INTERACTIONS BETWEEN TWO NATURALLY CO-INFECTING MYCOVIRUSES IN THE CHESTNUT BLIGHT FUNGUS *Cryphonectria parasitica*

March, 2020

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CHAPTER 1. GENERAL INTRODUCTION

A. VIRUSES INFECTING FUNGI

Viruses are obligate parasites that infect cellular organisms belonging to both prokaryotes and eukaryotes (Koonin and Dolja, 2006). Virus infections are common among fungi and have been reported in species belonging to all major taxonomic classes of true fungi (Said and Nobuhiro, 2009). In contrast to viruses of plants, animals and bacteria that were first described around the beginning of 19th century (Artenstein, 2012), fungal viruses (mycoviruses) could not be detected by scientists until 1960s. The first mycovirus was discovered from a cultivated basidiomycete mushroom, *Agaricus bisporus,* that associated with "die-back disease". The symptoms of the disease include morphological distortion and premature deterioration of mushroom tissue that contributed to significant losses in mushroom cultivation (Gandy and Hollings, 1962). Indeed, mycoviruses received less attention compared to other viruses due to their cryptic mode of infections (Said and Nobuhiro, 2009).

The majority of the mycoviruses infect their hosts without producing any perceptible disease symptoms. Nevertheless, some viruses upon infection can bring about phenotypic changes in their hosts. These include abnormal colony morphology, reduced hyphal growth rate, sporulation, pigmentation, and hypovirulence (reduced disease-causing ability than normal) (Dawe and Nuss, 2001). Mycovirus-mediated hypovirulence phenomenon found in fungi is transmissible to closely related strains of the same fungus. This leads to the idea of exploiting mycoviruses as biocontrol agents against phytopathogenic fungi. The most successful case is exploiting Cryphonectria hypovirus 1 (CHV1) to manage chestnut blight disease caused by the ascomycete fungus *Cryphonectria parasitica* (MacDonald and Fulbright, 1991). Other examples of mycovirus-mediated hypovirulence were reported in ascomycete (*Helicobasidium mompa*, *Rhizoctonia solani*, *Slerotinia sclerotium, Rosellinia necatrix,* and *Botrytis porii*) (Chiba et al., 2009; Osaki et al., 2006; Wu et al., 2012; Xiao et al., 2014; Zheng et al., 2014).

In addition to hypovirulence, some viruses can also enhance the virulence (hypervirulence) of their hosts. Hypervirulence is characterized by enhanced sporulation, aggressiveness and growth (Ahn and Lee, 2001). Talaromyces marneffei partitivirus 1 (TmPV1) enhanced the virulence of its host and consequently resulted in shorter survival time and higher fungal burden in the experimental mice inoculated with TmPV1-infected *Talaromyces marneffei* (formerly *Penicillium marneffei*, an opportunistic human pathogen ascomycete) (Lau et al., 2018). In addition, two different mycoviruses, Aspergillus fumigatus partitivirus 1 (AfuPV1) and an uncharacterized virus infecting *Aspergillus fumigatus* (a saprotroph ascomycete and cause

opportunistic human disease), were shown to induce hypervirulence in this fungus (Özkan and Coutts, 2015).

The influence of mycovirus infections on host fungal ecology is not clearly understood and therefore could be an interesting topic of research. There are several studies that suggested possible roles of mycoviruses in regulating the ecology of their host fungi. For instance, *Curvularia protuberata* an ascomycete fungal symbiont of tropical panic grass (*Dichanthelium lanuginosum*) allowed its host plant to grow at high temperatures only when it was infected with a double-stranded RNA (dsRNA) mycovirus named Culvularia thermal tolerance virus (an unclassified virus) (Márquez et al., 2007). Environmental factors can also affect the influence of mycoviruses on their hosts. For example, Heterobasidion RNA virus 6 (HetRV6) that infect *Heterobasidion annosum* (a basidiomycete forest pathogen) showed a considerable degree of geographical and host-related differentiation (Vainio et al., 2012). Further studies showed that HetRV6 could produce different effects on different *Heterobasidion* hosts, such as beneficial, cryptic or detrimental effects depending on the environmental and ecological condition (Hyder et al., 2013).

Mycoviruses can be transmitted either vertically via spores or horizontally via hyphal fusion (also known as anastomosis). Anastomosis generally occurs between fungal strains belonging to the same somatic compatibility group of a given fungus (Choi et al., 2012; Zhang et al., 2014a). Mycoviruses are believed not to have any extracellular phases in their life cycle (Ghabrial and Suzuki, 2008). Interestingly, Sclerotinia sclerotiorum hypovirulence associated DNA virus 1 (SsHADV1), a recently discovered circular ssDNA mycovirus (belonging to the family *Genomoviridae*) has been shown to replicate in and transmitted by a mycophagous insect *Lycoriella ingenua*. Therefore, SsHADV1 is regarded as a fungal virus and an insect virus as well (Liu et al., 2016).

B. MYCOVIRUS TAXONOMY

Mycoviruses have diverse genome types, such as double-stranded RNA (dsRNA), positive sense (+) single-stranded RNA (ssRNA), linear negative-sense (–) ssRNA, and circular single-stranded DNA (ssDNA). Mycoviruses with dsDNA have not yet been discovered in fungi (Lefkowitz et al., 2017; Liu et al., 2014). The majority of the fungal viruses have either (+)ssRNA or dsRNA genomes. The known dsRNA mycoviruses are currently classified into six linear dsRNA virus families (*Chrysoviridae, Megabirnaviridae, Quadriviridae, Partitiviridae, Reoviridae, Totiviridae*) and one linear dsRNA virus genus (*Botybirnavirus*) (Lefkowitz et al., 2017). Most of the dsRNA mycoviruses have genetic materials that are packaged in isometric particles (Ghabrial et al., 2015; Ghabrial and Suzuki, 2009). Although many dsRNA mycoviruses are packaged in isometric particles, a recent study on Aspergillus fumigatus tetramycovirus-1 (AfuTmV-1, a polymycovirus) that infect human pathogenic fungus *A. fumigatus* showed that

viral genome was not encapsidated but only coated by a virus-encoded protein PAS (proline, alanine, and serine residues)-rich protein (Kanhayuwa et al., 2015).

The (+)ssRNA mycoviruses are classified into nine virus families (*Alphaflexiviridae, Barnaviridae, Deltaflexiviridae, Gammaflexiviridae, Hypoviridae, Endornaviridae*, *Narnaviridae, Mymonaviridae* and *Botourmiaviridae*) (King et al., 2018). Previously, it was reported that (+)ssRNA mycoviruses in phytopathogenic fungi lacked capsid protein (CP) genes and therefore failed to form true virions (Liu et al., 2015). However, Botrytis virus F and Botrytis virus X are exceptions to this rule: both viruses form flexous rod-shaped virions (Pearson and Bailey, 2013).

