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Molecular characterizations of two mitoviruses co-infecting a hyovirulent isolate of the plant pathogenic fungus *Sclerotinia sclerotiorum*

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ABSTRACT

The complete nucleotide sequences of two double-stranded RNA (dsRNA) segments, isolated from the same hypovirulent strain (KL-1) of *Sclerotinia sclerotiorum*, were determined. Sequence analysis showed that dsRNAs 1 to be 2513 nts long and is A–U rich (61.7%). Excluding the poly(A) tail, dsRNAs2 is 2421 nts long and its AU content is 53.1%. The 5′ and 3′-terminal sequences of the positive-strand of each dsRNA could be folded into predicted stable stem–loop structures. Mitochondrial codon usage revealed that each dsRNA has a single large open reading frame coding for a protein containing RNA-dependent RNA polymerase conserved motifs. Furthermore, dsRNAs 1 and 2 share sequence similarities with other mitoviruses. These results suggest that dsRNAs 1 and 2 represent two distinct new mitoviruses, designated Sclerotinia sclerotiorum mitovirus 1 (SsMV1/KL-1) and SsMV2/KL-1, respectively. The hypovirulence traits of strain KL-1 and the two mitoviruses could be co-transmitted to a virus-free virulent strain via hyphal anastomosis.

Introduction

Mycoviruses (or fungal viruses) are prevalent in all major groups of plant pathogenic fungi (Ghabrial and Suzuki, 2009). Although the majority of dsRNA mycoviruses have been reported to be associated with symptomless infections of their hosts, there are well documented cases for mycoviruses that induce hypovirulence in their plant pathogenic fungal hosts and are currently being exploited as means of biological control for combating plant diseases (Pearson et al., 2009; Ghabrial and Suzuki, 2009). The hypovirus-mediated hypovirulence in *Cryphonectria parasitica* was applied effectively to control the chestnut blight disease in European chestnut (Anagnostakis, 1982; Nuss, 1992). In addition, another hypovirulence-associated bipartite dsRNA virus, *Rosellinia necatrix* megabirnavirus 1, is a potential viral agent for biological control of the white root rot disease caused by *Rosellinia necatrix* (Chiba et al., 2009). Moreover, fungus–mycovirus systems present an opportunity to understand the molecular basis of virulence in phytopathogenic fungi and provide insight into the fundamental features of virus–fungus–plant interactions (Nuss, 2005; Li et al., 2008). The discovery of more novel mycoviruses including latent viruses might help to expand our understanding of the origin, ecology and evolutionary pathways of mycoviruses (Ghabrial, 1998; Ghabrial and Suzuki, 2009).

The family *Narnaviridae* of single-stranded (ss) RNA viruses comprise two genera, *Narnavirus* and *Mitovirus* (Hillman and Esteban, 2011). The genomes of narnaviruses and mitoviruses are unencapsidated and consist of only a single open reading frame (ORF) coding for a putative RNA-dependent RNA polymerase (RdRp) that is required for RNA replication. The genomes of mitoviruses and narnaviruses represent the simplest of all known autonomously replicating viruses (Deng et al., 2003; Hillman and Esteban, 2011). The mitoviruses described so far, infect only filamentous fungi and have been reported in numerous plant pathogenic fungi (Table 1).

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen that causes important diseases in a wide variety of broadleaf crops including soybean and sunflower (Boland and Hall, 1994). The diseases caused by this pathogen are difficult to control via cultural practices or fungicide application. Furthermore, resistant cultivars are not available for most crops. Annual losses to crop production have exceeded $200 million in the United States (Bolton et al., 2006). As is the case with some other fungi, several different mycoviruses including ssRNA, dsRNA and ssDNA mycoviruses have been isolated and characterized from *S. sclerotiorum* (Xie et al., 2006; Liu et al., 2009, 2010; Yu et al., 2010; Xie et al., 2011). Examples of viruses that are associated with hypovirulence of *S. sclerotiorum* include the ssRNA Sclerotinia sclerotiorum

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Table 1
Lengths of 3′-UTR and AU content of *Mitoviruses* and *Narnaviruses*.

Mitovirus	Acronym	3′-UTR length (nt)	AU content (%)	Poly(A)	GenBank accession no.
Sclerotinia sclerotiorum mitovirus 1/KL-1	SsMV1/KL-1	16	61.7	N	JQ013377
Sclerotinia sclerotiorum mitovirus 2/KL-1	SsMV2/KL-1	82	53.1	Y	JQ013378
Ophiostoma novo-ulmi mitovirus 1a	OnMV1b	394	64.3	N	AM087548
Ophiostoma novo-ulmi mitovirus 1b	OnMV1a	170	63.5	N	AM087549
Ophiostoma novo-ulmi mitovirus 3a	OnMV3a	195	62.8	N	AJ004930
Ophiostoma novo-ulmi mitovirus 3b	OnMV3b	88	67.1	N	AM087550
Ophiostoma novo-ulmi mitovirus 4	OnuMV 4	46	73.3	N	NC_004052
Ophiostoma novo-ulmi mitovirus 5	OnuMV 5	60	73.6	N	NC_004053
Ophiostoma novo-ulmi mitovirus 6	OnuMV 6	117	70.7	N	NC_004054
Tuber aestivum mitovirues	TaMV	952	60.1	N	HQ992989
Botrytis cinerea mitovirus 1	BcMV1	114	66.8	N	EF580100
Thielaviopsis basicola mitovirus	TbMV	351	67.4	N	AY563138
Sclerotinia homoeocarpa mitovirus	ShMV	201	61.0	N	AY172454
Gremmeniella abietina mitochondrial RNA virus S1	GaMRV-S1	96	69.4	N	AF534641
Cryphonectria cubensis mitovirus 1a	CcMV1a	186	50.5	Y	AY328476
Cryphonectria cubensis mitovirus 2a	CcMV2a	79	62.4	N	AY328479
Cryphonectria parasitica mitovirus 1-NB631	CpMV1	225	63.5	N	NC_004046
Helicobasidium mompa mitovirus 1-18	HmMV1-18	115	58.2	N	AB110977
Gremmeniella abietina mitochondrial RNA virus S2	GaRV-MS2	93	69.0	N	NC_006264
Thanatephorus cucumeris mitovirus	TcMV	884	57.7	N	U51331
Saccharomyces 20S RNA *narnavirus*	ScNV-20S	–	41.7	N	AF039063
Saccharomyces 23S RNA *narnavirus*	ScNV-23S	–	41.0	N	U90136

debilitation associated RNA virus (SsDRV; genus *Sclerodarnavirus*, family *Alphaflexiviridae*) and the ssDNA Sclerotinia sclerotiorum hypovirlence associated DNA virus 1 (SsHDV1; unclassified) (Xie et al., 2006). The mycovirus SsRV-L, which co-infects strain Ep-1PN with SsDRV, is related to a human viral pathogen and has a slight adverse effect on its host (Liu et al., 2009). The partitivirus Sclerotinia sclerotiorum partitivirus S (SsPV-S) shares high sequence identity with the *ILR2* gene of *Arabidopsis thaliana*, thus revealing horizontal gene transfer from double-stranded RNA viruses to eukaryotic nuclear genomes (Liu et al., 2010).

