



RESEARCH ARTICLE



Blue-White Colony Selection of Virus-Infected Isogenic Recipients Based on a Chrysovirus Isolated from *Penicillium italicum*

Tingfu Zhang¹ · Na Li^{1,2} · Yongze Yuan¹ · Qianwen Cao¹ · Yanfen Chen¹ · Binglan Tan¹ · Guoqi Li¹ · Deli Liu¹

Received: 16 February 2019 / Accepted: 14 May 2019 / Published online: 2 August 2019
© Wuhan Institute of Virology, CAS 2019

Abstract

Mycoviruses have been found to infect more than 12 species of *Penicillium*, but have not been isolated from *Penicillium italicum* (*P. italicum*). In this study, we isolated and characterized a new double-stranded RNA (dsRNA) virus, designated *Penicillium italicum* chrysovirus 1 (PiCV1), from the citrus pathogen *P. italicum* HSPi-YN1. Viral genome sequencing and molecular characterization indicated that PiCV1 was highly homologous to the previously described *Penicillium chrysogenum* virus. We further constructed the mutant HSPi-YN1 $\Delta pksP$ defective in the polyketide synthase gene (*pksP*), which is involved in pigment biosynthesis, and these mutants formed albino (white) colonies. Then we applied hyphal anastomosis method to horizontally transmit PiCV1 from the white virus-donors (i.e., HSPi-YN1 mutants) to wild-type recipients (i.e., *P. italicum* strains HSPi-CQ54, HSPi-HB4, and HSPi-HN1), and the desirable PiCV1-infected isogenic recipients, a certain part of blue wild-type strains, can be eventually selected and confirmed by viral genomic dsRNA profile analysis. This blue-white colony screening would be an easier method to select virus-infected *P. italicum* recipients, according to distinguishable color phenotypes between blue virus-recipients and white virus-donors. In summary, the current work newly isolated and characterized PiCV1, verified its horizontal transmission among dually cultured *P. italicum* isolates, and based on these, established an effective and simplified approach to screen PiCV1-infected isogenic recipients.

Keywords *Penicillium italicum* chrysovirus 1 (PiCV1) · *pksP* knockout · White-blue colony screening · Isogenic recipients · Horizontal transmission

Introduction

Penicillium is a major pathogen causing huge losses during post-harvest handling of citrus fruits. *P. digitatum* and *P. italicum* are representative *Penicillium* pathogens because

they have the strongest pathogenicity in citrus fruits (Prusky *et al.* 2004; Boubaker *et al.* 2009). Artificial synthetic fungicides, including demethylase inhibitors, have been used to control these phytopathogens (Korsten 2006; Liu *et al.* 2015). However, directed evolution of resistant strains and the failure to control green and blue molds have been attributed to the excessive use of fungicides (Wang *et al.* 2014). Therefore, it is necessary to develop alternative strategies, such as hypovirulent viruses, to control these post-harvest diseases.

Viruses have been discovered in all major groups of fungi since the first definitive report of a virus infecting the cultivated button mushroom *Agaricus bisporus* 56 years ago (Hollings 1962). In 1967, the first evidence of mycoviruses infecting *P. stoloniferum* was reported (Ellis and Kleinschmidt 1967). Subsequently, mycoviruses were identified in *P. funiculosum*, *P. chrysogenum*, *P. cyaneofulvum*, *P. brevi-compactum*, *P. citrinum*, and *P. variable* (Banks *et al.* 1969; Wood *et al.* 1971. Borré *et al.* 1971;

Tingfu Zhang, Na Li and Yongze Yuan have contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12250-019-00150-z>) contains supplementary material, which is available to authorized users.

✉ Deli Liu
ldl@mail.ccnu.edu.cn; deliliu2013@163.com

¹ Hubei Key Laboratory of Genetic Regulation and Integrative Biology, School of Life Sciences, Central China Normal University, Wuhan 430079, China

² Yunnan Higher Education Institutions, College of Life Science and Technology, Honghe University, Mengzi 661199, China

Border *et al.* 1972). Nevertheless, few studies have reported the identification and classification of viruses at the molecular level. Kim *et al.* (2003) reported that the complete genome sequence of *P. stoloniferum* virus (PsV-S) contained S1 and S2 double-stranded RNA (dsRNA). Phylogenetic analysis revealed that PsV-S was a definite partitivirus in the family *Partitiviridae* (Kim *et al.* 2003). With regard to *Chrysovirus*, Jiang and Ghabrial first published the whole-genome sequence of *P. chrysogenum* virus (PcV) and suggested that PcV may be subordinate to the genus *Chrysovirus* rather than the family *Partitiviridae* (Jiang and Ghabrial 2004). With the rapid development of sequencing technology, more than 10 *Penicillium* viruses infecting *P. aurantiogriseum* (Nerva *et al.* 2016), *P. janczewskii* (Nerva *et al.* 2016), and *P. digitatum* (Niu *et al.* 2016, 2018; Yang *et al.* 2018) have been successively identified, some of which are still unclassified or have not been approved by the International Committee on Taxonomy of Viruses (ICTV). We recently isolated viruses from *P. crustosum* and *P. italicum* (Niu *et al.* 2018). To date, mycoviruses have been found in more than 12 species of *Penicillium*. These results have expanded our understanding of taxonomic groups of *Penicillium* mycoviruses.

Mycoviruses have been reported to include dsRNA, single-stranded RNA, and single-stranded DNA genomic types (Yu *et al.* 2010). *Chrysoviridae* family members belong to the dsRNA virus. Phylogenetic analysis of them and related, unclassified viruses showed that all these viruses can be divided into two clusters; cluster I contains viruses with three or four genome segments, whereas cluster II contains viruses with four or five genome segments (Ghabrial *et al.* 2018). Nine classified species of *Chrysoviridae* can infect ascomycetous or basidiomycetous fungi, harboring a typical quadripartite genome (11.5–12.8 kb) that is individually encapsidated with virions (isometric, nonenveloped, and approximately 40 nm in diameter) (Ghabrial *et al.* 2018). The capsid of PcV, a representative species of *Chrysoviridae*, comprises 60 subunits of a 109 kDa polypeptide arranged on a T = 1 icosahedral lattice (Castón *et al.* 2003). Unclassified, chrysovirus-related viruses with three-segmented dsRNA genomes infect plants but do not induce obvious damage (Li *et al.* 2013). However, some chrysovirus-related viruses with five-segmented dsRNA genomes cause deleterious effects in their fungal hosts (Urayama *et al.* 2014).

RNA mycoviruses can be transmitted vertically via sporulation and horizontally via hyphal anastomosis between mycelial compatible strains of the same fungus (Pearson *et al.* 2009). Transfection of virions into recipient protoplasts or protoplast fusion between donors and recipients has greatly improved the efficiency of virus infection between host fungi (Hillman *et al.* 2004; Sasaki *et al.* 2006; Lee *et al.* 2014; Niu *et al.* 2016). In addition,

dual culture based on mycelia anastomosis of virus donors and recipients has become the main technique for studying mycovirus infections between fungi. Although mycoviruses are thought to exhibit host specificity, the ability of fungal viruses to transmit among fungal individuals (even via interspecies transmission) is determined by virus characteristics and differences in host mycelial compatibility (Ghabrial and Suzuki 2009). Mycovirus transmission via hyphal anastomosis has been reported in the same fungal species, including *Botrytis cinerea* (Wu *et al.* 2010), *Fusarium graminearum* (Lee *et al.* 2014), *Ustilaginoidea virens* (Zhang *et al.* 2014), and *Rosellinia necatrix* (Sasaki *et al.* 2016), and via interspecies transmission in *Aspergillus* (Coenen *et al.* 1997), *Sclerotinia* (Melzer *et al.* 2002), *Cryphonectria* (Liu *et al.* 2003), *Heterobasidion* (Vainio *et al.* 2010), and *P. aurantiogriseum* (Nerva *et al.* 2017) under laboratory experiment conditions. Chrysoviruses could be transmitted via intracellular routes within an individual during hyphal growth, in asexual or sexual spores, or between individuals via hyphal anastomosis (Ghabrial *et al.* 2018). However, exploration of host/chrysovirus interactions has been hampered in *Penicillium* spp. because of difficulties in curing fungi of viral infections and a lack of simple methods for artificial inoculation of mycoviruses (Kim *et al.* 2013). Based on hyphal anastomosis or protoplast fusion, established methods for screening virus-infected isogenic recipient strains are mainly dependent on antibiotic (hygromycin B or geneticin G418) resistance selection after coculture (Lee *et al.* 2011; Kim *et al.* 2013). Therefore, optimization or construction of a simple and convenient screening method/system for horizontal transmission of viruses is essential for studies of the interactions between *Penicillium* spp. and chrysoviruses.

