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Novel mitoviruses in *Rhizoctonia solani* AG-3PT infecting potato

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ABSTRACT

Double-stranded RNA (dsRNA) elements are ubiquitous in *Rhizoctonia solani*. Total dsRNA was randomly amplified from a *R. solani* isolate (RS002) belonging to anastomosis group-3PT (AG-3PT), associated with black scurf in potato. Assembly of resulting cDNA sequences identified a nearly complete genome of a novel virus related to the genus *Mitovirus* (family *Narnaviridae*), herein named Rhizoctonia mitovirus 1 RS002 (RMV-1-RS002). The 2797 nucleotide partial genome of RMV-1-RS002 is A-U rich (59.06 %), and can be folded into stable stem-loop structures at 5′ and 3′ ends. Universal and mold mitochondrial codon usages revealed a large open reading frame in the genome, putatively encoding an 826 amino acid polypeptide, which has conserved motifs for mitoviral RNA-dependent RNA polymerase. The full length putative polypeptide shared 25.6 % sequence identity with the corresponding region of Tuber excavatum mitovirus (TeMV). The partial genome of a second mitovirus (proposed name Rhizoctonia mitovirus 2 RS002 (RMV-2-RS002)) was also amplified from RS002. A nearly identical copy of RMV-1-RS002 was detected in two additional AG-3PT isolates. These data indicate that multiple mitoviruses can exist in a single isolate of *R. solani* AG-3PT, and that mitoviruses such as RMV-1-RS002 are probably widespread in this pathogen. The roles of mitoviruses in the biology of *R. solani* AG-3PT remain unknown.

Introduction

Mitoviruses are unencapsidated positive sense single-stranded RNA (ssRNA) viruses belonging to the family *Narnaviridae*. They are considered to be the simplest among all mycoviruses described to date (Ghabrial & Suzuki 2009), comprising of AU-rich genomes (mostly greater than 60 %), with an abundance of either an A or U at the third base position of codons (Hong *et al.* 1998; Park *et al.* 2006; Wu *et al.* 2010). Their genomes have a single large open reading frame (ORF), which encodes a putative polypeptide with motifs conserved in RNA-dependent RNA polymerases (RdRps) (Ghabrial & Suzuki 2009). Despite having ssRNA genomes, mitoviruses exist in host cells mostly as double-stranded RNA (dsRNA) replicative forms; although a number of studies have reported an over abundance of single-stranded copies (Hong *et al.* 1998; Deng *et al.* 2003; Park *et al.* 2006). Mitoviruses are predominantly localised to the mitochondria (Koonin & Dolja 2014).

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Mitoviruses have been reported in several economically important plant pathogenic fungi, including the chestnut blight pathogen *Cryphonectria parasitica* (Polashock & Hillman 1994), the Dutch elm disease pathogen *Ophiostoma novo-ulmi* (Hong *et al.* 1998, 1999), the violet root rot pathogen *Helicobasidium mompa* (Osaki *et al.* 2005), the conifer pathogen *Gremmeniella abietina* (Botella *et al.* 2012), the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Xie & Ghabrial 2012; Khalifa & Pearson 2014), the pine pitch canker disease pathogen *Fusarium circinatum* (Martínez-Álvarez *et al.* 2014), and the cosmopolitan fungal pathogen *Botrytis cinerea* (Wu *et al.* 2010). In several cases, the mitoviruses have been associated with diminished virulence (hypovirulence) of their hosts (Hong *et al.* 1999; Wu *et al.* 2010; Xie & Ghabrial 2012). Furthermore, their potential to reduce host virulence upon transfer into virus-free hosts by hyphal fusion (anastomosis) has been demonstrated experimentally (Wu *et al.* 2007; Wu *et al.* 2010; Xie & Ghabrial 2012).

Rhizoctonia solani Kuhn (teleomorph = *Thanatephorus cucumeris*) is a cosmopolitan, soilborne basidiomycete that causes diseases to several economically important crops worldwide (Anderson 1982). *Rhizoctonia solani* has been proposed to be a collective species comprising of at least 13 anastomosis groups (AGs). AGs are mostly non-interbreeding populations with distinct morphologies, host ranges and epidemiology (Carling *et al.* 2002). Rhizoctonia-related diseases of potato include black scurf, stem canker and tuber malformation. These diseases remain major concerns for potato production in New Zealand (Das *et al.* 2014a), and collectively may be responsible for losses of up to $NZ 75 million each year (equivalent of up to 20 % of the total value of the industry) (Potatoes New Zealand, unpublished data).

