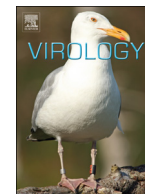




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A novel mycovirus closely related to viruses in the genus *Alphapartitivirus* confers hypovirulence in the phytopathogenic fungus *Rhizoctonia solani*

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ABSTRACT

We report here the biological and molecular attributes of a novel dsRNA mycovirus designated *Rhizoctonia solani* partitivirus 2 (RsPV2) from strain GD-11 of *R. solani* AG-1 IA, the causal agent of rice sheath blight. The RsPV2 genome comprises two dsRNAs, each possessing a single ORF. Phylogenetic analyses indicated that this novel virus species RsPV2 showed a high sequence identity with the members of genus *Alphapartitivirus* in the family *Partitiviridae*, and formed a distinct clade distantly related to the other genera of *Partitiviridae*. Introduction of purified RsPV2 virus particles into protoplasts of a virus-free virulent strain GD-118 of *R. solani* AG-1 IA resulted in a derivative isogenic strain GD-118T with reduced mycelial growth and hypovirulence to rice leaves. Taken together, it is concluded that RsPV2 is a novel dsRNA virus belonging to *Alphapartitivirus*, with potential role in biological control of *R. solani*.

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Introduction

Mycoviruses (fungal viruses) are widespread in all major taxonomic groups of filamentous fungi, yeasts and oomycetes, and an increasing number of novel mycoviruses have been reported recently (Ghabrial and Suzuki, 2009; Pearson et al., 2009). The mycoviruses with RNA genomes are now classified into 11 families, among them, five families (*Chysoviridae*, *Partitiviridae*, *Reoviridae*, *Totiviridae* and *Megabirnaviridae*) accommodate undivided (family *Totiviridae*) or divided (4 segments for family *Chysoviridae*, 2 segments for families *Partitiviridae* and *Megabirnaviridae*, and 11 or 12 segments for family *Reoviridae*) double-stranded RNA (dsRNA) genomes which are encapsidated within capsid proteins with the formation of rigid virus particles, and the remaining six families (*Alphaflexiviridae*, *Barnaviridae*, *Endornaviridae*, *Gammaplexiviridae*, *Hypoviridae* and *Narnaviridae*) accommodate single-stranded RNA (ssRNA) genomes, of which only two families (*Alphaflexiviridae* and *Gammaplexiviridae*) form filamentous particles, and the other four families do not form typical virus particles (Ghabrial and Suzuki, 2009; Lin et al., 2012). Most mycoviruses do not cause any visible abnormal symptoms (cryptic infections) for their host fungi; however, some mycoviruses are known to cause phenotypic alterations including hypovirulence and debilitation (Nuss, 2010).

The basidiomycetous fungus *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a notorious soil-borne plant pathogen causing consistent economic losses in a wide host range of vegetable and field crops, ornamentals and tree species worldwide (Zheng et al., 2013). *R. solani* is a collective species, consisting of at least 14 genetically isolated anastomosis groups (AGs) defined by their hyphal interactions (Strauss et al., 2000; Cubeta and Vilgalys, 1997; Carling, 1996). It does not produce any asexual spores, nevertheless, the sexual stage (teleomorph) is also hard to induce *in vitro* (Sneh et al., 1996). A cytoplasmically controlled degenerative disease of *R. solani* was first reported in 1978 (Castanho et al., 1978). Previous studies showed that dsRNAs are commonly detected in natural populations of *R. solani* AG-2 to -13 (Bharathan et al., 2005). Indirect evidence suggested that the 3.6-kbp (M2) and 6.4-kbp (M1) dsRNAs are associated with diminished or enhanced virulence in *R. solani* AG-3 (Jian et al., 1997). Furthermore, it was reported that M2 dsRNA influenced the disease-causing activity of the fungus, a phenomenon referred to as hypovirulence (Liu et al., 2003). More recently, a novel unclassified dsRNA has been found in *R. solani* AG-1 IA strain B275 in our laboratory (Zheng et al., 2013). Although some progress in the research of dsRNA mycoviruses in many fungi has been made, we have known little about the genome organizations of dsRNAs found in *R. solani* because most dsRNA mycoviruses infect *R. solani* asymptotically (Kousik et al., 1994; Zanzinger et al., 1984). These cryptic mycoviruses are mostly found in the families *Totiviridae* and *Partitiviridae* frequently (Ghabrial, 1998). However, notably previous studies showed that some *Partitivirus* currently were shown to induce

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symptoms in some cases like the *Aspergillus fumigatus* (Bhatti et al., 2011), *Heterobasidium* sp. (Vainio et al., 2010; Ihrmark et al., 2004) and *Cryphonectria parasitica* (Chiba et al., 2013a). These, particularly the last one, provided solid evidence for a viral etiology. The family *Partitiviridae* currently comprises four approved genera, of which *Alphapartitivirus* and *Betapartitivirus* infect plants and fungi, *Gammapartitivirus* infects only fungi, whereas *Deltapartitivirus* so far comprises only viruses isolated from plant host (http://talk.ictvonline.org/files/proposals/taxonomy_proposals_fungal1/m/fung04/4772.aspx).