In the present study, we determined the sequences of two novel and distinct mitoviruses, Sclerotinia sclerotiorum mitovirus 1 (SsMV1/KL-1) and SsMV2/KL-1, isolated from *S. sclerotiorum* hypovirulent strain KL-1. Our results support a causal role of mitovirus infection in reducing the growth and pathogenicity of strain KL-1. Sequence comparisons and phylogenetic analysis of deduced amino acid sequences of the putative RdRp revealed that SsMV1/KL-1 and SsMV2/KL-1 are closely related to members of the genus *Mitovirus*.

Results

S. sclerotiorum strain KL-1 exhibits hypovirulence traits

The genome of the virulent strain 1980^{hyg}, selected for this study, has been completely sequenced (Amselem et al., 2011). It is vegetatively compatible with the hypovirulent strain KL-1 (see Material and Methods). Compared to the virulent strain 1980^{hyg}, strain KL-1 grew slowly (1.2 cm/d versus 2.5 cm/d for the virulent strain), and produced less sclerotia on PDA medium (Fig. 1A and B). The sclerotia of strain KL-1 can germinate in culture and produce a small ascogonidium, but no apothecium is produced. When dsRNA was isolated from mycelial extracts of strains KL-1 and 1980^{hyg} and subjected to digestion with RNase-free DNase I and S1 followed by agarose gel electrophoresis, one distinct dsRNA band could be detected in strain KL-1 under UV light, but no bands were detected in strain 1980^{hyg} (Fig. 1C; upper panel). The hypovirulence-associated traits (slow growth and low production of sclerotia) and dsRNA segments were co-transmitted from strain KL-1 to strain 1980^{hyg} when mycelia from these two strains were in contact using the dual-culture method (Fig. 1A–C). These experiments were repeated at least 6 times and the resultant hygromycin-resistant 1980^{hyg} converted isolates exhibited the hypovirulence traits of strain KL-1 and contained the two mitoviruses. The transfer of the two mitoviruses from strain KL-1 to strain 1980^{hyg} was confirmed by RT-PCR analysis in all 1980^{hyg} converted isolates (Fig. 1C; lower panel). Moreover, strain KL-1 and the 1980^{hyg} converted isolates showed lower levels of virulence on detached soybean leaves and lettuce seedlings than the virulent strain 1980^{hyg} (Fig. 2A and B). These results suggest that strain KL-1 is a hypovirulent strain of *S. sclerotiorum* and that the dsRNA segments are likely the causal agents of hypovirulence in strain KL-1.

Molecular cloning and sequencing of dsRNA from S. sclerotiorum strain KL-1

Fourteen cDNA clones derived from strain KL-1 dsRNA were obtained using tagged random primers-generated cDNA library and sequenced. A search of NCBI database with BLASTX program using the sequences of the 14 cDNA clones revealed that all sequences were related to mitoviral RdRp sequences. Full-length cDNA sequence of dsRNA was obtained using sequence-specific primers for RT-PCR and RACE protocols. Nucleotide sequence analysis revealed the presence of two distinct dsRNA segments (dsRNA1 and dsRNA2) in the mycelium of strain KL-1. The two dsRNAs, which co-migrated on agarose gels, differed in length by only 92 nts, excluding the poly(A) tail. Although the two dsRNAs extracted from the mycelium of strain KL-1 were resolved only as one band, as revealed by agarose gel electrophoresis (Fig. 1B), two dsRNA segments could be separated clearly by electrophoresis on a 15% nondenaturing polyacrylamide gel (Fig. 3A).

Molecular characterization of two mitoviruses

The complete nucleotide sequences of the two dsRNAs segments isolated from the mycelium of strain KL-1 were determined and the genetic organization of each was shown in Fig. 3B. A blastp search of NCBI protein database was conducted using the proteins encoded by dsRNA1 and dsRNA2. Each of these two putative proteins contains the six conserved motifs (I–VI) including the highly conserved GDD motif typical of RdRp. The RdRps encoded by dsRNA1 and dsRNA2 showed significantly high

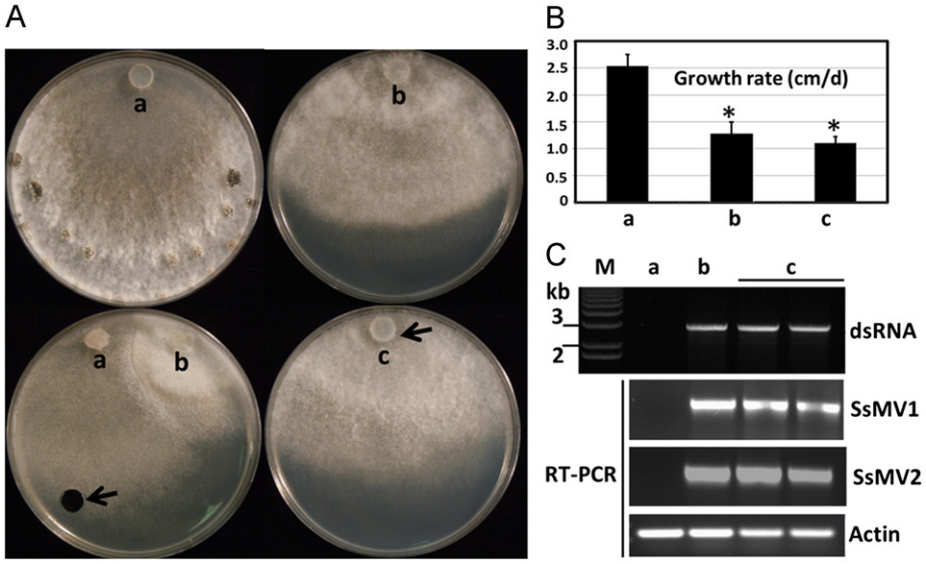

Fig. 1. Colony morphology, growth rate and dsRNA content of *S. sclerotiorum* strains. (A) Colony morphology of strains 1980^{hyg} and KL-1 (a and b, respectively). A dual culture of strains 1980^{hyg} and KL-1 is shown at left in the lower panel. A mycelial agar plug (marked with a black arrow) was removed from the colony margin of strain 1980^{hyg} (black arrow) and transferred to a fresh PDA plate containing 25 μg/ml hygromycin to establish derivative isolate 1980^{hyg}-converted (c). All *S. sclerotiorum* strains were grown on potato dextrose agar for 5 day at 20 °C prior to photography. (B) Comparative growth rate of *S. sclerotiorum* strains 1980^{hyg}, KL-1 and 1980^{hyg}-converted (a, b and c, respectively). The growth rate was calculated based on measurements made 24 and 48 h post-inoculation; values that are significantly different ($P < 0.05$) were indicated by asterisks. (C) RT-PCR detection of mitoviruses in strains 1980^{hyg} (a), KL-1 (b) and two representative 1980^{hyg}-converted isolates (c). All dsRNA samples were treated with DNase I and S1 nuclease prior to electrophoresis. The actin gene of *S. sclerotiorum* was used as an internal control. The predicted lengths of the RT-PCR products for SsMV1/KL-1 and SsMV2/KL-1 are 587 and 321 nts, respectively.

