Viral cross-class transmission results in disease of a phytopathogenic fungus

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INTRODUCTION

Virulence is a key trait of pathogens, but some scientists believe that virulence brings very few benefits for pathogens, unless it is necessary for their transmission [1]. According to “conventional wisdom”, parasites should evolve to be increasingly benign or less virulent, as diseases can decrease the fitness of hosts and may lead to the extinction of possibly both the hosts and the microbes [2–4]. However, the evolution of virulence is also influenced by many factors, such as host condition [5, 6], host density [7, 8], or interactions with other species [9]. In some cases, increased virulence may be accompanied with host shift [10], changes in host population [11], or the competition with other parasites [12]. For instance, the dense and uniform host populations in an agro-ecosystem favor the emergence of highly virulent, host-specialized plant pathogens, and this is an important factor driving the origins of plant pathogens in agro-ecosystems [13]. In contrast, long-term co-evolution between hosts and pathogens may favor decreased virulence [14, 15], in this case, mycoviruses are considered to be a good example [16, 17].

Mycoviruses are viruses infecting fungi, yeasts, and oomycetes, which are ubiquitous in all major fungal groups. They were first reported in 1962 causing a disease of the cultivated button mushroom Agaricus bisporus [18]. Since then, a large number of mycoviruses have been discovered, and most of them have double-strand (ds) or single-strand (ss) RNA genomes, while a few possess genomes of DNA. In some cases, mycoviruses can significantly alter the biology of their hosts, such as reduction of mycelial growth rate and attenuation of virulence (e.g., hypovirulence). In contrast, some others are able to enhance the virulence of their fungal hosts [19–23]. Furthermore, mycoviruses can participate in some complex mutualistic symbiosis, enhancing the survival of host fungi and plants under extreme conditions [24]. In addition, mycoviruses may also be involved in the interaction between biocontrol agents and target phytopathogenic fungi [25]. Although mycoviruses having biological effects are frequently observed and widely investigated, most mycoviral infections are asymptomatic or cryptic, and this may be related to their lifestyle. Different from animal or plant viruses, mycoviruses are deficient of extracellular stage in their lifecycles [26], with a few exceptions [27]. Most of them are still believed to be mainly transmitted vertically through spores and horizontally via hyphal anastomosis between vegetatively compatible individuals [28]. As a consequence, their lack of virulence may be due to their limited transmission among different individuals, therefore, in the long run, mycoviral infection is likely to be relatively benign, or possibly even beneficial [3, 4, 16, 29–32].

Although most mycoviral infections are asymptomatic, hypovirulence-associated mycoviruses are widely reported in many phytopathogenic fungi, and they are also the most
investigated viral group [33, 34], as some of them could be used or have the potential for the control of plant fungal diseases [35]. This raises the question that why this viral group is widely detected in different fungal species, as this is contrary to the above-mentioned arguments. Moreover, many hypovirulent fungal strains carrying mycoviruses can be initially isolated from diseased plants with typical symptoms. These strains were less virulent or even avirulent on plants when being re-inoculated on the same host plants, and some biological characteristics may change as well, such as sporulation and sclerotial formation [36, 37]. If these strains were hypovirulent or avirulent at the beginning, how could these strains cause severe damage on plants? To answer this question, one explanation is that these fungal strains may have acquired the mycoviruses recently between species [38]. Similar cases have been widely investigated in animal viruses [39].

Compared with plant and fungal viruses, animal viruses are the most intensively studied viral group, as some of them can cause infectious diseases that threaten humans and animals [40–42]. Although some animal viruses have a wide host range, they do not cause significant symptoms in all hosts [43, 44], and one typical example is bat viruses. Due to recent studies of virus tracing, bats are considered as reservoirs for many emerging zoonotic viruses [45], including rabies virus [46], Nipah virus [47], Hendra virus [48], Marburg virus [49], Zaire Ebola virus [50], and coronaviruses such as severe acute respiratory syndrome coronavirus (SARS-CoV) [40, 51], Middle East respiratory coronavirus [52], and recently discovered SARS-CoV-2 which causes the current pandemic of COVID-19 [53]. Although bats harbor so many zoonotic viruses, most of them do not seem to show ostensible symptoms [54].

