Hypovirulence of the phytopathogenic fungus *Botryosphaeria dothidea*: association with a co-infecting chrysovirus and a partitivirus

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Abstract

Botryosphaeria dothidea is an important pathogenic fungus causing fruit rot, leaf and stem ring spots and die-back, stem canker, stem death or stool mortality, and decline of pear trees. Seven double-stranded RNAs (dsRNA1 to 7, with sizes of 3654, 2773, 2597, 2574, 1823, 1623 and 511 bp, respectively) were identified in an isolate of B. dothidea exhibiting attenuated growth and virulence, and sectoring phenotype. Characterization of the dsRNAs revealed that they belong to two dsRNA mycoviruses. The four largest dsRNAs (1 to 4) are the genomic components of a novel member of the family Chrysoviridae (tentatively designated as Botryosphaeria dothidea chrysovirus 1, BdCV1), a view supported by the virion morphology and the phylogenetic analysis of the putative RNA-dependent RNA polymerases (RdRp). Two other dsRNAs (5 and 6) are the genomic components of a novel member of the family Partitiviridae (tentatively designated as Botryosphaeria dothidea partitivirus 1, BdPV1), which is placed in a distinct clade from other established partitivirus genera based on the phylogenetic analysis of its RdRp. The smallest dsRNA7 seems to be a non-coding satellite RNA of BdPV1 based on its terminal sequences being conserved in the BdPV1 genomic segments and on co-segregation with BdPV1 after horizontal transmission. This is the first report of a chrysovirus and a partitivirus infecting B. dothidea, and of a chrysovirus associated with the hypovirulence of a phytopathogenic fungus.
Importance
Our studies identified and characterized two novel mycoviruses, *Botryosphaeria dothidea* chrysovirus 1 (BdCV1) and *Botryosphaeria dothidea* partitivirus 1 (BdPV1) associated with the hypovirulence of an important fungus pathogenic to fruit trees. This is the first report of a chrysovirus and a partitivirus infecting *B. dothidea*, and of a chrysovirus associated with the hypovirulence of a phytopathogenic fungus. BdCV1 appears a good candidate for the biological control of the serious disease induced by *B. dothidea*. Additionally, BdPV1 is placed in a distinct clade from the established genera. BdCV1 capsid has two major structural proteins, which is distinct from that made up by one single polypeptide of the typical chrysoviruses. BdPV1 is the second partitivirus with the putative CP sharing no significant identity with any mycovirus protein. A small accompanying dsRNA, presumed to be a non-coding satellite RNA of BdPV1, is the first of such kind reported for a partitivirus.

Introduction
Mycoviruses, widespread in all major groups of plant pathogenic fungi, associated with latent infections of their hosts. Mycoviruses that debilitate the virulence of their phytopathogenic fungal hosts are valuable for the development of novel bio-control strategies (1) and represent an important way to combat fungal diseases, as exemplified by the successful control of chestnut
blight caused by virulent strains of *Cryphonectria (Endothia) parasitica* with hypovirulent strains of this pathogen from Europe (2-4). Recently, a growing number of this kind of mycoviruses has been reported (5): *Rosellinia necatrix megabirnavirus* 1 (RnMBV1) shows significant potential for biological control of apple white root rot disease induced by *Rosellinia necatrix*, as does *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1) for biological control of the diseases induced by *Sclerotinia sclerotiorum* (6, 7). Key to this purpose is to find mycoviruses debilitating specific phytopathogenic fungi, because the natural host range of the former is limited to individuals within the same or closely related vegetative compatibility groups (1).

Pear is the third most important temperate fruit species, after grape and apple, and has widespread cultivation on the five continents, with major production in China, USA, Italy, Argentina and Spain (8). *Botryosphaeria dothidea* (Moug.: Fr.) Cesati & De Notaris (anamorph *Fusicoccum aesculi* Corda) is the causal agent of pear ring spot, an important disease characterized by ring spots on leaves and stems, fruit rot, die-back, stem canker, stem death or stool mortality, and decline. This fungus has a worldwide distribution and an extremely broad host range, being almost ubiquitous in endophytic communities of woody plants (9). The fungus results in damage to host as the host is stressed by environmental conditions, competition, insect injury, or mechanical damage (10). Presently, control of the disease is restricted to cultural and chemical
approaches, with the long lifespan of woody plants adding further difficulties. Biocontrol measures may represent an important way to combat fungal diseases. Apart from \textit{R. necatrix} and \textit{Helicobasidium mompa}, no other fungi attacking fruit trees have been reported as being infected by mycoviruses (6, 11-17). Here we report a strain of \textit{B. dothidea} (LW-1), isolated from a pear tree from Wuhan (China). On culture media LW-1 grows very slowly and with sectoring phenotypes, and on pear is hypovirulent compared to other strains of \textit{B. dothidea}. Seven dsRNAs were detected in mycelia of strain LW-1, but not in virulent strains, suggesting that strain LW-1 might be infected with one or more mycoviruses. Supporting this view, we have identified and characterized in strain LW-1 two novel mycoviruses, \textit{Botryosphaeria dothidea} chrysovirus 1 (BdCV1) and \textit{Botryosphaeria dothidea} partitivirus 1 (BdPV1) associated with the hypovirulence of this strain. Our result may provide a new approach for the biocontrol of pear ring spot disease.