Recent advancement on the high-throughput RNA deep sequencing technology along with the availability of fungal genomic and transcriptomic data allowed researchers to discover several putative negative-stranded (−)ssRNA mycoviruses using plant and animal (−)ssRNA virus sequences as queries (Kondo et al., 2013). The first existence of first (−)ssRNA mycovirus existence was reported from *Sclerotinia sclerotiorum* (a notorious plant fungal pathogen with a broad host range) which infected with Sclerotinia sclerotiorum negative-stranded RNA virus 1 (SsNSRV-1) (Liu et al., 2014). SsNSRV-1 is closely related to the members of the *Nyamiviridae* and *Bornaviridae* families (Liu et al., 2014) and taxonomically classified into a novel family, *Mymonaviridae* (genus *Sclerotimonavirus*) in the order *Mononegavirales*. Recently novel (−)ssRNA viruses related to mymonaviruses and phenuiviruses (order *Bunyavirales*) were found in edible basidiomycete mushroom, *Lentinula edodes* (Lin et al., 2019) Although, there are many mononegaviruses found in vertebrates and invertebrates, only a limited number appears to infect plants and a very few to infect fungi (Dietzgen et al., 2017; Kondo et al., 2013; Walker et al., 2015).

C. CHESTNUT BLIGHT DISEASE AND DISCOVERY OF HYPOVIRULENCE

Chestnut blight disease has received a great attention because of the destruction of the chestnut stands native to eastern North America (Anagnostakis, 1987). The causal agent of this disease is an ascomycete fungus *C. parasitica*, an ascomycete belonging to the family *Cryphonectriaceae*. *C. parasitica* quickly devastated the American chestnut (*Castanea dentata*) population and killed almost all the susceptible trees. The introduction of *C. parasitica* to the United States and Europe in the 20th century was from East Asia (most likely from Japan) and resulted in destruction of the chestnut trees in those areas (Anagnostakis, 1982; Griffin et al., 1986; Milgroom, 1996). In late 1930, European foresters were aware of the damage being caused by *C. parasitica* and took several actions to prevent the pandemic (Heiniger and Rigling, 1994).

Wounds in the trees facilitate *C. parasitica* to enter the host. The fungus forms sunken cankers on the stem of the affected tree that continues to expand and penetrate, and eventually demolishing the cambium layer of the affected tree(Eusebio-Cope et al., 2015). Chestnut blight is

considered as one of the three most destructive tree diseases of the world along with Dutch elm disease and white pine blister rust (Tainter and Baker, 1996).

However, eventually less aggressive *C. parasitica* strains were found in Europe and extensive studies showed that such strains were infected with mycovirus (Heiniger and Rigling, 1994). The virus was later referred as CHV1 (Nuss, 2000). The first discovery of hypovirulent *C. parasitica* strains was made in a chestnut coppice showing approx. 85% infection rate in the shoot by *C. parasitica*, yet looking "surprisingly healthy" (Biraghi, 1951; Hillman and Suzuki, 2004). It is noteworthy that no mycovirus as a physical entity was identified until 1962 (Gandy and Hollings, 1962). In 1964, Grente et al. isolated atypical strains of *C. parasitica* from healing cankers in northern Italy. These strains had lower pigmentation and sporulation rates relative to their virulent counterparts when grown on potato dextrose agar (PDA). The atypical strains reduced pathogenicity when inoculated in chestnut trees, and the phenomenon became popular as hypovirulence (Grente and Berthelay-Sauret, 1978).

D. VIRUSES INFECTING *Cryphonectria parasitica*

Since the discovery of CHV1-induced hypovirulence in *C. parasitica,* several other mycoviruses were discovered subsequently in this pathogen. Natural virus infection in *C. parasitica* populations could be found in Japan and China (2% and 6%, respectively) and in North America (28%) (Milgroom, 1996; Park et al., 2008; Peever et al., 1997; Peever et al., 1998). However, virus infection rate in *C. parasitica* appears to be lower compared to the cases of others fungal pathogen such as the violet root rot fungus *H. mompa* (a basidiomycete) and rice false smut fungus *Ustilaginoidea virens* (an ascomycete), having much higher virus incidences under the natural conditions (approximately 70% and 90%, respectively) (Ikeda et al., 2004; Xie and Jiang, 2014).

So far, naturally infecting viruses found in *C. parasitica* are classified into at least four families *Hypoviridae, Reoviridae, Narnaviridae,* and *Partitiviridae* (Hillman and Suzuki, 2004). In addition to these, heterologous viruses, originally isolated from other fungi have also been demonstrated to replicate in *C. parasitica*. These include several dsRNA viruses: a mycoreovirus (mycoreovirus 3, MyRV3), a megabirnavirus (Rosellinia necatrix megabirnavirus 1, RnMBV1), a victorivirus (Rosellinia necatrix victorivirus 1, RnVV1), and two partitiviruses (Rosellinia necatrix partitivirus 1 and 2, RnPV1 and RnPV2) from the white root rot pathogen, *Rosellinia necatrix* (Chiba et al., 2013a, b; Kanematsu et al., 2010; Salaipeth, 2014), a fusarivirus (Fusarium graminearum virus 1, FgV1) that isolated from *Fusarium graminearum* (a plant-pathogenic ascomycete causes fusarium head blight), and Helminthosporium victoriae virus 190S (a victorivirus) from *Helminthosporium victorae* (telemorph: *Cochliobolus victoriae,* a plant-pathogenic basidiomycete) (Lee et al., 2011). Furthermore, many isolates of *C. parasitica* isolates

from different geographical regions were examined for virus infections (Chung et al., 1994; Heiniger and Rigling, 1994; Liu et al., 2007; Peever et al., 1997; Peever et al., 1998; Quan et al., 1994). This help the finding of a US strain, EP155, which have been extensively studied (including its draft whole genome sequencing and development of several mutants) and later served as the standard strain for exploration of virus replication and pathogenesis. Importantly, the viruses mentioned above could be experimentally introduced into EP155 and were found to replicate in it. In fact, these viruses, upon infection, could lead to distinct symptoms and different host responses (Eusebio-Cope et al., 2015).

E. VIRUS/VIRUS INTERACTIONS

Mixed viral infections are common among fungi with a single fungal strain can be infected simultaneously by several unrelated viruses (Hao et al., 2018; Osaki et al., 2016; Ran et al., 2016). The effect of mixed infection to the co-infecting viruses as well as to their fungal host could be different. Co-infection of multiple viruses in the single host may lead to virus-virus interaction that can be either synergistic, mutualistic, or antagonistic. Under the mixed infection conditions, one virus can enhance accumulation of another co-infecting virus. In addition, co-infecting viruses can also suppress the vegetative incompatibility and thereby can increase the horizontal transmission of viruses through hyphal anastomosis (Wu et al., 2017).

