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A symptomless hypovirus, CHV4, facilitates stable infection of the chestnut blight fungus by a coinfecting reovirus likely through suppression of antiviral RNA silencing

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ABSTRACT

Field-collected US strain C18 of *Cryphonectria parasitica*, the chestnut blight fungus, was earlier reported to be infected by a double-stranded RNA virus, mycoreovirus 2 (MyRV2). Next-generation sequencing has revealed co-infection of C18 by a positive-strand RNA virus, hypovirus 4 (CHV4). The current molecular and genetic analyses showed interesting commensal interactions between the two viruses. CHV4 facilitated the stable infection and enhanced vertical transmission of MyRV2, which was readily lost during subculturing and showed reduced vertical transmission in single infections. Deletion of a key antiviral RNA silencing gene, *dcl2*, in isolate C18 increased stability of MyRV2 in single infections. The ability of CHV4 to facilitate stable infection with MyRV2 appears to be associated with the inhibitory effect of CHV4 on RNA silencing via compromising the induction of transcriptional up-regulation of *dcl2*. These results suggest that natural infection of isolate C18 by MyRV2 in the field was facilitated by CHV4 co-infection.

1. Introduction

Fungal viruses (mycoviruses) are widespread in all major groups of fungi and mixed infections of single host fungi are common phenomena (Ghabrial and Suzuki, 2009; Ghabrial et al., 2015). There are a few types of interesting virus/virus interplays in coinfected fungal host organisms (Hillman et al., 2018). For example, a positive strand, single-stranded RNA ((+)RNA) virus, Cryphonectria hypovirus 1 (CHV1, in the family *Hypoviridae*) exerts a one-way synergistic effect on a co-infecting double-stranded RNA (dsRNA) virus, mycoreovirus 1 (MyRV1, in the family *Reoviridae*), resulting in enhanced virus accumulation and increased vertical transmission of MyRV1 (Sun et al., 2006). By contrast, one virus, MyRV1 or a CHV1 mutant lacking an RNA silencing suppressor (CHV1-Δp69), interferes with the replication or transmission of a second virus, Rosellinia necatrix victorivirus 1 (RnVV1, the family *Totiviridae*) with an undivided dsRNA genome (Chiba and Suzuki, 2015). Recently, novel types of virus/virus interactions in fungi have attracted much attention where a capsidless (+)RNA virus is hosted by an unrelated dsRNA virus (Hisano et al., 2018; Zhang et al., 2016). In another example, one virus facilitated horizontal transfer of a coinfecting virus between fungal strains belonging to different vegetative incompatibility groups, normally a barrier to such transmission (Wu et al., 2017).

Some of the aforementioned virus/virus interactions occurring in a model filamentous fungus host, the chestnut blight fungus (Eusebio-Cope et al., 2015), *Cryphonectria parasitica*, have been explored at the molecular level, and are known to be associated with antiviral RNA silencing (RNA interference). In *C. parasitica*, a number of viruses are targeted by RNA silencing in which two major players, Dicer-like protein 2 (DCL2) and Argonaute-like protein 2 (AGL2) are central (Segers et al., 2007; Sun et al., 2009). CHV1-encoded RNA silencing suppressor p29, a multifunctional papain-like protease, suppresses the induction of the RNA silencing key genes, *dcl2* and *agl2* (Chiba and Suzuki, 2015; Segers et al., 2006; Sun et al., 2009). This is likely involved in the CHV1/MyRV1 interactions where accumulation and transmission of MyRV1 is enhanced. Conversely, in the second example, infection of MyRV1 or CHV1-Δp69 induces antiviral RNA silencing via upregulation of transcription by ~40-fold of *dcl2* and *agl2*, and leads to inhibition of RnVV1 susceptible to RNA silencing (Chiba and Suzuki, 2015). These findings have been revealed using a US strain EP155 of *C. parasitica*, which has been broadly used as the standard virus-free strain of the fungus (Dawe and Nuss, 2013; Eusebio-Cope et al., 2015). Abundant

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genetic information and many mutants are available for EP155. In addition to EP155, many other *C. parasitica* field isolates have been isolated and studied to understand aspects of the fungus including virulence, genetic diversity, population structures, evolution, and virus presence in nature (Bryner et al., 2014; Hillman and Suzuki, 2004; Liu et al., 2007; Milgroom, 1995; Rigling and Prospero, 2018). Among them is the US strain C18 that is the subject of this study.

C. parasitica strain C18 was isolated in West Virginia by Dr. William MacDonald and colleagues, and shown to be infected by a mycoreovirus that was subsequently named mycoreovirus 2 (MyRV2) (Enebak et al., 1994a, 1994b). Sequence information for the virus MyRV2 is now available and will be published elsewhere, but its original host strain C18 has not been studied in depth. MyRV2 is a member of the genus *Mycoreovirus* (subfamily *Spinareovirinae*) (Carstens, 2010) and like MyRV1 its genome comprises 11 dsRNA segments (Enebak et al., 1994a). However, MyRV2 differs from MyRV1 in many properties: the viral dsRNAs are not closely related to each other, sharing less than 50% nucleotide sequence identity (Supyani et al., 2007); and the terminal sequences of MyRV1 and 2 are quite different from each other. Both reduce fungal virulence and alter colony morphology in culture, but MyRV1 causes greater reduction of aerial hyphal growth than MyRV2 while maintaining host sporulation levels (Hillman et al., 2004). Both MyRV1 and MyRV2 perturb fungal gene expression similarly, though not identically to each other, and differently from the hypovirus CHV1 (Deng et al., 2007). Importantly from the standpoint of this study, MyRV2 has always been more difficult to maintain in culture than MyRV1. Unlike EP155, C18 has not yet been explored as a virus host fungus. C18 is vegetatively incompatible with EP155 and supports the replication of mitovirus Cryphonectria parasitica mitovirus 1 (Shahi et al., 2019).

During the course of study on MyRV2/host interactions, we performed transcriptomic analysis and found Cryphonectria hypovirus 4 (CHV4) coinfecting the original C18 fungal strain. CHV4 is a (+)RNA virus, a member of the family *Hypoviridae* (Suzuki et al., 2018), and the most prevalent among the four known hypoviruses (CHV1 to CHV4) in the North American strains of *C. parasitica* (Linder-Basso et al., 2005). In this study we show that CHV4 suppresses antiviral RNA silencing of fungal strain C18 by cancelling induction of transcriptional up-regulation of *dcl2,* and thus facilitates stable infection with MyRV2 during co-infection.

