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ANNOTATED SEQUENCE RECORD

Complete genome sequence of a novel mitovirus from the phytopathogenic fungus *Rhizoctonia oryzae-sativae*

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Abstract A double-stranded RNA (dsRNA) segment was isolated from the filamentous phytopathogenic fungus *Rhizoctonia oryzae-sativae* and its full-length cDNA sequence (3038 nucleotides) was determined. Sequence analysis revealed that a large open reading frame (ORF) is present on the positive strand of this dsRNA segment when the mitochondrial genetic code was applied. The ORF encodes a putative RNA-dependent RNA polymerase, which shares the closest similarity with Rhizoctonia mitovirus 1 and Rhizophagus sp. RF1 mitovirus, with 43% and 29% identity, respectively. This dsRNA segment represents the replication form of a novel mitovirus that was temporarily designated Rhizoctonia oryzae-sativae mitovirus 1 (RoMV1). Phylogenetic analysis further suggested that RoMV1 belongs to the family *Narnaviridae*. This is the first study to report a mitovirus genome sequence in the phytopathogenic fungus *R. oryzae-sativae*.

Introduction

Rhizoctonia is considered ubiquitous in soils and represents an important plant pathogenic Basidiomycota species complex, which is capable of infecting a wide variety of plant species including rice, maize and wheat [1]. Three Rhizoctonia species comprising *Rhizoctonia solani*, *R. oryzae* and *R. oryzae-sativae* are responsible for sheath disease in rice. *R. solani* causes rice sheath blight, while *R. oryzae* and *R. oryzae-sativae* both cause similar disease symptoms with aggregate sheath spot. *R. oryzae-sativae* (whose teleomorph is known as *Ceratobasidium oryzae-sativae*) was first reported in China [2], and has been identified in the United States [3], Australia [4], and other Asian countries [5, 6]. *R. oryzae-sativae* survives overwintering periods as sclerotia or mycelium in rice crop debris or in the soil [7]. Although aggregate sheath spot disease is generally assumed to be a minor disease of rice worldwide, this disease has caused enormous yield losses in some geographic regions, such as Australia, with 20% yield losses [8], as well as Korea and California, though without specific reporting of the percentage yield losses [9].

Mycoviruses living within their hosts (fungi) for replication have been described in major important plant pathogen fungi [10]. *R. solani* has been confirmed to harbor diverse mycoviruses or dsRNA elements [11]. Nine mycoviruses have so far been characterized with complete genomes. Four of the nine mycoviruses have been assigned to three families: *Partitiviridae* (two mycoviruses infecting *R. solani* AG-3 and AG-1 IA) [12, 13], *Narnaviridae* (one mycoviruses infecting *R. solani* AG-3) [14], and *Endornaviridae* (one mycovirus infecting *R. solani* AG-3) [15]. Five of the nine mycoviruses have not been assigned into any approved genus or family [16–20]. Up to now, no known mycoviruses have been isolated and reported in *R. oryzae* and *R. oryzae-sativae*. Previous studies have shown that the simplest replicating viruses belong to the *Narnaviridae* family including the *Narnavirus* and *Mitovirus* genus [21]. The genome length of these mycoviruses is between 2.3 to 3.5 kilobases (kb) and unipartite,

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containing a single open reading frame encoded RNA-dependent RNA polymerase protein. Mitovirus is usually believed to locate in the mitochondria of the host and does not form any infectious virions [21]. We report here the first mycovirus infecting *R. oryzae-sativae*, which can, accordingly, advance our understanding of the ecology, evolution and classification of mycoviruses.

Provenance and sequencing of strains

Two *R. oryzae-sativae* strains 89-1 and 69 were isolated from a disease lesion of rice aggregate sheath spot in a rice plant collected from Wuxue County, Hubei Province, PR China. The mycelium (approximately 0.5 g) of *R. oryzae-sativae* strain was ground into fine powder in the presence of liquid nitrogen. The dsRNA segment was obtained from the mycelium powder in a dsRNA extraction buffer (0.2 Mm Glycine, 0.1 mM Na_2HPO_4, 0.6 mM NaCl, pH = 9.6), as previously described [22]. The extracted dsRNA segments were treated with DNase I and S1 nuclease as described by the manufacturer (Takara, Dalian, China). The treated dsRNA segment was separated on a 1% agarose gel (usually 120 V for 40 min) and purified with a gel extraction kit (Axygen® Brand Products). *R. oryzae-sativae* strain 69 was confirmed to be a dsRNA-free strain. The purified dsRNA from strain 89-1 was used for a reverse transcription reaction. A cDNA library of the purified dsRNA was constructed following a process described previously to develop cDNA [22]. To obtain the terminal sequences of the dsRNA, the purified dsRNA was ligated with an adaptor PC3-T7loop (5'-p-GGATCCCGGGAATTCGGTAATACGACTCACTA TATTTTTATAGTGAGTCGTATTA-OH-3') at 4°C for 18 h with T4 RNA ligase (Takara, Dalian, China) [22]. The PC3-T7loop-ligated dsRNA was purified by precipitation and dissolved in double-distilled water, and cDNA was later synthetized according to a previously reported protocol [22]. The terminal sequences were amplified with specific primers designed based on the already obtained dsRNA sequences and PC2 (5-p-CCGAATTCCCGGGATCC-3), which is complementary to the ligating adapter of the PC3-T7loop. The PCR product was purified, ligated into the pMD18-T Vector (Takara, Dalian, China) and then sequenced. Genome sequence assembly was conducted using DNAMAN 6.0 software (Lynnon Biosoft). Based on the obtained full-length cDNA sequences, gene-specific primers were designed and used for sequencing. Each base was determined by sequencing at least three independent overlapping clones in both orientations. Sequence analysis of the putative conserved domains was performed with the National Center for Biotechnology Information (NCBI) BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved domain and local sequence similarity information for the mitovirus were collected from the NCBI database and aligned using ClustalW software using the default parameters. Phylogenetic tree construction was performed with MEGA 6.0 software [23].