\textbf{Materials and methods}

\textbf{Fungal isolates and biological characterization}

Hypovirulent strain LW-1 and virulent strain HL-1 of \textit{Botryosphaeria dothidea} were isolated from sandy pear trunks (\textit{Pyrus pyrifolia} nakai cv. ‘Jinshuiyihao’ and ‘Hualiyihao’), respectively, collected in the Fruit and Tea Research Institute, Agricultural Scientific Academy, Wuhan, Hubei province, China. Strain LW-1 was further purified from a separately cultured single hyphal cell, which was
derived from mycelium protoplasts prepared according to a previous report (18). Virus-free strain LW-1-9 was obtained from LW-1 by the hyphal tipping technique (19), with the absence of dsRNAs being assessed by agarose gel electrophoresis. Virulent strain JS-1 was isolated from a sandy pear trunk (*P. pyrifolia nakai* cv. ‘Huanghua’) collected in Nanjing, Jiangsu province, China.

dsRNA extraction and purification

For dsRNA extraction, the virulent strains were cultured on cellophane membranes on PDA plates for 4-5 days, and the hypovirulent strains with slow growth rate for 10-15 days. The mycelia were collected and ground to a fine powder in liquid nitrogen, and subjected to dsRNA extraction using a patented method developed in our lab (unpublished data). The dsRNA preparation was digested with DNase I and S1 nuclease (New England Biolabs), electrophoresed on 1.2% agarose gel, and then visualized by staining with ethidium bromide. dsRNAs were separately excised and purified with a gel extraction kit (Qiagen, USA), dissolved in DEPC-treated water and kept at \(-70^\circ\text{C}\) until use.

cDNA synthesis and molecular cloning

The cDNA sequence of genomic dsRNA was determined following a reported method (20) with some modification. Briefly, purified dsRNAs were subjected to cDNA synthesis using *Moloney murine leukemia virus* (M-MLV) reverse transcriptase (Promega Corp., Madison, WI, USA) with tagged random
primers-dN6 (5’-CGATCGAT-CATGATGCAATGCNNNNNN-3’). The cDNAs were amplified using the tagged oligonucleotide (5’-CGATCGATCATGATGCAATGC-3’) in combination with end-filling with Taq (TaKaRa, Dalian, China). The amplified PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, China) and transformed into competent cells of Escherichia coli DH5a. Sequence gaps between clones were determined by RT-PCR using specific primers designed on obtained cDNA sequences. The 5’ and 3’ terminal sequence of dsRNA were determined as previously described (21). Briefly, the 3’ terminus of each strand of dsRNA was ligated with the 5’-end phosphorylated and 3’-end NH2 closed adaptor primer RACE-OLIGO (5’-p-GCATTCATCAGATCGATCGAATTCTTTAGTGAGGGTTAATTGC-(NH2)-3’) using T4 RNAligase (New England Biolabs, Beijing, Ltd, China) at 16 °C for 16 h, and the oligonucleotide-ligated dsRNA was reverse transcribed with M-MLV reverse transcriptase and 3 pmol of a primer complementary to the oligonucleotide used for the RNA ligation (oligo REV, 5’-GGCAATTAACCCTCACTAAAG-3’). The cDNA was amplified using another primer complementary to the RNA ligation oligonucleotide (O5RACE-2: 5’-TACTAAAGAATTGATCGATC-3’ or O5RACE-3: 5’-CGATCGATCATGATGCAATGC-3’) and the sequence-specific primer corresponding to the 5’- and 3’-terminal sequences of the dsRNA, respectively, and also cloned as above described.

Sequencing was performed at Nanjing Jinsirui Biotechnology Co., Ltd,
China, and every nucleotide was determined at least three independent overlapping clones in both orientations.

**Virus purification from mycelia**

Approximately 30 g fresh mycelia were homogenized in a mixer with 200 ml of phosphate buffer (PB, 8.0 mM Na₂HPO₄, 2.0 mM NaH₂PO₄, pH 7.2) containing 10 mM MgCl₂, 0.45% (w/v) DIECA and 10% (v/v) chloroform at room temperature after cultured in PB at 28 °C for 10 days. The homogenate was shaked at 150 rpm for 30 min at 10 °C and centrifuged at 5000g for 20 min. The resulting supernatant was made to contain NaCl and PEG-6000 to a final contraction of 1% (w/v) and 8% (w/v), respectively, left at 4 °C for 1h, and then centrifuged at 5500g for 15 min at 4 °C. The precipitate was resuspended in 10 mM PB, and subsequently subjected to ultracentrifugation at 148,400 g for 2 h (Optima LE-80K, Backman Coulter, Inc. USA). The resultant sediment was resuspended in 10 mM PB and centrifuged in sucrose density gradients (100-500 mg/ml, with intervals of 100 mg/ml) at 112 700 g for 4 h. An aliquot of each fraction was mixed with an equivalent volume of chloroform, briefly vortexed and centrifuged at 12 000 g for 10 min, and the resulting supernatant was subjected to viral dsRNAs precipitation with ethanol. The preparation was visualized by agarose gel electrophoresis after treatment with DNase I and S1 nuclease (New England Biolabs). The fractions containing viral dsRNAs were re-ultracentrifuged, with the pellets being resuspended in 200 µl 10 mM PB,
stained with 2% (w/v) uranyl acetate and observed with a transmission electron microscope (TEM, H7650; Hitachi).

