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MOLECULAR CHARACTERIZATION AND BIOLOGICAL CONTROL POTENTIAL OF AN EGYPTIAN ISOLATE OF SCLEROTINIA SCLEROTIORUM MITOVIRUS 1

Mahmoud E. Khalifa [1*], Eldessoky S. Dessoky [2] and Atef S. Sadik [3]

[1]Botany and Microbiology Department, Faculty of Science, Damietta University, Damietta, Egypt.
[2]Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia.
[3]Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, P.O. Box 68, Hadayek Shobra 11241, Cairo, Egypt.
[*] Corresponding author's email: mahmoud.khalifa@du.edu.eg

ABSTRACT

Several mycoviruses have potential to induce hypovirulence on their fungal pathogens and therefore the interest in mycoviruses has increased in recent years. In the current study, a single double-stranded RNA (dsRNA) molecule of 2531 nts was detected, sequenced and characterized from an Egyptian isolate (D7) of *Sclerotinia sclerotiorum* fungus. The dsRNA has one open reading frame (ORF), in its positive strand, encoding a protein with conserved motifs characteristic of viral RNA-dependent RNA-polymerases (RdRps). The RdRp encoded by the ORF shares 91.84% identity with that of isolate HC025 of sclerotinia sclerotiorum mitovirus 1 (SsMV1) and consequently it was tentatively named SsMV1-D7. As for previously described mitoviruses, the termini of the (+) strand of SsMV1-D7 RNA could potentially fold into stable secondary structures. Horizontal transmission and virulence experiments showed that SsMV1-D7 is probably responsible for the altered growth and virulence of *S. sclerotiorum*.

Keywords: dsRNA; Mycovirus; *Mitoviridae*; *Mitovirus*; *Sclerotinia sclerotiorum*; Hypovirulence

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INTRODUCTION

Viruses of fungi (mycoviruses) are widespread across all major fungal taxa (Myers *et al*., 2020; Pearson *et al*., 2009). Several mycoviruses with different genome types have been reported from several fungal species including *Sclerotinia sclerotiorum*. Mycovirus infection may negatively affect the fitness of their hosts and consequently have capacity to be able to control fungal pathogens (Muñoz-Adalia *et al*., 2016; Pearson *et al*., 2009). Among the simplest known mycoviral families is *Mitoviridae* which include members that severely debilitate fungi (Hillman and Esteban, 2011).

S. sclerotiorum is a devastating phytopathogenic fungus capable of attacking more than 400 plant species globally, some of which are crops of important economic value (Boland and Hall, 1994; Liang and Rollins, 2018). Due to the drawbacks associated with *S. sclerotiorum* chemical control (Bolton *et al*., 2006), researchers paid attention to the potential use of mycoviruses in controlling *S. sclerotiorum* diseases. Several mycoviruses have been identified from *S. sclerotiorum*, many of which are associated with hypovirulence (Jiang *et al*., 2013).

In recent years, the rate of virus discovery has significantly increased as novel viral screening and identification approaches are being developed including next-generation sequencing (NGS). The study's objectives were (i) to molecularly characterize the D7-dsRNA segment and (ii) to determine the effect of this dsRNA on *S. sclerotiorum* pathogenicity.

MATERIALS AND METHODS

***Isolates of Sclerotinia sclerotiorum*:** Isolate D7 was isolated in 2015 from a diseased legume of black-eyed pea (*Vigna unguiculata*) obtained from a grocery store in Damietta governorate, Egypt. Isolate 13844sh^{hyg} is a hygromycin-labeled, virus-free *S. sclerotiorum* used in virus transfer experiments (Khalifa and Pearson, 2013). All of the isolates were grown and kept on potato dextrose agar (PDA) media. For liquid cultures, potato dextrose broth (PDB) media was used and cultures grown for 4-5 days at 20^{o}C.

***Purification, sequencing and RT-PCR detection of dsRNA*:** A CF11 cellulose chromatography technique was used to extract dsRNA as previously reported by Valverde *et al*. (1990). Purified dsRNA was resolved by electrophoretic separation in 1% (w/v) agarose gel. Separated dsRNA was visualized, photographed under UV light and dsRNA nature confirmed using the method reported by Howitt *et al*. (1995). To determine the nucleotide sequence of dsRNA, the band was cut out of the gel, dsRNA extracted and then utilized as a template for random synthesization of cDNA prior to sequencing according to a modified method from that of Roossinck *et al*. (2010) as reported by Khalifa and Pearson (2013). The

sequence of the terminal regions of dsRNA was confirmed by adapter ligation at the 5' and 3' ends, RT-PCR and sequencing, as outlined previously by Khalifa and Pearson (2013).