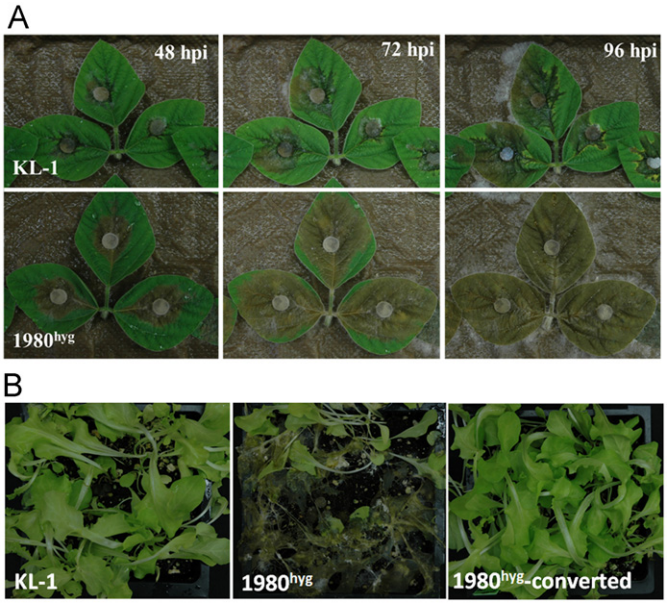

Fig. 2. Virulence assays of *S. sclerotiorum* strains. Assays were made on detached soybean leaves (A) and lettuce seedlings (B). The abbreviation hpi=hours post-inoculation.

sequence similarity to RdRps of viruses in the genus *Mitovirus* (Fig. 4). The two dsRNAs are predicted to replicate independently in their host strain KL-1 because each codes for its own RdRp. Furthermore, the overall nt sequence identity between dsRNAs 1 and 2 is 47.9%. At the deduced amino acid level, the dsRNA1 and dsRNA2 are 33.3% identical. These results indicate that the dsRNAs 1 and 2, isolated from the same *S. sclerotiorum* strain KL-1, represent replicative forms/intermediates of two distinct new members of the genus *Mitovirus* and are designated Sclerotinia sclerotiorum mitovirus 1 (SsMV1/KL-1) and SsMV2/KL-1, respectively. The full-length cDNA sequences of SsMV1/KL-1 and SsMV2/KL-1 were deposited in the Genbank database under accession numbers JQ013377 and JQ013378, respectively.

SsMV1/KL-1

Sequence analysis of full-length SsMV1/KL-1 cDNA indicated that it is 2513 nts in length. The complete genome sequence had a nucleotide composition of A (31.0%), C (18.0%), G (20.3%) and U (30.7%) with an overall A+U-rich content (61.7%). Sequence analysis using the DNAMAN and Genescan programs revealed the presence of several short open reading frames (ORFs) on either strand using the standard genetic code. However, a single large putative AUG-initiated ORF from nt positions 419 to 2494 was identified on the positive strand of cDNA of SsMV1/KL-1 when the fungal mitochondrial codon usage in which UGA was used as a tryptophan codon, not a stop codon, was applied (Osawa et al., 1992; Paquin et al., 1997). There are 8 UGA codons within the ORF region. The 5′- untranslated region (UTR) of SsMV1/KL-1 was determined to be 418 nts long. In contrast, the 3′-UTR, which is only 16 nts long, is relatively short. The large ORF contains two contiguous stop codons (^{2492}UAGUAA) to terminate translation and could encode a putative protein of 691 amino acid (aa) residues with calculated molecular mass of 79.43 kDa.

SsMV2/KL-1

The full-length nucleotide sequence of SsMV2/KL-1 cDNA was found to be 2421 nts in length excluding the poly(A) tail and has an A+U content of 53.1%. Similar to SsMV1/KL-1, a single large putative ORF, starting at nt position 312 and terminating at nt 2342, was identified on the positive strand of SsMV2/KL-1 when fungal mitochondrial codon usage was invoked. There are 6 UGA codons within the ORF. The 5′- and 3′-UTRs are 311 and 82 nts in length, respectively. The deduced amino acid sequence was found to code for a protein of 676 aa residues with a calculated molecular mass of 75.75 kDa.

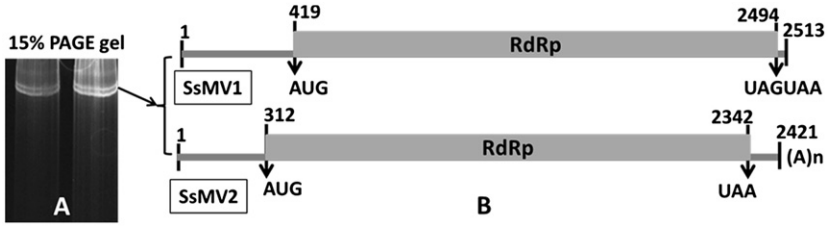

Fig. 3. Polyacrylamide gel electrophoresis of dsRNA on nondenaturing 15% polyacrylamide gel and genomic organization of SsMV1/KL-1 and SsMV2/KL-1. (A). Ethidium bromide stained gel showing two dsRNA segments (upper: SsMV1/KL-1; lower: SsMV2/KL-1). All dsRNA samples were treated with both DNase I and S1 nuclease prior to electrophoresis. (B) Schematic representation of the genomic organization of the mitoviruses SsMV1 (SsMV1/KL-1) and SsMV2 (SsMV2/KL-1).

Phylogenetic analysis of mitovirus RdRps

To define the relationship of SsMV1/KL-1 and SsMV2/KL-1 with the other mitoviruses listed in Table 1, we produced multiple alignments of the putative RdRps. The six conserved motifs characteristic of mitovirus RdRps (I to VI) were found in the putative RdRps of SsMV1/KL-1 and SsMV2/KL-1 (Fig. 4). Alignment of the full-length amino acid sequence of the mitoviruses listed in Table1 showed that SsMV1/KL-1 shared the highest sequence identity with OnuMV5 (40%), whereas SsMV2/KL-1 RdRp was most closely related to CcMV1a (30%) among members of the genus *Mitovirus* (Table 1). Phylogenetic analysis based on multiple alignments of full length RdRp sequences further supported these results (Fig. 5). The neighbor-joining phylogenetic tree also showed that members of the genus *Mitovirus* were divided into two clusters I and II). SsMV1/KL-1 and SsMV2/KL-1 were both placed in one clade (cluster I) with seven other members including CcMV1a, HmMV1-18, OnuMV6, OnuMV4, TbMV, GaMRV and OnuMV5 with 97% bootstrap support. SsMV1/KL-1 and SsMV2/KL-1, however, were distantly related to other mitoviruses belonging to cluster II of the genus *Mitovirus* as well as to members of the genus *Narnavirus* (Fig. 5).