SDS-PAGE and peptide mass fingerprinting (PMF) analysis of viral proteins

Proteins extracted from each fraction were analyzed by 12% SDS-PAGE with 25 mM Tris/glycine and 0.1% SDS. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Bio-Safe CBB; Bio-Rad, USA). The protein bands on the gel were individually excised and subjected to PMF analysis at Sangon Biotech (Shanghai) Co., Ltd, China, according to a reported method (22).

Horizontal transmission of hypovirulence traits

Horizontal transmission of hypovirulence traits of strain LW-1 was assessed according to a previous method (23). Strain LW-1 and LW-1-9 or HL-1 were dually cultured at 28°C for 7 days to allow the two colonies to contact each other in each dish (9 cm in diameter); the dsRNA-containing hypovirulent strain LW-1 served as the donor, whereas virus-free strain LW-1-9 or HL-1 served as recipients. After incubation of the contact cultures, mycelial agar plugs from the colony margin of strain LW-1-9 or HL-1 were placed on to a fresh PDA plate, and three or four derived isolates were obtained from each recipient strain in the contact cultures. Their biological characterization and virulence were analyzed.
as described in the section of biological characterization. The parental strains LW-1, LW-1-9 and HL-1 were included as controls.

**Growth rate and virulence assay**

Mycelial agar plugs (5 mm in diameter) punched from the colony margin of a 5-day-old culture of each strain or isolate were placed on potato dextrose agar (PDA) in petri dishes (9 cm in diameter), and incubated at 28°C in the dark for determination of the mycelial growth rate and for observation of the colony morphology in quadruplicate. Virulence of each strain was determined by inoculating detached fruits in quadruplicate or branches in triplicate of cultivar ‘Hongxiangsu’ (*P. pyrifolia* nakai cv. ‘Hongxiangsu’) according to a reported procedure unless otherwise statement (1). Briefly, fruits or branches were inoculated with agar plugs of actively growing mycelia, placed in a styrofoam chamber and covered with plastic membrane to keep a constant humid atmosphere (90% RH) between 25°C and 30°C. Non-colonized PDA discs were also inoculated and incubated in parallel as control. The lesions developed from the inoculated samples were measured and photographed at 7 days post inoculation (dpi) for the inoculated fruits and 10 dpi for the inoculated branches.

**Sequence analysis**

Sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI) databases with the BLAST program.
Multiple alignments of nucleic and amino acid sequences were conducted using MAFFT version 6.85 as implemented at http://www.ebi.ac.uk/Tools/msa/mafft/ with default settings except for refinement with 10 iterations. The resulting data were shaded in GeneDoc software (24). Identity analyses were performed with the Molecular Evolutionary Genetic Analysis MEGA4 program (25). The phylogenetic trees for RdRp and CP sequences were constructed according to M. Nibert et al. (a taxonomic proposal for the family Partitiviridae by M. Nibert et al. in 2013, code assigned: 2013.001a-kkF, [http://talk.ictvonline.org/files/proposals/taxonomy_proposals_fungal1/m/fung02/4734.aspx]). Briefly, trees were generated at http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html using the LG substitution model, empirical equilibrium frequencies, program-estimated invariant-proportion value (0.013) and gamma-shape value (1.509), and 4 rate categories. The starting trees were obtained by BioNJ, optimized by both branch length and tree topology, and improved according to the Best of NNI and SPR. Branch support values (%) were estimated by the approximate likelihood ratio test (aLRT) with SH-like criteria. The secondary structures of terminal sequences of the dsRNAs were determined online at the web site (http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold) (26). Open reading frame (ORF) was deduced using DNAMAN DNA analysis software package (DNAMAN version 6.0; Lynnon Biosoft, Montreal, Canada).
Protoplast transfection

Protoplast preparation and transfection was performed according to a previous method (22). The virus-free strain HL-1 was transfected with the purified virus particles of BdPV1, extracted from LW-1-9a, or mixed particles of BdPV1 and BdCV1, extracted from strain LW-1. Mycelium colonies generated from the protoplasts were individually transferred to new PDA plates. The dishes were incubated at 28°C in the dark for 7 days, and the resulting cultures were screened the presence of dsRNAs.

Data analysis

The data were statistically analyzed using SPSS Statistics 17.0 (WinWrap Basic; http://www.winwrap.com) with descriptive statistic, chi-square test, one-way ANOVA and Tukey post-hoc test at significance level of $P = 0.05$. 

Results

LW-1 strain displays hypovirulence traits in vitro and in vivo

Compared to standard *B. dothidea* strains HL-1 and JS-1, LW-1 shows an abnormal phenotype with irregular colony margins with sectoring regions (Fig. 1A, top panel). At 28 °C in darkness, the in vitro growth rate of LW-1 strain is 1.5 mm per day versus 20.8-21.9 mm per day for the standard strains. Importantly, LW-1 strain exhibits no or very weak virulence on sandy pear, with a lesion size less than 5.0 mm on the fruits and branches versus more than
LW-1 strain is associated with a complex pattern of dsRNAs

To investigate whether one or more mycoviruses were responsible for the abnormal phenotype of strain LW-1, mycelia of this and HL-1 strain were subjected to dsRNA extraction, digestion with DNase I and S1 nuclease, and agarose gel electrophoresis. While seven dsRNAs (termed 1 to 7 according with their decreasing sizes) were detected in preparations of LW-1 strain, no dsRNA was observed in preparations from HL-1 strain (Fig. 1B).