Analysis of dsRNA sequence and its phylogeny: Nucleotide sequences were assembled by Geneious R8.1software (Kearse *et al*., 2012). Open reading frame (ORF) detection was carried out with the ORFfinder tool of NCBI. MFOLD software was used to predict potential stem-loop and panhandle secondary structures (Mathews *et al*., 1999). MUSCLE sequence alignment software was used to align amino acid (aa) sequences of RNA-dependent RNA-polymerase (RdRp) in order to detect the protein conserved motifs. MEGA 5 software (Tamura *et al*., 2011) was used to execute the neighbor joining phylogenetic tree.

Virus transmission and biological characteristics: Dual-culture technique was used to transfer dsRNA from isolate D7 to isolate 13844sh^{hyg} for producing isolate 13844sh^{hyg}-D7 as previously reported by Khalifa and Pearson (2013). RNA was extracted from isolates sub-cultured on PDA and used as template for the detection of dsRNA associated with isolate D7 (dsRNA-D7), using RT-PCR. To assess the growth rate of *S. sclerotiorum* isolates, mycelium discs were taken from the growing margins, sub-cultured on PDA plates (three replicates), plates incubated at 20^{o}C for a period of 4-5 days and the rate of colony growth was measured daily. For virulence assessment, mycelium discs were used to infect tomato detached leaves (three replicates) and lesion diameters were measured three days post incubation. Data obtained from growth rate and virulence assement experiments were analyzed using one-way analysis of variance (ANOVA) and statistically significant different values were considered if $P<0.05$.

***RT-PCR detection of dsRNA associated with S. sclerotiorum isolate D7*:** To detect dsRNA-D7 in different isolates of *S. sclerotiorum*, RT-PCR was performed on extracted RNA using specific primers for dsRNA-D7 (Forward: 5′-CCTGGGATAAAAGTTTTGATCG-3′; Reverse: 5′-AGAGATGAGTAAGGAAAGGCGG-3′) to amplify a 219 nucleotides (nts) long fragment. As an internal control for RT-PCR, a 146 nts long sequence of the host actin gene was amplified via primers (forward: 5′-GAGCTGTTTTCCCTTCCATTGTC-3′) and (reverse: 5′-GACGACACCGTGCTCGATTGG-3′) (Sexton *et al*., 2009). Amplification products were resolved by electrophoresis, visualized and photographed under UV.

RESULTS

***DsRNA in isolate D7 of S. sclerotiorum*:** DsRNA associated with isolate D7 of *S. sclerotiorum* was purified using CF11 chromatography. Purified RNA has double-stranded nature as proved by nuclease (DNase and RNase) treatments at high and low-salt buffer solutions. Gel electrophoresis of purified dsRNA showed a single dsRNA band of ~2.5kb (Fig. 1). The band was gel-extracted and RT-PCR performed to amplify random cDNAs that were cloned and sequenced. RACE-PCR was used to complete the nucleotide sequence of the D7-dsRNA molecule.

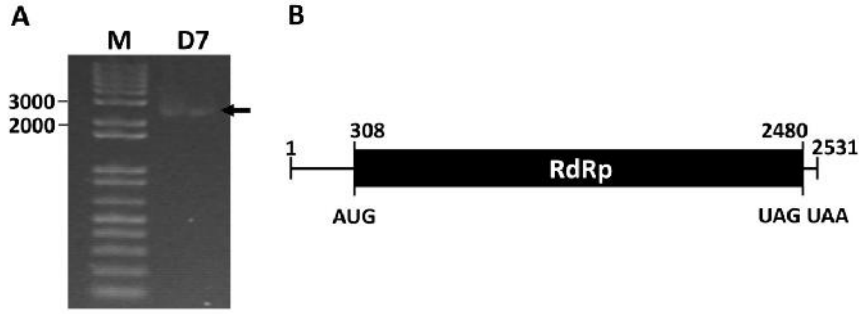

Fig. 1. (A) Agarose gel electrophoresis of *Sclerotinia sclerotiorum* isolate D7 dsRNA. M: 1 kb plus marker. (B) The genome organization of sclerotinia sclerotiorum mitovirus 1 (SsMV1-D7) (+) strand. Numbers indicate nucleotide positions. Start and stop codons and their positions are indicated.