Recently, reproducible transfection protocols with purified rigid virus particles have been reported for some mycoviruses in the families *Reoviridae* (Hillman et al., 2004; Sasaki et al., 2007), *Megabirnaviridae* (Chiba et al., 2009), *Totiviridae* (Chiba et al., 2013b) and *Partitiviridae* (Sasaki et al., 2007). The advanced technique has helped to clarify the relationship of virus–host interactions and virocontrol (Ghabrial and Suzuki, 2009).

Here we describe the discovery of a novel virus species observed in *R. solani* AG-1 IA strain GD-11, the causal agent of rice sheath blight. In addition, we studied viral genome organization, phylogeny and particle morphology. Furthermore, the virus was transmitted to virus-free strain via protoplast transfection with virus particles. In addition, we characterized the effects of viral infection on colony phenotype and virulence level of the fungal host.

Results

Nucleotide sequences of RsPV2 genomic dsRNAs

The complete nucleotide sequences of two dsRNA segments of RsPV2 were determined 2020 bp (dsRNA-1) and 1790 bp (dsRNA-2) in length (Fig. 1A). The full-length cDNA sequences for the two segments of dsRNA were deposited in GenBank under accession numbers KF372436 and KF372437 for dsRNA-1 and dsRNA-2, respectively. The 5'- and 3'-untranslated region (UTR) of these two dsRNAs were 88 and 60 bp long in dsRNA-1 and 107 and 213 bp long in dsRNA-2, respectively. Moreover, the 5'-UTRs of both dsRNAs were highly conserved, which may be involved in the replication cycle of the dsRNAs (Fig. 1B). It is notable that adenine-rich regions were detected in the 3'-UTRs of dsRNA-1 and dsRNA-2

(Fig. 1B), as shown in other members of *Partitiviridae* (Lim et al., 2005; Osaki et al., 2002; Strauss et al., 2000), which were similar to interrupted poly (A) tails. Additionally, the 5'- and 3'-UTRs of dsRNA-1 and dsRNA-2 were detected to form stem-loop structures using the RNA structure software version 4.6 (data not shown), and the stem-loop structures may play an important role in dsRNA replication and virus assembly (Compel and Fekete, 1999).

Amino acid sequence and phylogenetic analyses

Analysis of RsPV2 organization indicated that dsRNA-1 genome contains a single open reading frame (ORF1) starting at nt 89 and ending at nt 1960 on its plus strand (Fig. 1A). The single ORF1 potentially encodes a 623-amino-acid (aa) protein with a predicted molecular mass of 72.59 kDa. A sequence search with BLASTp suggested that this protein was most closely related to the RdRps of *Diuris pendunculata* cryptic virus (DpCV) (Wylie et al., 2012), cherry chlorotic rusty spot associated partitivirus (CrSPV) (Coutts et al., 2004), and Amasya cherry disease-associated mycovirus (AcPV) (Coutts et al., 2004). Furthermore, a search of the conserved domain database (CDD) and multiple protein alignment confirmed that the predicted RdRp domain includes the six conserved motifs (III to VIII) similar to the RdRp sequences of members of family *Partitiviridae* (Fig. 2). With the exception of RdRp sequence, no proteins homologous to ORF1-encoded polypeptide were detected in the NCBI database. The sequence of dsRNA-2 contained a single open reading frame, ORF2, starting at nt 108 and ending at nt 1577 (Fig. 1A). The deduced amino acid sequence coded for a 489-aa protein with a molecular mass of 53.27 kDa. BLASTp searches of the deduced amino acid sequence of dsRNA-2 ORF2 showed relatively high sequence identity with the coat protein (CP) of the family *Partitiviridae*.