The sequence of full-length cDNAs of dsRNA1 to 7 was determined assembling partial-length cDNAs amplified from the purified dsRNAs using RT-PCR with tagged random primers and RACE protocols. The corresponding sequences have been deposited in GenBank with accession numbers KF688736-KF688742.

Analysis of dsRNA1 to 4 reveals that they are the genomic components of a novel chrysovirus

Sequence analysis of the full length cDNAs of dsRNA1 to 4 showed sizes of 3654, 2773, 2597 and 2574 bp respectively, each containing a single ORF in one of the strands (Fig. 1C). The 5'-untranslated regions (5'-UTRs) of the coding strands of dsRNA1 to 4 are 231, 273, 294 and 293 nt long (Fig. 1C), respectively, and share 53.3 to 72.5% identity, while the corresponding 3'-UTRs
are 73, 254, 63 and 128 nt long (Fig. 1C), respectively, and share 41.7 to 75% identity among themselves. Both termini of the coding strands of the four dsRNAs contain conserved sequences: 21 nt (CGCAAAAAAGAAGAAAAGGGG) at the 5’- termini, and seven nt (AUUGUGU) at the 3’- termini (Fig. 2A), and were predicted to fold into stable stem-loop structures, as illustrated for dsRNA1 (Fig. 2C, left panel).

BLASTp searches of the deduced amino acid sequences of ORF1 unveiled the highest identity (45 to 35%) with the RdRps of some tentative members of the family Chrysoviridae. Specifically, alignment of the amino acid sequence of the putative RdRp encoded by ORF1 revealed the eight motifs conserved in members of family Chrysoviridae (see Fig. S1A in the supplemental material) (27-30). Moreover, phylogenetic reconstruction of the complete sequence of the RdRp encoded by ORF1 with the RdRps of selected members of the families of Totiviridae and Chrysoviridae indicated that the former clustered together with the tentative members of the family Chrysoviridae (Fig. 3A). In agreement with this view, BLASTp searches of the deduced amino acid sequences of ORF 2 to 4 showed the highest identity (27%, 31% and 33%, respectively) with the homologous ORFs of a tentative member (Magnaporthe oryzae chrysovirus 1, MOCV1) of the family Chrysoviridae. Based on this evidence, we propose that dsRNAs 1 to 4 are the genomic components of a novel chrysovirus designated as Botryosphaeria dothidea chrysovirus 1 (BdCV1).
The genetic organization of dsRNA5 to 7 reveals their partitivirus-related origin

Analysis of the full-length cDNA sequences of dsRNA5 and dsRNA6 showed sizes of 1823 and 1623 bp respectively, each containing a single ORF in the protein-coding strand (Fig. 1C). The 5′-UTRs of dsRNA5 and dsRNA6 are 35 and 52 nt long (Fig. 1C), respectively, and share 85.3% identity, while the corresponding 3′-UTRs are 70 and 87 nt long (Fig. 1C), respectively, and share 62.9% identity. More specifically, they contain conserved sequences (CGAAAAUGAGUCACAACAUUACA) and (CUCACCCMUACACCA) at their 5′- and 3′-termini, respectively (Fig. 2B). The 5′-UTRs of both dsRNAs are predicted to fold into unstable stem-loop structures, in contrast with the corresponding 3′-UTRs adopting stable stem-loop structures, as illustrated for dsRNA5 (Fig. 2C, right panel).

BLASTp searches of the deduced amino acid sequence of the dsRNA5 ORF1 revealed low identity (22-29%) with partial RdRps of members in the family Partitiviridae and Totiviridae, and with those of a few unassigned mycovirus taxa; it also shared similar low identity (23-28%) with polyproteins or NIb proteins of plant or animal viruses in the family Potyviridae, Caliciviridae and Astroviridae (Table S1). However, alignment of the putative RdRp encoded by dsRNA5 ORF1 revealed six motifs conserved in members of family Partitiviridae (see Fig. S1B in the supplemental material) (31-33).

Moreover, a phylogenetic tree of the putative RdRp encoded by dsRNA5
ORF1 with the RdRps of representative members of the family *Partitiviridae* (Table 1) indicated that the former is clustered as a separate clade together with members of the five genera of the family *Partitiviridae* (Fig. 3B). BLASTp searches of the deduced amino acid sequence encoded by dsRNA6 ORF2 revealed no detectable sequence similarity with any mycovirus protein, while, remarkably, it displayed 46% identity and 59% similarity with a hypothetical protein of *Exophiala dermatitidis* (EHY58581.1, score 343, E value of 2e-109, coverage 91%).

*dsRNA7,* of 511 bp, encodes no ORF and has a 5' (CGAAAAU) and 3' (CA) termini identical to those of *dsRNA5* and *dsRNA6*. Its sequence shares no similarity with the sequences deposited in NCBI database and those of the co-infecting dsRNAs, and it is predicted to fold into a highly branched secondary structure (data not shown).

Based on these analyses, we propose that *dsRNA5* and *dsRNA6* are the genomic components, while *dsRNA7* is a related dsRNA, of a novel partitivirus: *Botryosphaeria dothidea* Partitivirus 1 (BdPV1).