Predicted secondary structures of the 5′ and 3′ terminal regions

The potential secondary structures of the 5′ and 3′-terminal sequences of the positive-strand of SsMV1/KL-1 and SsMV2/KL-1 were predicted using the MFOLD software. The results showed that the terminal sequences of the two mitoviruses could be folded into potential stable stem–loop structures. The SsMV1/KL-1 5′-terminal sequence (nt positions 1–60) and the 3′-terminal sequence (nt positions 2470–2513) could be folded into a double stem–loop structure with the ΔG value of −16.5 kcal/mol and −16.80 kcal/mol, respectively (Fig. 6A and B). The 5′-terminal sequence of SsMV2/KL-1 (nt positions 1–27) could also be folded into a potential double stem–loop structure with the ΔG value of −13.00 kcal/mol. The 3′-terminal sequence (nt positions 2361–2421) of SsMV2/KL-1 could be folded into a potential stable stem–loop structure with the ΔG value of −23.10 kcal/mol (Fig. 6C and D). However, since the two terminal sequences lack inverted complementarity, the 5′- and 3′-terminal sequence of SsMV1/KL-1 and SsMV2/KL-1 could not be folded into a potentially stable panhandle structure.

Stability of the two mitoviruses in strain KL-1

To eliminate one or both mitoviruses from strain KL-1, several approaches were attempted including hyphal tipping, single sclerotia isolation, thermotherapy (growth at the relatively high temperature of 30 °C) and chemotherapy (incorporation of cycloheximide or chloramphenicol in the culture medium). Regardless of the treatment applied, the two mitoviruses (SsMV1/KL-1 and SsMV2/KL-1) were retained in *S. sclerotiorum* strain KL-1 (Fig. 7).

Discussion

In the present study, we report the molecular characterization of two novel mitoviruses, SsMV1/KL-1 and SsMV2/KL-1, and describe the biological properties of their hypovirulent host, strain KL-1 of *S. sclerotiorum*. The full-length nucleotide sequences of the two mitoviruses were determined and their genome organizations were characterized. Mitochondrial codon usage revealed that the genome of each of the two mitoviruses comprises a single unique ORF. BLAST searches with the deduced aa sequences showed that SsMV1/KL-1 and SsMV2/KL-1 each encodes a putative RdRp with six conserved RdRp motifs (I–VI) (Fig. 4). The RdRps of SsMV1/KL-1 and SsMV2/KL-1 are most closely related to those of OnMV5 and CcMV1a, respectively, with amino acid sequence identity of 40% and 30%, respectively. Based on these results as well as those of phylogenetic analysis and genome organization, we propose that SsMV1/KL-1 and SsMV2/KL-1 represent two new members of the genus *Mitovirus* in the family *Narnaviridae*.

Previous studies showed that mitoviruses are naked RNA viruses with a positive single stranded RNA genome containing a single ORF, as revealed by invoking fungal mitochondrial codon usage (Hillman and Esteban, 2011; Ghabrial and Suzuki, 2009). Moreover, mitoviruses infect solely fungi and have been found in at least twelve independent species of filamentous fungi (Table 1). This study represents the first report of mitoviruses in the plant pathogenic fungus *S. sclerotiorum*.

Mitovirues are confined to their host's mitochondria where their genomes are translated. Mitovirues are co-purified with mitochondria and RNA-dependent RNA polymerase (RdRp) activity has been detected in mitochondria from an isolate of *Ophiostoma novo-ulmi* infected with OnuMV6 (Cole et al., 2000). Furthermore, most of the genomes of viruses in the genus *Mitovirus*, like fungal and plant mitochondrial genomes, have a common property of being A–U rich (usually > 60%) (Paquin et al., 1997; Hong et al., 1998; Table 1). Our results showed that the genome of SsMV1/KL-1 is indeed A–U rich (61.7%). Moreover, the third position of each genetic codon in the ORF region of SsMV1/KL-1 has a preference for either A or U and the frequency of codons XYA+XYU is 66.3%, which is considered to be characteristic of mitochondrial codons (Paquin et al., 1997). It is noteworthy that SsMV2/KL-1 has a lower A–U content of 53.1% (and its XYA+XYU content is 50%), which is lower than that of most of other mitoviruses, but similar to the A–U content of CcMV1a (50.5%) (Table 1).

The 3′- UTRs of mitoviruses so far reported are variable in length (Stielow et al., 2011). Our present report indicated that the length of the 3′-UTRs of SsMV1/KL-1 (16 nts) and SsMV2/KL-1 (82 nts) are relatively short, compared with other mitoviruses (Table 1). Moreover, the length of the 3′-UTR of SsMV1/KL-1 was the shortest among all known mitoviruses (Table 1). Another characteristic feature of SsMV2/KL-1 was the presence of a poly(A) tail at its 3′-terminal sequence, while other mitoviruses (with the exception CcMV1a) do not have this structure (Table 1).