2. Materials and methods

2.1. Viral and fungal strain

Fungal and viral strains used in this study are listed in Table 1. MyRV2-infected *C. parasitica* strain C18 was describe earlier (Enebak et al., 1994a, 1994b). *C. parasitica* standard EP155 (ATCC® 38755™), and its RNA silencing-defective derivatives, Δ*dcl2* (Segers et al., 2007) were a generous gift from Donald L. Nuss. *CpMK1* (mitogen-activated protein kinase)-knockout (KD) strain with EP155 background was described earlier (Chiba and Suzuki, 2015). CpMK1 is one of the scrutinized mitogen-activated protein kinases of *C. parasitica* (Park et al., 2004). An ORF A mutant of CHV1 lacking the viral silencing suppressor, p29 (CHV1-Δp69, GenBank accession no. M57938 with an internal deletion), was described previously (Suzuki and Nuss, 2002). Fungal cultures were grown at 22–27 °C on potato dextrose agar (PDA) plates on the bench-top for maintenance and colony phenotypic observations and in potato dextrose broth liquid media or on PDA plates layered with cellophane for RNA preparation.

2.2. Spheroplast preparation and fungal transformation

Spheroplast preparation and transformation were performed as described previously (Eusebio-Cope and Suzuki, 2015). Strain C18-CpMK-1 was generated by transformation of *C. parasitica* strain C18 with plasmid pCPXHY2-IR:CpMK1:IR (Chiba and Suzuki, 2015).

2.3. Generation of dcl2 knock out mutant

Deletion of *dcl2* gene in C18 strain was generated by the method of homologous recombination (Faruk et al., 2008). DNA fragments consisting of a neomycin resistance gene (neomycin phosphotransferase II gene, NPT II) cassette flanked with 2000-bp sequences derived from the both upstream and downstream sequences of the coding region of the C18 *dcl2* gene were produced by ligating the NPT II cassette to the vector pGEMT-easy (Promega, Madison, WI, USA) and then inserting both upstream and downstream DNA fragments into the *Sph*I and *Sal*I sites of the vector using the in-fusion cloning system (Takara Bio, Shiga, Japan). This plasmid (pGEMT- Neo-Pdcl2D2) was used to transform strain C18. All transgenic strains were subjected to single conidial spore isolation, and representative homokaryon strains were selected for use.

2.4. Virus inoculation and horizontal and vertical transmission

CHV1-Δp69 was introduced to strain C18 by spheroplast transformation with plasmid pCPXHY-CHV1-Δp69 (Andika et al., 2017). Horizontal viral transmission was examined by co-culture of viral-donor and -recipient fungal strains on a PDA plate (9 cm in diameter) as described previously (Andika et al., 2017). Vertical transmission rates were determined as described by Sun et al. (2006). Single conidial suspensions from two-week-old fungal cultures were spread on PDA at an appropriate spore concentration. Conidial germlings were subcultured on PDA for phenotype observation and cellophane overlaid

Table 1
Fungal and viral strains used in this study.

Strain	Description	Reference or Source
Fungal		
C18	*Cryphonectria parasitica* field strain doubly infected by MyRV2 and CHV4–C18	Enebak et al. (1994a)
C18-VF	Virus-free single conidial isolate of C18	This study
C18/MyRV2	C18 singly infected by MyRV2	This study
C18/CHV4	C18 singly infected by CHV4–C18	This study
C18/CHV1-Δp69	C18 singly infected by CHV1-Δp69	This study
EP155	Standard strain of *Cryphonectria parasitica* (virus free)	ATCC 38755
C18 Δ*dcl2*	*dcl2* knock-out mutant of C18 (RNA silencing defective, virus free)	This study
C18/IR-Cpmk1	Transformant expressing CpMK1-derived inverted repeat	This study
Viral		
MyRV1	Strain belonging to the type species *Mycoreovirus 1* within the genus *Mycoreovirus*	Hillman et al. (2004)
MyRV2	Strain belonging to the species *Mycoreovirus 2* within the genus *Mycoreovirus*	Enebak et al. (1994a)
CHV4–C18	Novel strain of the species *Cryphonectria hypovirus 4*	This study
CHV1-EP713	Prototype of the family *Hypoviridae*	Choi and Nuss (1992)
CHV1-Δp69	ORF-A deletion mutant of CHV1-EP713 lacking the p29 and p40 coding domains	Suzuki and Nuss (2002)

PDA for RNA extraction. Resultant RNA was examined for presence of virus sequence.

2.5. RNA extraction, RT-PCR and RNA blotting

Total RNA was extracted as described previously (Eusebio-Cope and Suzuki, 2015). For reverse transcription (RT)-polymerase chain reaction (PCR) analysis, first cDNA strands were synthesized by M-MLV or SuperScript II reverse transcriptase (Thermo Fisher Scientific, Carlsbad, CA, USA). PCR amplification using QuickTaq (Toyobo, Osaka, Japan). Alternatively, for virus detection after vertical virus transmission through conidia, rapid one step RT-PCR method using PrimeScript® OneStep RT-PCR Ver.2 (Takara Bio) and carried out as described previously (Urayama et al., 2015). 5′ RACE of the CHV4 genome was carried out as described by Suzuki et al. (2004). RNA blot analysis was performed as described previously (Eusebio-Cope and Suzuki, 2015) using digoxigenin (DIG)-labeled DNA probes prepared by PCR (PCR DIG Labeling mix, Roche, Risch-Rotkreuz, Swizerland). All primers used in the present study are listed in Table S1.