1. Synergistic Interactions

Synergism between co-infecting viruses refers to a phenomena in which coinfection by two or more viruses causes more severe symptoms or increases viral titer of either one or both the viruses (Pruss et al., 1997; Scheets, 1998). In plant virus system, virus synergism is well known, and many examples have been documented. One of such examples of virus synergism is the co-infection of tobacco by a potexvirus (potato virus X, PVX) and a *potyvirus*, (potato virus Y, PVY), enhancing viral symptoms expression with increased accumulation of PVX in tobacco (bance, 1991). In fungi, one-way synergism was observed during co-infections of CHV1 and MyRV1 in *C. parasitica.* CHV1 infection enhanced MyRV1 virus accumulation, but not vice versa. The MyRV1 accumulation was partially elevated by transgenic expression of p29, an RNA silencing suppressor encoded by CHV1, without CHV1 infection (Sun et al., 2006). Another example of viral synergism in fungus was reported for *R. necatrix* doubly infected by a megabirnavirus, RnMBV2 and a partitivirus, RnPV1 (Sasaki et al., 2016). Single infection by either RnMBV2 or RnPV1 did not cause any phenotypic alterations, but co-infection by both viruses induced hypovirulence in the host, regardless of the fungal strains.

2. Mutualistic Interactions

Mutualistic interactions between bona fide viruses are different from satellite-helper viruses or from defective RNA-helper virus interactions. The interaction of a helper virus with its satellite or defective RNA is not a virus-virus interaction but an interaction between viruses and subviral elements, since satellite viruses do not have the replicase, an essential enzyme for virus replication (Hillman et al. 2017). Virus-virus mutualistic interactions usually occur between two independent viruses. A novel mutualistic interaction between two unrelated viruses has recently been discovered in a debilitated field isolate of *R. necatrix* where a capsidless (+ss)RNA virus, Yado-kari virus 1 (YkV1, a calivi-like virus) is hosted by an encapsidated dsRNA virus, Yado-nushi virus 1 (YnV1, a toti-like virus) (Zhang et al., 2016). Like other bona fide dsRNA viruses, YnV1 can replicate and survive independently in its host, while for survival, YkV1 needs constant presence of YnV1. In this novel mutualistic interaction, YkV1 hijacks YnV1's capsids to encase its genomic RNA and replicase, and probably utilizing the capsids as a site of its replication as if it the virus is an encapsidated dsRNA virus. However, YkV1 depends on YnV1 for trans-encapsidation, but it probably uses its own RdRP for replication and transcription. To return the favor, YkV1 trans-enhance accumulation of YnV1. There are may be similar mutualistic interactions existing among other fungi such as *A. foetidus* (Kozlakidis et al., 2013)

3. Antagonistic Interactions

Most of the antagonistic virus-virus interactions are beneficial to the infected hosts. Antagonistic interactions occur when a less-pathogenic virus strain prevents or reduces subsequent infection by a highly pathogenic strain that shares sequence homology with the protective virus, the phenomenon commonly known as cross-protection (Syller, 2012). In plants, antagonistic interactions involves the protective virus triggering the plant RNA silencing (or RNA interference, RNAi) machinery directed by small interfering RNAs (siRNAs), which due to the genetic similarity between the two viruses, prevents a secondary virus infection (Melcher and Ali, 2018; Syller and Grupa, 2016). An interesting antagonistic phenomenon was found in *C. parasitica,* where two heterologous viruses, MyRV1 or a p29-lacking mutant of CHV1 (CHV1-Δp69) interferes with the replication and horizontal transmission of another virus, a victorivirus (RnVV1) (Chiba and Suzuki, 2015). MyRV1 and CHV1-Δp69 induce high level of the key RNA silencing gene, dicer-like 2 (*dcl2*) transcript accumulation, thereby interfering with the multiplication of RnVV1. Interestingly, pre-existing RnVV1 also be eliminated by introducing MyRV1 or CHV1-Δp69. Hence, the mechanism is different than that of previously known cross protection-based antagonistic interactions occurring between closely related viruses.

F. RESEARCH OBJECTIVE

Virus co-infections in fungi are quite common, however virus-virus interactions in animals or plants were studied in more detail than that in fungi. Previous studies showed many interesting and novel interactions between viruses in fungi. As aforementioned, several research groups have reported many virus-infected *C. parasitica* strains including a US strain C18. This strain was earlier shown by Hillman and colleagues (Enebak et al., 1994a) to be infected by a dsRNA virus, Mycoreovirus 2 (MyRV2) and a (+ss) RNA virus Cryphonectria hypovirus 4-C18 (CHV4-C18). These two viruses showed interesting mutualistic interactions in which CHV4-C18 infection is required for stable infection of MyRV2. The main objective of this research is therefore to elucidate the underlying molecular mechanism of the interaction between MyRV2 and CHV4-C18 and also the effect of co-infection of these two viruses to the fungal host.

CHAPTER 2. CO-INFECTION OF DOUBLE-STRANDED RNA VIRUS AND A POSITIVE STRAND RNA VIRUS IN *Cryphonectria parasitica* STRAIN C18

A. INTRODUCTION

1. Family *Reoviridae*

Viruses belonging to the family *Reoviridae* consists of two subfamilies with 15 distinct genera (subfamily *Sedoreovirinae*: *Cardoreovirus, Mimoreovirus, Orbivirus, Phytoreovirus, Rotavirus,* and *Seadornavirus*; subfamily *Spinareovirinae*: *Aquareovirus, Coltivirus, Cypovirus, Dinovernavirus, Fijivirus, Idnoreovirus, Mycoreovirus, Orthoreovirus,* and *Oryzavirus*) that infect a wide range of hosts including mammals, birds, reptiles, fish, crustaceans, insects, ticks, arachnids, plants, and fungi, making it as the largest virus family known so far (Fauquet et al., 2005). Reoviruses are important as pathogens and as tools for virologists and molecular biologists. Human reoviruses and Cytoplasmic polyhedrosis viruses are used as a model for the study of eukaryotic mRNA synthesis and capping mechanism (Furuichi and Shatkin, 2000). Rice dwarf virus (a phytoreovirus) was the first virus, known to be transmitted by arthropods (Fukushi, 1969). Reoviruses have a special architecture and construction, making them a renowned subject for structural studies (Prasad et al., 2000)

The members of the family *Reoviridae* share common properties. They have 9–12 dsRNA with a size ranging between 0.2 and 3.0 kb. The genome segments are mostly with monocistronic in nature, virus particles are multi-shelled and mRNAs are synthesized within virus core particles that serve as RNA transcription factory (Olland et al., 2001). There are three major structural patterns in the member of *Reoviridae*. The example of the first pattern is exemplified by orthoreoviruses and phytoreoviruses. The second pattern is exemplified by orbiviruses and rotaviruses and the third pattern is represented by cypoviruses and fijiviruses.