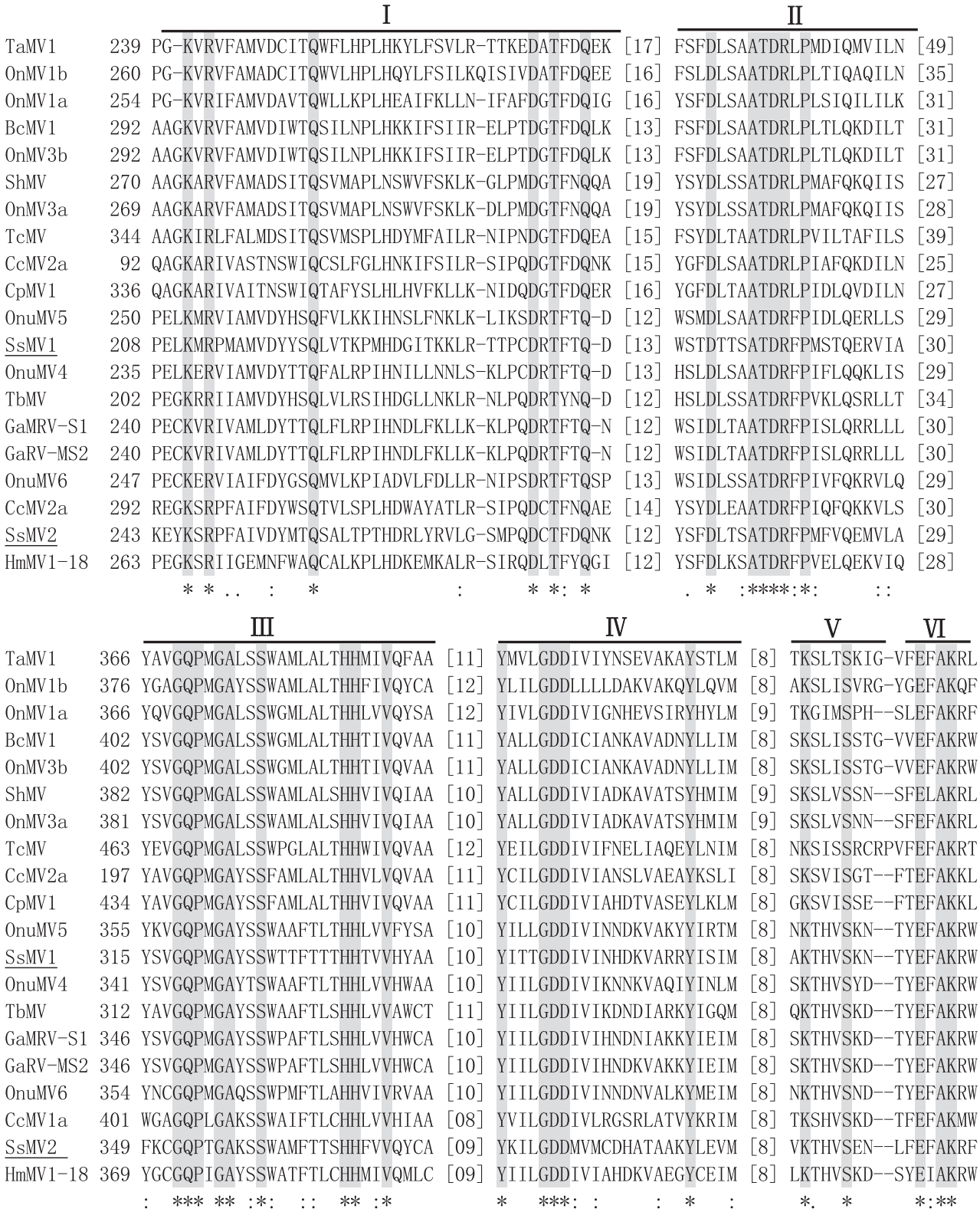

Fig. 4. Multiple alignment of conserved amino acid motifs in RNA-dependent RNA polymerase (RdRp) regions of SsMV1 (SsMV1/KL-1), SsMV2 (SsMV2/KL-1) and corresponding regions in other mitoviruses. The positions of motifs I–VI are indicated by lines above the sequences. Identical residues are shaded and indicated by asterisks; conserved and semi-conserved amino acid residues are indicated by colons and dots, respectively. Numbers in square brackets correspond to the number of amino acid residues separating the motifs.

The stem–loop structure at the termini may play an important function in the replication and translation of single stranded RNA viruses. These secondary structures may also have a function in protecting the naked single stranded RNA from degradation (Hong et al., 1999; 1998). In the present study, we demonstrated that the terminal sequence of RNA from SsMV1/KL-1 and SsMV2/KL-1, like other mitoviruses, could be folded into potentially stable stem–loop structures (Fig. 6); this is another characteristic

feature of mitovirus genomic RNA. In addition, the potential stable panhandle structure predicted at the terminal sequences of the positive strand of some mitoviruses, such as HmMV1–18 (Osaki et al., 2005), could not be predicted for SsMV1/KL-1 or SsMV2/KL-1 because of the lack of the inverted complementarity that is required for formation of panhandle structures. This is similar to some previously reported mitoviruses (Wu et al., 2010).

Mixed infections with two or more related or unrelated viruses have been detected in some phytopathoogenic fungi, such as *Helmintbosporium victoriae* (Ghabrial et al., 2002), *R. necatrix* (Sasaki et al., 2005) and *S. sclerotiorum* (Xie et al., 2006; 2011). Multiple mitochondrial viruses co-infecting the same fungal strain was also reported. For example, at least seven independent species of mitoviruses co-infect the hypovirulent strain Ld of *O. novo-ulmi* (Cole et al., 1998; Doherty et al., 2006). Our results showed that *S. sclerotiorum* strain KL-1 harbors two distinct mitoviruses (SsMV1/KL-1 and SsMV2/KL-1) based on sequence analysis. However, it is not known whether there is an interaction between the co-infecting mitoviruses in the same fungal strain.

The biological effects of mitoviruses on their fungal hosts appear to be variable. Mitovirus-associated hypovirulence has been described in *Botrytis cinerea* (Wu et al., 2007), *C. parasitica* (Polashock and Hillman, 1994), *O. novo-ulmi* (Hong et al., 1999), *S. homoeocarpa* (Deng and Boland, 2006) and *R. solani* (Lakshman and Tavantzis,

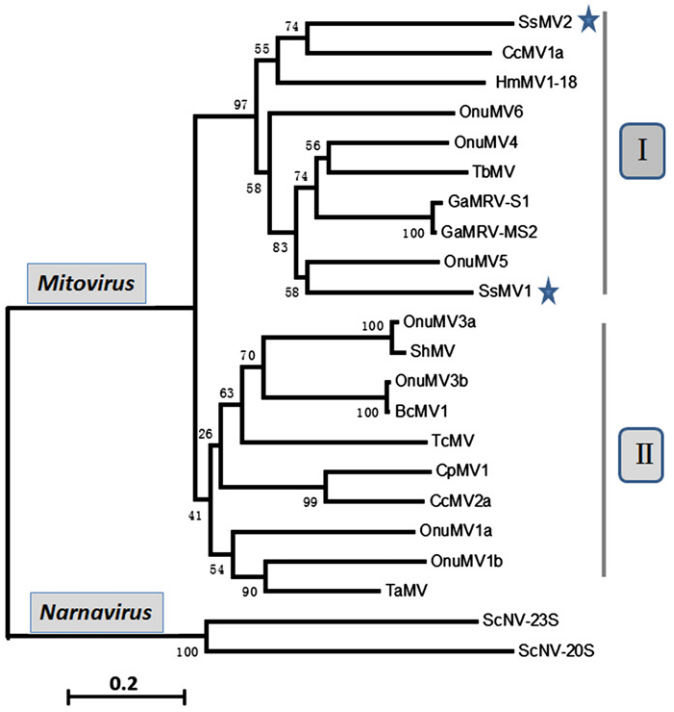

Fig. 5. An unrooted phylogenetic tree constructed based an alignment of the respective RdRp amino acid sequences of SsMV1 (SsMV1/KL-1), SsMV2 (SsMV2/KL-1) and other mitoviruses. A neighbor joining unrooted tree is shown. Bootstrap values (%) obtained with 1000 replicates are indicated on branches and branch lengths correspond to genetic distance; scale bar at lower left corresponds to a genetic distance of 0.2. Abbreviations of virus names and sequence accession numbers are as indicated in Table 1. The results of phylogenetic analysis indicate that mitoviruses could be separated into two large clusters (I and II).

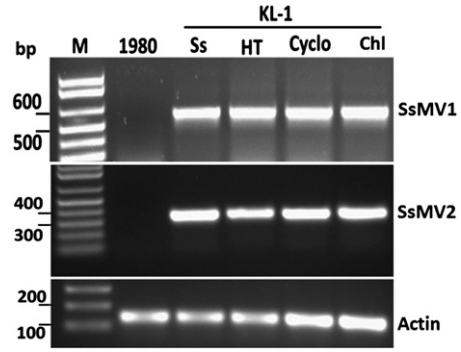

Fig. 7. RT-PCR analysis for detection of the mitoviruses SsMV1/KL-1 and SsMV2/KL-1 in individual isolates of *S. sclerotiorum* subjected to different treatments aimed to eliminate the mitoviruses. The actin gene of *S. sclerotiorum* was used as an internal control. The predicted lengths of the RT-PCR products for SsMV1/KL-1 and SsMV2/KL-1 are 587 and 321 nts, respectively. The treatments applied included: single sclerotia isolation (SS); High temperature (HT); cyclhoheximide (Cyclo) and chloramphicol (Chl).