To test whether similar situation occurs in fungi, we investigated the possibility of viral transmission between two fungal species, Leptosphaeria biglobosa and Botrytis cinerea, which have similar ecological niches on oilseed rape (Brassica napus L.). L. biglobosa, together with its sister species L. maculans, is the causative agent of black leg disease in Br. napus and many other Brassica species worldwide [55]. Both two fungal pathogens could cause lesions on leaves or cankers on stems, of which L. maculans is more aggressive than L. biglobosa. These two related species co-exist in most Br. napus growing regions of the world except in China where only L. biglobosa has been reported. B. cinerea has a wide host range including more 1400 plant species, and causes great losses on many economically important crops [56]. Similarly, oilseed rape and other Brassica vegetables are also ideal hosts for B. cinerea. Both fungal pathogens infect similar tissues of Brassica crops, including leaves and stems. Sometimes, co-infection of two fungi on the same plant tissue could be observed (Fig. 1). Therefore, we speculate that cross-species transmission of mycoviruses may occur between the two species.

In the present study, a mycovirus namely Leptosphaeria biglobosa botyrbirnavirus 1 (LbBV1) was used for testing viral cross-species transmission between L. biglobosa and B. cinerea. We first characterized LbBV1, and investigated the effects of LbBV1 on its native host L. biglobosa. Then, the transmission of LbBV1 from L. biglobosa to B. cinerea was also tested, as well as the biological changes mediated by LbBV1 on B. cinerea. Moreover, viral cross-species transmission between B. cinerea and L. biglobosa under field conditions was investigated by using high-throughput sequencing. Our results indicate that viral cross-species transmission may occur frequently in nature if two fungal species share the same ecological niche, and this may lead to hypovirulence of newly invaded phytopathogenic fungi.

MATERIALS AND METHODS
Fungal strains and biological characterization
L. biglobosa strain GZJS19 was originally isolated from diseased oilseed rape stem with “blackleg” symptom collected in Jinsha County, Guizhou Province, China following the method previously described [57]. All fungal strains (Table S1) were cultured on potato dextrose agar (PDA) at 20 °C and stored at 4 °C, or fungal mycelial plugs were stored in 20% sterilized glycerol at –80 °C. To remove LbBV1 from strain GZJS19, the hyphal tips of strain GZJS19 were picked out and cultured on PDA for ten generations, and a LbBV1-free strain GZJS19-VF was obtained.

B. cinerea and L. biglobosa strains on PDA were incubated at 20 °C (12 h light/12 h dark) for determination of mycelial growth rate and for observation of colony morphology. Virulence assay of L. biglobosa on Br. napus (20 °C, 7 day) and B. cinerea on Nicotiana benthamiana (20 °C, 72 h) were performed according to established protocols [58, 59]. The radial mycelial growth rate of two fungal species on PDA was determined using the procedures described in our previous studies [59].

DNA Extraction and fungal strain identification
Total DNA of fungal mycelia grown on PDA was extracted by using the cetyltrimethylammonium bromide method. The simple sequence repeat (SSR) analysis was used to distinguish different L. biglobosa strains with the primer pair SSR17-F/SSR17-R (Table S2). The identities of L. biglobosa and B. cinerea strains were confirm by using PCR with species-specific primer pairs LbF/LmacR and Bc-F/Bc-R (Table S2), respectively.

RNA extraction, viral genome sequencing, and RT-PCR detection
Extraction and purification of dsRNA and total RNA from mycelia of B. cinerea and L. biglobosa were performed as described previously [60]. The dsRNA was further confirmed based on the resistance to DNase I and RNase A (Promega, Madison, WI, USA). All RNA samples were stored at –80 °C, and was fractionated by agarose gel (1%, w/v) electrophoresis and visualized by staining with ethidium bromide (1.5 μg/L) and viewing on a UV trans-illuminator.

To determine the genome of LbBV1, the purified dsRNAs were excised from the gel and purified using AxyPrepTM DNA Gel Extraction Kit (Axygen Scientific, Inc.; Union City, CA, USA). The cDNA library of two dsRNA bands was produced using a random primer-mediated PCR amplification protocol [61] and sequenced as previously described [62]. The terminal sequences of each dsRNA were cloned through ligating the 3’-terminus for each strand of each dsRNA with the 5’-terminus of the 110A adaptor (Table S3) using T4 RNA ligase (Promega Corporation, Madison, WI, USA) at 16 °C for
18 h, and then reverse transcribed using primer RC110A (Table S3). All these amplicons were detected by agarose gel electrophoresis, gel-purified, and cloned into E. coli DH5α, sequenced, and assembled as previously described [61] to obtain the full-length cDNA sequences of LbBV1.

For reverse transcription-PCR (RT-PCR) detection of LbBV1, cDNA was synthesized using Reverse Transcriptase M-MLV (RNase H-), and two pairs of specific primers (RT-1-F/RT-1-R, RT-2-F/RT-2-R, Table S3), which were designed based on the sequences of dsRNA-1 and dsRNA-2, generating amplicons of 871 bp and 870 bp in size, respectively. PCR amplification was carried out by using 2x PCR Mix (Tsingke Biotechnology Co., Ltd.) with the above specific primer pairs.