Sequence properties of D7-dsRNA: The complete sequence of D7-dsRNA is 2531 nts in length and codes for a single ORF, when translated according to the mitochondrial genetic code as the UGA stop codon encodes tryptophan amino acid. The sequence is high in A+U content (60.4%) and the ORF is bordered by 5' and 3' untranslated regions (UTRs) that are 307 and 52 nts in length, respectively. The ORF uses an AUG as a start codon (nt positions 308-310), terminates at two adjacent UAG UAA codons (nt positions 2477-2479 and 2480-2482) and codes for a protein that is 723 aa long and has a molecular mass estimation of 83.85 kDa. BLAST search of D7-dsRNA-ORF against the non-redundant protein sequences (nr) revealed identities to RdRps of several mitoviruses. The highest aa sequence identity was shared between the RdRp of dsRNA-D7 and that of

isolate HC025 of sclerotinia sclerotiorum mitovirus 1 (SsMV1) and therefore, it was considered as an isolate of SsMV1, tentatively named SsMV1-D7. The nt sequence was deposited in GenBank database under accession number MW161169.

Prediction of secondary structures formed by SsMV1-D7 RNA termini: The 5' and 3' UTRs of SsMV1-D7 (+) RNA have the potential to fold and form stem-loop secondary structures that have ΔG values of -28.6 and -39.0 kcal/mole, respectively. The sequences of the 5' and 3' termini are inverted complementary and therefore have potential to fold into a panhandle-like duplex structure (ΔG= -70.7 kcal/mole) (Fig. 2).

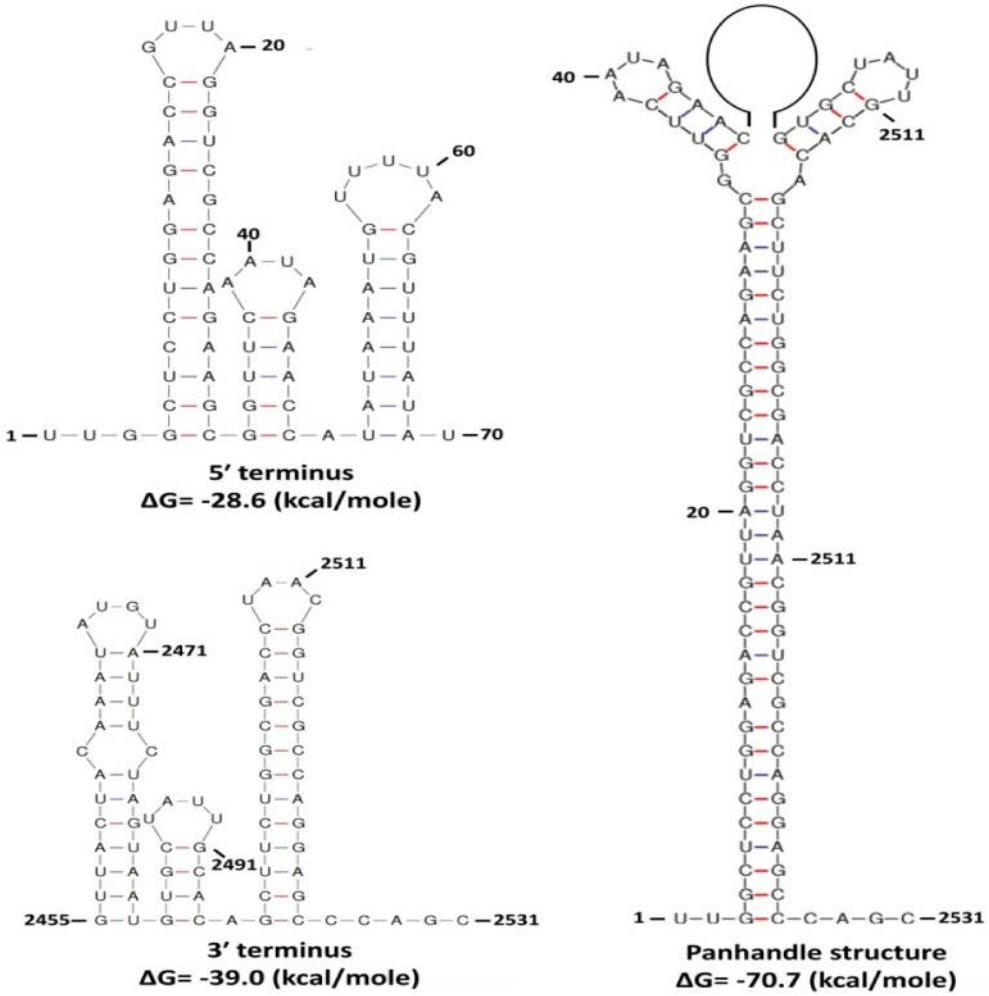

Fig. 2. Potential secondary structures formed by the 5' and 3' termini of sclerotinia sclerotiorum mitovirus 1 (SsMV1-D7) (+) strand. Secondary structures were predicted and ΔG values (kcal/mole) calculated using MFOLD software. Numbers indicate nucleotide positions.