To analyze the relationship between RsPV2 and other dsRNA mycoviruses, phylogenetic trees (Fig. 3A and B) based on the RdRp and CP sequences of RsPV2 and the different members of the family *Partitiviridae* were constructed using the neighbour-joining method (Tamura et al., 2011). The phylogenetic tree clearly placed dsRNA-1 of RsPV2 in a distinctive cluster containing *Diuris pendunculata* cryptic virus (DpCV), cherry chlorotic rusty spot associated partitivirus (CrSPV), Amasya

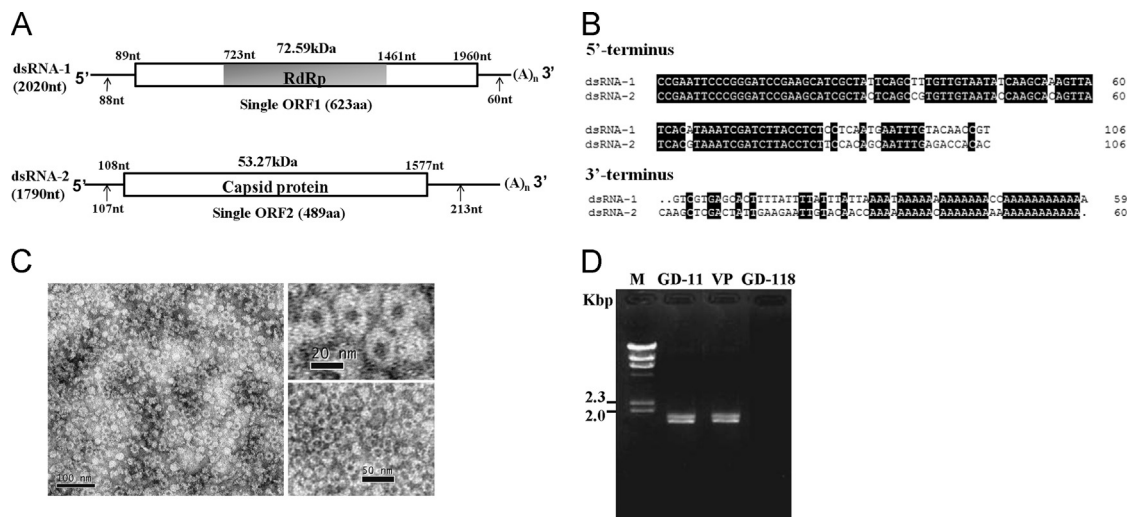


Fig. 1. (A) Genomic organizations of dsRNA-1 and dsRNA-2 of a novel dsRNA mycovirus RsPV2 in *Rhizoctonia solani*. The open reading frame (ORF) and the untranslated regions (UTRs) are indicated by an open bar and a single line, respectively. The shadowing part showed the conserved RNA-dependent RNA polymerase domain (RdRp). (B) Terminal sequence domains of RsPV2 genome. Identical sequences of the 5'-UTR and 3'-UTR of the two dsRNAs are reverse highlighted. (C) RsPV2 particles from strain GD-11. TEM images (negative staining) of the virus particles of RsPV2. (D) Agarose gel electrophoresis of dsRNA extracted from purified virus particles of RsPV2 and from mycelia of strains GD-11 (virus-containing strain) and GD-118 (virus-free strain). Note the similar sizes of dsRNA segments extracted from the purified virus particles and from the mycelia. M indicates molecular markers (λ DNA digested with *Hind* III).

	Motif-III	Motif-IV	Motif-V	Motif-VI	Motif-VII	Motif-VIII
BCV1	VKQDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
DCV1	VKHNDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
WCCV1	VKNDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
VCV	VKQDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
HetPV4	VKQDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
HetPV5	VKQDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
RSPV2	VKQDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
DpCV	VKQDDPNKMRITWGSKFPW [52]	LDWRSRFDKRAY [70]	GIPSGFLITQLLDSWYNTML [16]	KVGGDDSIIRL [24]	NAVNVKSEVFNGLNGREVLVSY [8]	RDEIMMLAQFYHTKA
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Fig. 2. Alignment of amino acid sequences of the RdRp motifs of RSPV2 and those of selected viruses in the genus *Alphapartitivirus*. Conserved amino acids, motifs III–VIII, are shown in horizontal lines above the amino acid areas. Numbers in brackets indicate the position of the amino acid in the ORF. Asterisks indicate identical amino acid residues, and colons show the similar residues. The abbreviations of virus names are as follows: BCV1, beet cryptic virus 1; DCV1, dill cryptic virus 1; WCCV1, white clover cryptic virus 1; VCV, vicia cryptic virus; HetPV4, Heterobasidion partitivirus 4; HetPV5, Heterobasidion partitivirus 5; RSPV2, Rhizoctonia solani partitivirus 2; DpCV, Diuris pendunculata cryptic virus.