**Sequence analysis of LbBV1**

The full-length cDNA sequences of two dsRNAs were used as queries to BLAST search the public database at National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). ORFs were deduced using the ORF Finder program in NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Conserved domains of each dsRNA were deduced using CDD database (http://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi). Multiple alignments of the sequences of RNA-dependent RNA polymerase (RdRp) were accomplished using the DNAAMAN program and phylogenetic trees based on the sequences of RdRp domain were constructed using the neighbor-joining (NJ) method and tested with a bootstrap of 1000 replicates to determine the reliability of a given branch pattern in MEGA 7.0.

**Purification of viral particles and TEM**

Viral particles were purified as described previously [62] with some modifications. Mycelial plugs of strain GZJS19 were inoculated in potato dextrose broth in shake culture 150 rpm/min at 20 °C for 7 days. Mycelia were harvested by filtration with three layers of lens tissue. About 30 g of mycelia were ground into fine powder in liquid nitrogen, and then transferred to a 1000 ml-blender containing 200 ml extraction solution of 0.1 M sodium phosphate with 3% (wt/vol) Triton X-100 (pH 7.0). The mixture was blended twice for 2 min each. Then, the suspension was transferred to 50 ml plastic tubes, followed by centrifugation at 10,000 × g for 20 min to remove the hyphal cell debris. The supernatant was then carefully layered out and ultracentrifuged at 119,000 × g under 4 °C for 2 h. The pellet was re-suspended in 0.5 ml of sodium phosphate buffer (0.05 M), and the re-suspended solution was then overlaid on a centrifuge tube containing sucrose solutions with a concentration gradient ranging from 10 to 50% (wt/vol), and centrifuged at 70,000 × g under 4 °C for 2 h. Each fraction was carefully collected and suspended in 150 μl of 0.05 M sodium phosphate buffer (pH 7.4) and then individually tested for the presence of dsRNA by agarose gel electrophoresis. The purified virus particles were stained with phosphotungstic acid solution (20 g/l, pH 7.4) and observed under a transmission electron microscopy (TEM, Hitachi HT7800/HT7700). The structure proteins from viral particles were detected by electrophoresis on a 12% (wt/vol) polyacrylamide gel (PAGE) amended with 1% (wt/vol) sodium dodecyl sulfate (SDS).

**Northern hybridization**

Northern hybridization was performed to confirm the authenticity of the cDNA sequences generated from dsRNA-1 (6.2 kb) and dsRNA-2 (5.9 kb) in strains GZJS19 of L. biglobosa and t-459 of B. cinerea. Two DNA probes, nt positions 1228–1697 for Probe 1 and nt positions 2318–2957 for Probe 2 (Table S3), were designed based on full-length cDNA sequences of dsRNA-1 and dsRNA-2, respectively. The purified dsRNA-1 and dsRNA-2 were separated in 1% (wt/vol) agarose gel and transferred to Immobilon-NE membranes (Millipore, Bedford, MA, USA) by the capillary transfer method using 20 × SSC as transfer buffer [62]. Probe 1 and Probe 2 were pre-labeled as described by the manufacturers (GE Healthcare, Little Chalfont, United Kingdom) for hybridization with the denatured dsRNAs blotted on two membranes, respectively. The chemiluminescent signals of the probe-RNA hybrids were detected by using a CDP-Star kit (GE Healthcare Life Sciences, China).

**Vertical transmission of LbBV1 in L. biglobosa**

Vertical transmission refers to transmission of hypovirulence-associated dsRNAs from mycelia to the next generation via sexual or asexual spores [33]. Here, vertical transmission of LbBV1 refers to viral transmission through the asexual spore—conidia of L. biglobosa. Conidial suspension (1 × 107 conidia/ml) was evenly spread on PDA plates (150 μl/plate) for the generation single conidial colonies. All single conidial (SC) strains were subjected to LbBV1 detection with RT-PCR. Six SC strains (VT-1, VT-2, VT-3, VT-4, VT-5, and VT-6) were selected for investigation of biological property.