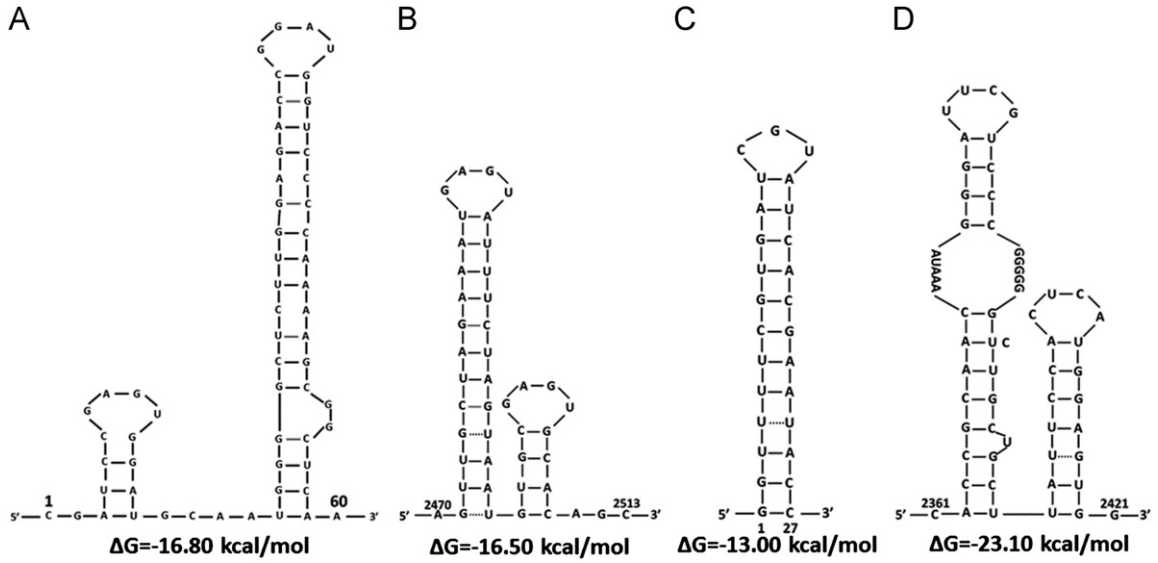

Fig. 6. Predicted secondary structures of the terminal sequences. The 5′ and 3′-terminal sequences of the positive-strand of both SsMV1/KL-1 and SsMV2/KL-1 dsRNAs could be folded into potential stable stem–loop structures. The predicted secondary structures of the 5′- (A and C) and 3′- (B and D) terminal sequences of SsMV1/KL-1 and SsMV2/KL-1, respectively, are shown. The MFOLD program was used for predicting the secondary structures of the terminal sequences and for calculating the free energy.

1994). Furthermore, Wu et al. (2007, 2010) and Polashock et al. (1997) demonstrated that hypovirulence traits and mitoviruses can be co-transferred via hyphal anastomosis in *B. cinerea* and *C. parasitic*, respectively. The present study also presented evidence that SsMV1/KL-1 and SsMV2/KL-1 can be co-transferred to virus-free strain via hyphal anastomosis. Polashock et al. (1997) reported that mitovirus transmission via hyphal anastomosis was accompanied by mitochondrial movement and recombination. The authors proposed that mitochondrial fusion is a possible mechanism for mitovirus transmission. The mitochondrial movement mechanism is predicted to be important in the transmission of SsMV1/KL-1and SSMV2/KL-1 in *S. sclerotiorum*. The results of the pathogenicity assays in the present study suggest that SsMV1/KL-1, SsMV2/KL-1 or a combination of the two mitoviruses may play a significant role in reducing the pathogenicity of *S. sclerotiorum* KL-1. Therefore SsMV1/KL-1 and/or SsMV2/KL-1 could be exploited as biocontrol agents in the management of Sclerotinia diseases. Furthermore, the completely sequenced strain 1980^{hyg} that exhibits the hypovirulence traits when infected with the two mitoviruses might prove valuable in studying virus–host interactions and the molecular basis of virulence in *S. sclerotinia*.

Materials and methods

Fungal isolates

S. sclerotiorum strain KL-1 was isolated from diseased lettuce in a farm in Kentucky, USA. The identity of isolate KL-1 as a strain of *S. sclerotiorum* was verified by amplifying and sequencing the contiguous internal transcribed spacer (ITS) region, ITS 1–5.8S–ITS 2, and comparing the sequence (GenBank accession # JQ739461) with those of other strains in the Genbank. Strain 1980, a virus-free strain isolated from *Phaseolus vulgaris* in Nebraska, USA (Amselem et al., 2011), was kindly supplied by Jeffrey A. Rollins (Department of plant pathology, University of Florida, USA). Strains KL-1 and strain 1980 were determined to be vegetatively compatible since no barrage formation was observed when mycelia from the two strains were in contact with each other and examined with a microscope. Strain 1980 was labeled with a hygromycin-resistance gene (*hyg*) using the PEG-mediated transformation method (Zhao et al., 2006). These two strains were cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit) at 20 °C and stored at 4 °C.

Comparison of biological characteristics

To compare growth rates, 5-mm-diameter mycelial agar plugs from actively growing colony margins of KL-1 and 1980^{hyg} were transferred onto PDA plates, and then incubated at 20 °C. The diameter of colonies of each strain was measured at 24 and 48 h post inoculation (hpi). Colony morphology of each strain was examined daily until mature sclerotia were produced. Six replicates were used for each strain.

To assess the difference in virulence between strain KL-1 and strain 1980^{hyg}, actively-growing mycelial agar plugs were inoculated onto lettuce seedlings and detached leaves of soybean, and then the inoculated hosts were placed in an incubator at 20 °C and 100% relative humidity. The disease lesion development on soybean leaves was examined and photographed at 48, 72 and 96 hpi. There were nine replicates for each treatment. The virulence to the lettuce seedlings was examined at 8 day post inoculation prior to photography.

Horizontal transmission of hypovirulence traits

The methods of Zhang et al. (2009) were followed to assess potential horizontal transmission of hypovirulence traits of strain KL-1. Dual culturing of strain 1980^{hyg} and strain KL-1 on a PDA plate was used to allow the two colonies to contact each other. Following contact, mycelial agar plugs from the colony margin of strain 1980^{hyg} were placed onto a fresh PDA plate containing 25 μg/mL hygromycin; this would only allow labeled strains to grow. Mycelial plugs were taken from the new colony and transferred into fresh PDA plates without hygromycin. The characteristics of subcultures of 1980^{hyg} after contacting strain KL-1 were tested for hypovirulence traits, as described above.