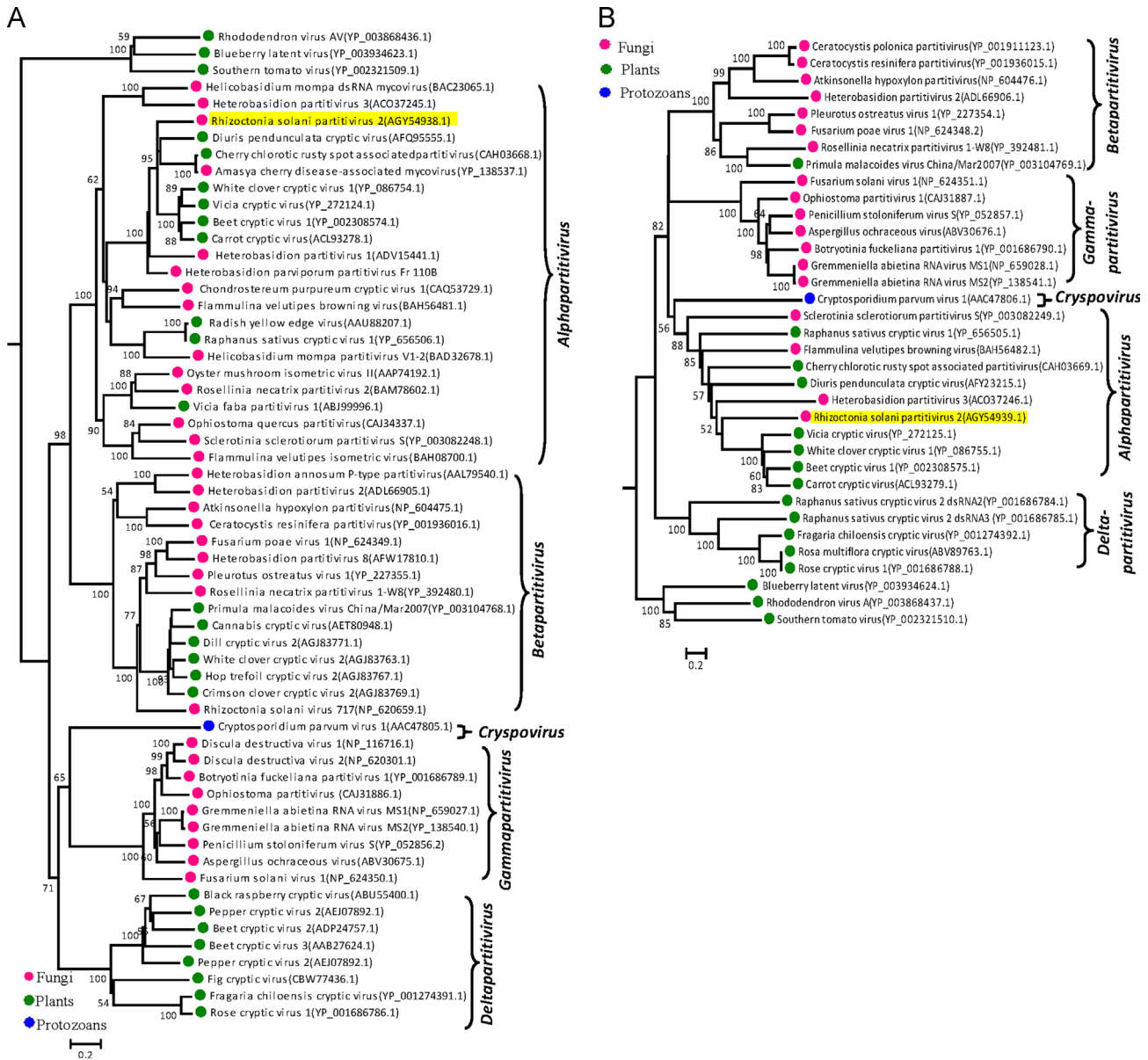


Fig. 3. A phylogenetic trees constructed based on the deduced amino acid sequences of the putative RdRp (Fig. 3A) and CP (Fig. 3B) using the neighbor-joining method with 1000 bootstrap replicates. The scale bar represents a genetic distance of 0.2 amino acid substitutions per site. The trees were rooted with totivirus-like group. Viral lineages were shown in color-dots so as to reflect their host range. Yellow highlighted indicates the novel mycovirus RSPV2 in the present study.

cherry disease-associated mycovirus (AcPV), white clover cryptic virus 1 (WCCV1), vicia cryptic virus (VCV), beet cryptic virus 1 (BCV1) and carrot cryptic virus (CCV) in the genus *Alphapartitivirus*. In addition, the results of phylogenetic analysis with dsRNA-2 and members of the family *Partitiviridae* also indicated that it is grouped with members of *Alphapartitivirus*, and is most closely related to the members of *Alphapartitivirus*

(Fig. 3B). In addition, the comparison between RSPV2 and other representative partitiviruses was conducted and the results were listed in [Supplementary material \(S1\)](#). Therefore, the genome organization, amino acid sequence alignments and phylogenetic analyses all indicated that RSPV2 is a new member of the genus *Alphapartitivirus* within the family *Partitiviridae*.