**Intra- and interspecies transmission of LbBV1**

For interspecies transmission, the pairwise culture technique as previously described [63] was used to test the viral transmission from L. biglobosa strain GZJS19 to strains Lb731 and W10, and from LbBV1 infected derivative strain HT-2 to W10. Strain W10 was resistant to hygromycin, which could be used to rule out the contamination by strain GZJS19 as both strains shared the same SSR marker. The derivative strains of W10 were cultured on PDA containing hygromycin B (50 μg/ml). For interspecies transmission, three methods were explored to test the viral transmission from L. biglobosa strain GZJS19 to B. cinerea strain t-459. Besides pairing culture technique, conidia of strain GZJS19 and t-459 were mixed and co-cultured on PDA to evaluate the efficiency of interspecies transmission of LbBV1. Conidal suspension of B. cinerea strain t-459 (200 μl, 1 × 107 conidia/ml) was mixed with that of L. biglobosa strain GZJS19 (800 μl, 1 × 107 conidia/ml), and 100 μl of the mixed conidial suspension were evenly spread on a PDA plate with ten repeats and inoculated at 20 °C for 24 h (Fig. S1). Moreover, mixed conidial suspension of the same composition was also inoculated on sterilized healthy oilseed rape stems (8 repeats) in a sealed flask at 20 °C for 2 weeks (Fig. S2). The presence of LbBV1 in all derivative strains and biological properties of partial derivative strains were determined as described above.

**Protoplast transfection**

Protoplast transfection was used to introduce viral particle of LbBV1 into protoplast of B. cinerea strain t-459 in the presence of polyethylene glycol (PEG) 4000 [63]. LbBV1 particles were mixed gently with 100 μl t-459 protoplast suspension and inoculated in ice bath for 30 min, then 200 μl, 200 μl, and 600 μl PEG solutions were gradually added to the above-mixed suspension in three steps and inoculated at 20 °C for 20 min. The mixed suspension of 200 μl was evenly mixed with 20 ml regeneration medium and then poured in six Petri plates and inoculated at 20 °C for 2 days. Single regenerated colonies were picked up from the surface of regeneration medium and subsequently cultured before detection of LbBV1.

**High-throughput sequencing and sequence analysis**

To test whether viral cross-species transmission occurs between B. cinerea and L. biglobosa under natural conditions, two fungal species from same infected tissues of Brassica crops were isolated and used for RNA-seq. Sixteen B. cinerea strains and 15 L. biglobosa strains (Table S4) were finally obtained. Total RNA samples were extracted as described above. One microgram of RNA was taken from each strain, and RNA from two fungal species were then mixed respectively into two samples (B. cinerea and L. biglobosa) and sent to GENEWIZ (Suzhou, China) for RNA sequencing. Ribosomal RNA depletion (Ribo-Zero RNA Removal Kit, Illumina, Inc.), library preparations (~1 μg RNA, TruSeq RNA Sample Preparation Kit, Illumina, Inc.), and high-throughput sequencing in a HiSeq X ten system (Illumina, Inc.) were accomplished by GENEWIZ. The unqualified reads were filtered out, then clean reads were spliced from scratch using software Trinity (version: 2.3.3), and the resulting sequences were then deduplicated with cd-hit (version: 4.6). Finally, the software Diamond (version: 0.8.22) and the non-redundant protein database in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) were used for BLASTx annotation, and the viral sequences were selected. The presence of each virus was also confirmed by using RT-PCR with specific primers designed according to the viral sequences.

**Statistical analysis**

Fungal mycelial growth rate and lesion diameter of strains of L. biglobosa and B. cinerea were analyzed by using analysis of variance in SAS V8.0 (SAS Institute, Cary, NC, USA, 1999). Treatment means on each of these two parameters for the tested strains or isolates were compared using the least-significant-difference test at α = 0.05.

**RESULTS**

**Viral genome and particle of LbBV1**

LbBV1 has two dsRNA segments, namely dsRNA-1 and dsRNA-2, with the sizes of 6190 and 5900 bp, respectively (Fig. 2a).
Moreover, the nucleotide sequences of dsRNA-1 and dsRNA-2 at the 3′-terminus (80 bp) and the 5′-terminus (500 bp) shared sequence identities of 57% and 84%, respectively (Fig. S3). Each dsRNA segment contained one large open reading frame (ORF), namely ORF1 and ORF2, putatively encoding two polypeptides of 202 and 192 kDa for dsRNA 1 and dsRNA 2, respectively (Fig. 2b). The ORF2-encoded polypeptide contained a proline-rich region and an RdRp domain (Fig. 2b). The cDNA sequences of LbBV1 were most related to those of Alternaria botybirnavirus 1 (ABV1) with the identity of 78% and 81% to dsRNA-1 and dsRNA-2, respectively. Moreover, they also showed relative high sequence similarity to the dsRNA-2 and dsRNA-1 of Botrytis cinerea botybirnavirus 1 (BcBV1) with the nucleotide identity of 56% and 48%, respectively. The eight conserved motifs (I–VIII) botybirnaviral RdRp sequences were also detected (Fig. S4). Therefore, this virus was named as Leptosphaeria biglobosa botybirnavirus 1 (LbBV1). Phylogenetic analysis showed that LbBV1 and ABV1 formed a clade with the bootstrap of 100%, then clustered with BcBV1, and finally formed an independent clade together with other botybirnaviruses with the bootstrap of 100% (Fig. 2c). Viral particles purified from strain GZJS19 of L. biglobosa were isometric and ~37 nm in diameter (Fig. 2d), accommodating two dsRNA segments with the sizes similar to those isolated from the mycelium of strain GZJS19 (Fig. 2e). SDS-PAGE analysis of the purified particles revealed the presence of two major structure proteins with estimated molecular weight of about 100 and 90 kDa, respectively (Fig. 2f).