SsMV1-D7 RdRp and its relatedness to other mitoviruses: Multiple aa sequence alignments of SsMV1-D7 RdRp and corresponding sequences of previously described mitoviruses revealed that it contains the aa conserved motifs of RdRp proteins, including GDD region which is highly conserved in motif IV (Fig. 3). The RdRp aa sequence identities of SsMV1-D7 and other representative mitoviruses are presented in Table 1. SsMV1-D7 RdRp showed the greatest identity (91.84%) with SsMV1-HC025. SsMV1-D7 was considered as an isolate of SsMV1 based on the ICTV rules for species demarcation of mitoviruses, as isolates of the same species have RdRp aa identities over 90% (Hillman and Esteban, 2011). Mitoviral RdRp phylogenetic analysis divided the sequences into several clades with SsMV1-D7 clustering with other isolates of SsMV1 and other viruses from *Ophiostoma novo-ulmi*, *Botrytis cinerea* and *Colletotrichum falcatum*. Other mitovirus species isolated from *S. sclerotiorum* were randomly scattered in different clades (Fig. 4).

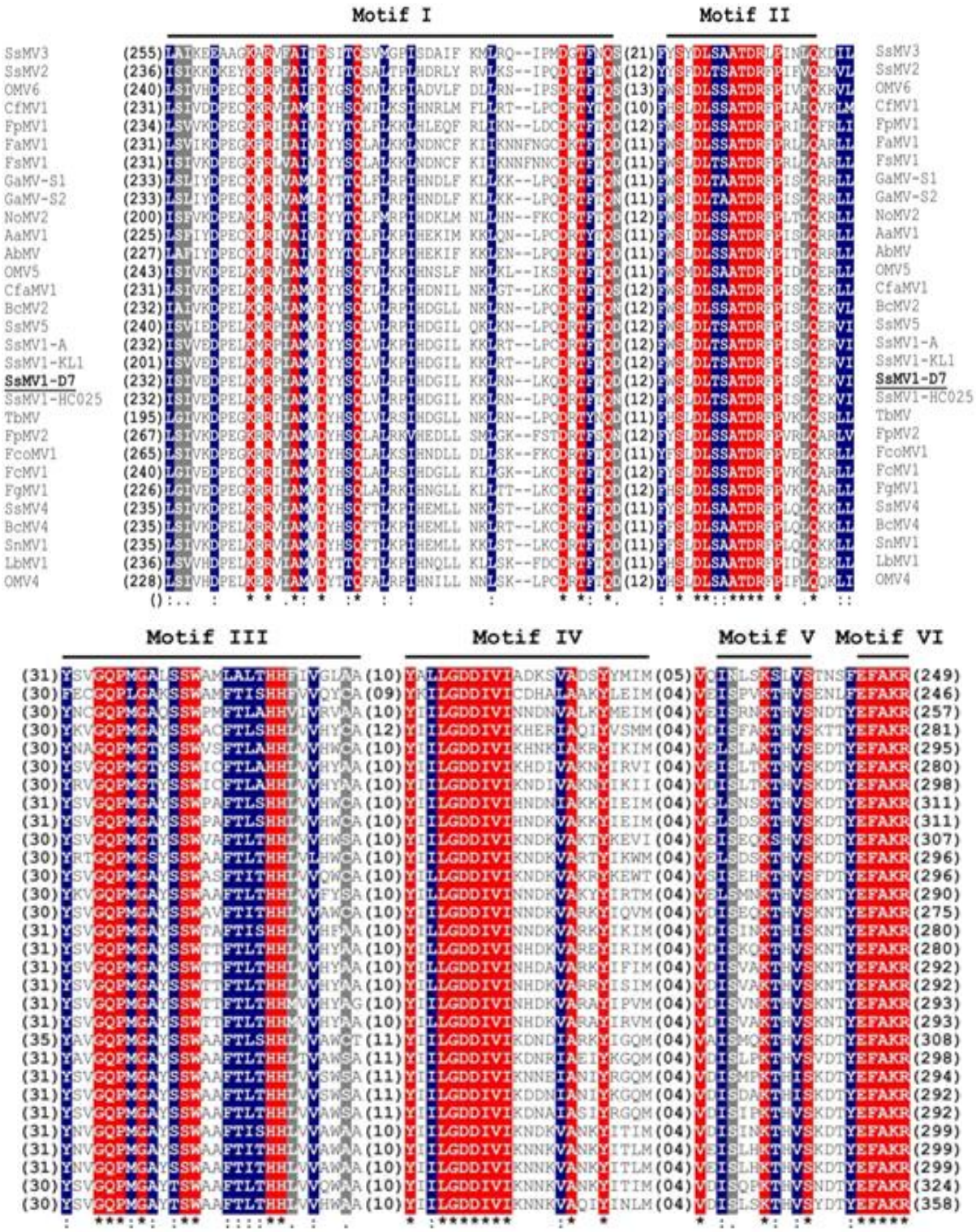