The outer capsid shells of reoviruses tend to be unstable because they are sensitive to the physical or chemical agents. Rotaviruses' outer capsid shells are sensitive to proteolytic enzymes, whereas orbiviruses and phytoreoviruses are sensitive to high salt, Cypoviruses and others phytoreoviruses are sensitive to fluorocarbon (Joklik, 2013).

Depending on the genus, the number of genome segments ranging between nine and 12. The positive strand of each RNA duplex has a 5′-terminal cap structure(m7 GpppN-), while the 5′-termini on negative strands are phosphorylated. Nevertheless, in some cases, they have 'blocked' 5′-structure. Both the RNA strands have 3′OH and viral mRNAs lack 3′poly (A) tails (Fauquet et al., 2005)

Reoviruses have been known to infect wide range of organisms, but only in the early 1990s two reoviruses were identified from a chestnut blight fungus from West Virginia, US. Infection by these two reoviruses caused hypovirulence in their fungal hosts. Two strains that harbored

reoviruses were strain 9B21 and strain C18, each was infected with MyRV1 and MyRV2, respectively (Hillman et al., 2004). This finding led to the proposal of a new genus *Mycoreovirus* in the *Reoviridae* family. Subsequent identification of additional mycoreoviruses from the fungi *R. necatrix* (MyRV3) and from *Sclerotinia sclerotium* (Sclerotinia sclerotiorum reovirus 1, SsMyRV1) further added new members to this genus (Liu et al., 2017; Mertens et al., 2005; Yaegashi et al., 2013)

2. Family *Hypoviridae*

Viruses belonging to the family *Hypoviridae* were the first viruses found to infected *C. parasitica*. Many hypoviruses caused a strong debilitation to the pathogen that led to virulence attenuation (hypovirulence) (Dawe and Nuss, 2001). Hypoviruses are phylogenetically related to plant potyviruses (Koonin et al., 1991).

So far, the most extensively studied *Hypovirus* is CHV1, which was originally isolated in Europe (France). Additional *C. parasitica*-infecting hypoviruses are CHV2, CHV3, and CHV4. These were isolated from the US. Strain NB58 was isolated from New Jersey and it was infected with CHV2, strain GH2 was isolated from Michigan and it harbored CHV3, and strain SR2, which was infected with CHV4 was isolated from Maryland (Linder-Basso et al., 2005; Milgroom and Lipari, 1995; Milgroom et al., 1996; Nuss et al., 2005; Paul and Fulbright, 1988; Peever et al., 1998; Smart et al., 1999).

CHV1 is widespread in Europe and has been associated with the decline of chestnut blight in many regions (Dawe and Nuss, 2001; Heiniger and Rigling, 1994; Robin et al., 2000). CHV1 infection of *C. parasitica* reduces fungal virulence, conidiation, pigmentation and female fertility. Moreover, infection of CHV1 also modulates approximately 13% of *C. parasitica* transcripts (Allen et al., 2003). The complete sequence of CHV1 has been determined and is publicly available online (GenBank accession number: M57938.1). The infectious cDNA clone of CHV1 has been constructed in 1992 (Choi and Nuss, 1992).

The genome size of CHV1 is 12.7 kbp, comprising of two continuous open reading frames (ORFs), ORF A and ORF B. Each ORF encodes a papain-like protease, p29, and p48, respectively. These proteases are responsible for self-cleavage of the respective polyproteins. The papain-like protease p29 is a multifunctional protein, derived from polyprotein p69 at the N-terminal portion of the ORF A (Dawe and Nuss, 2001). Sequence analysis of p29 suggested its resemblance with HC-Pro, a plant potyvirus multifunctional protein having papain-like protease and RNA silencing suppressor (RSS) activities (Choi et al., 1991a; Choi et al., 1991b). Further studies showed that p29 functions as an RSS (Segers et al., 2006; Sun and Suzuki, 2008).

CHV2 was first identified in hypovirulent isolates from New Jersey and has been less thoroughly studied compared with CHV1. The genome size of CHV2 is 12.7 kbp, which is slightly larger than CHV1. CHV2 also consists of two ORFs. Although the ORF A of CHV2-NB58 encodes a protein that showed sequence similarity to the p29 and to the highly basic p40

region of CHV1 ORF A, lacking the p29 papain-like catalytic or cleavage site sequences (Hillman et al., 1994).

Unlike CHV1 and CHV2, CHV3 has a single ORF genome organization. The size of the CHV3 genome is 9.8 kb and it encodes a large polyprotein with a cis-acting papain-like protease at the N-terminus followed by conserved RdRp and Hel domains. This virus was predominantly found in Michigan and in Ontario, US (Paul and Fulbright, 1988; Yuan and Hillman, 2001). Unlike CHV1 and CHV2, host phenotypic changes caused by CHV3 is milder than that of CHV1 and CHV2, but still reduces *C. parasitica* virulence substantially (Smart et al., 1999).

CHV4 was initially isolated from *C. parasitica* strain SR2 (Linder-Basso et al., 2005). CHV4 accumulates at a very low titer and during northern blot analysis does not cross-hybridize with others *C. parasitica* hypoviruses. CHV4 infection does not cause any phenotypic changes and/or hypovirulence of the fungal host (Enebak et al., 1994b). In this regard, CHV4 is one of many mycoviruses that show latent or cryptic infection.

3. Virus co-infection in fungi

Multiple virus infections in fungi is a common and well-documented phenomenon (Ikeda et al., 2004; Tuomivirta and Hantula, 2005). With the advancement of sequencing techniques, more mixed viral infections have been identified from various fungal species (Lin et al., 2019; Ohkita et al., 2019; Velasco et al., 2019). Some studies have also reported that virus co-infection can also lead to virus genome re-arrangement (Hillman et al., 2018; Sun and Suzuki, 2008).

Mixed viral infection in fungi is an important topic and detailed knowledge about which may help manipulate virulence of pathogenic fungi. The diversity of coinfecting mycoviruses and the diverse nature of their interactions thought to be occurring freely without any constraint in fungi. However, *in vitro* experiments suggested that the frequency of co-infection varies within fungal systems, and in many cases, is lower than what would be expected from random incidences. Thus, the co-infection scenarios are likely influenced by interactions between co-infecting viruses (Thapa and Roossinck, 2019). There are several examples of mixed viral infections in fungi and the interactions between co-infecting viruses and mycovirus transmission influenced the coinfection setup. For example, the suppression of the fungal host non-self-recognition by one of the mycovirus in the co-infection system. *Sclerotinia sclerotiorum mycoreovirus 4*, a mycoreovirus infecting *S. sclerotiorum* was found to suppress non-self-recognition of the host fungus and facilitate co-infection through horizontal transmission of mycoviruses across vegetatively incompatible group (Wu et al., 2017).