2.6. Next-generation sequencing and sequence analysis

A total RNA sample (62 ng/μl) was used for cDNA library construction using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) with the Ribo-Zero kit used to deplete the host rRNA. The library was subjected to single-end sequencing of 75 nucleotide reads using Illumina HiSeq. 2000 technology (Illumina). The cDNA library construction and sequencing were performed by the Research Institute for Microbial Diseases of Osaka University. After deep sequencing, the adaptor sequences were trimmed and then the reads (41,755,609 reads) were assembled *de novo* into 21,261 contigs (162–9126 nt in length) using CLC Genomics Workbench (version 11, CLC Bio-Qiagen, Aarhus, Demark). The contigs were then subjected to local BLAST searches against the viral reference sequence dataset obtained from National Center for Biotechnology Information (NCBI). Viral sequence data were analyzed using GENETYX-MAC (Software Development Co., Ltd., Tokyo, Japan) or Enzyme X v3.3.3 (nucleobytes.com/enzymex/index.html). The conserved protein domains were predicted from the NCBI conserved domain database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

3.1. Cryphonectria parasitica strain C18 is coinfected by a mycoreovirus and a hypovirus

C. parasitica strain C18 was previously shown to be infected by MyRV2 (Enebak et al., 1994a, 1994b). A next-generation sequencing (NGS) approach taken in this study revealed another co-infecting RNA virus, termed CHV4 strain C18 (CHV4–C18). It should be noted that a replicative dsRNA form of CHV4–C18 was very difficult to detect by a conventional dsRNA extraction and gel electrophoretic analyses (data not shown), as previously noted for other CHV4 strains including SR2 (Linder-Basso et al., 2005), and previous studies of *C. parasitica* strain C18 overlooked the co-infection by CHV4–C18. CHV4–C18 shared a high level of sequence identity to CHV4-SR2. Their overall nucleotide sequence identity is 99.4%. A total of 54 nucleotide substitutions were detected, 17 of which involved amino acid sequence substitutions (Fig. 1A). CHV4–C18 has common features of the genome organization to CHV4-SR2. Both the strains have five predicted functional domains of putative protease (prot?), glucosyltransferase (UGT), permuted papain fold peptidases of dsRNA viruses and eukaryotes (PPPDE). RNA-dependent RNA polymerase (Pol), and RNA helicase (Hel) on the single long polyprotein (Iyer et al., 2004; Linder-Basso et al., 2005) (Fig. 1A). An interesting difference is that the CHV4–C18 ORF starts at nucleotide position 287 unlike the CHV4-SR2 ORF starting at nucleotide position 194. Thus, the CHV4–C18 ORF is predicted to encode a polyprotein of 2817 aa, 317.2 kDa with a shorter N-terminal (30 aa) portion than that of CHV4-SR2 ORF (Fig. 1B), but a longer 5′-untranslated region (UTR). This difference in the ORF size appears not to exert any effect on the symptom expression because both strains show no or little overt phenotypic effect on the fungal host. Its effect on virus accumulation needs to be investigated.

3.2. Isolation of virus-free and singly infected fungal strains with the C18 genetic background

In order to confirm the effects of each virus onto the host fungus strain and investigate their possible interactions, we attempted to isolate fungal strains infected singly by CHV4–C18 and MyRV2 via single conidial isolation. Over 100 single conidial isolates of *C. parasitica* C18 strain were tested for CHV4–C18 and/or MyRV2 infection. While MyRV2-free strains could readily be obtained, CHV4–C18-free ones were relatively more difficult to isolate (see below). This approach allowed us to obtain multiple fungal conidial isolates of 1) virus-free strains, 2) single infectants by MyRV2, 3) single infectants by CHV4–C18, and 4) double infectants by the two viruses (CHV4–C18 + MyRV2). Their infection status was confirmed by RT-PCR (Fig. 2A). These obtained fungal strains were cultured on filter discs and stored at −20 °C until use for subsequent stability tests. Virus-free C18 and CHV4–C18-infected C18 were indistinguishable from each other phenotypically (Fig. 2B). MyRV2-infected C18 strains showed reduced mycelial growth rate, which was similar to that of the double infectant by CHV4–C18 + MyRV2, while its pigmentation phenotype is slightly different from that of the double infectant (Fig. 2B). CHV4–C18 promoted the growth of aerial mycelia when it co-infected with MyRV2. This contributed to the apparent difference in colony morphology (Fig. 2B).

3.3. CHV4–C18 facilitates stable maintenance of MyRV2 infection during subculturing and vertical transmission via asexual sporulation

While maintaining MyRV2-infected C18 without coinfecting CHV4–C18, we noticed that MyRV2 often was lost during subculturing of the fungal strain. This is consistent with past observations that MyRV2 is easily lost in culture (Enebak, 1992; Hillman and Suzuki, 2004). We systematically analyzed this phenomenon by subculturing 5 replicates of each of the above fungal strains and monitoring for virus infection during subculturing at one-week intervals. Interestingly, MyRV2 single infectants lost virus infection as subculture proceeded (Fig. 3A, red column). After the 10th subculture MyRV2 was retained in only one (Fig. 3A, red column, Experiments I and III) or zero subculture (Fig. 3A, red column, Experiments II). By contrast, MyRV2 was stably maintained in doubly-infected fungal isolates during subculturing (Fig. 3A, green column). CHV4–C18 was maintained stably irrespective of whether fungal isolates were singly infected by CHV4–C18 or doubly infected by MyRV2 and CHV4–C18 (Fig. 3A). The instability of MyRV2 to be retained in singly infected isolates was confirmed by three repeated experiments. The absence of MyRV2 in the fungal subcultures tested in Fig. 3A were confirmed by RT-PCR analyses (Fig. S1).

Vertical transmission via asexual sporulation was compared among single and double infections. Consequently, a tendency similar to that observed for horizontal transmission via subculturing was detected. Co-infection with CHV4 led to a higher transmission rate of MyRV2 compared to that for single MyRV2 infection (Fig. 3B and 25.6% vs. 9%). The total MyRV2 transmission rate (25.6%) represents the sum of singly-infected single-conidial isolates (4.3%, red area) plus doubly infected ones (21.3%, green area). CHV4–C18 transmission rates were much higher and close between doubly-infected isolates (93.6% total, = 72.3% singly-infected progenies [yellow area] plus 21.3% doubly-infected progenies [green area]) and singly-infected isolates (98.9%, yellow area) (Fig. 3B).