Fig. 3. Amino acid (aa) sequence alignments of RNA-dependent RNA-polymerase (RdRp) sequences of sclerotinia sclerotiorum mitovirus 1 (SsMV1-D7) and other mitoviruses. Sequences were aligned using MUSCLE tool. Identical residues are denoted by asterisks "*", whereas higher and lower chemically similar residues are denoted by colons ":" and dots ".", respectively. Conserved motifs (I-VI) of RdRps of mitoviruses are indicated. Virus notations are as shown in Table 1.

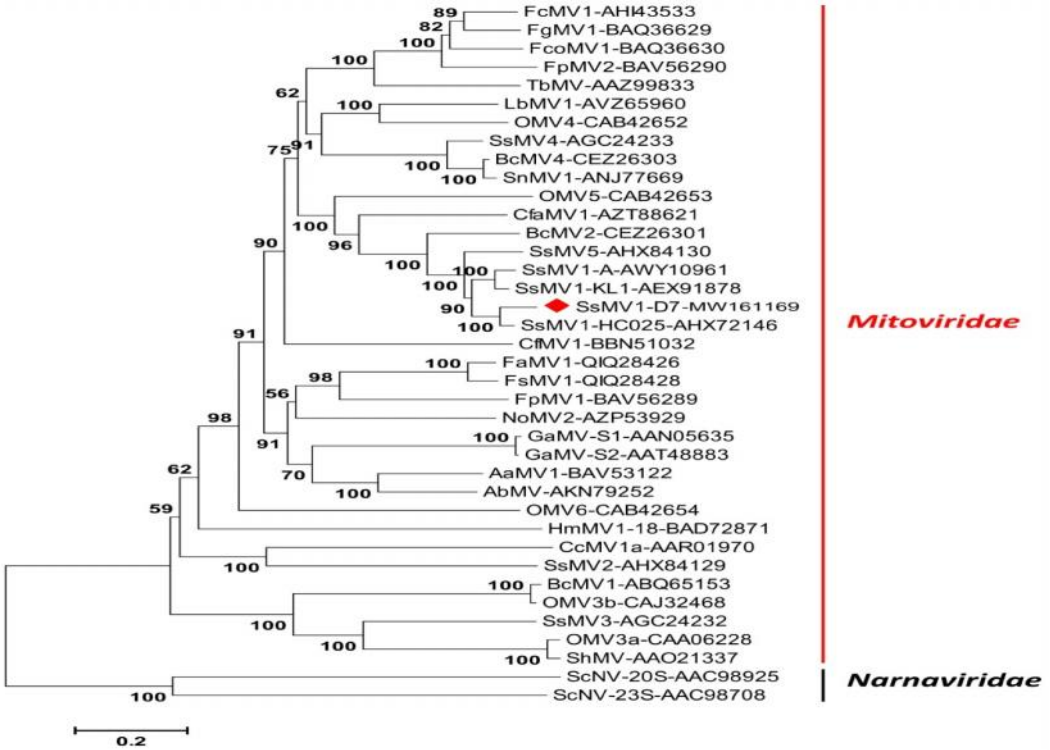

Fig. 4. Neighbor-joining phylogenetic tree based on multiple alignments of RdRp aa sequences of sclerotinia sclerotiorum mitovirus 1 (SsMV1-D7) and other mitoviruses. Sequences of two members of *Narnaviridae* were used as an outgroup. Sequence alignment was performed using MUSCLE tool and the tree constructed using MEGA 5 software using Poisson model. Values on the branches represent the percentage of 1000 bootstrap replicates. Virus notations are as shown in Table 1. ScNV-20S and ScNV-23S are abbreviations for saccharomyces cervisiae 20S narnavirus and saccharomyces cervisiae 23S narnavirus, respectively.