Viral particles

The virus particles purified from *R. solani* AG-1 IA strain GD-11 were isometric and approximately 30 nm in diameter observed under a transmission electron microscope (TEM) (Fig. 1C). The virus particles accommodate two segments of dsRNA with sizes similar to dsRNA-1 and dsRNA-2 extracted directly from mycelia of strain GD-11 (Fig. 1D). Interestingly, the nucleic acid from virus particles showed brightness similar to that of the two dsRNAs extracted directly from the mycelia of strain GD-11. The results indicated that the molar ratio of the two dsRNAs extracted from virus particles is similar to that of dsRNAs extracted directly from mycelia.

Transfection with viral particles of RsPV2

In order to determine the effect of the virus RsPV2 on the fungal host, we tried to transfect the protoplasts of the virus-free strain GD-118 of *R. solani* with the purified virus particles of RsPV2 in the presence of PEG 4000. After protoplast transfection and regeneration, a derivative virus-transfected strain GD-118T was finally obtained. Colony morphologies of these two isogenic strains GD-118 and GD-118T grown under the same conditions were compared. The results showed that GD-118T had thinner mycelia, fewer and smaller sclerotia, and darker pigmentation on PDA plate when compared with GD-118 (Fig. 4A). RsPV2 infection reduces the mycelial growth (Fig. 4C) and resulted in decline of sclerotial dry weight (Fig. 4D) in strain GD-118T. In addition, the effect of RsPV2 on fungal virulence was evaluated based on lesion sizes on rice leaves caused by the two isogenic strains GD-118 and GD-118T (Fig. 4B). Three days after inoculation, the average lesion areas caused by GD-118T were smaller than those caused by GD-118 (Fig. 4E), indicating that RsPV2 induced hypovirulence in the virus-transfected strain GD-118T.

The two dsRNA segments presented in strain GD-11 were detected consistently in the mycelia of GD-118T (data not shown). The identity of the dsRNA-1 and dsRNA-2 segments in strain GD-118T as the genome of RsPV2 was confirmed by RT-PCR (Fig. 4F-a) with the RsPV2-specific primers (PV2F1/PV2R1 and PV2F2/PV2R2) using total RNA as the templates (Fig. 4F-b).

Discussion

Characterization of newly isolated mycoviruses has contributed to our understanding of the molecular evolution and diversity of mycoviruses, and will lead to the discoveries of novel virion structures and genome organizations (Nibert et al., 2013; Castón et al., 2013; Chiba et al., 2009; Liu et al., 2009). The present study revealed the complete nucleotide sequence, genome organization and viral particle morphology of a novel dsRNA mycovirus RsPV2 infecting *R. solani* AG-1 IA strain GD-11, the causal agent of rice sheath blight. In addition, purified RsPV2 particles were successfully introduced into protoplasts of virus-free strain GD-118 and led to hypovirulence. To the best of our knowledge, this is the first report of a novel dsRNA mycovirus in *Alphapartitivirus* causing hypovirulence in *R. solani* AG-1 IA strains.

Sequence analysis indicated that RsPV2 genome was divided into two segments of 2020 bp and 1790 bp with a single ORF in each segment. The deduced amino acid sequences of the two ORFs of RsPV2 encoded proteins showed a high sequence identity with the RdRp and CP sequences of *Alphapartitivirus*. It is reported that the above-mentioned structure of genome organization is the characteristic of the family *Partitiviridae* (Ghabrial et al., 2011). The phylogenetic analysis with RdRp and CP sequences placed RsPV2 in a distinctive clade with members of

Alphapartitivirus in the family *Partitiviridae* (Fig. 3A and B). In the present study, the RdRps were detected by a small set of conserved motifs and the evolutionary relationship about dsRNAs has been deduced on the basis of homologies found in the conserved motifs of RdRp, because dsRNA mycoviruses evolve and diverge very rapidly (Ghabrial, 1998). Moreover, the RsPV2 virions were isometric particles approximately 30 nm in diameter. The dsRNA segments for the genomes of the mycoviruses in *Partitiviridae* are separately encapsidated (Ghabrial et al., 2011). Therefore, the two segments for RsPV2 were regarded as to be packaged separately.