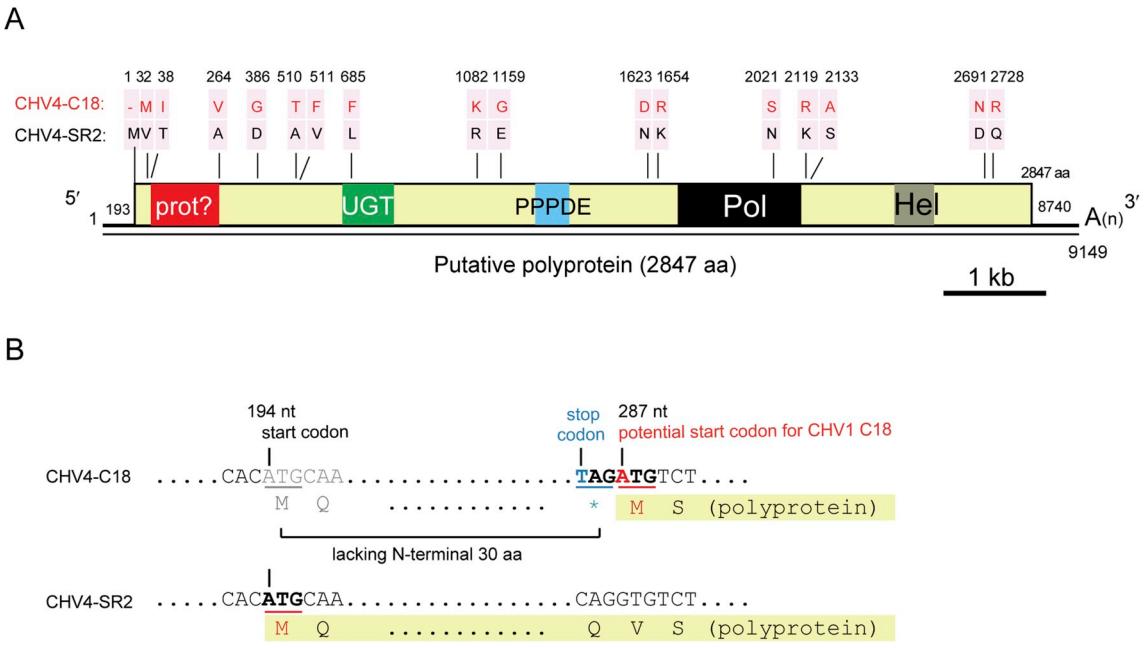
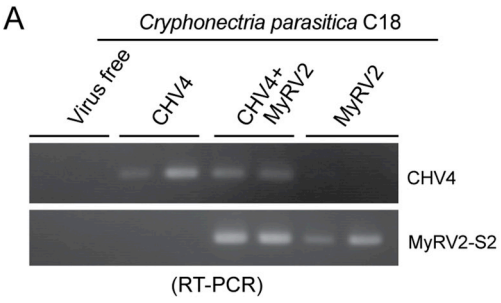


Fig. 1. Genome map and features of CHV4 isolated from *C. parasitica* strain C18. (A) Genome organization of CHV4. The five known domains of putative protease (prot?), UDP-glucose/sterol glucosyltransferase (UGT), permuted papain fold peptidases of dsRNA viruses and eukaryotes (PPPDE), RNA-dependent RNA polymerase (Pol), and RNA helicase (Hel) were indicated (Iyer et al., 2004; Linder-Basso et al., 2005). Amino acid sequence differences between CHV4–C18 and CHV4-SR2 are presented. (B) Predicted difference in translational initiation sites of CHV4–C18 and CHV4-SR2 ORFs. CHV4-SR2 ORF is initiated at nucleotide position 194, whereas CHV4–C18 ORF is initiated at nucleotide position 287.

3.4. Antiviral RNA silencing targets MyRV2 and reduces its stability in the C18 strain

We anticipated that antiviral RNA silencing contributed to the reduced stability of MyRV2. To test this possibility, we first prepared a deletion mutant of an antiviral silencing key gene *dcl2*, Δ*dcl2*, in the virus-free C18 strain. Screening of about 300 independent transformants with a *dcl2* disruption construct (Fig. S2A) allowed us to obtain three independent Δ*dcl2* strains. These mutants were validated by PCR-based genotyping (Fig. S2B) and RNA blotting of *dcl2* transcript expression, as well as phenotypic observation following hypovirus infection (Fig. S2C). Three independent Δ*dcl2* strains in the C18 genetic background with/without virus infection were isolated. Two Δ*dcl2* strains were singly and doubly infected with MyRV2 and MyRV2 and



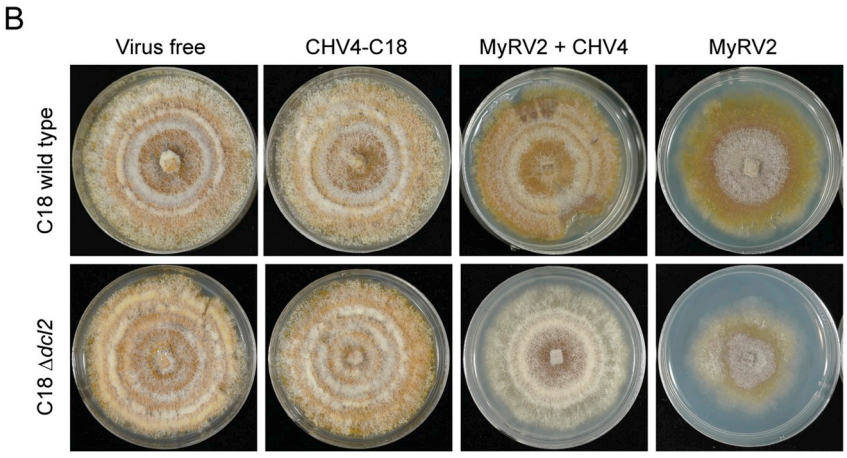


Fig. 2. Single and double infection of MyRV2 and CHV4–C18 in C18 strain. (A) RT-PCR detection of MyRV2 and CHV4–C18 in single conidial isolates of the C18 strain. (B) Colony morohology of wild type and Δ*dcl2* of C18 uninfected or infected with MyRV2 alone, CHV4–C18 alone or together. Colonies were grown on PDA for 6 days on the bench-top and photographed.