The obtained full-length cDNA sequences were further confirmed by northern blotting. The northern blotting analysis of RoMV1 was carried out following a standard protocol, as previously described, with minor adjustments [24]. The extracted dsRNA samples were separated on a 1% agarose gel for 3 hours at 65 volts. The gel was successively treated in 0.05 M NaOH for 30 min, and then rinsed with ddH_2O and 20 × SSC buffer (pH = 7). RNA was then transferred from the treated gel to a Hybond-N membrane (Amersham) in 20 × SSC buffer (pH = 7) using the capillary method. The fragment was amplified with specific primers (F1: 5'-AAACCAGTTAGTTGGCGTGATTGC-3' and R1: 5'-CACATGATTGAGCTGTCTTTGATTT-TACCC-3') based on the genome sequence of RoMV1 and used with a probe labeled with digoxigenin.

Sequence properties

A dsRNA segment, the genome replication form of a novel mitovirus Rhizoctonia oryzae-stativae mitovirus 1 (RoMV1), was successfully extracted from the mycelium of *R. oryzae-sativae* strain 89-1 (Fig. 1A). The full-length cDNA sequence of RoMV1 was determined via a combination of methods including conventional random priming cDNA synthesis, RT-PCR and RACE cloning. The genome of RoMV1 was confirmed by northern blotting with the specific probe for RoMV1 (Fig.1A), which revealed that strain 89-1 harbors RoMV1. The genome sequence of RoMV1 was deposited in NCBI database under accession number KU057949. The genetic organization of RoMV1 is shown in Fig. 1B. RoMV1 consists of 3038 nucleotides (nts) and has a rich A + U content of 60.7%. The lengths of the 5'- and 3' -untranslated regions (UTRs) of the positive strand are 231 and 346 nts, respectively. Like other previously reported mitoviruses [25, 26], two potential stem-loop structures in the 5'- and 3'-UTRs of the positive strand of RoMV1 were predicted, with an initial ΔG value of −19.50 kcal/mol and −35.20 kcal/mol, respectively. The 5' and 3' terminal sequences also formed a potential panhandle structure with an initial ΔG value of −33.80 kcal/mol (Fig. 1C).

Analysis of the complete cDNA sequence revealed that RoMV1 contains a single large putative ORF when the mitochondrial genetic code was invoked. It is worth noting that the same ORF was predicted using the standard genetic code, since the RoMV1 CDS does not have UGA as a stop codon as can be found in standard codon usage. Since RoMV1 was phylogenetically related to the members of