Accordingly, in this study, we isolated and sequenced a chrysovirus, designated PiCV1, from *P. italicum* (HSPi-YN1). Molecular characterization and phylogenetic analysis of PiCV1 were performed to evaluate the taxonomic status. Based on the PiCV1 (virus)-HSPi-YN1 (host) system, we then established a blue–white colony screening method using a PiCV1-infected HSPi-YN1 mutant defective in the *pkpP* gene. Overall, our findings provided important insights into the detection, isolation, and characterization of *P. italicum* isolates infected by PiCV1.

Materials and Methods

Isolation and Characterization of *Penicillium* Strains

In total, 148 strains of *Penicillium* were isolated from the epidermis of mildew citrus collected from markets or fruits

packing houses in Hubei, Jiangxi, and Yunnan provinces and Chongqing municipality, China from November 2015 to March 2016. These strains included 28 *P. italicum* strains, such as the virus-infected strain HSPi-YN1 and the virus-free strains HSPi-CQ54, HSPi-HB4, and HSPi-HN1. The three virus-free strains were selected as recipients in dual culture for the current study. Conidia and mycelia from all strains were cultured on potato dextrose agar (PDA) medium and in potato dextrose broth on a rotary shaker (180 rpm) at 28 °C. Total genomic DNA was extracted from ground mycelia powder in liquid nitrogen using a Biospin Fungus Genomic DNA Extraction Kit (Bio Flux) following the manufacturer's protocol. The virus-harboring strains were primarily screened through genomic composition detection and confirmation using nuclease digestion analysis by agarose gel electrophoresis and staining with ethidium bromide. For species determination of fungal isolates, internal transcribed spacer (ITS) sequences of ribosomal DNA were amplified by PCR using universal primer pairs (Supplementary Table S1). PCR products were sequenced using an ABI3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Extraction and Purification of dsRNA

Mycelia were frozen with liquid nitrogen and ground to a fine powder in a mortar. dsRNA was then extracted with phenol–chloroform and isolated by CF-11 cellulose (Sigma, St. Louis, MO, USA) chromatography, according to a previously described method (Morris and Dodds 1979). The nucleic acid sample was treated with RNase-free DNase I and S1 nuclease (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 30 min to digest genomic DNA and single-stranded RNAs (rRNAs and mRNAs) and then separated on 1% agarose gels stained with ethidium bromide. The dsRNA gel bands were excised and purified with a gel extraction kit (Axygen, Union City, CA, USA), dissolved in appropriate volume of RNase-free double-distilled water, and stored at –70 °C. This dsRNA sample was later used for high-throughput sequencing and as a template for synthesizing cDNA.

Viral Genome Sequencing, Full-Length cDNA Confirmation, and Northern Blot Hybridization Analysis

Purified dsRNA (8 µg) was used for cDNA library construction with a TruSeq RNASample Preparation Kit (Illumina) following the manufacturer's protocol. Paired-end sequencing with a 150-bp read length was conducted on an Illumina HiSeq 2500 machine. cDNA library construction, sequence assembly, and evaluation of deep sequencing were carried out by GENEWIZ Solid Science. Sequenced

reads matching the host *P. italicum* databases (isolate B3) were removed, and the remaining reads were *de novo* assembled using Velvet (Zerbino and Birney 2008) (version 1.2.10) with default parameters. All contigs were subjected to BLASTN and BLASTX alignment against the NCBI database to search for viral sequences.

The complete 5'- and 3'-terminal sequences were further determined using rapid amplification of cDNA ends (RACE)-PCR. First, the 3'-terminus of each strand of dsRNA was ligated to the closed adaptor primer PC3-T7 loop (Supplementary Table S1) by T4 RNA ligase (Thermo Fisher Scientific) at 16 °C for 18 h. Next, the oligonucleotide-ligated dsRNA was reverse-transcribed using primer PC2 (Supplementary Table S1), which was complementary to the oligonucleotide used for RNA ligation, and sequence-specific primers corresponding to the 5'- and 3'-end sequences of the dsRNA in the presence of M-MLV reverse transcriptase, as described previously (Liu *et al.* 2009), with minor modifications. Additionally, fragments of dsRNAs between the 5'- and 3'-ends were subjected to reverse transcription (RT)-PCR using sequence-specific primer pairs to obtain cDNA that overlapped with RACE sequences of the 5'- and 3'-ends. All amplified cDNA products were cloned into the pMD18-T vector (TaKaRa, Shiga, Japan), sequenced using an ABI3100 DNA sequencer, and subsequently assembled by DNAMAN (version 7.0) to obtain and confirm the full-length sequences of all dsRNAs. At least three independent clones were analyzed for sequence determination at all nucleotide positions.

For Northern blot hybridization analysis, dsRNAs isolated from mycelia of virus hosts were separated on 1% agarose gels for 3 h at 80 V. The gels were then soaked for 20 min in 0.1 mol/L NaOH and neutralized twice in 0.1 mol/L Tris–HCl (pH 8.0) for 20 min, and the RNAs were transferred by capillary action to a Hybond-N⁺ nylon membrane in 20 × SSC buffer overnight. The RNA was fixed to the membrane by ultraviolet (UV) crosslinking following exposure to UV irradiation for 60 s at 120 mJ/c². Prehybridization and hybridization were performed under stringent conditions in hybridization solution using a BCIP-NBT DIG Detection Kit (TIANDZ, Beijing, China) according to the manufacturer's instructions. The RNA blots were probed with digoxigenin-labeled probes (500–700 bp) prepared by oligolabeling of cloned cDNA to the four dsRNAs (Jamal *et al.* 2010).

Viral dsRNA Sequence Analysis

Sequence similarity was assessed using the BLAST tool on the NCBI website. Analysis of conserved sequences at the 5'- and 3'-untranslated regions (UTRs) in dsRNAs was performed with DNAMAN (version 7.0) by manually

adjustment. Coding open reading frames (ORFs) in dsRNA sequences were detected with ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi>), deduced, and translated by DNAMAN (version 7.0) with default parameters. Multiple sequence alignments of the amino acid sequences were generated using CLUSTAL_X (Thompson *et al.* 1997). Phylogenetic tree construction was based on the neighbor-joining method, as described previously with boot-trapping analysis of 1000 replicates by MEGA (version 5.0) (Tamura *et al.* 2011).

Purification of Virus Particles and Composition Analysis

Approximately 80 g of mycelia mixed with 320 mL of 0.05 mol/L sodium phosphate buffer (pH 7.4) was levigated for 3 min using a precooled grinder. Isolation of virus-like particles (VLPs) was performed as previously described using differential centrifugation and ultracentrifugation in sucrose density gradients (Niu *et al.* 2016). Briefly, the crude extracts were obtained by differential centrifugation, and the supernatants were subjected to ultracentrifugation in sucrose density gradients (200–600 mg/mL with intervals of 100 mg/mL). The UV absorbance at 260 nm for all 12 fractions was measured using a spectrophotometer, and fractions with a peak absorption corresponding to the sucrose gradient were supplemented with 0.05 mol/L sodium phosphate buffer (pH 7.4) and subjected to ultracentrifugation once again to remove the sucrose. The VLPs were suspended in 100 μ L of 0.01 mol/L sodium phosphate buffer (pH 7.4) and then used for transmission electron microscopy observations and SDS-PAGE analysis.

Virus Transmission by Dual Culture

The method of blue–white colony screening was established based on hyphal anastomosis for virus transmission between *P. italicum* individuals. Briefly, the virus-free recipient was streak-inoculated at an interval of 0.5 cm with virus donor harboring deletion of the *pksP* gene, resulting in the albino colony phenotype. In order to obtain the *pksP*-knockout *P. italicum* strain, we introduced approximately 1.0 μ g of the *pksP* L-hyg-R fragment (i.e., approximately 1.2-kb upstream and downstream sequences of the *pksP* ORF were selected as the L- and R-arms, respectively, establishing a knockout site to fuse with the *hyg* cassette) into wild-type donor (HSPi-YN1) protoplasts (100 μ L, 1×10^8 protoplasts/mL) by polyethylene glycol (PEG)-mediated transformation (Zhao *et al.* 2016). Regenerative transformants with the albino phenotype were verified using negative and positive-selection by PCR

amplification. Primer pairs used to construct and verify the HSPi-YN1 Δ *pksP* mutant are listed in Supplementary Table S1.