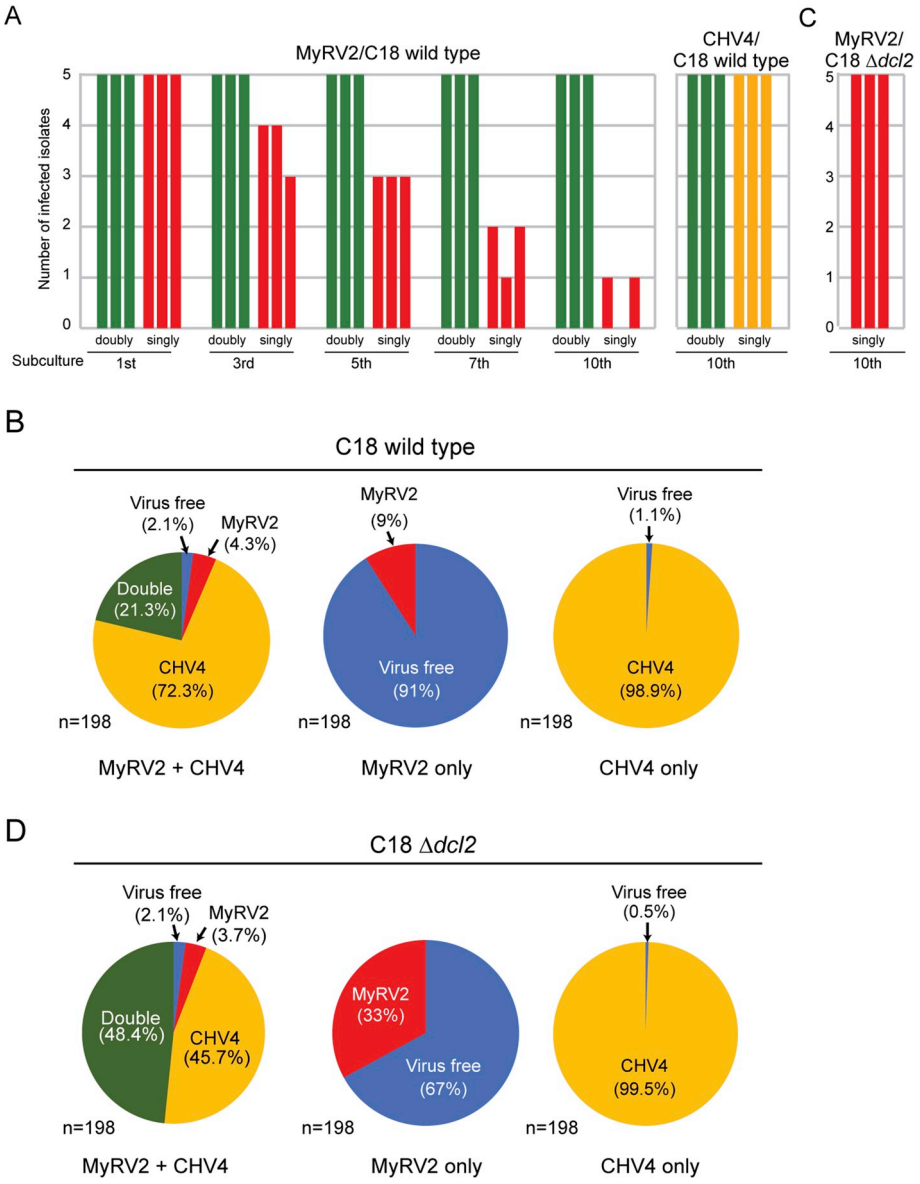


Fig. 3. Stability and vertical transmission of MyRV2 and CHV4–C18. (A and C) Infectivity of viruses after successive fungal subculture in wild type C18 (A) and its *dcl2* knockout mutant (Δ*dcl2*) (C). (B and D) Efficiency of virus transmission through conidia in wild type C18 (B) and Δ*dcl2* (D).

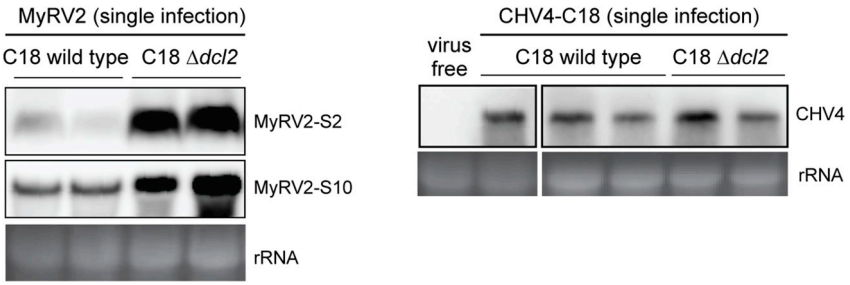


Fig. 4. Viral accumulation in C18 *dcl2* knockout mutant (KO) mutant (Δ*dcl2*). RNA blot analysis of the MyRV2 (S2 and S10 mRNAs, left panel) and CHV4–C18 (right panel) accumulation in wild type and *dcl2* KO mutant. VF: virus free C18 strain. The EtBr-stained rRNAs are shown as loading controls.

CHV4–C18, respectively. The Δ*dcl2* strain showed more severe symptoms induced by MyRV2 relative to wild type C18 as in the case for the standard fungal strain EP155 infected by viruses (Chiba et al., 2013b; Salaipeth et al., 2014; Segers et al., 2007), whereas CHV4–C18-infected Δ*dcl2* was indistinguishable from CHV4–C18-infected wild-type C18 (Fig. 2B). Northern blotting showed great enhancement of accumulation of MyRV2 S2 and S10 transcripts in Δ*dcl2* compared to in wild-type C18 (Fig. 4). However, the accumulation of CHV4–C18 in Δ*dcl2* was comparable to that in wild-type C18 (Fig. 4). These results indicate that MyRV2, but probably not CHV4–C18, is susceptible to host RNA silencing.

Deletion of *dcl2* resulted in enhanced stability of MyRV2 during

subculturing and showed 100% maintenance even after the 10th subculture in the absence of CHV4–C18 (Fig. 3C, red column). That is, MyRV2 was retained as well in Δ*dcl2* as it was in the CHV4–C18/MyRV2 co-infections. Similarly, the vertical transmission rate of MyRV2 in Δ*dcl2* was increased from 9% for wild type C18 (Fig. 3B, red area in MyRV2 only) to 33% for Δ*dcl2* (Fig. 3D, red area). In doubly infected Δ*dcl2*, MyRV2 was transmitted at 52.1%: singly-infected single conidial isolates (3.7%, red area) plus doubly infected ones (48.4%, green area) (Fig. 3D). The MyRV2 frequency 52.1% was higher than that (33%) in singly infected strains. Possible tripartite interactions among the host Δ*dcl2* and the two viruses may lead to the enhanced vertical transmission.