Other examples showed that RNA silencing counter defense protein (RSS) of one mycovirus can help another mycovirus that get suppressed by the host RNA silencing. A victorivirus (RnVV1) that originally isolated from *R. necatrix* could replicate in *C. parasitica* strain EP155 when co-infected with CHV1 (Chiba and Suzuki, 2015). Furthermore, the co-

infection of CHV1 also enhanced MyRV1 virus accumulation in *C. parasitica* strain EP155 (Sun et al., 2006).

B. MATERIAL METHODS

1. Fungal strains and culturing

C. parasitica strain C18 (carrying CHV4-C18 and MyRV2) was originally obtained from William MacDonald (West Virginia University, Morgantown). The virus-free and single infectant of strains of C18 were obtained via single spore isolation. Fungal cultures were grown at 22-27^{o}C on potato dextrose agar (PDA) plates (Difco, Radnor, PA, USA) and incubated on the bench-top for maintenance and colony phenotypic observations. For RNA preparation, fungal cultures were grown in potato dextrose broth (PDB, Difco) liquid media (Difco, USA) or on cellophane-overlaid PDA plates.

2. RNA preparation

Fungal mycelia grown on PDA overlaid with cellophane were harvested after three days of culturing. Total RNA was extracted following the methods described by Suzuki and Nuss (2002). Briefly, mycelia were harvested and ground to a fine powder with pestle and mortar using liquid nitrogen and homogenized with an extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM NaCl, 4 mM EDTA, and 2% SDS (sodium dodecyl sulfate)) followed by phenol-chloroform and chloroform extraction. RNA was precipitated in 2 M lithium chloride followed by another round of chloroform extractions. Finally, RNA was precipitated in 100% ethanol and 0.3 M sodium acetate and washed with 70% ethanol.

To extract dsRNA, the supernatant obtained from chloroform-isoamyl alcohol extraction was incubated with CC41 cellulose (Whatman, Piscataway, NJ, USA) in STE buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 mM EDTA) containing 15% (v/v) ethanol for one hour. After washing in STE-15% ethanol for three times, dsRNA was eluted with STE buffer and precipitated with absolute ethanol. The dsRNAs were analyzed by 1% agarose gel electrophoresis in 0.5×TAE (Tris/acetic acid/EDTA).

The non-phenol extraction of dsRNA was carried out following a modified version of the method described previously (Balijja et al., 2008). The grounded mycelia were homogenized with EBA buffer containing 5mM Tris-HCl (pH 8.5), 50mM EDTA pH 8.0, 3% SDS, 1% Polyvinyl polypyrrolidone (PVPP) and 50 mM DTT. The mixtures were centrifuged at maximum speed at 4^{o}C for 10 minutes. Resulting supernatants were collected and incubated with CC41 cellulose. The following steps were carried out as mentioned in the phenol-chloroform method earlier.

3. Next-generation sequencing

Total RNAs samples (62 ng/µl) was used for cDNA library construction using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) with Ribo-Zero kit

was used to deplete the host rRNAs. 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 then assembled de novo into 21,261 contigs (162 to 9,126 nt in length) using CLC Genomics Workbench (version 11, CLC Bio-Qiagen, Redwood City, CA, US). The contigs were then subjected to BLAST searches against the viral reference sequence dataset obtained from the National Center for Biotechnology Information (NCBI).

4. Terminal sequence determination

The terminal sequences were determined by RLM-RACE (RNA ligase mediated rapid amplification cDNA ends). 5′-phosphorylated oligodeoxynucleotide (5′-PO_4 CAATACCTTCTGACCATGCGTGACAGTCAGCATG-3′) (3′ RACE-adaptor) was ligated to each of the 3′-terminus with T4 RNA ligase in 40% PEG6000 at 16^{o}C for 16h. Ligated dsRNAs were denatured in 90% DMSO at 65^{o}C for 20 minutes followed by precipitation. Denatured dsRNAs were then reverse-transcribed by using MMLV reverse transcriptase (Invitrogen, CA, USA) following the manufacturer's protocol and using the 3′RACE-1st primer (5′-CATGCTGACTGTCACTGCAT-3′) that is complementary to the 3′-half of the 3′RACE-adaptor. The resulting cDNAs were amplified by PCR (Blend Taq, Toyobo, Osaka, Japan) with the primer set 3′RACE-2nd (5′-TGCATGGTCAGAAGGTATTG-3′) that complementary to the 5′-half of 3′RACE-adaptor and virus sequence-specific primer (listed in Table 2.1). Amplified DNA fragments were cloned into pGEM-T Easy (Promega, Madison, WI, US) for bacterial transformation and sequence analysis. Primers that used in this chapter are listed in Table 2.1.

Table 2.1 List of primers used for detection co-infection viruses in C*ryphonectria parasitica* strain C18

Primer Name	Sequence (5`-3`)	Direction	Remarks
CHV4 5'RACE inner_R	AAGTGAGCCGCAAACACATGGT	Reverse	CHV4 5' end
CHV4 5'RACE outer_R	TCCAACCATCATCGGTCAAAAA	Reverse	CHV4 5' end
MyRV2_S2_3294_F	AGGAGTTTATGAGCCTTACGAG	Forward	MyRV2 RT-PCR and S2 probe
MyRV2_S2_3828_R	AGTATCAGCGAGAATAGGGC	Reverse	MyRV2 RT-PCR and S2 probe
CHV4_1180_F	TGAAGGAACTGCGTCGTCT	Forward	CHV4 RT-PCR
CHV4_1632_R	CTTGGTGCTTATCGTCCATT	Reverse	CHV4 RT-PCR
MyRV2-E2-10_300_F	AATTCAATTCCGCGCGAAGGGG	Forward	MyRV2 S10 probe
MyRV2-E2-10_1200_R	TTCATTTTCACGTTGTTAAAAC	Reverse	MyRV2 S10 probe

5. Asexual sporulation assays

Asexual sporulation assay was carried out by growing (on PDA) the fungal strains on bench top for three days and followed by exposing culture plates under high-light intensity for 10 days to stimulate sporulation. Spores were harvested by using sterilized water inside the laminar air flow to minimize the risk of contamination. Spore suspensions were observed under light microscope, and as per requirement, were diluted serially with autoclaved water. The spore suspensions were then plated onto PDA and incubated at 25–26^{o}C for two days. Germinated spores were transferred onto new PDA plates. After three days of incubation, the germlings generated from individual spores were examined for virus infections by virus-specific One-step reverse transcriptase polymerase chain reaction (RT-PCR) (described below).