**Sucrose gradient centrifugation reveals that dsRNA1 to 7 are encapsidated in two different kinds of virus-like particles**

To determine whether the dsRNAs associated with LW-1 strain were encapsidated, the presumed virus particles were analyzed by sucrose gradient centrifugation. Examination of the gradient fractions by TEM revealed two
kinds of isometric virus-like particles with a diameter of ~35 and ~40 nm in the fraction corresponding to 400 and 500 mg/ml sucrose (Fig. 4A). Agarose gel electrophoresis of the dsRNAs extracted from each fraction revealed that dsRNA1 to 7 were concentrated in the fractions containing the virus-like particles (Fig. 4C, lane V-BdPV1+BdCV1). SDS-PAGE analysis of the proteins of the fraction corresponding to 400 mg/ml of sucrose (containing both kinds of virus-like particles) revealed the presence of seven bands, five of them with estimated molecular masses of 125, 82, 72, 70, and 54 kDa in very good agreement with the deduced 125, 82, 79, 77, and 55 kDa proteins encoded by the ORFs of dsRNA1, dsRNA3, dsRNA2, dsRNA4, and dsRNA6, respectively (Fig. 4D, lane BdPV1+BdCV1).

When the analysis was extended to over-cultured (about 1.5 months) mycelium of LW-1 in liquid medium or to the derived isolates with only BdPV1 obtained from the horizontal transmission assay (see below), only the ~35 nm virus-like particles were observed by TEM (Fig. 4B), and only dsRNA5 to 7 were detected on agarose gel electrophoresis (Fig. 4C, lane V-BdPV1). Moreover, SDS-PAGE analysis of proteins from the purified particles revealed a major band of 54 kDa, similar to the size deduced for the capsid protein (CP, 55 kDa) of BdPV1 (Fig. 4D, lane BdPV1). When the extracted proteins were stored over long time, e.g., more than 2 days at 4 °C, an additional band corresponding to a protein with a molecular mass about 50 kDa was observed (data not shown); this protein is most likely a degradation product of p55, and
corresponds to the lowest band observed in the SDS-PAGE protein analysis of the sample containing of BdCV1 and BdPV1 (Fig. 4D, lane BdPV1+BdCV1).

To further verify the presence of the deduced proteins on SDS-PAGE analysis, those two (p72 and p70) with sizes different from the deduced ones were purified and subjected to PMF analysis. The results showed that p72 and p70 generated a total of 18 and 17 peptide fragments, respectively (Tables S2 and S3). Of these peptide fragments, 12 from p72 matched the partial sequence encoded by ORF2 of dsRNA2 delimited between amino acids 2 to 591, accounting for 25 % of the entire coverage. And fourteen peptide fragments from p70 matched the partial sequence encoded by ORF4 of dsRNA4 between amino acids 6 to 591, accounting for 21% of the entire coverage, respectively. All peptide fragments matched the deduced sequences with ion scores higher than 37 (Tables S2 and S3); therefore, p72 and p70 were confirmed to correspond to the deduced 79 and 77 kDa proteins encoded by the ORFs of dsRNA2 and dsRNA4, respectively (Fig. 4D, lane BdPV1+BdCV1).

Hypovirulence of *B. dothidea* is associated with the horizontal transmission of BdCV1

In contact cultures between strains LW-1 and LW-1-9 (a virulent sub-isolate obtained from LW-1 by hypal tipping), and between LW-1 and HL-1 (also virulent), strains LW-1-9 and HL-1 grew rapidly and covered the entire plates after 7 days, like in single cultures, while strain LW-1 grew slowly and formed
small colonies in dual or single cultures (Fig. 5AI and II).

Additionally, four mycelial derivative subisolates (LW-1-9a, LW-1-9b, LW-1-9c and LW-1-9d) were obtained from four subcolonies of LW-1-9 in four contact cultures of LW-1/ LW-1-9 (Fig. 5AIII). Isolate LW-1-9b was similar to the donor strain LW-1 in mycelial growth on PDA (2.9 to 4.2 mm/day), morphological features (small colonies), pathogenicity on pear (fruits and branches with lesions of 0 to 10 mm and 2.3 to 5.5 mm, respectively) (Fig. 5BI to III, and C). However, the other three subisolates showed no significant difference in growth (21.25 to 21.81 mm/day) and virulence on pear (fruits and branches with lesions of 28.0 to 41.5 mm and 61.7 to 115.0 mm, respectively) with their parental strain LW-1-9 (Fig. 5BI to III, and C). Examination by agarose gel electrophoresis disclosed the presence of dsRNAs 1 to 4, and 5 to 7 (associated with BdCV1 and BdPV1, respectively) in LW-1-9b, but only the dsRNAs associated with BdPV1 in subisolates LW-1-9a, LW-1-9c, and LW-1-9d (Fig. 5BIV). Therefore, BdCV1 and BdPV1 from strain LW-1 can be horizontally co-transmitted to strain LW-1-9 through hyphal contact, and the hypovirulence and impaired growth rate appear qualitatively correlated with this transmission. On the other hand, when only BdPV1 was horizontally transmitted to strain LW-1-9, no obvious change on virulence or phenotype was observed in the derivatives, thus indicating the major role of BdCV1 in the change of biological properties of *B. dothidea*.