In *R. solani*, dsRNA elements have been associated with hypovirulence and increased virulence (hypervirulence) (Castanho *et al.* 1978; Finkler *et al.* 1985; Jian *et al.* 1997). However, the molecular identities of these elements associated with hypo- and hypervirulence of *R. solani* have remained unknown in the majority of cases. Yet, a variety of mycoviruses or mycovirus-related dsRNA elements have been detected in this pathogen (Bharathan & Tavantzis 1990, 1991; Kousik *et al.* 1994; Jian *et al.* 1998; Lakshman *et al.* 1998). Das *et al.* (2014b) recently showed that dsRNA elements are ubiquitous in potato-infecting *R. solani* isolates belonging to AG-3PT and AG-2Nt in New Zealand, and identified that the large dsRNA elements in selected isolates were genomes of endornaviruses. In the same study, dsRNA elements of c. 2.9 kb (typical of *Mitovirus* genomes) were also detected in all *R. solani* isolates belonging to a widely distributed AG-3PT group in New Zealand (Das *et al.* 2014b). Here, we describe the characterisation of these dsRNAs as genomes of novel mitoviruses, and demonstrate that multiple mitoviruses can co-inhabit individual isolates of *R. solani* AG-3PT.

Materials and methods

Fungal isolates

The dsRNA elements were characterised from three representative isolates (RS002, RS006-2 and RS058-1) of *Rhizoctonia solani* AG-3PT internal transcribed spacer (ITS) type m1. These isolates were collected from black scurf on potato tubers obtained from three different potato growing localities in New Zealand. Information regarding locations of isolation, ITS type, and the dsRNA content for each isolate are described in Table 1. Cultures of these isolates are held in the New Zealand Institute for Plant & Food Research Lincoln Culture Collection.

Extraction and purification of total dsRNA

DsRNA extractions were carried out following the methods described by Das *et al.* (2014b). In brief, all the isolates were grown in potato dextrose broth (PDB) (Difco) amended with tetracycline (0.015 g L^{-1}) (Sigma–Aldrich) at 22 °C for 25–30 d. Mycelial mats (4–6 g) were collected and ground to a fine powder with liquid nitrogen. Total dsRNA was then isolated by Whatman CF-11 cellulose affinity chromatography. DsRNA fractions were subsequently treated with DNase I, amplification grade (Life Technologies), RNase A (Qiagen), and purified following the methods mentioned previously (Das *et al.* 2014b).

Synthesis, cloning and sequencing of cDNA amplicons

Reverse transcription of total dsRNA was carried out with the random priming method using an adapter-tagged random hexamer (Primer A, 5′-CCTGAATTCGGATCCTCCNNNNNN-3′) (Darissa *et al.* 2010). The amplification of total dsRNA and second strand synthesis were conducted following the methods described by Das *et al.* (2014b). A-tailing reactions were carried out to add A overhangs at the 3′ ends of cDNA amplicons before cloning. Cloning of A-tailed cDNA amplicons was carried out using pGEM-T Easy vector system II (Promega), following the manufacturer's protocol. Bacterial transformation, selection of recombinant plasmids and plasmid isolations from selected transformed colonies were conducted as described by Das *et al.* (2014b). The cDNA inserts cloned in recombinant plasmids were sequenced (using universal primers, M13F and M13R) in both directions using the commercial sequencing service (Macrogen, Korea).

Sequence analysis and phylogenetic studies

Plasmid vector sequences were detected in cDNA sequences using the VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html), and were subsequently removed. Consensus sequences were constructed from overlapping cDNA clones using Geneious Pro 5.5.7 software (Biomatters). BLAST (http://blast.ncbi.nlm.nih.gov/) analyses were carried out to examine sequence similarities.

The ORF finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used for prediction of the presence of open reading frames (ORFs). The expected AU content at the third base position (Wobble position) of codons within the ORF was calculated using the EMBOSS 6.3.1: wobble program (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::wobble). The presence of conserved domains in putative amino acid (aa) sequences were examined using CD-search with default settings (http://www.ncbi.nlm.nih.gov/Structure/cdd/