We used three virus-free *P. italicum* strains, i.e., HSPi-CQ54, HSPi-HB4, and HSPi-HN1, as original recipients in dual cultivation with the albino donor (HSPi-YN1 Δ *pksP*). Virus donors and recipients were incubated at 28 °C for one week on dishes containing 20 mL PDA. When the spores and hyphae were grown together, spores were randomly selected at the edge of the border to separate single spore isolates on fresh PDA plates by spore gradient dilution with dilution ratios of 10^3 – 10^8 . Next, 10 blue isolates derived from single spores were visually selected for every recipient and cultured on PDA plates for DNA and dsRNA extraction and subsequent virus detection by gel electrophoresis analysis and RT-PCR. In addition, the wild-type virus donor HSPi-YN1 was evaluated using the same methods as for the recipient strains (CQ54*hyg*, HB4*hyg*, and HN1*hyg*) harboring the hygromycin B resistance gene. Recipients overexpressing the hygromycin B-resistant gene were obtained by *Agrobacterium*-mediated genetic transformation (Wu *et al.* 2016). Single spore isolates separated from cocultures were selected on PDA containing 50 μ g/mL hygromycin B by spore gradient dilution with dilution ratios of 10^3 – 10^8 . Virus detection was performed using the blue–white colony screening method.

Analysis of Growth Rate, Virulence, and Prochloraz Half-Maximal Effective Concentration (EC₅₀)

The conidial suspension (1×10^7 spores/mL) of each isolate strain (10 μ L) was inoculated into a hole in the epidermis of citrus fruit and cultured several days at 25 °C. Lesions in the citrus fruits were measured at 7 days after inoculation. In addition, 100 μ L of the conidial suspension was coated onto PDA media plates and precultured overnight at 28 °C. Mycelia plugs (\sim 8 mm in diameter) were shaped from the plate using a punch and transplanted on the center of fresh PDA or treated with different concentrations of prochloraz (i.e., 0–0.01 mg/mL for HSPi-HN1, 0–0.1 μ g/mL for HSPi-CQ54, and 0–10 μ g/mL for HSPi-HB4) for assays of vegetative growth and prochloraz EC₅₀, respectively. The plaques were measured at 7 days after inoculation, and the colony areas were measured once every day for 7 consecutive days at 28 °C or according to the experimental need. The diameters of different colonies were measured. Each assay was performed with three replicates and independent samples *t* test was applied in the SPSS Statistics 17.0 context to assess the significance of differences between the means.

Results

Screening and Clustering of *Penicillium* Strains with Extra Genome Elements

In total, 148 strains of *Penicillium* were isolated from the epidermis of post-harvest mildew citrus fruit in Hubei, Jiangxi, and Yunnan provinces and Chongqing municipality, China (Fig. 1A). There were 39 isolates harboring extra genome segments in profile analyses of the fungal total genome, suggesting that these fungi harbored viruses (Fig. 1B). Phylogenetic analysis of ITSs showed that one, four, and 34 isolates were clustered to branches with previously identified *P. italicum*, *P. crustosum*, and *P. digitatum* based on sequences of accession numbers KU561924.1, KU714642, KX507075.1, MF188258.1, KJ834506.1, and AY373910.1 (Fig. 1C). The extra genome fragments of *P. italicum* strain HSPi-YN1 were confirmed to be the dsRNA genome of a virus infecting the host fungus HSPi-YN1 by nuclease digestion analysis (Fig. 2A).

PiCV1 Genome Sequencing and Molecular Characterization

dsRNA extracts from the fungal mycelia of *P. italicum* strain HSPi-YN1 were treated with DNase I and S1 nuclease, and four clear bands of approximately 3.0–4.0 kb were observed after 1% agarose gel electrophoresis (Fig. 2A). The purified dsRNA sample was used to construct a cDNA library for cluster preparation and subsequent sequencing using an Illumina next-generation sequencing platform. Sequences from the fungal host were removed, and the remaining reads were *de novo* assembled to four segments, which were similar to those of PcV, with sequence identity of approximately 83%–84% by BLAST. For convenience, the virus from HSPi-YN1 strains was named PiCV1, and these four dsRNAs were numbered dsRNA1–4 based on their molecular masses. The complete 5'- and 3'-terminal sequences of each segment were further determined using RACE-PCR, and all full-length sequences were verified by PCR amplification and cloning. The sequences of these dsRNAs were deposited in GenBank (accession numbers: MK214380, MK214381, MK214382,

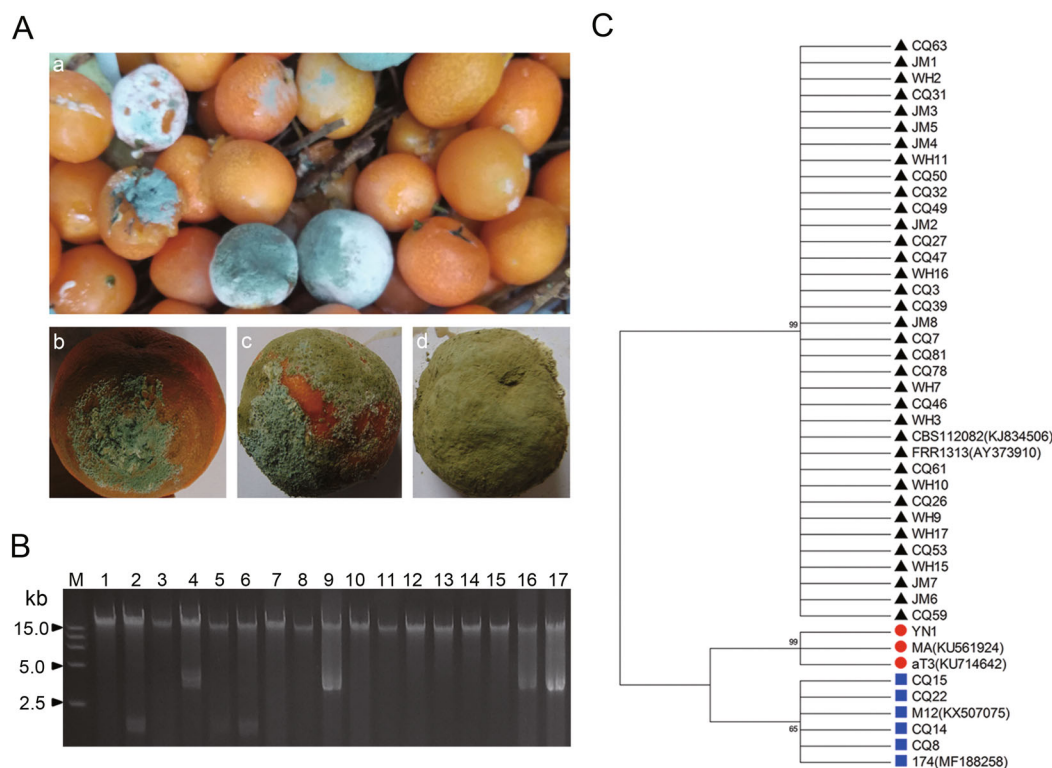
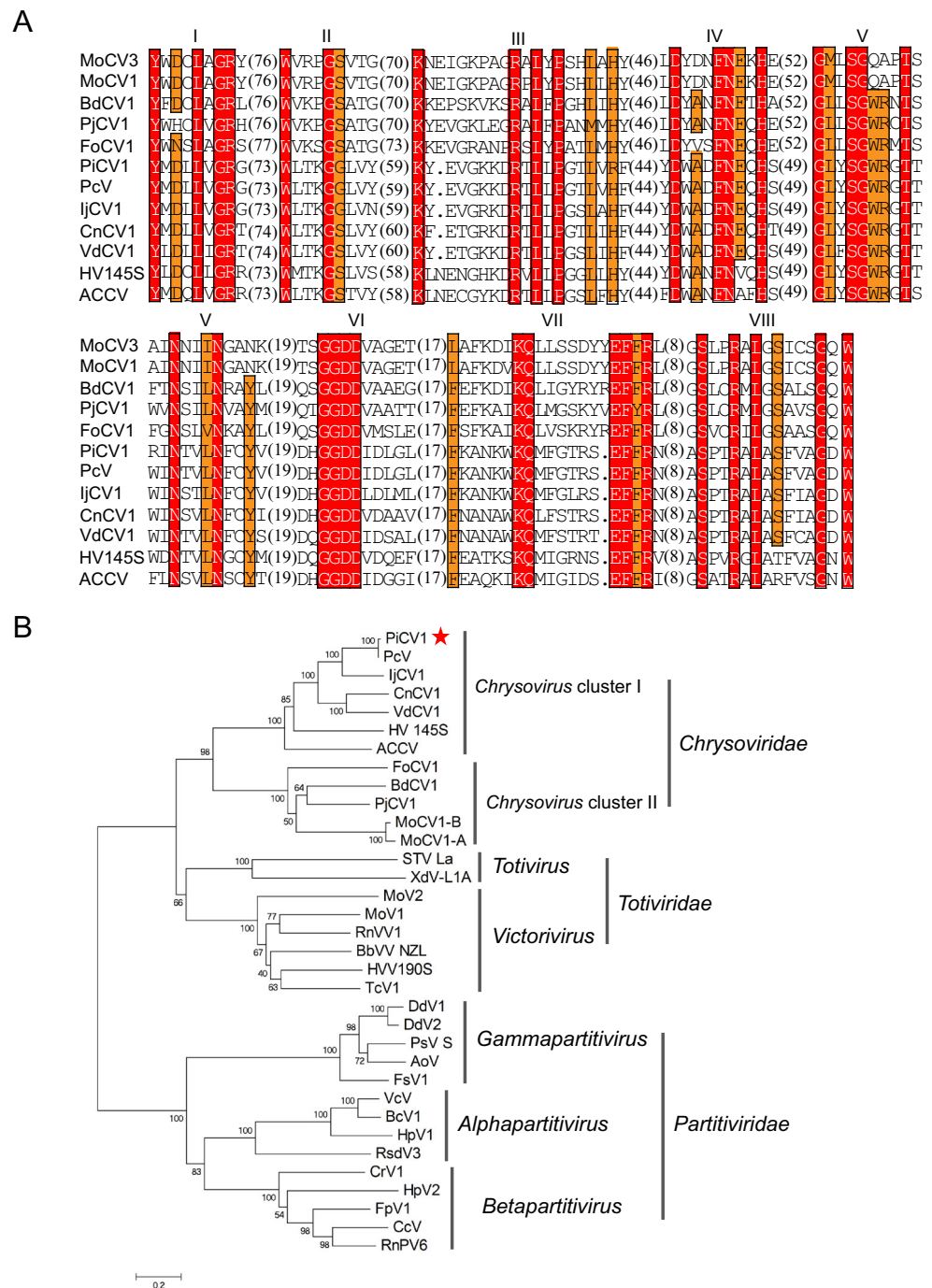