Mitovirus	Acronym	Identity (%)	GenBank Accession No.
alternaria arborescens mitovirus 1	AaMV1	35.22	BAV53122
alternaria brassicicola mitovirus	AbMV	40.80	AKN79252
botrytis cinerea mitovirus 1	BcMV1	29.88	ABQ65153
botrytis cinerea mitovirus 2	BcMV2	64.73	CEZ26301
botrytis cinerea mitovirus 4	BcMV4	39.81	CEZ26303
colletotrichum falcatum mitovirus 1	CfaMV1	46.02	AZT88621
colletotrichum fructicola mitovirus 1	CfMV1	38.17	BBN51032
cryphonectria cubensis mitovirus 1a	CcMV1a	40.52	AAR01970
fusarium andiyazi mitovirus 1	FaMV1	36.88	QIQ28426
fusarium circinatum mitovirus 1	FcMV1	37.69	AHI43533
fusarium coeruleum mitovirus 1	FcoMV1	36.85	BAQ36630
fusarium globosum mitovirus 1	FgMV1	37.38	BAQ36629
fusarium poae mitovirus 1	FpMV1	34.44	BAV56289
fusarium poae mitovirus 2	FpMV2	35.23	BAV56290
fusarium sacchari mitovirus 1	FsMV1	37.50	QIQ28428
gremmeniella abietina mitovirus S1	GaMV-S1	37.41	AAN05635
gremmeniella abietina mitovirus S2	GaMV-S2	37.76	AAT48883
helicobasidium mompa mitovirus 1-18	HmMV1-18	34.68	BAD72871
leptosphaeria biglobosa mitovirus 1	LbMV1	42.67	AVZ65960
nigrospora oryzae mitovirus 2	NoMV2	33.53	AZP53929
ophiostoma mitovirus 3a	OMV3a	28.64	CAA06228
ophiostoma mitovirus 3b	OMV3b	28.84	CAJ32468
ophiostoma mitovirus 4	OMV4	41.19	CAB42652
ophiostoma mitovirus 5	OMV5	41.35	CAB42653

ophiostoma mitovirus 6	OMV6	35.71	CAB42654
sclerotinia homoeocarpa mitovirus	ShMV	27.70	AAO21337
sclerotinia nivalis mitovirus 1	SnMV1	39.34	ANJ77669
sclerotinia sclerotiorum mitovirus 1-A	SsMV1-A	78.56	AWY10961
sclerotinia sclerotiorum mitovirus 1-HC025	SsMV1-HC025	91.84	AHX72146
sclerotinia sclerotiorum mitovirus 1-KL1	SsMV1-KL1	79.19	AEX91878
sclerotinia sclerotiorum mitovirus 2	SsMV2	33.04	AHX84129
sclerotinia sclerotiorum mitovirus 3	SsMV3	31.41	AGC24232
sclerotinia sclerotiorum mitovirus 4	SsMV4	41.54	AGC24233
sclerotinia sclerotiorum mitovirus 5	SsMV5	74.31	AHX84130
thielaviopsis basicola mitovirus	TbMV	40.26	AAZ99833

Biological characteristics of SsMV1-D7: To study the effect of SsMV1-D7 on S. sclerotiorum, dual-culture technique was used to transfer the virus from its parental isolate D7 to a virus-free isolate (13844sh^{hyg}) via anastomosis. Successful virus movement from isolate D7 to isolate 13844sh^{hyg} was confirmed using RT-PCR detection with SsMV1-D7 specific primers (Fig. 5). Isolate 13844sh^{hyg} infected with SsMV1-D7 was labeled 13844sh^{hyg}-D7. Growth rate and virulence of the parental and transfected isolates were compared. Growth rates of isolates D7, 13844sh^{hyg}, and 13844sh^{hyg}-D7 were 1.8, 2.15 and 1.9 cm/d, respectively. Moreover, lesion diameter produced by the newly infected isolate 13844sh^{hyg}-D7 on tomato detached leaves was reduced to 1.75 cm, which is comparable with that of SsMV1-D7 naturally-infected isolate D7 (1.65 cm) and less than that of SsMV1-D7-free isolate 13844sh^{hyg} (2 cm) (Fig. 5).