3.5. CHV4–C18 impairs transcriptional upregulation of dcl2 in the C18 strain

The aforementioned observations suggest that CHV4–C18 inhibits RNA silencing and leads to stable infection and enhanced vertical transmission of MyRV2. Only a limited number of fungal viruses have been shown experimentally to have RNA silencing suppressor activities. Among them is CHV1, which represses upregulation of the antiviral RNA silencing key genes, *dcl2* and *agl2* via the activity of the multifunctional viral protein p29 (Chiba and Suzuki, 2015; Sun et al., 2009). To examine whether CHV4–C18 has similar activities, northern blotting of *dcl2* transcripts was performed.

Like CHV1-Δp69 (a mutant devoid of RNA silencing suppressor activity) and MyRV1 (the prototype mycoreovirus) (Chiba and Suzuki, 2015; Zhang et al., 2008), MyRV2 alone greatly induced transcript levels of *dcl2*, relative to virus-free C18, which is not detectable in the blot (Fig. 5A). Interestingly, co-infection with CHV4–C18 and MyRV2 revealed the weak induction of *dcl2* transcripts, although not to the level of virus-free strain C18 (Fig. 5A).

3.6. Coinfection by CHV4–C18 leads to stable accumulation level of MyRV2

We anticipated that targeting of MyRV2 by antiviral RNA silencing and reduction of the transcriptional upregulation of *dcl2* by CHV4–C18 might lead to enhanced accumulation of MyRV2 in doubly infected fungal colonies. To test this hypothesis, MyRV2 accumulation was compared among fungal strains. At the first subculture, no discernible influence on MyRV2 accumulation by co-infection with CHV4–C18 was observed between singly and doubly infected fungal strains, but after the 5th subculture, MyRV2 accumulation was slightly lower in singly infected than in doubly infected isolates (Fig. 5B). In contrast, CHV4–C18 accumulation was also not affected by co-infecting MyRV2 (Fig. 5C).

3.7. MyRV2 is very susceptible to antiviral RNA silencing and its induced state impairs replication and horizontal transmission

Unstable maintenance of MyRV2 was shown to be caused at least in part by antiviral RNA silencing, suggesting that it is susceptible to antiviral silencing in the fungus. To further support this, we carried out a horizontal transfer assay using two different systems. The first system involves pairing of MyRV2-infected C18 with CHV1-Δp69-infected C18 in which *dcl2* was induced slightly more highly than in the first strain (Fig. S3A). Subcultures obtained from the MyRV2-infected strain that received CHV1-Δp69 showed reduced levels of MyRV2 accumulation (Fig. 6A, MyRV2-side). However, subcultures obtained from the CHV1-Δp69-infected strain failed to support introduction of MyRV2, but retained CHV1-Δp69 (Fig. 6A, CHV1-Δp69-side).

We previously developed an RNA silencing induced strain in which *dcl2* transcript levels were highly increased by transgenic expression of dsRNA of an endogenous gene *CpMK1*, triggering the *dcl2* induction by the hairpin RNAs without virus infection (Chiba and Suzuki, 2015). We

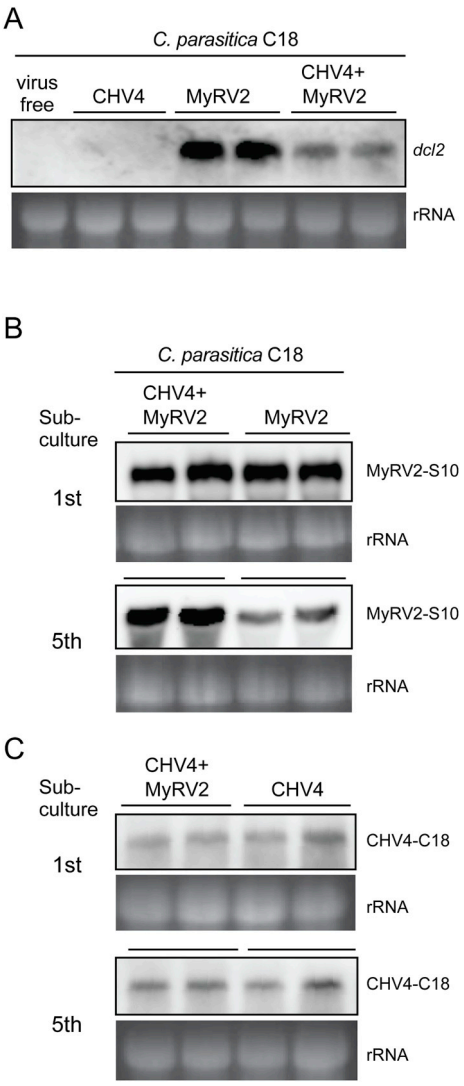


Fig. 5. Effect of single and double infection on *dcl2* transcript and virus RNA accumulation in the C18 strain. (A) RNA blot showing *dcl2* transcript accumulation in singly and doubly infected C18 strains. (B and C) RNA blotting analyses of the MyRV2 (S10 mRNA, B) and CHV4–C18 (C) accumulation in singly and doubly infected strains with 1st (top row) and 5th (bottom row) subcultured samples. The EtBr-stained rRNAs are shown as loading controls.

transformed virus-free C18 with the same hairpin construct, pCPXHY2-CpMK1-IR, used in the earlier study, and tested a few transformants (C18/IR-CpMK1 lines) for *dcl2* upregulation. As was observed for the standard strain EP155 with CHV1-Δp69, *dcl2* was highly induced in the C18/IR-CpMK1 similar to that of EP155 genetic background (EP155/CpMK1-IR) (Fig. S3B). These strains were then fused with MyRV2 singly-infected C18 as a donor. Subcultures derived from the donor carried MyRV2, but no MyRV2 was detected by either northern blotting or RT-PCR in subcultures from the C18/IR-CpMK1 recipient strain (Fig. 6B and data not shown).