The 5' terminal sequences of multipartite viruses are conserved among their RNA genomic segments, and several satellite RNAs have showed sequence similarity in 5' and 3' terminal sequences with their helper viruses (Strauss et al., 2000). It was reported that the 5' terminal sequence of *Pleurotus ostreatus* virus 1 (PoV1) was highly conserved and contained inverted repeats capable of forming stem-loop structure (Lim et al., 2005). As expected, the 5' terminal sequences of the two segments of RsPV2 were also highly conserved and formed stable RNA stem-loop structures using RNA structure software version 4.6 (data not shown). Stem-loop structure plays an important role in dsRNA replication and virus assembly (Compel and Fekete, 1999). Significantly, an AC-rich sequence was found in dsRNA-2 before the translation initiation region but not in dsRNA-1. Little knowledge is available for the potential role of AC-rich in mycoviruses except in some plant viruses including tobacco mosaic virus and potato virus X (Zaccomer et al., 1995; Kim et al., 2002). Therefore, the conserved sequence and stem-loop structure of RsPV2 are suspected to be related to the replication and assembly of the virus. In the 3' terminal sequences of dsRNA-1 and dsRNA-2 of RsPV2, there is an adenine-rich sequence in them. The 'interrupted' poly A tails were reported in other members of *Partitiviridae* and were responsible for virus replication cycle. Based on the information about the dsRNA segments, the dsRNA genetic organization, the RdRp sequences and the phylogenetic analyses, we believe that RsPV2 is a novel mycovirus in *Alphapartitivirus* of the family *Partitiviridae* in *R. solani*.

RsPV2 infection is very stable in the mycelia of *R. solani*. An attempt to obtain virus-free strain by hyphal tipping of virus-harboring strain GD-11 was unsuccessful in our laboratory. Furthermore, *R. solani* does not produce any asexual spores and the sexual form is also hard to induce *in vitro* (Sneh et al., 1996). In order to clarify the relationship of virus–host interactions, we successfully introduced the purified RsPV2 virus particles into the healthy virus-free strain GD-118 by means of PEG-mediated mycovirus transfection technique. This useful technique might play an important role in the further determination of experimental host range of RsPV2 for the biological control of rice sheath blight in the near future.

Materials and methods

Fungal strain and cultural conditions

Strain GD-11 of *R. solani* AG-1 IA, which contains viral dsRNA, was isolated from rice sheath with sheath blight symptom in Ruyuan County, Guangdong Province, China. Strain GD-118, a virus-free virulent strain maintained in our laboratory (Yang et al., 2012), was used as a virus recipient in protoplast transfection experiment aimed at obtaining virus-containing isogenic strain GD-118T. All strains used in this study were grown on potato dextrose agar (PDA) medium at 28–30 °C and stored on PDA slants at 4–8 °C.