Fig. 1 Screening and identification of *Penicillium* strains harboring virus. **A** Citrus fruits were decayed by infection with *Penicillium* pathogens during the post-harvest stage. (a) The decayed citrus in the fruit heap; (b) citrus fruits infected by *P. italicum*; (c) citrus fruits infected by *P. digitatum* and *P. italicum*; (d) citrus fruits infected by *P. digitatum*. **B** Total genome extracts was detected using 1% agarose gel electrophoresis. Lanes 2, 4, 5, 6, 9, 16, 17 correspond to isolates

WH7, YN1, WH2, WH3, CQ8, CQ15, and CQ16, respectively, which had extra genome elements; other lanes corresponded to virus-free strains. Lane M: DS1500 DNA marker. **C** Thirty-nine wild-type strains of *Penicillium* harboring virus were clustered using ITS sequences by the neighbor-joining method. Black triangles, blue squares, and red dots represent *P. digitatum*, *P. crustosum*, and *P. italicum*, respectively.

Fig. 3 Comparison of RdRp conserved motifs among different members in the *Chrysoviridae* family and phylogenetic analysis of dsRNA mycovirus RdRps from three related families. **A** Comparison of RdRp conserved motifs encoded by PiCV1 dsRNA1 and other selected mycoviruses. The Roman numerals I–VIII refer to the eight conserved motifs characteristic of RdRps in the selected dsRNA viruses. Alignment was performed, and the accession numbers used to analyze conserved motifs are listed in Supplementary Table S2. **B** Phylogenetic analysis of the PiCV1 RdRp sequence. A phylogenetic tree was constructed using the neighbor-joining method. Member information for the three families and six genera is listed in Supplementary Table S2 in the phylogenetic tree.



were different. Phylogenetic analysis based on multiple alignments of the deduced amino acid sequences of the RdRp in PiCV1 and other selected viruses in the families *Chrysoviridae*, *Totiviridae*, and *Partitiviridae* (Supplementary Table S2) demonstrated that PiCV1 was clustered closely to *Chrysovirus* member PcV, with a support coefficient of 100 in the same sub-branch (Fig. 3B). According to this analysis, we propose that PiCV1 may be a new strain of *P. chrysoygenum* virus from *P. italicum*.

Morphology and Composition of Virus Particles from HSPi-YN1

PiCV1 particles were purified by differential centrifugation and sucrose density gradient centrifugation. The absorbance profile at 260 nm showed a single peak for the fractions corresponding to 300 mg/mL sucrose (Supplementary Figure S1). Observation of these gradient fractions using transmission electron microscopy uncovered

isometric virus-like particles with a diameter of approximately 40 nm (Fig. 4A), consistent with the morphology and size of virions from chrysovirus (Jiang and Ghabrial 2004; Ghabrial *et al.* 2018). Profile analysis showed that dsRNAs extracted from the purified particles were indistinguishable from dsRNAs isolated from the mycelia of the same HSPi-YN1 culture used for particle preparations (Fig. 4B). SDS-PAGE analysis of the purified virions showed a major band at ~ 116 kDa representing the CP, which was close to the putative molecular weight (108.4 kDa) of the major CP encoded by PiCV1 genome segment dsRNA2 (Fig. 4C).

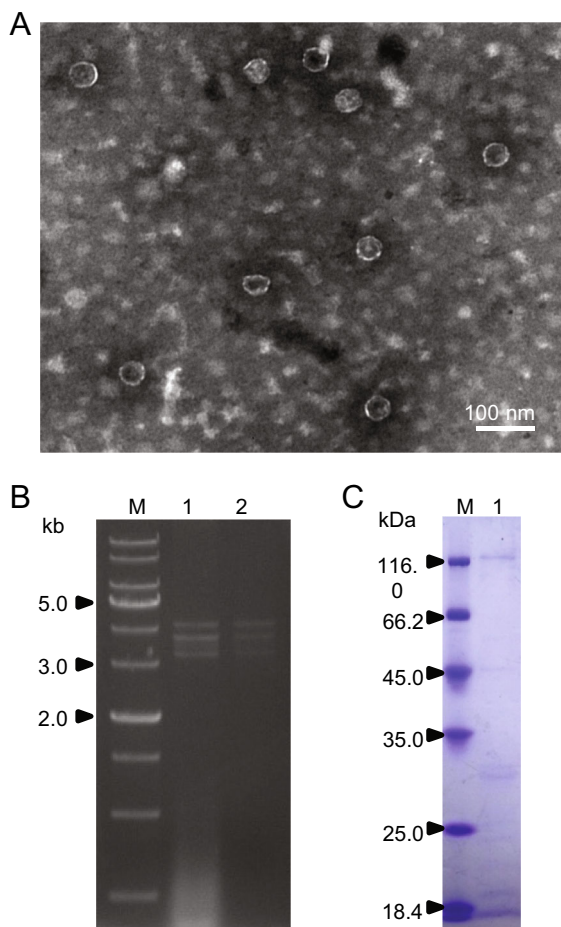


Fig. 4 Compositions of virus particles separated from *P. italicum* strain HSPi-YN1. **A** Electron micrograph of virus particles. The purified virus particles were negatively stained with 2% phosphomolybdic tungstic acid and observed by transmission electron microscopy. Scale bar: 100 nm. **B** dsRNA detection in HSPi-YN1 virus particles. Lane 1: dsRNA isolated from the same hyphae of HSPi-YN1 after virion extraction and purification; lane 2: dsRNA isolated from virus particles. Lane M: 1-kb ladder DNA marker. **C** SDS-PAGE was used to detect protein components of purified particles. The purified virus particles were mixed with loading buffer, denatured, and electrophoresed on 10% polyacrylamide gels (lane 1). Proteins were stained with Coomassie brilliant blue R-250. Lane M: protein molecular weight marker.