4. Discussion

Here we reported an interesting virus/virus interaction, where the stable infection by a dsRNA reovirus, MyRV2, was facilitated by a co-infecting (+)RNA hypovirus, CHV4–C18, which appears to impair antiviral RNA silencing by suppressing upregulation of its key gene, *dcl2*. Because no beneficial or interfering effects of MyRV2 on CHV4–C18 were observed, the interactions can be regarded as commensalism. Without the aid of CHV4–C18, MyRV2 was readily eliminated from the

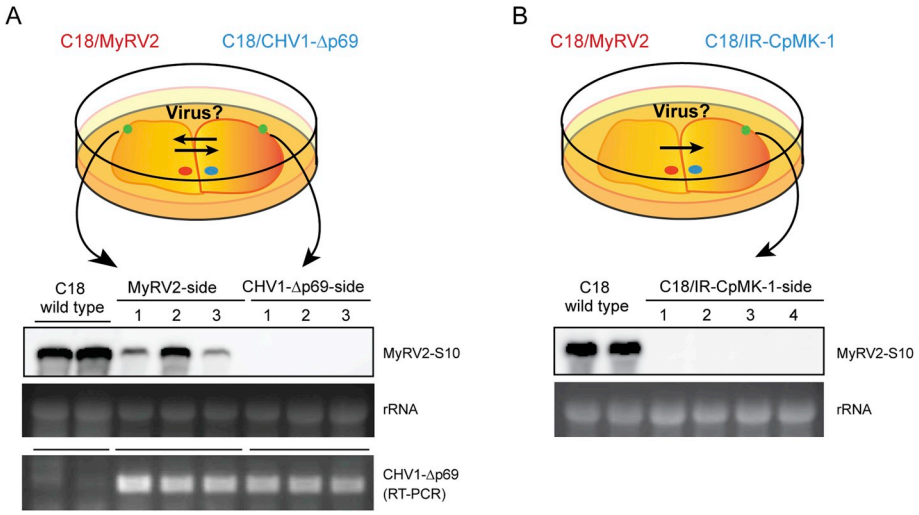


Fig. 6. High induction of *dcl2* inhibits MyRV2 horizontal transmission through hyphal fusion in the C18 strain. (A) Co-culture of MyRV2 and CHV1-Δp69 infected strains and the virus accumulation in their subcultured strains obtained from the both sides (MyRV2-side and CHV1-Δp69-side) was analyzed. MyRV2 (S10 mRNA, top row) and CHV1-Δp69 (bottom row) accumulation in the subcultured strains from the both sides were analyzed by RNA blotting and RT-PCR. Each lane (lane no. 1 to 3) represents RNA samples from three independent experiments. Fungal strains were co-cultured for 7 days and the mycelial plugs of the growing edge (shown as green spot regions in the cartoon) were cultured. (B) MyRV2 singly-infected C18 strain was used as donor and virus-free CpMK1-IR transformant of C18 was used as the recipient in virus horizontal transmission assay. MyRV2 RNA accumulation (S10 mRNA) in their subcultured strains obtained from the recipient site was analyzed by RNA blotting. Each line (1–4) represents RNA samples from four independent experiments. Fungal strains were co-cultured for 7 days and the mycelial plugs of the growing edge were cultured.

C18 host strain under the laboratory conditions, resulting in unstable maintenance during subculturing and lower rates of vertical transmission via conidia. As MyRV2 has been isolated only once in an otherwise deeply sampled population (Enebak, 1992), it is reasonable to suggest that such coinfection may be a prerequisite for MyRV2 in natural settings as well.

RNA silencing is the primary antivirus defense in fungi and targets many viruses (Chiba et al., 2013b, 2016; Nuss, 2011; Salaipeth et al., 2014). Which RNA silencing-related genes are upregulated and responsible for antiviral defense appears to depend on viruses and host fungi (Mochama et al., 2018; Yaegashi et al., 2016; Yu et al., 2018). In the case of *C. parasitica*, two genes, *dcl2* and *agl2,* play major roles (Segers et al., 2007; Sun et al., 2009), while more genes appear to play roles in antiviral RNA silencing in other filamentous fungi *Sclerotinia sclerotiorum* and *Fusarium graminearum* (Mochama et al., 2018; Yu et al., 2018). Many of these genes are transcriptionally induced upon virus infection, and their regulation requires the general transcriptional activator, SAGA (*S*pt-*A*da-*G*cn5 *a*cetyltransferase) complex, and DCL2 in *C. parasitica* (Andika et al., 2017, 2019). However, the extent to which the RNA silencing key genes are induced depends on specific properties of the viruses, ranging from apparently zero to ~40-fold induction relative to those in virus-free strains (Chiba and Suzuki, 2015; Salaipeth et al., 2014; Shahi et al., 2019a; Sun et al., 2009; Zhang et al., 2012). The observation that deletion of *dcl2* restored maintenance stability and increased transmission frequency of MyRV2 (Fig. 3) indicates that RNA silencing is most likely the cause of the low stability of MyRV2 in C18. Therefore, the ability of CHV4–C18 to increase MyRV2 stability is likely associated with its suppressor activity against antiviral RNA silencing. The observation that single infection by CHV4–C18 does not strongly induce either the *dcl2* or *agl2* gene, whereas coinfection by CHV4–C18 suppressed, to some degree, the upregulation of *dcl2* by infection by MyRV2 (Fig. 5A and data not shown) suggests the presence of an RNA silencing suppressor encoded by CHV4–C18. However, note that coinfection by CHV4–C18 did not increase the accumulation of MyRV2, but still helped the host strain C18 to maintain the relatively high virus accumulation level even after several subcultures (Fig. 5B). The mechanism by which MyRV2 reproducibly accumulates less in the 5th subculture than in the first subculture in single infections (Fig. 5) is unknown. Host antiviral RNA silencing and CHV4–C18 counter-measure are likely involved in this phenomenon.

Only a few mycovirus proteins have been identified as RNA silencing suppressors; among them are VP10 of another mycoreovirus, MyRV3 and p29 of the hypovirus CHV1 (Segers et al., 2006; Yaegashi et al., 2013). VP10 can suppress transgene (GFP) RNA silencing in plant and possibly in fungi. The multifunctional protein p29 RNA serves as a silencing suppressor of for CHV1 (Segers et al., 2006), as a protease that plays roles in polyprotein processing (Choi et al., 1991a, 1991b), and as a symptom determinant (Craven et al., 1993; Suzuki et al., 1999). Furthermore, its coding sequence is part of the internal ribosome entry site (IRES) of the viral RNA (Chiba et al., 2018). Identification of RNA silencing suppressor in CHV4–C18 is not easy based solely on sequence comparison, because no clear homolog of CHV1 p29 is detectable on CHV4–C18 (Linder-Basso et al., 2005). It was hypothesized that CHV1 p29 blocks either DCL2 or downstream of the SAGA-mediated transcriptional upregulation for which DCL2 plays a positive feedback role (Andika et al., 2017, 2019; Chiba and Suzuki, 2015). Note that a protease may be encoded on the 5′-proximal coding domain of the CHV4–C18 genome (Hillman and Suzuki, 2004; Linder-Basso et al., 2005). Protease activity has been demonstrated for the related species CHV3 (Yuan and Hillman, 2001), but silencing suppressor activity has not yet been investigated for that species. Functional mapping to identify a protease in CHV4–C18 or an RNA silencing suppressor that possibly potentiates the observed enhanced transmission and stability of MyRV2 have not been performed.