In another experiment, three mycelial subisolates of strain HL-1 (HL-1a, HL-1b, and HL-1c) were obtained from four subcolonies of HL-1 in four contact cultures of HL-1/ HL-1-9. Isolate HL-1a was similar to the donor strain HL-1 in mycelial growth on PDA (2.2 to 3.5 mm/day), morphological features (small colonies), pathogenicity on pear (fruits and branches with lesions of 0 to 10 mm and 2.2 to 5.5 mm, respectively) (Fig. 5BI to III, and C). However, the other two subisolates showed no significant difference in growth (21.25 to 21.81 mm/day) and virulence on pear (fruits and branches with lesions of 28.0 to 41.5 mm and 61.7 to 115.0 mm, respectively) with their parental strain HL-1-9 (Fig. 5BI to III, and C). Examination by agarose gel electrophoresis disclosed the presence of dsRNAs 1 to 4, and 5 to 7 (associated with BdCV1 and BdPV1, respectively) in HL-1a, but only the dsRNAs associated with BdPV1 in subisolates HL-1-9a, HL-1-9b, and HL-1-9c (Fig. 5BIV). Therefore, BdCV1 and BdPV1 from strain HL-1 can be horizontally co-transmitted to strain HL-1-9 through hyphal contact, and the hypovirulence and impaired growth rate appear qualitatively correlated with this transmission. On the other hand, when only BdPV1 was horizontally transmitted to strain HL-1-9, no obvious change on virulence or phenotype was observed in the derivatives, thus indicating the major role of BdCV1 in the change of biological properties of *B. dothidea*. 
HL-1b, and HL-1c) were obtained from the three colonies of strain HL-1 in contact cultures with LW-1 (Fig. 5A). Three subisolates were similar to the parental strain HL-1 both in mycelial growth rate on PDA and in virulence on pear fruits and branches (Fig. 5BI to III, and C). However the dsRNAs 1 to 7 were never detected in these subisolates, nor in their parental strain HL-1 (Fig. 5BIV), thus confirming the association of at least some of these dsRNAs with hypovirulence of *B. dothidea*.

Protoplast transfection

As hypovirulence-associated dsRNAs could not be transmitted from LW-1 to strain HL-1, we tried to transflect protoplast of HL-1 with the mixed particles of BdPV1 and BdCV1. Twenty-two smaller mycelium colonies generated from the protoplasts were individually cultured and screened the presence of dsRNAs. The results indicated that BdCV1 was not transmitted to these derivative isolates, excepting only the 3654bp dsRNA of BdCV1 that was transmitted to a derivative colony HL-1d together with BdPV1, while BdPV1 was transmitted into seven derivative colonies. In the transfection with only the BdPV1 particles, three of twelve derivative colonies contained the expected dsRNAs, and one of them, HL-1e, was chosen as a representative isolate for further assays. Mycelial growth rate test of HL-1d (22.97 mm/day) and HL-1e (25.80 mm/day) revealed that they had similar growth rate with that of HL-1 (24.40 mm/day), which was considerably bigger than that of LW-1 (8.35 mm/day). Pathogenicity test on
Discussion

In this study, we also tried to isolate viral origin DNA or ssRNA of viral origin from B. dothidea strain LW-1, but none of them was found in this strain (data not shown), suggesting the involvement of the mycoviral dsRNAs in the hypovirulence and abnormal phenotypes. Two virus-like particles (~35 and ~40 nm in diameter) were purified (and visualized via TEM) from strain LW-1 mycelia co-infected by BdCV1 and BdPV1, while only the ~35 nm particles were observed from the strain just containing BdPV1 (Fig. 4A and B).

Additionally, dsRNAs 1 to 7 were recovered from preparations containing both particles, whereas only the dsRNAs 5 to 7 were recovered from the ~35 nm particles (Fig. 4C). Furthermore, proteins with the size corresponding to the ORFs of BdCV1 and BdPV1 were revealed by SDS-PAGE or PMF analysis (Tables S2 and S3), with the predicted CPs matching the major protein bands in the gel (Fig. 4D). Altogether these results support that the dsRNAs 1 to 4 are encapsidated in the ~40 nm viral particles of BdCV1, and dsRNAs 5 to 7 in the ~35 nm viral particles of BdPV1; their putative RdRps and CPs are encoded by the ORFs of the dsRNAs 1 and 2 (BdCV1) and of the dsRNAs 5 and 6 (BdPV1), respectively. Two major structural proteins encoded by BdCV1 dsRNAs 2 and 4...
suggest an icosahedral T=1 capsid consisting of 60 coat protein heterodimers. This situation is similar to those of some recently discovered dsRNA mycoviruses such as *Botrytis porri* RNA virus 1 (BpRV1) (22) and quadriviruses (34), but distinct from those of typical chrysoviruses including *Penicillium chrysogenum virus* (PcV) (35) and *Cryphonectria nitschkei* virus 1 (CnV1) (36), reported to have a T=1 capsid made up by 60 copies of one single polypeptide. Multiple protein components of chryso-like viruses were also reported for MoCV1 (28, 37).