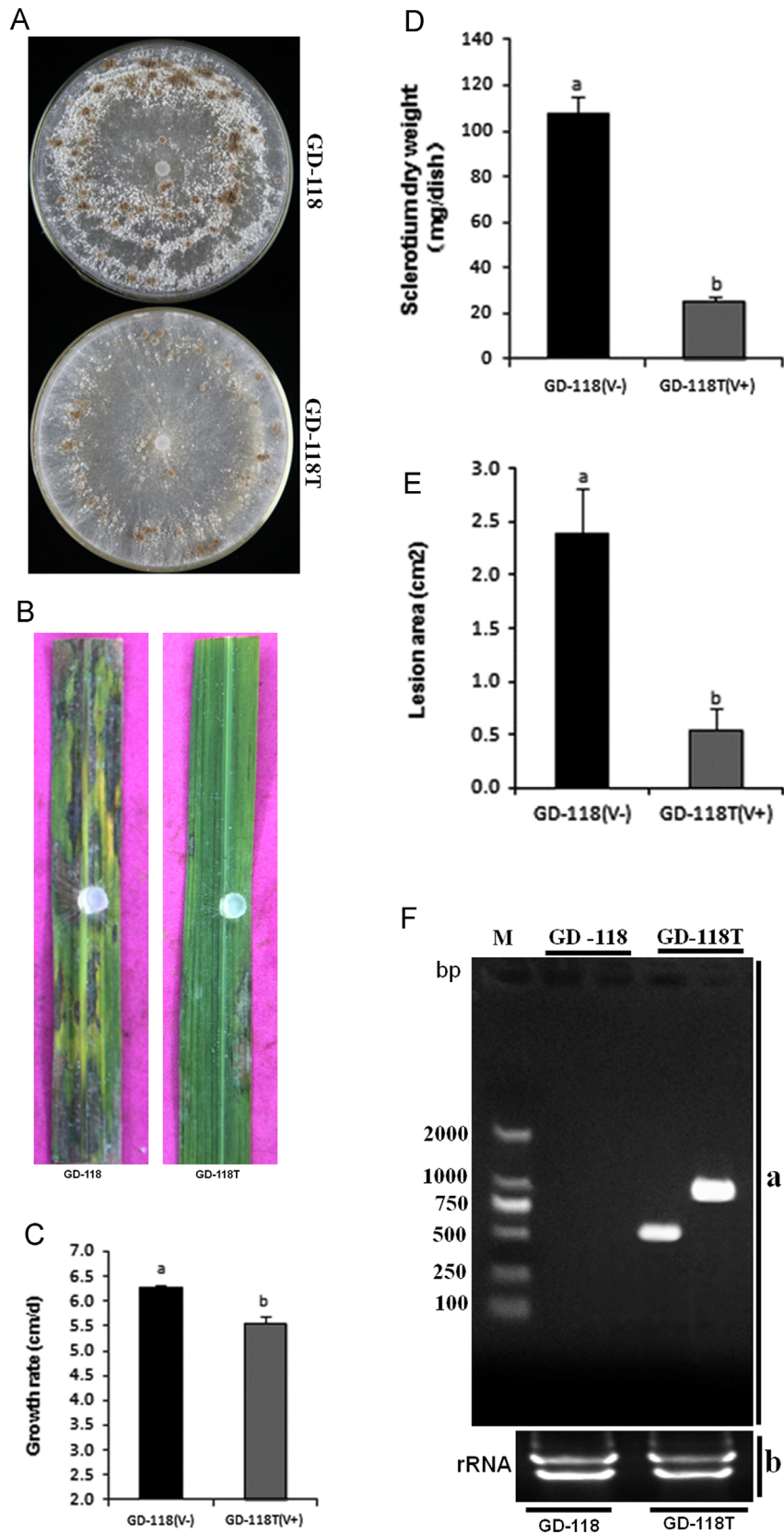


Fig. 4. Hypovirulence-associated traits of strain GD-118T of *Rhizoctonia solani*. (A) Colony morphology. Cultural characteristics of strains GD-118 and GD-118T on PDA plates at 28 °C for 6 days. (B) Pathogenicity. The symptoms on detached rice leaves caused by isogenic strains GD-118 (left) and GD-118T (right) incubated at 28 °C for 72 h. (C) and (D) Comparison of average mycelial growth rates and sclerotium dry weight on PDA plates of the isogenic strains GD-118 and GD-118T. (E) Average lesion areas caused by two isogenic strains GD-118 and GD-118T on detached rice leaves. In (C), (D) and (E), the data were indicated as arithmetic means \pm standard error, and the significant differences were assessed by using Student *t* test. Bars in each histogram labeled with the different letters are significantly different. (F-a) Total RNA samples of isogenic strains GD-118 and GD-118T were reversely transcribed with RevertAid M-MuLV reverse transcriptase and the specific RT primers PV2F1 (5'-CCT CAA CCA AAC CGT CCC T-3')/PV2R1 (5'-GAC TTG ATT AGG CAT TCG-3') and PV2F2 (5'-GCA TCC CCG ATC AAG AAG-3')/PV2R2 (5'-GAC GGT GTC CTT GAG CAT-3') designed based on the sequences of dsRNA-1 and dsRNA-2 of RsPV2 were used to amplify the corresponding conserved fragment of the RsPV2 in GD-118 and GD-118T, respectively. (F-b) Total RNA samples of isogenic strains GD-118 and GD-118T.

Extraction of dsRNA

The Viral dsRNA was extracted from 15 g of frozen mycelia by selective absorption to the columns of cellulose powder CF-11 (Whatman, UK) using the method described by Morris and Dodds (1979) with minor modifications. For the preparation of mycelia from strain GD-11, a mycelium agar plug (5 mm in diameter) cut from colony margin of a 2-day-old culture was placed onto the surface of each PDA plate covered with cellophane membrane and cultured for 5 days, and then harvested the mycelia and stored at -80°C until use.