PiCV1 Horizontal Transmission between Individuals

In order to generate a morphological selection marker phenotype, the cassette *pksP* L-*hyg*-R was amplified using overlapped PCR and introduced into protoplasts of the wild-type virus donor (HSPi-YN1) by PEG-mediated transformation. The *pksP* disruption mutant (HSPi-YN1 Δ *pksP*) was generated through homologous recombination and exhibited albino colonies that could be visually distinguished from the wild-type colonies (Fig. 5A, 5C). A segment of approximately 1.1 kb in the knockout sequence was amplified in the wild-type strain or mutant harboring ectopic expression of *hyg* but not in mutants with *pksP* gene knockout by negative PCR. The *hyg* cassette (~ 2.1 kb) was amplified from a mutant of *pksP* gene disruption or ectopic expression of *hyg* but not the wild-type strain by positive PCR (primers at two terminuses of the *hyg* cassette; Fig. 5B). These results demonstrated that the *pksP* gene was successfully knocked out in the wild-type strain HSPi-YN1. Subsequently, viral genome profile analysis and RT-PCR verification revealed that the albino mutant YN1 Δ *pksP* still harbored PiCV1 and became a modified virus donor (Fig. 5D), which was easily distinguished from the wild-type recipient strain based on colony color. Thus, we established a blue–white colony screening system by dual culture of the albino virus donor strain with wild-type recipient strains (blue colony) for the first time.

Based on colony color differences combined with genomic profile analyses, we observed PiCV1 horizontal transmission among *P. italicum* individuals with different genetic backgrounds, generating isogenic and virus-infected fungal isolates derived from wild-type recipients. After coculture of the albino mutant (HSPi-YN1 Δ *pksP*) with wild-type recipients (HSPi-CQ54, HSPi-HB4, and HSPi-HN1), isogenic and virus-infected recipient candidates from these cocultures were selected based on colony color (blue) by spore gradient dilution on PDA (Fig. 6A). All candidate single-spore isolates (30 isolates in three groups of paired cultures) were screened and identified by total genome and viral dsRNA profile analyses. The results indicated that PiCV1 could be horizontally transmitted between different individuals of *P. italicum* via hyphal anastomosis (Fig. 6B). The proportion of virus-infected isolates was 10%–50% (1–5 of 10 randomly picked single-spore isolates) in every plate coated with an appropriate dilution ratio (10^5 – 10^6) of the spore suspension after dual culture (Fig. 6B). These virus-infected *P. italicum* isolates were further confirmed by RT-PCR with specific probe primers targeting the four dsRNA segments of the PiCV1 genome (Fig. 6C).

Additionally, we performed hygromycin B screening for recipient strains with overexpression of the *hyg* marker

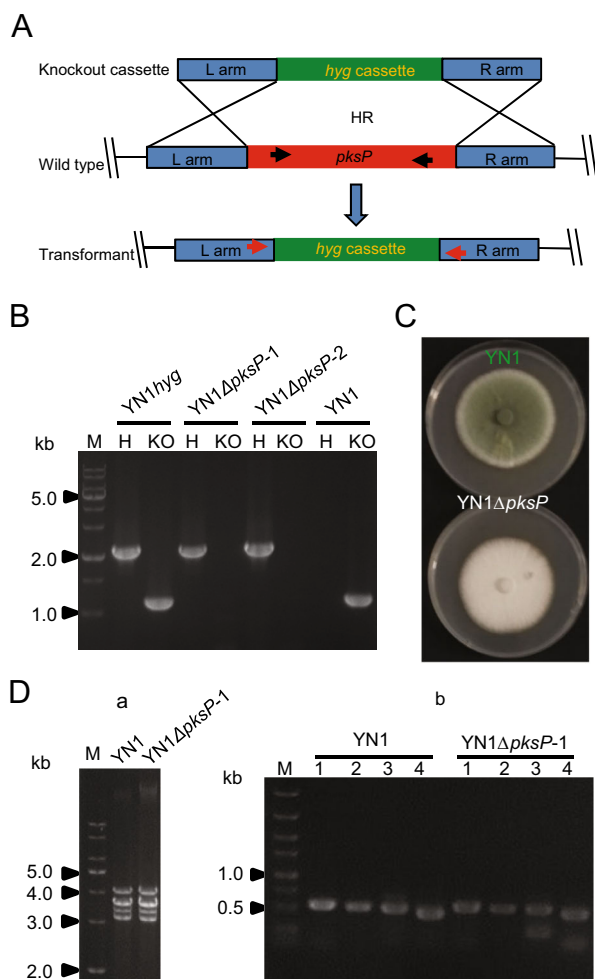


Fig. 5 *pksP* gene disruption in the wild-type strain HSPi-YN1. **A** Schematic showing that the target gene *pksP* was replaced with the *hyg* cassette through homologous recombination (HR). The black and red arrows represent negative and positive PCR primers, respectively. **B** Mutants with *pksP* gene disruption were confirmed by negative and positive PCR. Lane M: 1-kb DNA marker. H and KO represent lanes loaded with amplification products using positive and negative PCR primers, respectively. **C** Wild-type HSPi-YN1 and HSPi-YN1 Δ *pksP* morphological phenotypes on PDA. **D** Viral genomic profile analysis and RT-PCR verification confirmed that the modified virus donor (albino mutant) harbored PiCV1. (a) dsRNA genome of PiCV1 isolated from the wild-type strain and albino mutant, as detected by electrophoresis analysis. (b) PiCV1 found in the wild-type strain and the modified virus donor by RT-PCR. Lanes 1–4: PiCV1 dsRNA segments; lane M: DNA marker DS5000.

(HSPi-CQ54*hyg*, HSPi-HN1*hyg*, and HSPi-HB4*hyg*) cocultured with the wild-type virus donor (HSPi-YN1). Based on hygromycin B resistance, genomic profile analysis, and RT-PCR, genetic backgrounds of donor and recipients were distinguished, and the horizontal transmission of PiCV1 between these strains of *P. italicum* was achieved via hyphal anastomosis. The efficiency was similar to that observed by blue–white colony screening (Supplementary

Figure S2). Thus, *pksP* gene knockout did not affect the ability of PiCV1 to inhabit the virus host (*P. italicum*) or the efficiency of horizontal transmission between different *P. italicum* individuals via hyphal anastomosis. Furthermore, blue–white colony screening for generation of isogenic and virus-infected recipients was simpler and more economically feasible than antibiotic screening.

Biological Effects of PiCV1 Infection

To investigate biological effects of PiCV1 infection, we compared vegetative growth and virulence between isogenic virus-free and virus-infected strains, i.e., HSPi-CQ54 versus HSPi-CQ54V, HSPi-HB4 versus HSPi-HB4V, and HSPi-HN1 versus HSPi-HN1V. The results showed no obvious difference in mycelia growth, colony morphologies and lesions on 7-day incubated citrus fruits for each comparison (Fig. 7A, 7C, 7D, 7F). There were also no difference in pigment accumulation between HSPi-CQ54 and its isogenic virus-infected strain HSPi-CQ54V (Fig. 7A). In addition, all the PiCV1-infected recipients including HSPi-CQ54V, HSPi-HN1V, and HSPi-HB4V strains exhibited little difference in fungicide prochloraz resistance as compared to the corresponding virus-free recipients, i.e., HSPi-CQ54, HSPi-HN1, and HSPi-HB4, respectively (Fig. 7B, 7E). These results together indicated no significant hypovirulent and drug-conditioned hypovirulent effects of PiCV1 infection on the present *P. italicum* strains.

Discussion

Mycoviruses are widely distributed in different fungal groups (Ghabrial and Suzuki 2009). To the best of our knowledge, approximately 12 species of *Penicillium* fungi have been found to harbor viruses, and chrysovirus have been detected in various *Penicillium* species, including *P. cyaneofulvum*, *P. brevicompactum*, *P. chrysogenum*, *P. janczewskii*, *P. crustosum*, and *P. italicum* (Banks *et al.* 1969; Wood *et al.* 1971; Jiang and Ghabrial 2004; Nerva *et al.* 2016; Ghabrial *et al.* 2018; Niu *et al.* 2018). Interestingly, PiCV1 and *Penicillium crustosum* chrysovirus 1 (PcCV1), which were isolated from the citrus pathogens *P. italicum* and *P. crustosum*, respectively, shared approximately 83% identity in the quadripartite genome with the PcV. However, when the PiCV1 host *P. italicum* strain (HSPi-YN1 or HSPi-YN1 Δ *pksP*) and virus-free *P. crustosum* strain (HSPc-CQ4) or PcCV1 wild-type host *P. crustosum* strain (HSPc-CQ15) and virus-free *P. italicum* strain (HSPi-HB4) were cocultured, no virus-infected isolates derived from HSPc-CQ4 or HSPi-HB4 were detected