Therefore, based on the dsRNA number, RdRp global amino acid sequence similarity and presence of specific motifs, genomic organization, and virion size, BdCV1 and BdPV1 were identified as belonging to the families *Chrysoviridae* and *Partitiviridae* respectively, according to the demarcation criteria for these mycoviruses (1). Phylogenetic analysis of the deduced RdRp of BdCV1 suggested that it is a new tentative member of family *Chrysoviridae* differing from those of the genus *Chrysovirus*, a view further supported by the absence of the (CAA)$_n$ repeats at the 5'-UTRs of the genomic RNAs characteristic of typical members of this genus (27, 29). In the phylogenetic tree, BdCV1 is most closely related with MoCV1. Furthermore, we observed that while only BdPV1 remained in mycelia after prolonged culturing, co-infecting BdCV1 was not detected. This suggests a similar behavior of BdCV1 to MoCV1 that is present in culture media after long culturing (28). It revealed that BdCV1 bears similar molecular and biological features with MoCV1, although they were isolated
Evaluation of the taxonomic status of BdPV1 revealed that it cannot be assigned to any known genera of the family *Partitiviridae* according to the new being proposed criteria (see the taxonomic proposal for the family *Partitiviridae* by M. Nibert et al. in 2013, code assigned: 2013.001a-kkF, [http://talk.ictvonline.org/files/proposals/taxonomy_proposals_fungal1/m/fung02/4734.aspx](http://talk.ictvonline.org/files/proposals/taxonomy_proposals_fungal1/m/fung02/4734.aspx)). Firstly, in the phylogenetic tree inferred from the RdRp amino acid sequences, BdPV1 forms a clade apart from representative members of the known genera (Fig. 3B; Table 1). Secondly, the RdRp amino acid sequence of BdPV1 shows low identity (10.7-21.7%) with those of the other members of the family, less than the identity (>24.7%) shared by the members within each genus (see Table S4 in the supplemental material). Thirdly, the size of dsRNAs and proteins from BdPV1 does not match the size range characteristic of each known genus (Table S5). Moreover, the RdRp of BdPV1 has a characteristic substitution (L instead of F) in one of the six conserved motifs (IV) (Fig. S1B), and a parallel analysis with the putative CP of BdPV1 produced essentially the same results (Tables S4 and Fig. S2).

Together with PsV-F, BdPV1 is only partitivirus with the putative CP sharing no significant identity with any mycovirus protein (38). Consequently, BdPV1 appears separated from the other members of the family *Partitiviridae* in the phylogenetic tree of putative CP sequences (Fig. S2), suggesting that the CP of BdPV1 might have a unique origin. Moreover, the CP of BdPV1 shares a 46%
identity with a hypothetical protein of *E. dermatitidis*, suggesting that a horizontal gene transmission may have occurred between the mycovirus and its host (39-41). Regarding dsRNA7, it is encapsidated in BdPV1 but lacks detectable identity with the coinfecting dsRNAs and with sequences deposited in the NCBI database, indicating dsRNA7 is not a defective interfering RNA. However, unlike the small dsRNA F3 (670 bp, GenBank acc. no. AY738338) of PsV-F (38) and dsRNA 4 (308 bp, no. AF316995) of *Discula destructiva virus 1* (DdV1) (42) encoding a small putative protein, dsRNA7 encodes no protein, suggesting that it could be a non-coding satellite RNA. Although a non-coding satellite RNA (1970 bp, no. L3912) had been stated in *Atkinsonella hypoxylon virus* (AhV) (43), it was deduced to encode five putative proteins ranging from 2.9 to 4.5 kDa using DNAMAN software.

It is worth noting that dsRNAs 1 to 7 are specifically encapsidated in their own viral particles, most likely because, like many other multipartite RNA viruses (6, 22, 27, 28, 38), they contain unique conserved terminal sequences playing an important role in packaging of viral RNA in addition to transcription and replication (44, 45). BdCV1 dsRNAs contain the sequence (CGCAAAAAAGAAAAAG) at the 5'-termini, similar to that (GCAAAAAAGAAAAAGAAAAAG) of MoCV1, a close tentative member of the genus *Chrysovirus* (28), and to that (GAUAAAAAAAA) of some other members of this genus, e.g., *Aspergillus fumigatus chrysovirus* (AfuCV) (46), PcV (27) and *Helminothosporium victoriae* virus 145S (HvV145S) (47). BdCV1 dsRNAs
also contains a defined sequence (GUGU) at their 3’-termini, the same as that of the dsRNAs of PcV, AfuCV and HvV145S. We conclude therefore that these 5’- and 3’- termini most likely mediate packaging of the genomic components of the proposed chrysoirus BdCV1. On the other hand, because dsRNA5, 6 and 7 contain identical sequences at their 5’ (CGAAAAU) and 3’ (CA) termini (Fig. 2B), we conclude that these conserved sequences act as a signal for guiding their co-package in the virions of BdPV1 instead of BdCV1.

In the horizontal transmission experiments by contact culture, both BdPV1 and BdCV1 from strain LW-1 did not infect HL-1 of B. dothidea. This negative result might be due to the vegetative incompatibility between the different stains, as proposed before for BpRV1 (22). In contrast, the two viruses were successfully transmitted from strain LW-1 to LW-1-9. Co-infection by BdPV1 and BdCV1 resulted in significant alteration in growth rate, virulence and sectoring phenotype, while infection by only BdPV1 induced no obvious changes of these biological features, suggesting that BdCV1 is responsible for the phenotypic alterations observed. In the transfection assays with purified virions, BdPV1 particles were transmitted into HL-1, while BdCV1 not. The unsuccessful transfection was not due to the failure of transfection manipulations, as BdPV1 particles can be successfully transmitted into HL-1 when it was mixed with those of BdCV1. The reason for unsuccessful transfection of BdCV1 needs further study, as there are no reports for successful chrysoirus transfection. The transfection assays using purified virions also
indicates that BdPV1 has no attenuated effect on the hypovirulence of its host fungus. It further supports that BdCV1 is closely associated with the hypovirulence of the phytopathogenic fungus, although we cannot eliminate the possibility of synergistic effects on the virulence attenuation by the two viruses at this stage.