**Biological properties of L. biglobosa strain GZJS19**

Four field strains of L. biglobosa free of LbBV1 isolated from diseased Br. napus were used for biological property analysis together with strain GZJS19 to investigate the biological effect of LbBV1. The results showed that strain GZJS19 produced fluffy white aerial mycelia and lots of pycnidia on both PDA and V8 plates (20 °C, 7 d), which is similar to the other four LbBV1-free strains (Fig. 3a). Moreover, the radial mycelial growth of strain GZJS19 on PDA and V8 was also comparable to the other four LbBV1-free strains (Fig. 3b). The pathogenicity assays showed that all strains of L. biglobosa could cause necrotic lesions on cotyledons of Br. napus, and the average lesion size caused by strain GZJS19 was not significantly different from those of the other four LbBV1-free strains (Fig. 3c, d). Furthermore, the LbBV1-free strain GZJS19-VF also showed similar radial mycelial growth and virulence to those of strain GZJS19, although the fungal colony of strain GZJS19-VF was slightly darker than that of strain GZJS19.
GZJS19 (Fig. S5). Overall, LbBV1 did not cause any significant phenotypical changes, especially on mycelial growth and virulence, in L. biglobosa, compared to the LbBV1-free strains.

**Vertical and horizontal transmission of LbBV1**

All 42 single conidium strains from the strain L. biglobosa GZJS19 carried LbBV1 by using RT-PCR detection (Fig. S6) and the 6 arbitrarily selected strains (VT-1, VT-2, VT-3, VT-4, VT-5, and VT-6) showed similar biological properties to those of strain GZJS19 (Fig. S7). These results suggested that LbBV1 could be efficiently transmitted vertically through conidia of L. biglobosa without causing any visible changes.

Nineteen derivative strains were obtained from the colonies of strain Lb731 of L. biglobosa after paring culture with strain GZJS19 for 14 days (Fig. 4). Two of the 19 derivative strains (HT-2 and HT-10) were infected by LbBV1 based on dsRNA detection and RT-PCR detection (Fig. 5a, b). The analysis of simple sequence repeat (SSR) confirmed that the genetic backgrounds of 8 derivative strains were consistent with that of strain Lb731, not of strain GZJS19 (Fig. 5c). Biological property tests showed that no significant difference in colony morphology (20 °C, 7 d) was observed between virus-infected and virus-free strains. Similarly, virus-infected and virus-free strains also showed similar radial mycelial growth and virulence (Fig. 5d, e, f). In addition, a LbBV1-free L. biglobosa strain W10 was used to compare the efficiency of LbBV1 horizontal transmission between L. biglobosa strains GZJS19 and HT-2 with different genetic background. Twenty derivative strains were obtained from the colonies of strain W10 after paring culture with strain GZJS19 and HT-2 for 14 days, respectively (Fig. 5b). Five of 20 and two of 20 derivative strains were infected by LbBV1 based on RT-PCR detection, respectively (Fig. 5b). These results indicated that LbBV1 could be horizontally transmitted to different L. biglobosa strains and LbBV1 are asymptomatic in L. biglobosa strains with different genetic backgrounds.