The CHV4/MyRV2 interactions are reminiscent of those between CHV1 and MyRV1 in which CHV1 enhances the vertical transmission via conidia (Sun et al., 2006) and represses *dcl2* transcripts levels (S. Chiba and N. Suzuki, unpublished data). One-way beneficial effects on reoviruses by hypoviruses are commonly observed in the two pathosystems. The CHV1 one-way synergistic activities are exerted by its RNA silencing suppressor, p29 (Sun et al., 2006). The observed activities of CHV4–C18 are similar to but different from those of CHV1. The activity of CHV4–C18 to suppress the transcriptional up-regulation of *dcl2* is smaller than CHV1 (Fig. 5A, data not shown). A great difference is also observed between their co-infecting partner reoviruses; MyRV1 is stably maintained in its original fungal host strain, 9B21, as well as strain EP155 (S. Supyani, B. I. Hillman, and N. Suzuki, unpublished data). By contrast, MyRV2 was shown to be less stable in its original host strain, C18, and this instability is more pronounced in EP155 (A. Aulia, B. I. Hillman, N. Suzuki, unpublished data). Whether this means that MyRV1 is more adjusted to *C. parasitica* than MyRV2 is a matter of conjecture: neither virus has been identified more than once in natural chestnut populations. It should be noted that MyRV2 is targeted by RNA silencing based on that the observation that MyRV2 accumulated much

more in Δ*dcl2* than in wild-type C18 (Fig. 4). In contrast, vertical and lateral transmission of CHV4–C18 were barely decreased or were unaltered by co-infecting MyRV2, just as MyRV1 has no appreciable effects on CHV1 transmission (Sun et al., 2006).

Previous comparison of the two reoviruses in a study that included partial microarray analysis revealed several interesting findings (Deng et al., 2007). Perhaps the most significant of these is that isolate EP155 infected with either MyRV1 or MyRV2 does not show loss of female-fertility, as is common with infection of *C. parasitica* by many other viruses. Approximately 60% of the single ascospore progeny derived from crosses in which either MyRV1 or MyRV2 served as the female parent were virus-infected. Consistent with these results, two genes that are associated with female fertility in fungi, the pheromone precursor gene, Mf2/1, and the yeast STE12-like transcriptional factor gene, cpst12, were not substantially downregulated in isolates infected with either reovirus. This is in contrast to infection by CHV1, which greatly downregulated those fertility-associated genes and results in female sterility. In the context of the current study, it is notable that the MyRV1 or MyRV2-infected EP155 strains used to initiate the study by Deng and colleagues were made by transfection of protoplasts with sucrose gradient-purified reovirus particles and subsequent regeneration of virus-infected colonies. Thus, both fungal isolates were singly infected. Investigation of possible differences in behavior in EP155 between MyRV1 and MyRV2 is underway.

Comparison of the two mycoreoviruses here reveals other interesting differences. MyRV1 can tolerate the highly induced state of antiviral RNA silencing mediated either by CHV1-Δp69 or transgenic expression of non-viral dsRNA (Chiba and Suzuki, 2015). However, MyRV2 transmission and replication were greatly reduced by co-infecting CHV1-Δp69 or transgenic expression of CpMK1-IR. Reduction of MyRV2 accumulation was also observed after invasion of the MyRV2-infected C18 by CHV1-Δp69 (Fig. 6A). These clearly indicate greater susceptibility of MyRV2 to RNA silencing compared to MyRV1, which can tolerates the highly induced antiviral RNA silencing state (Chiba and Suzuki, 2015). This supports the idea that MyRV1 may be better adapted to *C. parasitica* than MyRV2. In this regard, MyRV2 may be similar to the victorivirus RnVV1, derived from the fungal host *Rosellinia necatrix*, which shows impaired replication and horizontal transmission under an induced state of antiviral RNA silencing in the heterologous host *C. parasitica* (Chiba and Suzuki, 2015). Chiba and Suzuki (2015) hypothesized that RnVV1 is severely interfered because RnVV1 is not adapted to a newly extended host, *C. parasitica* and is susceptible to RNA silencing in the heterologous host system.

Molecular characterization revealed great similarity of CHV4–C18 to a previously reported strain, SR2, of CHV4 (Fig. 1): the two strains show 99% nucleotide sequence identity. Only 54 SNPs were found, among which 16 induce amino acid changes while the two strains retain five predicted functional domains of the putative viral polyprotein (Fig. 1) (Linder-Basso et al., 2005). It is not surprising that CHV4–C18 is more closely related to the type strain CHV4-SR2 than other CHV4 strains from diverse geographic regions investigated through partial sequence analysis in that study (Linder-Basso et al., 2005), as strains C18 and SR2 were isolated only a few miles from each other (Enebak, 1992). An interesting difference between the CHV4–C18 and CHV4-SR2 is that SNPs found in the 5′ proximal region of CHV4–C18 introduced a stop codon and move the predicted start codon 32 codons downstream of the start codon observed in CHV4-SR2. CHV4–C18 is also different in several respects from many other viruses infecting *C. parasitica*. First, CHV4–C18 shows similar levels of accumulation in the wild type C18 strain and in a mutant of strain C18 deficient in antiviral activity, Δ*dcl2* (Fig. 4). Second, CHV4–C18 induces no overt phenotypic alteration in strain Δ*dcl2* (Fig. 2B). Many dsRNA viruses such as victoriviruses, partitiviruses and megabirnaviruses, and (+)RNA viruses such as other hypoviruses, which have been tested in the EP155 genetic background, show enhanced replication and symptom induction in Δ*dcl2* (Chiba et al., 2013a, b, 2016; Salaipeth et al., 2014; Segers et al., 2007; Sun et al., 2009). However, the C18 Δ*dcl2* strain showed the same colony morphology regardless of whether it was infected by CHV4–C18 or uninfected (Fig. 4). These differences warrant further future investigation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.05.004.

Conflicts of interest

The authors declare that they have no conflict.

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