Many partitiviruses and chrysoviruses have been identified to infect phytopathogenic fungi, but few of them have been involved in the hypovirulence of their host fungi (1, 48-50). Some partitiviruses infect fruit pathogenic fungi, e.g., Helicobasidium mompa virus (HmV) (6, 17, 51), Rosellinia necatrix partitivirus 1 (RnPV1) (17), RnPV2 (32), and tentative species RnPV3, RnPV4, and RnPV5 (52), while no chrysovirus is known to infect fruit pathogenic fungi (12-17, 45). To our knowledge, this is the first report of a chrysovirus and a partitivirus infecting B. dothidea, and the first report of a chrysovirus associated with the hypovirulence of a phytopathogenic fungus (50). This chrysovirus, therefore, appears a good candidate for the biological control of a serious disease induced by B. dothidea.

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Figure legends

Fig. 1 Properties of the seven dsRNAs extracted from mycelium of strain LW-1 of *B. dothidea*. (A) Colony morphology of LW-1 (top) and HL-1 (bottom). (B) Electrophonic profiles of dsRNA preparations on a 1.2% agarose gel extracted from LW-1 and HL-1 after digested with DNase I and S1 nuclease. (C) Genomic organization of dsRNAs 1 to 4 of BdCV1, and of dsRNAs 5 to 7 of BdPV1.

Fig. 2 Multiple alignments and predicted secondary structures for the terminal regions of the coding strand of dsRNAs of BdCV1 and BdPV1. (A) and (B), conserved sequences of the 5'-termini (I) and 3'-termini (II) of the dsRNAs of BdCV1 and BdPV1, respectively. Black, grey and light grey backgrounds denote nucleotide identity no less than 100%, 80% and 60%, respectively. (C) Secondary structures proposed for the dsRNA1 and 5 with lowest energies ([http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold](http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold)).

Fig. 3 Phylogenetic analysis of the RdRp sequences of BdCV1, BdPV1 and selected members of family *Totiviridae*, *Chrysoviridae* and *Partitiviridae* listed in Table 1. The phylogenetic trees for RdRp sequences for BdCV1 (A) and BdPV1 (B) were constructed according to M. Nibert et al. (2013) completely. Two picobirnavirus sequences were used as outgroup for phylogenetic analysis of BdPV1.

Fig. 4 Virus-like particles, dsRNAs and proteins extracted from the fraction corresponding to 400 mg/ml sucrose following sucrose gradient centrifugation.
(A), electron micrograph of virus-like particles purified from strain LW-1 co-infected by BdPV1 and BdCV1, and (B), from over-cultured LW-1 or LW-1-9a containing only BdPV1. (C) agarose gel electrophoresis analysis of the dsRNAs extracted from purified virus-like particles of BdPV1 from over-cultured LW-1 (V-BdPV1), mixed BdPV1 and BdCV1 from LW-1(V-BdPV1+BdCV1), and mycelia of strain LW-1 (F-BdPV1+BdCV1). ‘V-’ and ‘F-’ indicated dsRNAs extracted from virus-like particles and fugal mycelia, respectively. M, DNA size marker. (D) SDS-PAGE analysis of proteins extracted from purified particles of BdPV1 from over-cultured LW-1 (BdPV1), and mixed BdPV1 and BdCV1 from LW-1 (BdPV1+BdCV1). M, protein molecular weight marker.

**Fig. 5** Horizontal transmission of BdCV1 and BdPV1, growth rate on PDA, and fruit lesion length and virulence tests of different strains of *B. dothidea* on pear. (A) Colony morphology of LW-1, LW-1-9 and HL-1 in single culture (I) and contact culture (II), and of the subisolates derived from the colony margins of the recipient strains (III). The symbol “*” indicates the place from where a mycelial agar plug was removed for the generation of a derivative isolate for HL-1 or LW-1-9. (B) Histograms of growth rates of subisolates derived from the contact cultures and the parent strains (I), and of the length of lesions induced on fruits (II) and branches (III) of pear (*P. pyrifolia* nakai cv. ‘Hongxiangsu’), and of the presence of BdPV1 and BdCV1 (IV). Symbols “+” and “-” indicate the presence and absence of BdPV1 or BdCV1, respectively, based on the
results of dsRNA detection by 1.2% agarose gel electrophoresis. (C) Virulence tests of HL-1, LW-1 and the subisolates on fruits (I) and branches (II) of pear (P. pyrifolia nakai cv. ‘Hongxiangsu’). (D) Histograms of growth rates of subisolates derived from the protoplast transfection and the parent strains (I), and of the length of lesions (II) showed by virulence tests (III) on fruits of pear (P. bretschneideri Rehd. cv. Mili). CK in (C) or (D) indicates treatments inoculated with non-colonized PDA plugs.
Table 1 Information of the virus isolates used for sequence alignment and phylogenetic analysis of their RdRps in Fig. 3 and S1

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<th>G&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>F, Family; <sup>b</sup>T, Totiviridae; C, Chrysoviridae; P, Partitiviridae; Pi, Picobirnaviridae.
<sup>b</sup>G, Genus; T, Totivirus; V, Victorivirus; C, chrysovirus; TC, Tentative chrysovirus; A, Alphapartitivirus; B, Betapartitivirus; D, Deltapartitivirus; G, Gammapartitivirus; PC, Cryspovirus, Pi, Picobirnavirus.