DISCUSSION

The current study describes some of the molecular and biological properties of an isolate of a previously described virus SsMV1 (SsMV1-D7). This isolate was found associated with an Egyptian isolate of *S. sclerotiorum*. Sequence analysis and BLASTP search showed that the (+) strand RNA of SsMV1-D7 encodes one ORF with similarities to mitoviruses. Phylogenetic analysis and aa identity comparisons supported our findings that it represents a novel Egyptian isolate of SsMV1. Mitoviruses were formerly classified as species of the genus *Mitovirus* within the family *Narnaviridae*. Currently, a new family, *Mitoviridae*, has been established to accommodate species of *Mitovirus* genus (Li *et al*., 2020; Nibert *et al*., 2018) as they appeared to have characters that make them distinct from *Narnaviridae* members. Mitoviruses were known to infect only fungi until they were recently discovered associated with plants such as chenopodium quinoa mitovirus 1 (CqMV1) (Nerva *et al*., 2019).

Mitoviridae include the simplest known members of mycoviruses with unencapsidated monopartite, positive sense, ssRNA genomes [(+)ssRNA)] of 2.3-3.6 kb in length (Hillman and Cai, 2013). The 3' end of SsMV1-D7 (+) RNA is not polyadenylated, which is a common property of most mitoviral genomes. However, some polyadenylated mitoviruses have been reported such as SsMV2/KL1 (Xie and Ghabrial, 2012). Different genomes of mitoviruses have one ORF, encodes only the RdRp. Replication of mitoviruses is restricted to their host mitochondria where the tryptophan aa could be coded for by the stop codon UGA (Cole *et al*., 2000). SsMV1-D7 ORF contains nine in-frame UGA codons, confirming its presence and replication within the host mitochondria. Some recently discovered mitoviruses, such as rhizoctonia solani mitovirus 39 (RsMV39), were found to encode the full-length RdRp when the standard or mitochondrial genetic codes were applied suggesting their ability to replicate within the cytoplasm and mitochondria (Li *et al*., 2020).

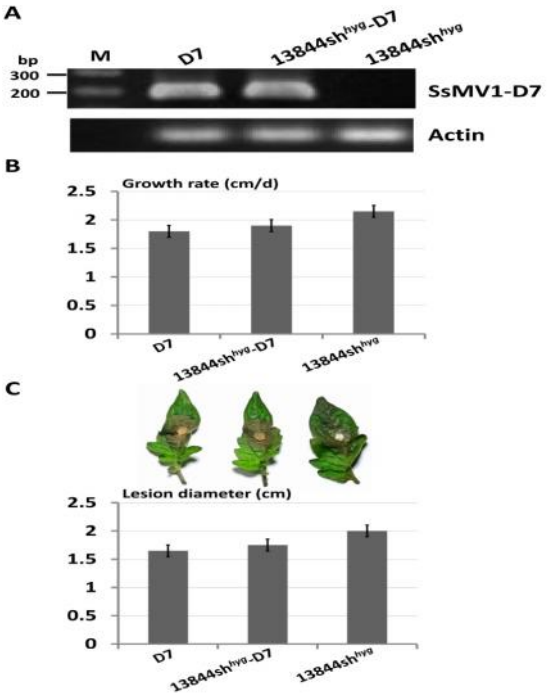

Fig. 5. (A) RT-PCR detection of sclerotinia sclerotiorum mitovirus 1 (SsMV1-D7) and actin gene of *Sclerotinia sclerotiorum* in different fungal isolates. (B) Growth rate and (C) lesion diameter assessments of virus-free and virus-containing isolates.

Plant and fungal mitochonderial genomes are rich in their content of A+U (>60%) probably due to their preference to have codons with either A or U nucleotide at the third wobble position (XYA+XYU) (Hong *et al*., 1998a). Although, in general, mitoviral genomes are characterized by having high A+U content of 62 to 73% (Hillman and Cai, 2013), some recently described mitoviruses have lower A+U content such as cryphonectria cubensis mitovirus 1a (CcMV1a; 50.5%) (Van Heerden, 2008), sclerotinia sclerotiorum mitovirus 2 (SsMV2/NZ1; 55.1%) (Khalifa and Pearson, 2013), SsMV2/KL1 (53.1%) (Xie and Ghabrial, 2012), and cronartium ribicola mitovirus 1 (CrMV1; 57.3%) (Liu *et al*., 2016). SsMV1-D7 genome is high in A+U percentage (60.4%) which is consistent with most mitoviruses and mitochondrial genomes. There is a preference for the UAA stop codon in fungal mitochondrial genomes (Paquin *et al*., 1997). This is also the case for most characterized mitoviruses. However, SsMV1/D7 ORF is terminated by UAG stop codon similar to several *S. sclerotiorum* mitoviruses (Khalifa and Pearson, 2014).