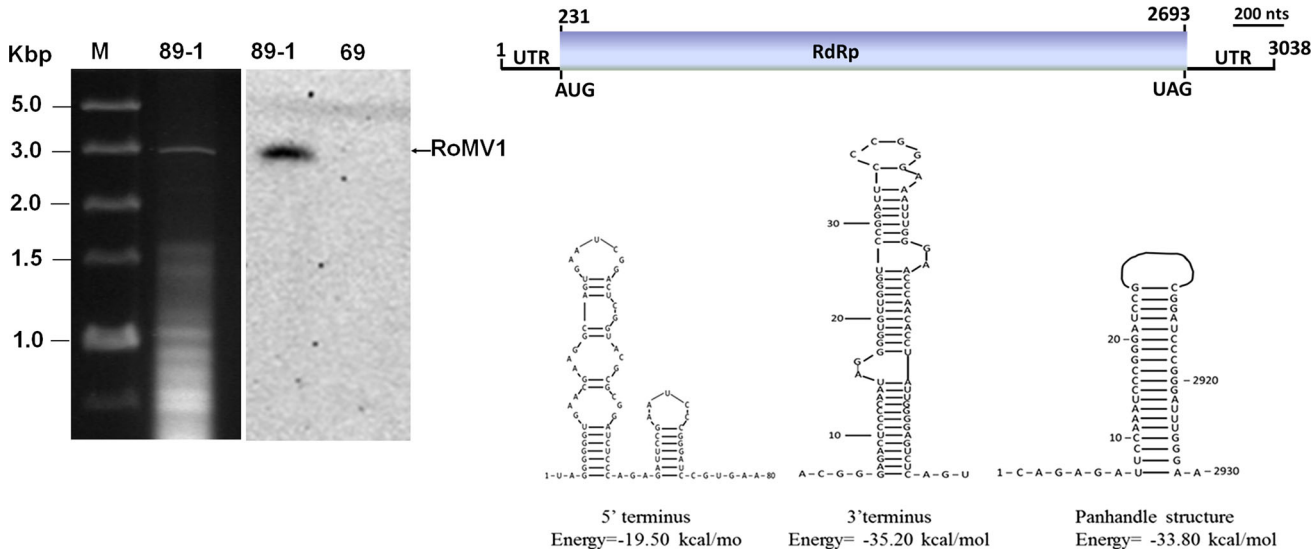

Fig. 1 (A) Electrophoresis of dsRNA extracted from strain 89 of *R. oryzae-sativae* in a 1% agarose gel (left figure) and hybridization blotting of the RoMV1 genomic RNA (right figure). Lane M, DNA molecular weight marker. (B) Molecular characterization of Rhizoctonia oryzae-sativae mitovirus 1,3038nt in length with a large ORF of 2460nt. The RdRp is represented by the rectangular box and the untranslated regions (UTR) at the termini are also shown. The bar (right upper) represents 200 nucleotides. (C) Predicted secondary structure of the 5'- (light) and 3'-termini (middle) from RoMV1. A putative panhandle structure (right) formed by the complementary 5' and 3' termini sequences is also shown. Mfold online software (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) was used to fold the RNA secondary structure and calculate the initial energy

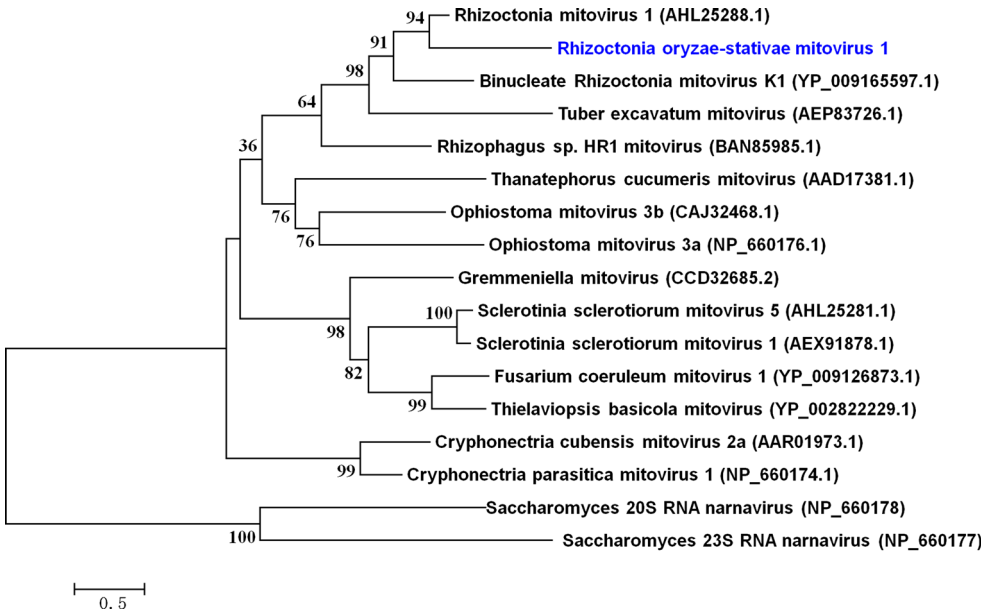

Fig. 2 A Neighbor-joining unrooted phylogenetic tree was made from the multiple alignment of the RdRps of RoMV1 and other mitoviruses. Individual accession numbers follow each mitovirus name. Bootstrap values (in percent) obtained after 1,000 replicates are indicated on the branches, and branch lengths correspond to genetic distance; the scale bar at the lower left corresponds to a genetic distance of 0.5

genus *Mitovirus*, the mitochondrial genetic code was applied for RoMV1. The RoMV1 ORF codes for a putative protein (RdRp) of 819 aa with an expected molecular mass of 91.983 kDa. Multiple alignment suggested that the RdRp of RoMV1 contains six conserved motifs (I-VI), which is a specific characteristic of the *Mitovirus* genus [27]. A BLASTP search showed that the RdRp of RoMV1 shares 43% and 29% identity with the RdRp of Rhizoctonia mitovirus 1 and Rhizophagus sp. RF1 mitovirus. Rhizoctonia mitovirus 1 infected *R. solani* AG-3 was isolated from New Zealand [14]. Rhizophagus sp. RF1 mitovirus was isolated from arbuscular mycorrhizal (AM) fungi in Japan [28]. Phylogenetic analysis of RoMV1 is presented in Fig.2 revealing that RoMV1 is a potential new member within the *Mitovirus* genus (Fig. 2). As far as we know, no mycovirus, so far, has been described to infect *R. oryzae-sativae* isolates. Thus RoMV1 is the first reported mitovirus in the plant pathogenic fungus *R. oryzae-sativae*.

Compliance with ethical standards

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Conflict of interest All authors declare that they have no conflicts 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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