6. One-step RT-PCR

To detect virus infections in single spore cultures, a One-step RT-PCR was carried out following the methods described in previous report (Urayama et al., 2015) with slight modifications. The young mycelia grown on an PDA plates were pricked with a toothpick for about 5mm deep with special care to avoid picking up any agar fragments. The toothpick with the mycelia was then dipped into 10 µl of PrimeScript^{TM} One-step RT-PCR premix (Takara, Kusatsu, Shiga, Japan) in a PCR tube and twisted three times. The one-step RT-PCR premix was performed following the manufacturer's protocol that is scaled down to 10 µl. Reverse transcription was conducted at 50^{o}C for 30 mins followed by DNA polymerase activation at 94^{o}C for 2 minutes. For PCR 30 cycles of denaturation at 94^{o}C for 30s, annealing at 60 ^{o}C for 30s, and extension at 72 ^{o}C for 1 min/1 Kb were followed by a final extension at 72 ^{o}C for 5 mins.

7. Spheroplast preparation

Spheroplasts were prepared from mycelia cultured in PDB media. A precultured was initially prepared by inoculating 150 ml of PDB (in 500 ml Erlenmeyer flasks) with several fungal plugs followed by incubating flasks in the dark for five days. The preculture media was then removed after centrifugation at 1300 *g* for 10 mins. Mycelia were then pipetted to break the hyphae and then cultured thereafter in the fresh media at 27^{o}C for three days in the dark conditions. The freshly-grown mycelia were harvested by filtering using two layers of Miracloth (Merck, Darmstadt, Germany) fitted on a Buchner funnel. After washing with 0.6 M $MgSO_4$, mycelia were suspended in an enzyme mixture containing 500 µl Sigma β glucuronidase (G7770) (Merck), 333mg Sigma lysing enzyme (L1412) (Merck), and 500 mg Bovine Serum Albumin (Nacalai Tesque, Kyoto, Japan) in 50 ml osmotic medium (1.2 M $MgSO_4$ in 10 mM NaH_2PO_4; adjusted pH to 5.8 with Na_2HPO_4) prepared by filter sterilization. Mycelia were then

incubated at 27°C for 2–3 h with gentle agitation for spheroplasting, which gives the fungal cell wall has been almost removed. Spheroplasts were harvested in a corex tube by filtering through two layers of nylon mesh. Spheroplast suspension (1.25 vol.) was then overlaid with trapping buffer (0.4 M Sorbitol in 100 mM Tris-HCl, pH 7.0) and centrifuged at 1300 *g* for 15 mins at 4°C. Spheroplasts were collected at the interface and diluted with two volumes of 1M Sorbitol. Spheroplasts were pelleted by centrifugation at 1300 *g* for 6 mins at 4°C. Supernatant was removed and pellet was suspended in 10 ml of STC buffer (1M Sorbitol in 100mM Tris-HCl (pH 8.0) and 100 mM $CaCl_2$); followed by another round of centrifugation at 1300 *g* for 6 mins at 4°C. Spheroplasts were suspended in 0.5 ml of STC and diluted to a final concentration 2×10^8/ml. For storage, mix four parts of spheroplast solution with one part of PTC (40% polyethylene glycol (PEG) 4000, 100 mM Tris-HCl (pH 8.0), 100 mM $CaCl_2$,) and 0.05 parts of DMSO. Divide the mixture into 100 µl for each tube and stored at -80°C until use.

8. Transformation of *C. parasitica* spheroplast

For transformation, a total of 10 µg of plasmid DNA was added into 100 µl of spheroplast suspension (contain approx. 2×10^8 cells) and mixed gently. Tubes were then incubated on ice for 30 mins. After incubation, 1 ml of PTC was added into the suspension and tubes were further incubated at room temperature for 25 min. Subsequently, spheroplasts were pelleted by centrifugation at 8,000 *g* for 5 min at 4°C and washed one time with 0.5 ml of STC buffer. Finally, the supernatant was removed by aspiration, and the pellet was resuspended in 0.5 ml of STC buffer. An aliquot of diluted spheroplast was distributed throughout the 90 mm Petri dishes (100 µl solution per Petri dish) with a sterilized pipette tip.

Approximately 20 ml of molting regeneration medium (0.1% yeast extract, 0.1% casein hydrolysate, 1M sucrose, and 1.6% agar) that has been cooled to approx. 50°C was poured into each plate. The next day, the Petri dishes were overlaid with 15 ml of 1% top agar containing the appropriate concentration of antibiotics. Colonies that appeared within 5–10 days after transformation, were transferred into PDA amended with the antibiotics.

C. RESULTS

1. *C. parasitica* C18 strain is co-infected with a mycoreovirus and a hypovirus

C. parasitica strain C18 showed phenotypical differences compared to the standard virus free strain EP155 (Fig. 2.1). Previous studies showed through dsRNA profiling that strain C18 was infected with a dsRNA genome virus belonging to the family *Reoviridae*, named "Mycoreovirus 2 (MyRV2)" which is closely related to MyRV1, a well-studied mycoreovirus (Enebak et al., 1994a; Enebak et al., 1994b). MyRV1 and MyRV2 shared a similar virion structure and had the same number of genome segments. However, the nucleotide sequence similarity of dsRNA segment 3 (S3) between these two viruses was less than 50% (Deng et al., 2007). MyRV2 complete genome sequence is not determined yet, except for the S3 segment. To determine the MyRV2 complete genome sequence, a next-generation sequencing (NGS) approach was taken using total RNA extracted from strain C18. Unexpectedly, BLAST analyses of NGS contigs showed that in addition to MyRV2, C18 was also harbored a *Hypovirus*, *Cryphonectria hypovirus 4* (CHV4-C18) (based on the high number of reads that are mapped to CHV4 genome). Strain C18 was previously thought to be infected only with MyRV2 as the routine dsRNA profiling was unable to detect the presence of CHV4-C18. To further confirm the presence of CHV4-C18, an RT-PCR analysis was performed with CHV4-C18-specific primers. The primers set (CHV4_1180_F and CHV4_1632_R) was designed based on the sequence that was obtained from NGS analysis. The primer pair successfully amplified a 452 base region of the CHV4-C18 genome in RT-PCR reactions. This result further confirmed CHV4 infection in strain C18 (Fig. 2.2).