The terminal sequences of all discovered mitoviruses have the potential to form stable stem-loop secondary structures. Moreover, genomes of some mitoviruses can fold into panhandle structures because of the presence of inverted complementary sequences at their termini (Hong *et al*., 1998b). Examples of the mitoviruses that have this potential are cronartium ribicola mitoviruses (CrMV1 to CrMV5) (Liu *et al*., 2016), ophiostoma mitoviruses 4 and 6 (OMV4 and OMV6) (Hong *et al*., 1999), RsMV39 (Li *et al*., 2020), SsMV1/HC025 (Xu *et al*., 2015), SsMV2/NZ1 (Khalifa and Pearson, 2013), and SsMV1-D7 of the current study. Such secondary structures are thought to (i) play a significant role in the replication process of mitoviral genomes, (ii) act as RdRp recognition sites and (iii) protect naked ssRNA genomes from degradation (Buck, 1996; Hong *et al*., 1999).

Hyphal anastomosis experiments are widely used to study the transmission of mycoviruses from a virus-harboring isolate to another virus-free isolate of fungal hosts. Mitovirus transmission through anastomosis is accompanied by mitochondrial movement and recombination such as in the case of cryphonectria parasitica mitovirus 1 (CpMV1) (Polashock *et al*., 1997). SsMV1-D7 was able to infect a virus-free fungal isolate through hyphal fusion. Its transmission might similarly involve movement and recombination of mitochondria.

The infection by mitoviruses leads to variable effects on their fungal hosts. Although mitoviruses that induce little or no effects on their natural hosts, such as cryphonectria cubensis mitovirus 1b (CMV1b) (Van Heerden, 2008) and sclerotinia sclerotiorum mitovirus 4 (SsMV4) (Khalifa and Pearson, 2013), have been reported, most mitovirus infections are associated with reduced fungal virulence, giving them the potential to be utilized as bio-control agents. Examples of hypovirulence-inducing mitoviruses have previously been reported in several fungal species including S. sclerotiorum (Jiang *et al*., 2013; Xu *et al*., 2015), B. cinerea (Pearson and Bailey, 2013), Cryphonectria parasitica (Dawe and Nuss, 2013), O. novo-ulmi (Hong *et al*., 1999) and Sclerotinia homoeocarpa (Deng and Boland, 2006). Infection of S. sclerotiorum with the Egyptian isolate of SsMV1 (SsMV1/D7) reduced its virulence and growth rate and hence SsMV1/D7 could be a potential candidate for controlling diseases caused by this fungal pathogen.

Some mitoviruses of S. sclerotiorum were phylogenitically related, whereas others are found in different phylogenetic clusters (Xu *et al*., 2015). Some mitoviruses showed great diversity in terms of species variability and geographical distribution. For example O. novo-ulmi was found to host at least nine mitoviruses from Europe and North America (Doherty *et al*., 2006; Hintz *et al*., 2013). Moreover, the NCBI database contained at least 34 species of mitoviruses sequenced from S. sclerotiorum fungus, the natural host of SsMV1-D7. Isolates of SsMV1 were identified in American (SsMV1/KL-1), Chinese (SsMV1/HC025) (Xie and Ghabrial, 2012; Xu *et al*., 2015) and Egyptian strains (SsMV1-D7). Territorial distribution of SsMV1 and association of its different isolates, including the one of the current study, with reduced host virulence provides alternative promising approach for the control of S. sclerotiorum fungus.

As a conclusion, in this study, we reported the isolation and characterization of an isolate of a mitovirus associated with an Egyptian isolate of *S. sclerotiorum*. Characteristics of the isolated mitovirus are in consistence with most previously described mitoviruses. The isolation of SsMV1-D7 from three different countries reveals that it has wide geographical distribution. Continuous isolation of mycoviruses from *S. sclerotiorum* indicates their great diversity. Since SsMV1-D7 confers hypovirulence in *S. sclerotiorum*, this research introduces the concept of studying mycoviruses and evaluating their possibility to be used in controlling fungal diseases in Egypt.

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