After extraction, the dsRNA fractions were further purified with DNase I and S1 nuclease so as to digest contaminating DNA and single-stranded RNA (ssRNA), respectively. The quality and concentration of purified dsRNAs were subjected to electrophoresis in 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.1) and visualized by 500 ng/ml ethidium bromide staining.

cDNA cloning, sequencing and sequence analysis

The dsRNAs extracted from *R. solani* AG-1 IA strain GD-11 were fractionated by agarose gel electrophoresis, and the individual dsRNA segments were cut off for further analysis. Cloning of the complementary DNAs (cDNA) for the dsRNAs from strain GD-11 by random primer-mediated PCR, sequencing and analysis of the sequences were done using the procedures described in our previous study (Zheng et al., 2013). To clone the terminal sequences of each dsRNA, the 5' and 3' ends cDNA amplifications were performed using a slightly modified protocol of rapid amplification of cDNA ends (Darissa et al., 2010). Every base was determined by sequencing at least three independent overlapping clones and by sequencing twice from a single clone.

Open reading frames (ORFs) in each full-length cDNA sequence were determined using the National Center for Biotechnology Information (NCBI) ORF Finder program (<http://www.ncbi.nlm.gov/gorf/gorf.html>). The deduced amino acid sequences were performed in the public database at NCBI using the program of BLASTp to search for similar sequences and conserved domains. Motif searches were carried out in three databases, including the CDD database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), the Pfam database (<http://pfam.sanger.ac.uk/>) (Finn et al., 2008) and the PROSITE database (<http://www.expasy.ch/>). Multiple alignments of the sequences of RdRp and CP were performed using the CLUSTAL-X program (Thompson et al., 1997). The phylogenetic trees were constructed with the neighbor-joining (NJ) method of the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al., 2011). Potential secondary structures at the 3'- and 5'-terminal sequences of RsPV2 were predicted using RNA structure software version 4.6 (Mathews et al., 2004).

Purification of viral particles and electron microscopy

Virus particles were purified using the method described by Sanderlin and Ghabrial (1978) with minor modifications. Strain GD-11 of *R. solani* AG-1 IA was grown on sterilized cellophane films placed on PDA at 28°C for 5 days. Mycelia (15 g) were harvested, and then ground to fine powder in the presence of liquid nitrogen using a sterilized mortar and pestle. The powder was mixed with 150 ml of extraction buffer (0.1 M sodium phosphate, pH 7.0 containing 3% Triton X-100). Then the suspension was centrifuged at 10,000g for 20 min to remove the hyphal cell debris. The supernatant was then subject to a 2-h ultracentrifugation at 119,000g under 4°C to precipitate all of the particles. Resultant pellets, suspended in 4 ml of sodium phosphate buffer

(0.1 M), were centrifuged at 16,000g for 20 min. The supernatant containing the virus particles was fractionated through a 10–40% (w/v) sucrose gradient by centrifugation at 70,000g under 4°C for 2 h. Fractions in the middle portion of the tube were carefully collected, and then resuspended in 100 μl of 0.05 M sodium phosphate buffer, pH 7.0. The fractions containing virus particles were observed under a transmission electron microscope (TEM) (Tecnai 12, The Netherlands) after staining with the phosphotungstic acid solution (20 g/L, pH 7.4). The nucleic acid from virus particles was extracted with phenol, chloroform and isoamyl alcohol, and separated by electrophoresis in 1% (w/v) agarose gel.

Protoplast transfection and RT-PCR determination

The virus-free strain GD-118 of *R. solani* was used as a virus recipient in this experiment. Transfection was conducted via the inoculation of protoplasts with purified virus particles (Hillman et al., 2004). The protoplasts of virus-free strain GD-118 were prepared using the method described previously by our laboratory (Yang et al., 2010). Purified virus particles of GD-11 were filtered with Ultrafree-MC sterile centrifugal filter units (Millipore, Tokyo, Japan) and introduced into protoplasts by using polyethylene glycol (PEG) 4000 mediated method described previously by Sasaki et al. (2007), so as to obtain the virus-containing isogenic strain GD-118T. Colonies of dsRNA-containing regenerants of GD-118T were subcultured for more than three generations to confirm whether the dsRNA is stable in each culture.

The total RNA samples were extracted using an E.Z.N.A. Fungal RNA Miniprep kit (Omega, GA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was carried out according to the method of Vainio et al. (2012) with minor modifications. The primers PV2F1/PV2R1 and PV2F2/PV2R2 were designed to amplify the corresponding conserved fragments of the dsRNA-1 and dsRNA-2 of RsPV2, respectively.

Statistical analysis

Analysis of variance in SAS V8.0 (SAS Institute, NC, USA) was conducted to analyze the data on mycelial growth rate and leaf lesion areas caused by strains of *R. solani*. Five replicative plates were made for each strain and each experiment was repeated twice. The statistical significant differences between strains GD-118 and GD-118T of *R. solani* on each of the two above-mentioned parameters were assessed by using Student *t* test at $\alpha=0.05$.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2014.03.029>.

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