**Infection of LbBV1 conferred hypovirulence in B. cinerea**

Virus particles of LbBV1 were successfully introduced into protoplasts of B. cinerea strain t-459, and one LbBV1-infected strain t-459-V was obtained. Three lines of evidence suggested that LbBV1 was successfully introduced to B. cinerea strain t-459. First, two specific PCR products corresponding to dsRNA-1 and dsRNA-2 of LbBV1 were detected in B. cinerea strain t-459-V as well as in L. biglobosa strain GZJS19, but not in B. cinerea strain t-459 (Fig. 6a). Second, two LbBV1 dsRNA segments with the same sizes were detected in both strains t-459-V and GZJS19 (Fig. 6b). Finally, northern blotting confirmed the authenticity of LbBV1 presence in strains t-459-V as well as in strain GZJS19 (Fig. 6b). In addition, the species specific PCR products of L. biglobosa (441 bp) and B. cinerea (327 bp) confirmed that all cultures were pure (Fig. 6c). The accumulation of LbBV1 appeared to be less in B. cinerea than in L. biglobosa (Fig. 6a, b). Furthermore, we found the ratios of dsRNA-1 and dsRNA-2 in B. cinerea and L. biglobosa were different, of which dsRNA-1 accumulated less in B. cinerea than in L. biglobosa (Fig. 6b). Generally, LbBV1 could replicate stably in B. cinerea strain t-459-V, as it could be stably detected after subculture six times continuously. Different from the asymptomatic nature of LbBV1 infection in L. biglobosa, LbBV1 infection in B. cinerea resulted in some phenotypical changes. Pathogenicity assay on detached N. benthamiana leaves showed that the average lesion diameter of strain t-459-V (20 mm) was significant smaller than that of strain t-459 (30 mm; Fig. 6d, e). Moreover, strain t-459-V also lost the ability to produce sclerotia on PDA (20 °C, 20 d) in comparison with strain t-459 (Fig. 6f). Although not statistically different, the average radial mycelial growth rate of strain t-459-V (11 mm/d) was slightly slower than that of strain t-459 (12 mm/d) (Fig. 6g).

**Cross-species transmission of LbBV1**

In the co-culture of conidia of both L. biglobosa strain GZJS19 and B. cinerea strain t-459 on PDA (Fig. S1), 65 derivative strains of B. cinerea were obtained and then subcultured on PDA for three generations. All these strains were detected for the presence of LbBV1 by RT-PCR using a specific primer pair RT-1-F/RT-1-R, and only 3 of 65 derivative strains (19-459-4-1, 19-459-4-3, and 19-459-4-5) showed the presence of LbBV1 (Fig. S1). Similarly, 16 derivative strains of B. cinerea were obtained from the parts of plant tissues showing the presence of conidiophores of B. cinerea after co-inoculation with conidia of both strains GZJS19 and t-459 (Fig. S2). In these derivative strains, three strains (Stem-2, Stem-3, and Stem-6) showed the presence of LbBV1 (Fig. S2). In addition, the identities of all derivative strains were also confirmed by using PCR with species-specific primers of L. biglobosa and B. cinerea, respectively (Fig. 6g). The stability of LbBV1 in these strains was also determined by using RT-PCR after sub-culturing on PDA for five generations, and LbBV1 could be detected in all derivative strains (Fig. 6g). Moreover, all these strains showed similar culture morphology to strain t-459-V, like the deficiency of sclerotium.
formation. However, the transmission of LbBV1 was not successful from *L. biglobosa* strain GZJS19 to *B. cinerea* strain t-459 by using pairing culture technique, as no LbBV1 was detected in all 50 derivative strains.

**Possible mycoviral cross-species transmission in nature**

Since co-infections of *Brassica* crops by both *B. cinerea* and *L. biglobosa* were observed under field conditions during disease surveys (Fig. 1), it is possible that viral cross-species transmission occurs between the two fungal pathogens. Total RNA of 15 *L. biglobosa* strains and 16 *B. cinerea* strains originated from co-infection samples were extracted and sequenced, and the presence of viruses in each fungal group was filtered out (Table S4). Moreover, the identities of all strains of *L. biglobosa* and *B. cinerea* were also confirmed by using PCR with the species-specific primers (Fig. S9). The results showed that *B. cinerea* strains have more potential viral species than *L. biglobosa* strains (Fig. 7a, b, c and Tables S5, 6). Combined with the results of PCR detection (Fig. S10), the 16 *B. cinerea* strains contained possibly 25 viral species, which could be assigned into 11 families (Table S6). By contrast, the 15 *L. biglobosa* strains contained possibly six viral species, which could be assigned into 4 families (Table S5). However, LbBV1 was not detected in either of the two fungal populations. Instead, 6 viral species were detected in both fungal pathogens with the high-throughput sequencing (Fig. 7d, Table S6). Specific primers were designed based on these viral sequences (Table S7), and the presence of the two viruses was confirmed by using RT-PCR. The results showed that Botrytis cinerea umbra-like virus 1 (BcUV1) and Botrytis cinerea mitovirus 4 (BcMV4) were detected in both *B. cinerea* and *L. biglobosa* (Fig. 7d, Table S8).