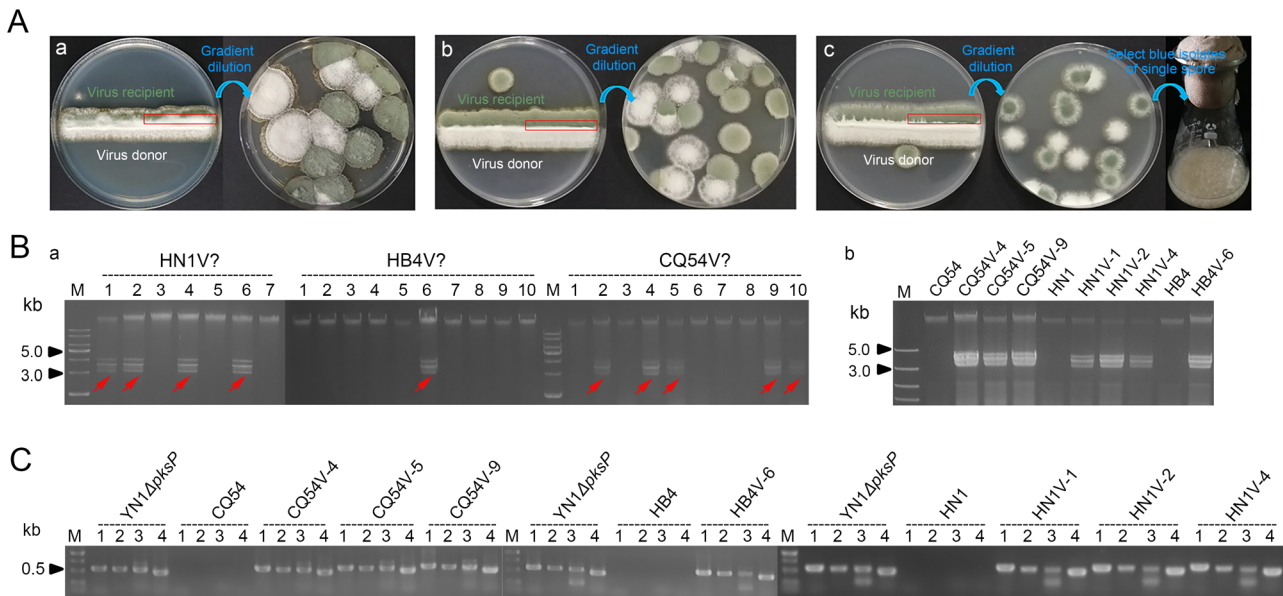


Fig. 6 Isogenic isolates infected with PiCV1 among wild-type recipients with different genetic backgrounds were screened based on the blue–white colony screening method. **A** Experimental procedure for the blue–white colony screening method based on the albino phenotype of the donor via hyphal anastomosis. (a–c) Wild-type recipients CQ54, HN1, and HB4 were cocultured with the albino virus donor YN1ΔpkSP, and corresponding single-spore isolates were generated by gradient dilution of the mixture spores whose blue isolates were selected as candidates for PiCV1 infection. The area in the red square is the colony border for the coculture. **B** PiCV1-infected isogenic isolates were screened and identified from candidates (blue colony) by total genome and viral genomic dsRNA profile

analyses. (a) The total genome profile analysis of all blue colony candidates derived from CQ54, HN1, and HB4. “V?” represents the status of PiCV1 infection, and lane numbers 1–10 represent corresponding blue colony isolate serial numbers. Lane M: DS5000 DNA marker. Red arrow: dsRNA from isolates infected with PiCV1. (b) Electrophoresis profile of dsRNA extracts from virus-free recipient strains and corresponding isogenic virus-infected isolates. Lane M: 1-kb ladder DNA marker. **C** Virus-infected isolates were further verified by RT-PCR. Lanes 1–4: serial numbers of PiCV1 dsRNA segments; lane M: DNA marker DS5000. For brevity, the strain numbers HSPi-CQ54, HSPi-HB4, and HSPi-HN1 are abbreviated as CQ54, HB4, and HN1, respectively, in **C**.

in single spore isolates of cocultures by hyphal anastomosis. Thus, taken together, our results indicated that PiCV1 could be horizontally transmitted between different individuals in intraspecies hosts but could not be transferred among different species of fungal hosts under natural culture conditions. These results suggested that *P. italicum* and *P. crustosum* each had their own unique chrysovirus, regardless of the considerably high genome identity observed in this study. Chrysovirus inhabiting different *Penicillium* species (i.e., *P. italicum*, *P. chrysogenum*, and *P. crustosum*) may be derived from the same original viral ancestor and adaptively evolved into new variants in different species of *Penicillium* hosts.

The *Chrysoviridae* family contains approved chrysovirus and chrysovirus-related viruses. The genome of the former viruses is comprised of four monocistronic dsRNA segments, whereas chrysovirus-related viruses infecting fungi have either four or five segments (Ghabrial *et al.* 2018). The PiCV1 genome includes four linear dsRNAs encoding proteins having high identity to corresponding proteins in PcV, as a classic chrysovirus. dsRNA1 encodes RdRp, which harbors conserved regions (motif I–VIII), and dsRNA2 encodes a major CP (approximately 109 kDa in

size), which assembles virions with a diameter of ~40 nm. p3 and p4 encoded by dsRNA3 and dsRNA4, respectively, are unknown functional proteins containing the phytoe S7 domain (found in phytoeovirus P7 core proteins) and a putative cysteine protease motif found in the ovarian tumor gene-like superfamily. All four dsRNA segments of PiCV1 were highly conserved in both the 5′- and 3′-UTRs. The 5′-terminus contained a 10-nt A-rich conserved sequence and a CCUGAGGA conserved sequence region, whereas the 3′-terminus contained a 9-nt U-rich conserved sequence and a 12-nt UA-rich conserved sequence. These conserved elements play important roles in virus replication, transcription, and packaging (Wei *et al.* 2003; Jiang and Ghabrial 2004). The analogous (CAA)_n repeats were similar to tobamovirus-like translational enhancer elements upstream of the AUG initiator codon of each dsRNA monocistronic in the PiCV1, similar to the typical chrysovirus PcV and Hv145SV, consistent with previously reported chrysovirus (Gallie and Walbot 1992; Jiang and Ghabrial 2004; Ghabrial and Suzuki 2009; Ghabrial *et al.* 2018). These enhancer elements may regulate genome replication and virion assembly for chrysovirus in their *Penicillium* hosts, which would explain

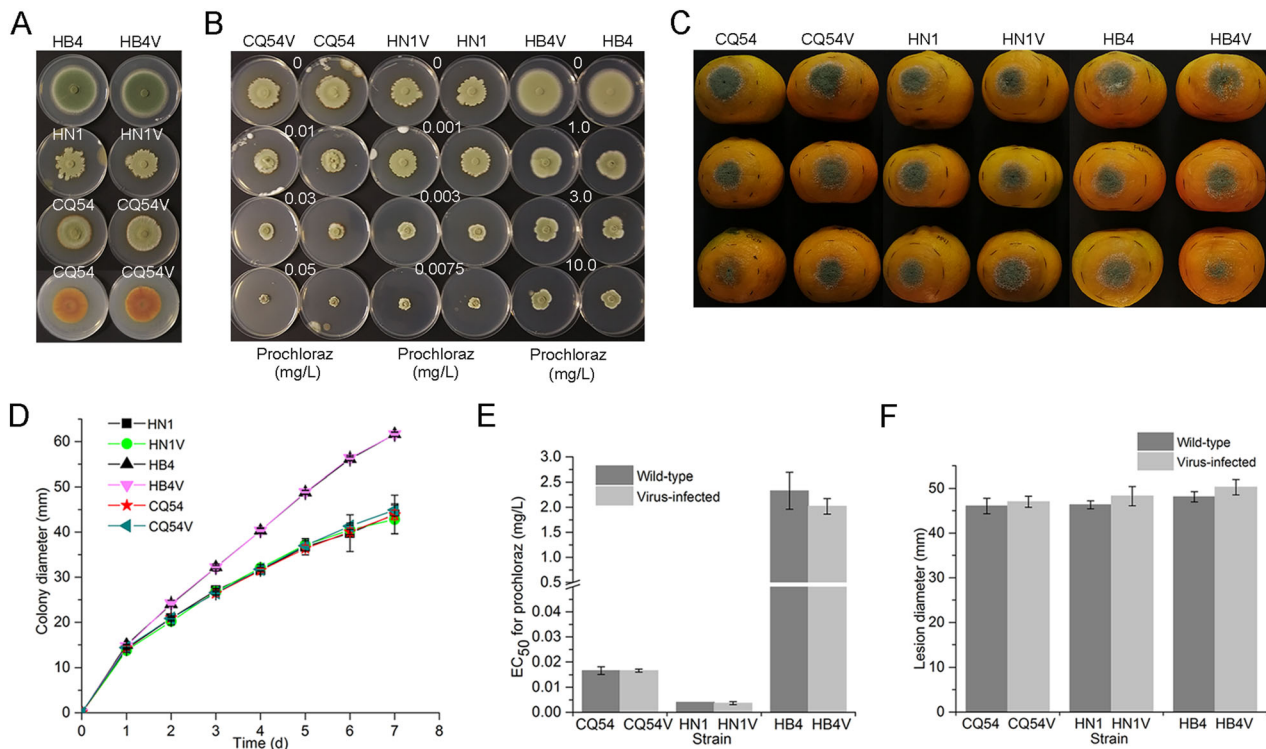


Fig. 7 Comparison of biological phenotypes between PiCV1-infected and virus-free *P. italicum* strains. **A** Colony morphology of growth assays for tested strains grown on PDA for 7 days. The bottom row shows the pigment on the back of the PDA plate. **B** Sizes of colonies grown on PDA with different concentrations of prochloraz. **C** Lesion area for virus-infected strains and virus free-strains on the surface of citrus fruits. **D** Line charts showing the mean diameters at different