DsRNA extraction and purification

For dsRNA extraction, all fungal isolates were grown on cellophane membranes that were overlaid on the surface of PDA plates for 4–5 day. The mycelia were collected and ground to a fine powder in liquid nitrogen with mortar and pestle, and dsRNA was subsequently isolated using CF-11 cellulose chromatography (Sigma-Aldrich, Dorset, England), as previously described (Jiang and Ghabrial, 2004). The dsRNA preparation was then treated with DNase I and S1 nuclease (NEB) and electrophoresed on 1% agarose gel or on nondenaturing 15% polyacrylamide gel, stained with ethidium bromide, and observed under UV illumination. DsRNA was purified with a gel extraction kit (QIAGEN, USA) and the purified dsRNA was kept in DEPC-treated water and used for making the cDNA clones.

cDNA synthesis and molecular cloning

The cDNA sequence of genomic dsRNA was performed following the method of Xie et al. (2011) using a cDNA synthesis kit (Invitrogen) with tagged random primers-dN6 (5′-CGATCGATCATGATGCAATGCNNNNNN-3′). Briefly, random cDNA products were amplified using a single specific primer (5′-CGATCGATCATGATGCAATGC-3′) based on tagged random primers-dN6. The amplified PCR products were cloned into the pGEM-T Easy vector (Promega) and transformed into competent cells of *Escherichia coli* DH5α and sequenced. Based on the cDNA sequences obtained from dsRNA, gene-specific primers were designed and used for RT-PCR to amplify parts of the genome that were not cloned by the initial random cDNA synthesis.

To determine the 5′ and 3′ terminal sequence of dsRNA, the 3′ terminus of each strand of dsRNA was ligated at 4–8 °C for 18 h with the 5′-end phosphorylated oligonucleotide 5′-GCATTGCATCATGATCGATCGAATTCTTTAGTGAGGGTTAATTGCC-(NH2)-3′ using T4 RNA ligase I (New England Biolabs), as previously described (Liu et al., 2009). The oligonucleotide-ligated dsRNA was denatured and used for the reverse transcription reaction with Superscript II reverse transcriptase and 3 pmol of a primer with sequence complementary to the oligonucleotide used for the RNA ligation (oligoREV, 5′-GGCAATTAACCCTCACTAAAG-3′). The reaction product was treated with RNase H as described above and the cDNA was amplified using another primer complementary to the RNA ligation oligonucleotide (5′-TCACTAAAGAATTCGATCGATC-3′) and the sequence-specific primer corresponding to the 5′- and 3′-terminal sequences of the dsRNA, respectively.

Nucleotide sequencing and sequence analysis.

All PCR products were fractionated by agarose gel electrophoresis and purified using a gel extraction kit (QIAGEN, USA). The PCR products were cloned into the pGEM-T Easy cloning vector and sequenced at the University of Kentucky Advanced Genetic Technologies Center (UK-AGTC). M13 universal primers or sequence-specific primers were used for sequencing and every base was determined by sequencing at least three independent overlapping clones in both orientations.

The deduced amino acid sequences of the two mitoviruses included in this study and those of other selected viruses were obtained from the NCBI database and aligned using Clustal W, and further used for phylogenetic analyses and tree construction. On the basis of the aligned sequences, phylogenetic trees were generated from multiple-alignment by the neighbor-joining method using the MEGA version 4.0 programs (Kumar et al., 2008). The secondary structures of 5′ and 3′-terminal sequence were predicted using the MFOLD software (version 3.5) at the MFOLD web site (http://mfold.rna.albany.edu/) (Matthews et al., 1999; Zuker et al., 1999).

Attempts to cure strain KL-1 from mitoviruses

To eliminate mitoviruses from strain KL-1, several approaches were applied. These include hyphal tipping, single sclerotia isolation, thermotherapy and chemotherapy. For heat treatment, mycelial plugs of strain KL-1 were cultured on PDA at 30 °C for 7–10 day. For chemotherapy, mycelial plugs of strain KL-1 were inoculated on PDA containing either cycloheximide (2, 4 or 8 μg/ml) or chloramphenicol (50, 100 μg/ml) and cultured for 3 day. Then, hyphal tips were transferred to fresh PDA medium containing the same chemicals for a second cycle of chemotherapy. Hyphal tip isolates of all cultures from the different treatments were grown on PDA and generated mycelia were used for total RNA isolation.

Detection of the mitoviruses in the different cultures was made by RT-PCR using gene-specific primers (SsMV1F: 5′-ACCGTGCATTCCATATTGGT-3′ and SsMV1R:5′-TGTCCACACCCAACTTGTTC-3′; SsMV2F: 5′-AAGAGTATAAGTCCCGAC-3 and SsMV2R: 5′-TGAACTCTACCTCGGGACCA-3′). A pair of primers (actin-qF2: 5′-GAGCTGTTTTCCCTTCCATTGTC-3′ and actin-qR4: 5′-GACGACACCGTGCTCGATTGG-3′) were used to amplify a fragment of the actin gene, which was used as internal control (Sexton et al., 2009).

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References

Amselem, J., Cuomo, C.A., van Kan, J.A.L., Viaud, M., Benito, E.P., et al., 2011. Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet. 7 (8), e1002230.

Anagnostakis, S.L., 1982. Biological control of chestnut blight. Science 215, 466–471.

Boland, G.J., Hall, R., 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 16 (2), 93–108.

Bolton, M.D., Thomma, B.P., Nelson, B.D., 2006. *Sclerotinia sclerotiorum* (Lib.) deBary: biology and molecular traits of a cosmopolitan pathogen. Mol. Plant Pathol. 7 (1), 1–16.

Chiba, S., Salaipeth, L., Lin, Y., Sasaki, A., Kanematsu, S., Suzuki, N., 2009. A novel bipartite double-stranded RNA mycovirus from the white root rot fungus *Rosellinia necatrix*: molecular and biological characterization, taxonomic considerations, and potential for biological control. J. Virol. 83 (24), 12801–12812.

Cole, T.E., Hong, Y., Brasier, C.M., Buck, K.W., 2000. Detection of an RNAdependent RNA polymerase in mitochondria from a mitovirus-infected isolate of the Dutch elm disease fungus, *Ophiostoma novo-ulmi*. Virology 268 (2), 239–243.

Cole, T.E., Mcller, B.M., Hong, Y., Brasier, C.M., Buck, K.W., 1998. Complexity of virus-like double-stranded RNA elements in a diseased isolate of the Dutch elm disease fungus, *Ophiostoma novo-ulmi*. J. Phytopathol. 146, 593–598.

Deng, F., Boland, G.J., 2006. Attenuation of virulence in *Sclerotinia homoeocarpa* during storage is associated with latent infection by Ophiostoma mitovirus 3a. Eur. J. Plant Pathol. 114, 127–137.

Deng, F., Xu, R., Boland, G.J., 2003. Hypovirulence-associated double-stranded RNA from *Sclerotinia homoeocarpa* is conspecific with *Ophiostoma novo-ulmi* mitovirus 3a-Ld. Phytopathology 93 (11), 1407–1414.

Doherty, M., Coutts, R.H., Brasier, C.M., Buck, K.W., 2006. Sequence of RNA-dependent RNA polymerase genes provides evidence for three more distinct mitoviruses in *Ophiostoma novo-ulmi* isolate Ld. Virus Genes 33 (1), 41–44.

Ghabrial, S.A., 1998. Origin, adaptation and evolutionary pathways of fungal viruses. Virus Genes 16 (1), 119–131.

Ghabrial, S.A., Soldevila, A.I., Havens, W.M., 2002. Molecular genetics of the viruses infecting the plant pathogenic fungus *Helminthosporium victoriae*. In Molecular Biology of Double-Stranded RNA: Concepts and Applications in Agriculture, Forestry and Medicine. In: Tavantzis, S. (Ed.), CRC Press, Boca Raton, pp. 213–236.