**DISCUSSION**

The results of genomic structure, sequence homology, and phylogenetic analysis supported that LbBV1 was a member in the genus *Botybirnavirus*. Besides two dsRNA genome segments, LbBV1 also had spherical virions (~37 nm) with similar size (35–40 nm in diameter) to other botybirnaviruses [64]. Two major structural proteins (~100 and 90 kDa) were detected from the purified particles of LbBV1 by using SDS-PAGE analysis. The structural proteins of other botybirnaviruses, including ABV1 [65], have been determined to be in the N-proximal regions of two polypeptides encoded by the two dsRNAs. As LbBV1 showed high sequence homology to ABV1, we suppose that the estimated coding regions for two structural proteins were also in the N-proximal polypeptides encoded by ORF1 and ORF2, respectively.

Mycoviral infection is generally thought to be benign due to their limited transmission capacity among different individuals. The belief is further enforced by the recent discovery of huge number of viral species in different fungal populations with the help of high-throughput sequencing technology [66–69]. In the present study, the infection of LbBV1 in the phytopathogenic fungus *L. biglobosa* was also asymptomatic, as all *L. biglobosa* strains with different genetic backgrounds carrying LbBV1 showed similar biological characteristics to those LbBV1-free strains of *L. biglobosa* (Fig. 3). However, LbBV1 could also infect *B. cinerea* and conferred hypovirulence to *B. cinerea*. Purified virions of LbBV1...
may help ensure the survival of LbBV1-infected pathogenicity was not fully lost. All these relatively mild changes mediated by LbBV1 in LbBV1 infection reduced sclerotium production and other fungi under laboratory conditions, like Aspergillus niger/Alternaria alternata and Sclerotinia sclerotiorum [72], and other fungi [74] in addition to its earliest recorded host—B. porri.

As co-infections of oilseed rape by both B. cinerea and L. biglobosa have been observed in the field, we hypothesize that
the transmission of LbBV1 from *L. biglobosa* to *B. cinerea* may also occur during the co-infection (Fig. 1). Furthermore, we also want to test whether other viruses may be transmitted from one species to the other during co-infection. To test this hypothesis, diseased samples of *Brassica* crops showing symptoms of co-infection by both pathogens were collected, and the *B. cinerea* and *L. biglobosa* strains were subsequently isolated and purified. Total RNAs were extracted from *B. cinerea* and *L. biglobosa* strains, and then used in RNA-seq. As LbBV1 was not detected in either of the two fungal populations, we also tried to collect and analyze samples from where *L. biglobosa* strain GZJS19 was originally collected, but no sample showing co-infection was obtained. However, two other mycoviruses, BcMV4, and BcUV1, were detected in both fungal species. This indicates that viral cross-species transmission may occur if two fungi share same or similar ecological niche. In this specific case, there is a question that which fungus is the original host of these two viruses. To address this question, the sequences of two viruses were searched in NCBI database, and all of them occurred if two fungi share same or similar ecological niche. In this case, there is a question that which fungus is the original host of these two viruses. To address this question, the sequences of two viruses were searched in NCBI database, and all of them occurred if two fungi share same or similar ecological niche.

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ulmi was observed for the two viruses [60, 63, 70]. In addition, some in-lab evidence also indicates that phenotypic alterations induced by same mycovirus may vary between different hosts [78, 79]. Different from mycoviruses, many solid lines of evidence confirm that cross-species transmission in animal viruses is often able to result in diseases of humans and animals, sometimes even human pandemics, such as influenza A virus [80, 81], human immunodeficiency virus-1 [82], and severe acute respiratory syndrome coronavirus-2—the causal agent of current pandemics [53]. Similarly, recent high-throughput sequencing studies revealed that diverse viruses persist in wild plants, and they are generally asymptomatic in wild plants [83], and are believed to be reservoirs of crop viruses, which may cause diseases on infected crops [84, 85]. Therefore, it is worthy to test whether similar cases also occur in fungi.

In the present study, we found that similar situation may also exist in mycoviruses. Mycoviral transmission can be accomplished between two fungal species distantly related to each other, and viruses having no apparent effects on one fungal host may cause significant phenotypical changes on the other one after interspecies transmission. This may happen under natural conditions if even two fungi share same or similar ecological niches, and the related clue can be also found in the literature. For example, the presence of Trichoderma koningiopsis totivirus 1 in Trichoderma koningiopsis strain Mg10 and Clonostachys rosea strain Mg06 isolated from the same soil sample suggest the viral interspecies transmission between the two fungi [86]. Moreover, some observations also implied that mycoviruses can be acquired during fungal vegetative growth under natural conditions without the limitation of vegetative incompatibility [87, 88]. In the present study, cross-species transmission of LbBV1 from L. biglobosa to B. cinerea was not successful by using pair-culture technique, but succeeded when co-inoculation of the conidia of the two fungi on PDA and on plants. This indicates that mycoviral interspecies transmission may occur under some circumstances beyond the familiar ways, as natural conditions are much more complex than