time points for growth rate assays shown in (A). Strains were evaluated using three independent replicates. **E** Column charts showing the mean EC_{50} s for all strains. All concentrations were measured with three plates for each strain ($P > 0.05$). **F** Column charts showing the mean diameters of lesions at 7 days after inoculation of *P. italicum* on the citrus surface. All strains were inoculated into three oranges ($P > 0.05$).

why chrysovirus cause latent persistent infections in fungal hosts (Ghabrial *et al.* 2018). In this study, we found it difficult to obtain PiCV1-abolished isolates from the host HSPi-YN1 (*P. italicum*) through ribavirin-based protoplast regeneration. Phylogenetic analysis based on RdRp indicated that the *Chrysoviridae* family included two clade groups; cluster I consisted of PiCV1 and typical chrysovirus members (PcV, Hv145S, IjCV1, CnCV1, VdCV1, and ACCV), whereas cluster II members were unclassified chrysovirus-related viruses, such as MoCV1, BdCV1, and PjCV1. These results were consistent with a recent report by the ICTV describing the *Chrysoviridae* family phylogenetic tree (Ghabrial *et al.* 2018). According to the PiCV1 selectivity of the host in *Penicillium* species, genomic characteristics, and phylogenetic analyses, we concluded that this was a new strain of *P. chrysogenum* virus.

To date, the reported mycoviruses in most cases cause little or no obvious symptoms in their fungal hosts, and some phytopathogenic fungi infected by chrysovirus can change their pathogenicity and pathogenic traits (Ghabrial and Suzuki 2009; Aihara *et al.* 2018; Okada *et al.* 2018). However, the PiCV1 infection caused no obvious change in the host *P. italicum* phenotypes including mycelia growth,

pigment production, virulence and fungicide resistance (Fig. 7). Our results were consistent with the previous report that some typical chrysovirus in *Penicillium* species (*P. chrysogenum*, *P. cyaneo-fulvum*, and *P. brevicompactum*) did have no obvious effects on their host fungi (Border *et al.* 1972). Infrequently, infection of specific mycovirus can enhance their host resistance to some abiotic stresses such as heat and salinity (Márquez *et al.* 2007; Nerva *et al.* 2017). Thus more biological effects of PiCV1 infection like those would be further studied.

In the current study, we constructed and applied two methods, i.e., the blue–white colony screening method and the hygromycin B screening method to generate recipients infected with PiCV1 via hyphae anastomosis. The former was based on the visual morphological marker of albino donor colonies to distinguish genetic backgrounds between virus donors and recipients, whereas the latter was based on the hygromycin B resistance of the recipient. Although these methods showed the same efficiency for detection of horizontal transmission between different *P. italicum* individuals via hyphal anastomosis, antibiotic screening was not economical in terms of cost and required complex steps. In contrast, the white–blue colony screening method showed the

following advantages. First, virus-infected recipient isolates were hyg-marker-free and had the same genetic background as the recipient strain before virus infection. Second, there was no need to consider the adverse effects of integration of the *hyg* gene into the fungal genome in the fungus/virus interaction system (Lee *et al.* 2014). In contrast, in hygromycin B screening, the virus must be retransmitted from the virus-infected recipient with overexpression of the *hyg* gene to the wild-type recipient (*hyg* sensitive) to exclude the influence of the *hyg* gene by back-introduction (Lee *et al.* 2014). Third, virus-infected isogenic candidate recipient isolates (blue) were visually selected based on the albino colony phenotype (white) of the virus donor in the coculture mixture, making this approach much simpler and more economical. Indeed, the process of hygromycin B screening was tedious and ineffective owing to the absence of a selective marker after coculture in back-introduction (Lee *et al.* 2014). Overall, our method could be tentatively applied to coculture screening of the horizontal transmission of mycoviruses to other filamentous fungi with pigmented colonies, such as *Penicillium* species and *Aspergillus*.

To make a conclusion, the present study isolated and characterized a new chrysovirus PiCV1 from the citrus pathogen *P. italicum* (HSPi-YN1), constructed *pksP*-knockout HSPi-YN1 and verified its horizontal transmission among dually cultured *P. italicum* isolates, and established a blue–white colony method to select virus-infected *P. italicum* recipients (blue colonies) using HSPi-YN1 $\Delta pksP$ (white colonies) as virus donors. Here we provide an effective and simplified approach to select virus-infected isogenic recipients based on the distinguishable colony-colors (i.e., blue virus-recipients versus white virus-donors), which would be applied to develop screening methods of virus-infected isogenic recipients for other filamentous fungi in future studies.

Acknowledgements This work was supported by the National Natural Science Foundations of China (No. 31371893), the Natural Science Fund of Hubei Province (No. 2018CFB676) and the Project of Hubei Key Laboratory of Genetic Regulation and Integrative Biology (Grant No. GRIB20184).

Author Contributions DL and YY conceived this study, acquired project funding, revised to complete final version of manuscript, and supervised all research activities. TZ and NL designed experiments and completed the data analysis. QC, YC, BT and GL performed most of the experiments. TZ wrote the paper draft. All authors read and approved the final manuscript.

Compliance with Ethics Standards

Conflict of interest The authors declare that they have no competing interests.

Animal and Human Rights Statement This article does not contain any studies with human or animal subjects performed by any of the authors.

References

- Aihara M, Urayama SI, Le MT, Katoh Y, Higashiura T, Fukuhara T, Arie T, Teraoka T, Komatsu K, Moriyama H (2018) Infection by *Magnaporthe oryzae* chrysovirus 1 strain A triggers reduced virulence and pathogenic race conversion of it host fungus, *Magnaporthe oryzae*. *J Gen Plant Pathol* 84:92–103
- Banks GT, Buck KW, Chain EB, Darbyshire JE, Himmelweit F (1969) *Penicillium cyaneo-fulvum* virus and interferon stimulation. *Nature* 223:155–158
- Border DJ, Buck KW, Chain EB, Kempson-Jones GF, Lhoas P, Ratti G (1972) Viruses of *Penicillium* and *Aspergillus* species. *Biochem J* 127:4P
- Borré E, Morgantini LE, Ortali V, Tonolo A (1971) Production of lytic plaques of viral origin in *Penicillium*. *Nature* 229:568
- Boubaker H, Saadi B, Boudyach EH, Benaoumar AA (2009) Sensitivity of *Penicillium digitatum* and *P. italicum* to imazalil and thiabendazole in Morocco. *Plant Pathol J* 8:152–158
- Castón JR, Ghabrial SA, Jiang D, Rivas G, Alfonso C, Roca R, Luque D, Carrascosa JL (2003) Three-dimensional structure of *Penicillium chrysogenum* virus: a double-stranded RNA virus with a genuine T = 1 capsid. *J Mol Biol* 331:417–431
- Coenen A, Kevei F, Hoekstra RF (1997) Factors affecting the spread of double-stranded RNA viruses in *Aspergillus nidulans*. *Genet Res* 69:1–10
- Ellis LF, Kleinschmidt WJ (1967) Virus-like particles of a fraction of statolon, a mould product. *Nature* 215:649
- Gallie DR, Walbot V (1992) Identification of the motifs within the tobacco mosaic virus 5'-leader responsible for enhancing translation. *Nucl Acids Res* 20:4631–4638
- Ghabrial SA, Suzuki N (2009) Viruses of plant pathogenic fungi. *Annu Rev Phytopathol* 47:353–384
- Ghabrial SA, Castón JR, Coutts RH, Hillman BI, Jiang D, Kim DH, Moriyama H (2018) ICTV virus taxonomy profile: *chrysoviridae*. *J Gen Virol* 99:19–20
- Hillman BI, Supyani S, Kondo H, Suzuki N (2004) A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. *J Virol* 78:892–898
- Hollings M (1962) Viruses associated with a die-back disease of cultivated mushroom. *Nature* 196:962
- Jamal A, Bignell EM, Coutts RH (2010) Complete nucleotide sequences of four dsRNAs associated with a new chrysovirus infecting *Aspergillus fumigatus*. *Virus Res* 153:64–70
- Jiang D, Ghabrial SA (2004) Molecular characterization of *Penicillium chrysogenum* virus: reconsideration of the taxonomy of the genus *Chrysovirus*. *J Gen Virol* 85:2111–2121
- Kim JW, Kim SY, Kim KM (2003) Genome organization and expression of the *Penicillium stoloniferum* virus S. *Virus Genes* 27:249–256
- Kim JM, Jung JE, Park JA, Park SM, Cha BJ, Kim DH (2013) Biological function of a novel chrysovirus, CnV1-BS122, in the Korean *Cryphonectria nitschkei* BS122 strain. *J Biosci Bioeng* 115:1–3
- Korsten L (2006) Advances in control of postharvest diseases in tropical fresh produce. *Int J Postharvest Technol Innov* 1:48–61
- Lee KM, Yu J, Son M, Lee YW, Kim KH (2011) Transmission of *Fusarium boothii* mycovirus via protoplast fusion causes