Ghabrial, S.A., Suzuki, N., 2009. Viruses of plant pathogenic fungi. Annu. Rev. Phytopathol. 47, 353–384.

Hillman, B.I., Esteban, R., 2011. Narnaviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, pp. 1055–1060.

Hong, Y., Dover, S.L., Cole, T.E., Brasier, C.M., Buck, K.W., 1999. Multiple mitochondrial viruses in an isolate of the Dutch Elm disease fungus *Ophiostoma novo-ulmi*. Virology 258 (1), 118–127.

Hong, Y., Cole, T.E., Brasier, C.M., Buck, K.W., 1998. Evolutionary relationships among putative RNA-dependent RNA polymerases encoded by a mitochondrial virus-like RNA in the Dutch elm disease fungus, *Ophiostoma novo-ulmi*, by other viruses and virus-like RNAs and by the Arabidopsis mitochondrial genome. Virology 246 (1), 158–169.

Jiang, D., Ghabrial, S.A., 2004. Molecular characterization of *Penicillium chrysogenum virus*: reconsideration of the taxonomy of the genus *Chrysovirus*. J. Gen. Virol. 85 (7), 2111–2121.

Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform. 9 (4), 299–306.

Lakshman, D.K., Tavantzis, S.M., 1994. Spontaneous appearance of genetically distinct double-stranded RNA elements in *Rhizoctonia solani*. Phytopathology 84, 633–639.

Li, H., Fu, Y., Jiang, D., Li, G., Ghabrial, S.A., Yi, X., 2008. Down-regulation of *Sclerotinia sclerotiorum* gene expression in response to infection with *Sclerotinia sclerotiorum* debilitation-associated RNA virus. Virus Res. 135 (1), 95–106.

Liu, H., Fu, Y., Jiang, D., Li, G., Xie, J., Cheng, J., Peng, Y., Ghabrial, S.A., Yi, X., 2010. Widespread horizontal gene transfer from double-stranded RNA viruses to eukaryotic nuclear genomes. J. Virol. 84 (22), 11876–11887.

Liu, H., Fu, Y., Jiang, D., Li, G., Xie, J., Peng, Y., Yi, X., Ghabrial, S.A., 2009. A novel mycovirus that is related to the human pathogen Hepatitis E virus and rubi-like viruses. J. Virol. 83 (4), 1981–1991.

Matthews, D.H., Sabina, J., Zuker, M., Turner, D.H., 1999. Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. J. Mol. Biol. 288 (5), 911–940.

Nuss, D.L., 1992. Biological control of chestnut blight: an example of virus-mediated attenuation of fungal pathogenesis. Microbiol. Mol. Biol. Rev 56 (4), 561–576.

Nuss, D.L., 2005. Hypovirulence: mycoviruses at the fungal–plant interface. Nat. Rev. Microbiol. 3 (8), 632–642.

Osaki, H., Nakamura, H., Nomura, K., Matsumoto, N., Yoshida, K., 2005. Nucleotide sequence of a mitochondrial RNA virus from the plant pathogenic fungus, *Helicobasidium mompa* Tanaka. Virus Res. 107 (1), 39–46.

Osawa, S., Jukes, T.H., Watanabe, K., Muto, A., 1992. Recent evidence for the evolution of the genetic code. Microbiol. Rev. 56 (1), 229–264.

Paquin, B., Laforest, M.J., Forget, L., Roewer, I., Wang, Z., Longcore, J., Lang, B.F., 1997. The fungal mitochondrial genome project: Evolution of fungal mitochondrial genomes and their gene expression. Curr. Genet. 31 (5), 380–395.

Pearson, M.N., Beever, R.E., Boine, B., Arthur, K., 2009. Mycoviruses of filamentous fungi and their relevance to plant pathology. Mol. Plant Pathol. 10 (1), 115–128.

Polashock, J.J., Hillman, B.I., 1994. A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. Proc. Natl. Acad. Sci. USA 91 (18), 8680–8684.

Polashock, J.J., Bedker, P.J., Hillman, B.I., 1997. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: ascospore inheritance and implications for mitochondrial recombination. Mol. Gen. Genet 256 (5), 566–571.

Sasaki, A., Miyanishi, M., Ozaki, K., Onoue, M., Yoshida, K., 2005. Molecular characterization of a partitivirus from the plant pathogenic ascomycete *Rosellinia necatrix*. Arch. Virol 150 (6), 1069–1083.

Sexton, A.C., Minic, Z., Cozijnsen, A.J., Pedras, M.S., Howlett, B.J., 2009. Cloning, purification and characterisation of brassinin glucosyltransferase, a phytoalexindetoxifying enzyme from the plant pathogen *Sclerotinia sclerotiorum*. Fungal Genet. Biol. 46 (2), 201–209.

Stielow, B., Klenk, H.P., Winter, S., Menzel, W., 2011. A novel *Tuber aestivum* (Vittad.) mitovirues. Arch. Virol. 156 (6), 1107–1110.

Wu, M., Zhang, L., Jiang, D., Hou, M., Huang, H.C., 2007. Hypovirulence and double stranded RNA in *Botrytis cinerea*. Phytopathology 97, 1590–1599.

Wu, M., Zhang, L., Li, G., Jiang, D., Ghabrial, S.A., 2010. Genome characterization of a debilitation-associated mitovirus infecting the phytopathogenic fungus *Botrytis cinerea*. Virology 406 (1), 117–126.

Xie, J., Xiao, X., Fu, Y., Liu, H., Cheng, J., Ghabrial, S.A., Li, G., Jiang, D., 2011. A novel mycovirus closely related to hypoviruses that infects the plant pathogenic fungus *Sclerotinia sclerotiorum*. Virology 418 (1), 49–56.

Xie, J., Wei, D., Jiang, D., Fu, Y., Li, G., Ghabrial, S.A., Peng, Y., 2006. Characterization of debilitation-associated mycovirus infecting the plant-pathogenic fungus *Sclerotinia sclerotiorum*. J. Gen. Virol. 87 (1), 241–249.

Yu, X., Li, B., Fu, Y., Jiang, D., Ghabrial, S.A., Li, G., Peng, Y., Xie, J., Cheng, J., Huang, J., Yi, X., 2010. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. Proc. Natl. Acad. Sci. USA 107 (18), 8387–8392.

Zhang, L., Fu, Y., Xie, J., Jiang, D., Li, G., Yi, X., 2009. A novel virus that infecting hypovirulent strain XG36-1 of plant fungal pathogen *Sclerotinia sclerotiorum*. Virol. J. 6, 96.

Zhao, T., Havens, W.M., Ghabrial, S.A., 2006. Disease phenotype of virus-infected *Helminthosporium victoria* is independent of overexpression of the cellular alcohol oxidase/RNA binding protein Hv-p68. Phytopathology 96, 326–332.

Zuker, M., Mathews, D.H., Turner, D.H., 1999. Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide in RNA biochemistry and biotechnology. In: Barciszewski, J., Clark, B.F.C. (Eds.), NATO ASI Series, 11-43. Kluwer Academic Publishers, Dordrecht, NL.