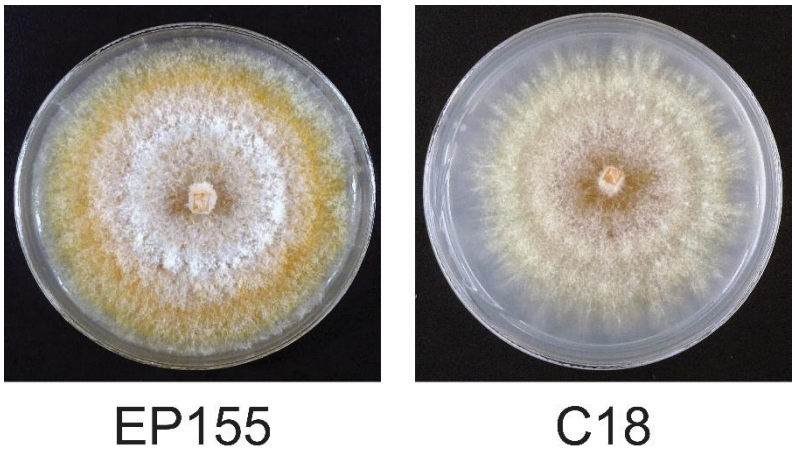

Fig. 2.1 Morphological differences between the *C. parasitica* strain EP155 (virus-free) and C18.

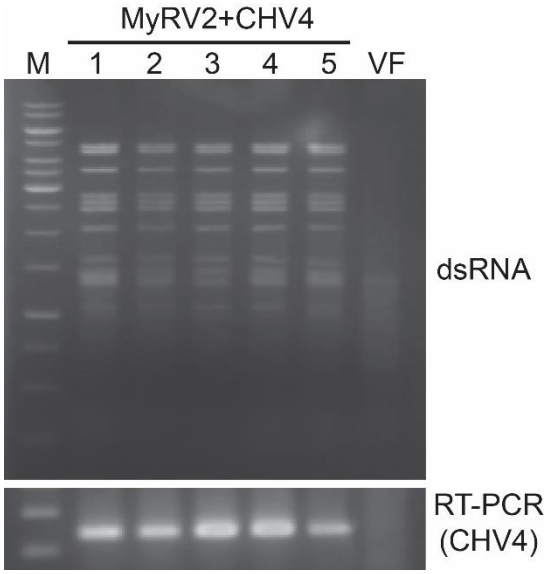

Fig. 2.2 Routine dsRNA extraction of *C. parasitica* C18 strain, infected with CHV4 and MyRV2. Routine dsRNA extraction failed to detect CHV4-C18 in strain C18. RT-PCR with CHV4-specific primers detected the presence of CHV4 in C18 strain.

2. Comparison of CHV4 genome sequence that infects strain C18 with previously reported CHV4-SR2 sequence

CHV4 was previously reported to infect strain SR2 (CHV4-SR2) (Linder-Basso et al., 2005). Sequence analysis revealed that CHV4-C18 and CHV4-SR2 shared high degree of sequence identity (99.4%) at the nucleotide level. In addition, amino acid sequence comparison between these two viruses showed a total of 54 nucleotide substitutions (Table 2.2), 17 of which leading to amino acid changes, of these, only three amino acid changes occured within the conserved motifs regions (Fig. 2.3). CHV4-C18 and CHV4-SR2 also shared common features in their functional domains. There are five predicted functional domains identified in the polyproteins of both CHV4-C18 and CHV4-SR2 (Fig. 2.3), including a putative protease (prot), a UDP-glucosyltransferase (UGT), a permuted papain fold peptidases of dsRNA viruses and eukaryotes (PPPDE), an RNA-dependent RNA polymerase (pol), and an RNA helicase (Hel) domain (Iyer et al., 2004; Linder-Basso et al., 2005).

Interestingly, due to sequence differences, CHV4-C18 and CHV4-SR2 have a different position of ORF start codon (AUG). CHV4-SR2's start codon is at the nucleotide position 194, while for CHV4-C18, it is at position 287, leading to a shorter

N-terminal (30 aa) portion of CHV4-C18 than that of CHV4-SR2. However, CHV4-C18 has a longer 5′-untranslated region (UTR) compared with the 5′ UTR of CHV4-SR2. Although the CHV4-C18 genome similarly contains the AUG at the nucleotide position 194, there are nucleic acid substitutions that introduced in-frame stop codon (UAG) and followed by AUG at the region around the nucleotide position 287 (Fig. 2.4). Thus, the polyprotein encoded by CHV4-C18 is approx. 2817 aa in size with an estimated molecular weight of 317.2 kDa, while that of encoded by CHV4-SR2 is 320.5 kDa. This difference in the size of ORF appears not to exert any effect on the symptom expression since both the strains induce either no or little perceptible phenotypic changes in their respective fungal hosts. The different size of ORF effect on virus accumulation needs to be investigated.

Table 2.2 Comparison of nucleotide sequence polymorphisms between previously reported CHV4-SR2 and characterized in the present study CHV4-C18. A total of 54 nucleotide polymorphisms were identified. The uracil (U) sequence are replaced with thymine (T)

Position	CHV4-SR2	CHV4-C18	Position	CHV4-SR2	CHV4-C18	Position	CHV4-SR2	CHV4-C18
245	A	G	4069	G	A	6544	A	G
284	C	T	4264	A	G	6549	A	G
287	G	A	4324	T	C	6590	T	G
306	C	T	4714	A	G	6643	A	G
984	C	T	4723	A	T	6958	C	T
1350	A	G	4777	A	G	7093	A	T
1615	G	A	4942	T	C	7246	G	A
1627	G	A	5060	A	G	7291	A	G
1721	G	A	5154	A	G	7384	A	G
1724	G	T	5212	C	T	7648	T	G
2246	C	T	5653	T	C	7762	C	T
2554	A	G	5725	T	C	8047	T	C
3438	G	A	5864	C	T	8236	G	A
3669	A	G	5896	A	G	8264	G	A
3802	C	T	5950	C	T	8376	A	G
3802	T	C	5983	G	A	8704	T	C
3877	G	A	6255	A	G	8793	T	C
3943	G	A	6271	G	A	8962	T	C

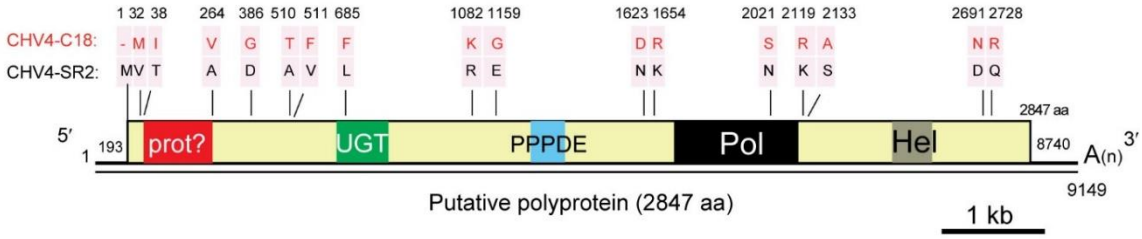

Fig. 2.3 Genome organization of CHV4. A total of five known conserved domains were identified in the CHV4-encoded polyprotein. These domains include a putative protease (prot), a UDP-glucose/sterol glucosyltransferase (UGT), a permuted papain fold peptidase of dsRNA viruses and eukaryotes (PPPDE), an RNA-dependent RNA polymerase (Pol), and an RNA helicase (Hel). The amino acid sequence differences between CHV4-C18 and CHV4-SR2 are spread along the genome (not only restricted to the conserved motifs). The figure is adopted from a recent publication by Aulia et al. (2019).