- hypovirulence in other phytopathogenic fungi. *PLoS ONE* 6:e21629
- Lee KM, Cho WK, Yu J, Son M, Choi H, Min K, Lee YW, Kim KH (2014) A comparison of transcriptional patterns and mycological phenotypes following infection of *Fusarium graminearum* by four mycoviruses. *PLoS ONE* 9:e100989
- Li L, Liu J, Xu A, Wang T, Chen J, Zhu X (2013) Molecular characterization of a trisegmented chrysovirus isolated from the radish *Raphanus sativus*. *Virus Res* 176:169–178
- Liu YC, Linder-Basso D, Hillman BI, Kaneko S, Milgroom MG (2003) Evidence for interspecies transmission of viruses in natural populations of filamentous fungi in the genus *Cryphonectria*. *Mol Ecol* 12:1619–1628
- Liu H, Fu Y, Jiang D, Li G, Xie J, Peng Y, Yi X, Ghabrial SA (2009) A novel mycovirus that is related to the human pathogen hepatitis E virus and rubi-like viruses. *J Virol* 83:1981–1991
- Liu J, Yuan Y, Wu Z, Li N, Chen Y, Qin T, Geng H, Xiong L, Liu D (2015) A novel sterol regulatory element-binding protein gene (*sreA*) identified in *Penicillium digitatum* is required for prochloraz resistance, full virulence and *erg11* (*cyp51*) regulation. *PLoS ONE* 10:e0117115
- Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315:513–515
- Melzer MS, Ikeda SS, Boland GJ (2002) Interspecific transmission of double-stranded RNA and hypovirulence from *Sclerotinia sclerotiorum* to *S. minor*. *Phytopathology* 92:780–784
- Morris TJ, Dodds JA (1979) Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology* 69:854–858
- Nerva L, Ciuffo M, Vallino M, Margaria P, Varese GC, Gnani G, Turina M (2016) Multiple approaches for the detection and characterization of viral and plasmid symbionts from a collection of marine fungi. *Virus Res* 219:22–38
- Nerva L, Silvestri A, Ciuffo M, Palmano S, Varese GC, Turina M (2017) Transmission of *Penicillium aurantiogriseum* partiti-like virus 1 to a new fungal host (*Cryphonectria parasitica*) confers higher resistance to salinity and reveals adaptive genomic changes. *Environ Microbiol* 19:4480–4492
- Niu Y, Zhang T, Zhu Y, Yuan Y, Wang S, Liu J, Liu D (2016) Isolation and characterization of a novel mycovirus from *Penicillium digitatum*. *Virology* 494:15–22
- Niu Y, Yuan Y, Mao J, Yang Z, Cao Q, Zhang T, Wang S, Liu D (2018) Characterization of two novel mycoviruses from *Penicillium digitatum* and the related fungicide resistance analysis. *Sci Rep* 8:5513
- Okada R, Ichinose S, Takeshita K, Urayama SI, Fukuhara T, Komatsu K, Arie T, Ishihara A, Egusa M, Kodama M, Moriyama H (2018) Molecular characterization of a novel mycovirus in *Alternaria alternata* manifesting two-sided effects: down-regulation of host growth and up-regulation of host plant pathogenicity. *Virology* 519:23–32
- Pearson MN, Beaver RE, Boine B, Arthur K (2009) Mycoviruses of filamentous fungi and their relevance to plant pathology. *Mol Plant Pathol* 10:115–128
- Prusky D, McEvoy JL, Saftner R, Conway WS, Jones R (2004) Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. *Phytopathology* 94:44–51
- Sasaki A, Kanematsu S, Onoue M, Oyama Y, Yoshida K (2006) Infection of *Rosellinia necatrix* with purified viral particles of a member of *Partitiviridae* (RnPV1-W8). *Arch Virol* 51:697–707
- Sasaki A, Nakamura H, Suzuki N, Kanematsu S (2016) Characterization of a new megabarnavirus that confers hypovirulence with the aid of a co-infecting partitivirus to the host fungus, *Rosellinia necatrix*. *Virus Res* 219:73–82
- Tamura K, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* 25:4876–4882
- Urayama SI, Sakoda H, Takai R, Katoh Y, Le TM, Fukuhara T, Arie T, Teraoka T, Moriyama H (2014) A dsRNA mycovirus, *Magnaporthe oryzae* chrysovirus 1-B, suppresses vegetative growth and development of the rice blast fungus. *Virology* 448:265–273
- Vainio EJ, Korhonen K, Tuomivirta TT, Hantula J (2010) A novel putative partitivirus of the saprotrophic fungus *Heterobasidium ecrustosum* infects pathogenic species of the *Heterobasidium annosum* complex. *Fungal Biol* 114:955–965
- Wang J, Yu J, Liu J, Yuan Y, Li N, He M, Qi T, Geng H, Xiong L, Liu D (2014) Novel mutations in CYP51B from *Penicillium digitatum* involved in prochloraz resistance. *J Microbiol* 52:762–770
- Wei CZ, Osaki H, Iwanami T, Matsumoto N, Ohtsu Y (2003) Molecular characterization of dsRNA segments 2 and 5 and electron microscopy of a novel reovirus from a hypovirulent isolate, W370, of the plant pathogen *Rosellinia necatrix*. *J Gen Virol* 84:2431–2437
- Wood HA, Bozarth RF, Mislivec PB (1971) Viruslike particles associated with an isolate of *Penicillium brevi-compactum*. *Virology* 44:592–598
- Wu M, Zhang L, Li G, Jiang D, Ghabrial SA (2010) Genome characterization of a debilitation-associated mitovirus infecting the phytopathogenic fungus *Botrytis cinerea*. *Virology* 406:117–126
- Wu Z, Wang S, Yuan Y, Zhang T, Liu J, Liu D (2016) A novel major facilitator superfamily transporter in *Penicillium digitatum* (PdMFS2) is required for prochloraz resistance, conidiation and full virulence. *Biotechnol Lett* 38:1349–1357
- Yang Z, Geng H, Zheng Y, Yuan Y, Wang M, Mao J, Zhang T, Niu Y, Liu D (2018) Molecular characterization of a new gamma-partitivirus isolated from the citrus-pathogenic fungus *Penicillium digitatum*. *Arch Virol* 163:3185–3189
- Yu X, Li B, Fu Y, Jiang D, Ghabrial SA, Li G, Peng Y, Xie J, Cheng J, Huang L, Yi X (2010) A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. *Proc Natl Acad Sci USA* 107:8387–8392
- Zerbino DR, Birney E (2008) Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 18:821–829
- Zhang T, Jiang Y, Dong W (2014) A novel monopartite dsRNA virus isolated from the phytopathogenic fungus *Ustilagoidea virescens* and ancestrally related to a mitochondria-associated dsRNA in the green alga *Bryopsis*. *Virology* 462:227–235
- Zhao S, Yan YS, He QP, Yang L, Yin X, Li CX, Mao LC, Liao LS, Huang JQ, Xie SB, Nong QD, Zhang Z, Jing L, Xiong YR, Duan CJ, Liu JL, Feng JX (2016) Comparative genomic, transcriptomic and secretomic profiling of *Penicillium oxalicum* HP7-1 and its cellulase and xylanase hyper-producing mutant EU2106, and identification of two novel regulatory genes of cellulase and xylanase gene expression. *Biotechnol Biofuels* 9:203