporium victoriae virus 190S (Hv190SV, U41345), Sphaeropsis sapinea RNA virus 1 (SsRV1, AF038665), Magnaporthe oryzae virus 1 (MoV1, AB176964), Magnaporthe oryzae virus 2 (MoV2, AB300379), Trichomonas vaginalis virus 1 (TVV1, U08999), Ustilago maydis virus H1 (UmV-H1, U01059), Giardia lamblia virus (GLV, L13218), Botryotinia fuckeliana totiviurs 1 (BfTV1, AM491608), Beauveria bassiana RNA virus 1 (BbRV1, HE572591), and Eimeria brunetti RNA virus 1 (EbRV1, AF356189). Penicillium chrysogenum virus (PcV, AF296439) was used as a outgroup

264-271, 56 nt upstream of the AUG initiation codon of ORF1 [17].

ORF1 was predicted to encode a protein of 776 amino acids (aa) with a molecular mass of 80.9 kDa. A BLASTP search indicated that this protein has significant similarity to CPs of viruses of the family *Totiviridae*: 80 % identity to the CP of CmRV and 60-65 % identity to those of related victoriviruses, including MoV2. Notably, the CP of AaVV1 has a proline-rich region near its carboxy terminus, which is also prominent in CmRV, EfV1, Gremmeniella abientina RNA virus L1 (GaRV-L1), MoV2, and Helminthosporium victoriae virus 190S (Hv190SV) (Fig. S2).

The predicted translation product of the ORF2 of AaVV1 is 829 aa in length with a calculated molecular mass of 92.5 kDa. This protein was most closely related to the putative RdRp of CmRV, which has 70 % identity (*E*-value of 0 and query cover of 100 %). It also showed considerable sequence similarity to the RdRps of EfV1 (54 %) and MoV2 (49 %), suggesting that AaVV1 is closely related to dsRNA mycoviruses in the genus *Victorivirus*. After we had begun our analysis, a partial sequence of a victorivirus isolated from *A. arborescens* was reported with the accession number KJ013354, but we found that the RdRp amino acid sequence of AaVV1 and the protein sequence of KJ013354 shared only 40 % identity, indicating that they belong to distinct virus species. A conserved domain search revealed that the ORF2 product of AaVV1 possessed the eight conserved motifs of RdRps of dsRNA viruses (RdRP_4; pfam02123), which was confirmed by an amino acid sequence alignment of RdRps of selected victoriviruses [3] (Fig. S3).

A phylogenetic tree based on RdRp amino acid sequences of AaVV1 and related mycoviruses demonstrated that AaVV1 belongs to a well-supported clade containing viruses of the genus *Victorivirus*, especially in a subclade containing CmRV, EfV1, MoV2 and GaRV-L1 (Fig. 1b). A phylogenetic tree based on CP amino acid sequences had a similarity topology to the one based on RdRp (Fig. S4). As mentioned above, viruses in the subclade containing AaVV1 have a proline-rich region at the carboxy terminus of their CP, which supports the idea that they are evolutionary closely related. However, the topology of the tree based on both CP and RdRp amino acid sequences of victoriviruses does not match the host phylogeny, suggesting that horizontal transfer between different host fungi could contribute to the evolution of victoriviruses.

Moreover, RNA secondary predictions revealed that AaVV1 has an H-type pseudoknot structure and a strong stem-loop structure upstream and downstream, respectively, of the tetranucleotide AUGA motif located at the junction of ORF1 and ORF2 (Fig. S5). These unique RNA structures may be important for the translation of ORF2 of AaVV1, as has been suggested for other victoriviruses [5].

Overall, based on the results of the present study, we propose that AaVV1 should be considered a distinct member of the genus *Victorivirus*. Further studies are needed to determine whether AaVV1-related viruses infect other *A. alternata* strains and to evaluated their effect on the growth and virulence traits of the host fungus.

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Compliance with ethical standards

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Conflict of interest The authors declare no conflict of interest.

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

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