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**Fig. 7** The diversity of mycoviruses detected by RNA-sequencing in *Leptosphaeria biglobosa* and *Botrytis cinerea* strains isolated from co-infection samples of *Brassica* crops. a The number of mycoviruses belonging to different genome types presents in each fungal population. b Venn diagram showed the number of mycoviruses present in *L. biglobosa* (Lb) and *B. cinerea* (Bc) populations. c Sankey diagram displaying the compositions of mycovirome from the populations of *L. biglobosa* and *B. cinerea*. d Detection of the presence of six mycoviruses in *L. biglobosa* and *B. cinerea* isolates by RT-PCR using the specific primers listed in Table S7. Note that two mycoviruses, Botrytis cinerea umbralike virus 1 (BcUV1) and Botrytis cinerea mitovirus 4 (BcMV4), were detected in both two fungal populations.
in the laboratory. For instance, one recent case showed that plant viruses may assist the transmission of hypoviruses between different fungal species [89]. As mentioned above, due to the limited transmissibility of mycoviruses, hypovirulence mediated by mycoviruses may be detrimental for both virus and host fungus, as they may not be able to survive in the long term. Therefore, most mycoviral infections are benign. However, hypovirulence-associated mycoviruses have been widely reported in many phytopathogenic fungi, which is contrary to their benign infection, and most of their host fungal strains were initially isolated from diseased plants showing typical symptoms. Therefore, the present study provides an explanation for why hypovirulence-associated mycoviruses were widely reported in many phytopathogenic fungi, as these viruses might invade their hosts recently. These results also support the hypothesis that hypovirulence traits mediated by viruses may be a consequence of viral invasions in new fungal hosts by transmission between species [38].

Cross-species transmission in fungi may be different from that in animals. In animal viruses, a few animals are considered as reservoirs of viruses, such as bats [45]. In contrast, we suppose that many fungi can serve as reservoirs to each other, as one fungal species may harbor numerous viruses as shown in recent high through-put sequencing analysis [66–69]. In the present study, LbBV1 could be transmitted from L. biglobosa to B. cinerea. In addition, the two viruses (BcMV4 and BcUV1) were also suggested to be transmitted from B. cinerea to L. biglobosa. Thus, both L. biglobosa and B. cinerea each can serve as reservoirs to the other. However, viral diversity in different fungal species vary greatly. In this study, B. cinerea contained more virus species than did L. biglobosa, although both fungal groups had almost same number of strains and were isolated from same geographic locations. Based on these observations, we speculate that phytopathogenic fungi with wide host range may tend to contain more species of viruses, as they should have more chances to contact with more diverse fungi and may have a wider geographic distribution. In addition, these phytopathogenic fungi, like B. cinerea and S. sclerotiorum, could also play as a bridge or link for viruses among different fungal species (Fig. 8). This may be helpful to explain why two fungi in different ecological niches can still carry the same virus. For example, O. novo-ulmi is a fungal pathogen causing Dutch elm disease on elm trees, whereas S. homoeocarpa is the causal agent of dollar spot on turfgrasses. Although they are in different ecological niches, the same virus—Ophiostoma novo-ulmi mitovirus 3a (OnMV3a) could be detected in both fungi [70]. In this case, one fungus living on both elm tree and turfgrass might be able to transmit OnMV3a between O. novo-ulmi and S. homoeocarpa. Therefore, we believe that further studies on fungal groups of saprophytes, epiphytes, and endophytes should be explored, as all of them have lots of chances to contact with other phytopathogenic fungi.

For a long time, investigations of mycoviruses have mostly focused on viral groups that manifest significant biological effects, especially hypovirulence-associated viruses in phytopathogenic fungi, as one important goal of mycoviral research is to explore viral resources for biocontrol of plant fungal disease. In contrast, mycoviruses that have no significant biological effects have received much less attention, and many of which just have sequence information. However, the present study provides a new insight for mycoviruses mostly seen to be asymptomatic. They may have no significant effects on one fungal host, but their host shifts may lead to great phenotypical changes on new fungal hosts, which may have the potential for further development as biocontrol agents against these new hosts.

**DATA AVAILABILITY**

The Genbank accession numbers of dsRNA 1 and dsRNA 2 of LbBV1 are MZ612795 and MZ612796, respectively. The raw sequencing reads reported in the present study have been deposited in the Sequence Read Archive (SRA) database: BioProject accession no. PRJNA778037, BioSample accession no. SAMN22899792 (L. biglobosa).
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