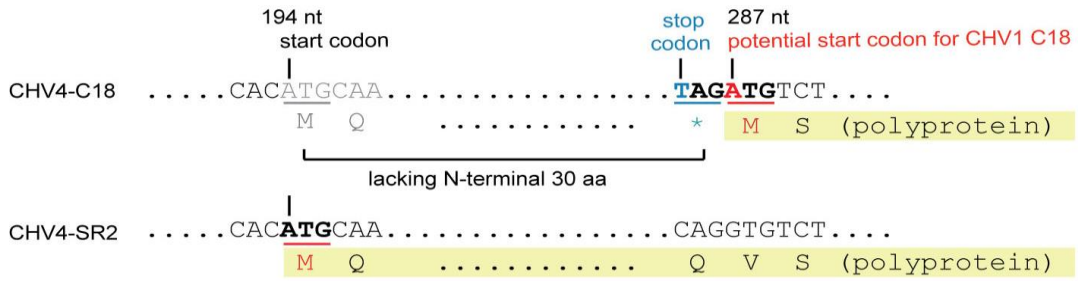

Fig. 2.4 Predicted differences among translational initiation sites between CHV4-C18 and CHV4-SR2 ORFs. CHV4-SR2 ORF starts at the nucleotide position 194, whereas CHV4-C18 ORF is initiated at the nucleotide position 287. Different translational initiation sites between these two viruses resulted in a polyprotein with 30aa shorter N-terminus for CHV4-C18. The uracil (U) sequence are replaced with thymine (T). The figure is adopted from a recent publication by Aulia et al. (2019).

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

Isolation of virus-free and singly infected C18 strains was carried out to investigate the effect of MyRV2 and CHV4-C18 infections on the host and also the possible interactions between these two viruses. Single spore isolation was carried out to obtain virus-free isolates as well as single infectants harboring either CHV4-C18 or MyRV2 isolates. During sporulation, usually, the virus is not 100% vertically transferred to the conidia. This gives a way to separate the co-infecting viruses or even to produce an isogenic virus-free line. Over 100 single conidial isolates of *C. parasitica* C18 strain were tested for CHV4-C18 and/or MyRV2 infections. While MyRV2-free strains could readily be obtained, CHV4-C18-free ones were relatively more difficult to isolate. 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 (CHV4-C18 primer sets: CHV4_1180_F and CHV4_1632_R; MyRV2 primer sets: MyRV2_S2_3294_F and MyRV2_S2_3828_R) (Fig. 2.5). Moreover, dsRNA profiling was carried out for further confirmation. MyRV2 had a similar dsRNA profile in singly or doubly infected strain of C18, while CHV4-C18 dsRNA was undetectable in both singly and doubly infected isolates (Fig. 2.5). These obtained fungal strains were cultured on filter discs and stored at −20 °C until use for subsequent stability tests.

The phenotypic comparison was carried following the isolation of either singly and doubly infected strains of C18. Virus-free C18 and CHV4-C18-infected C18 were indistinguishable from each other phenotypically. 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. CHV4-C18 promoted the growth of aerial mycelia when it co-infected with MyRV2. This contributed to the apparent difference in colony morphology (Fig. 2.6).

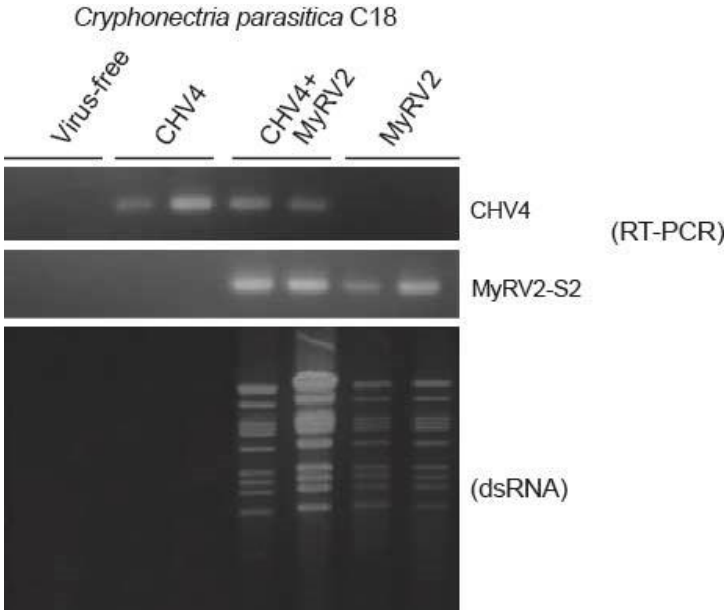

Fig. 2.5 Detection of MyRV2 and CHV4-C18 in single conidial isolates of *C. parasitica* strain C18 by sequence-specific RT-PCR and dsRNA profiling. CHV4-C18 dsRNA could not be detected in either singly or co-infected C18 strains (Adopted from Aulia et al., 2019).

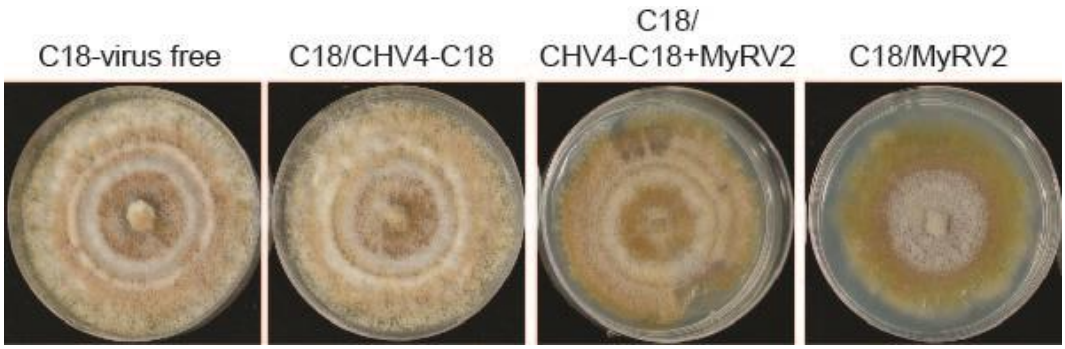

Fig. 2.6 Colony morphology of wild type *C. parasitica* C18 uninfected or infected with MyRV2 alone, CHV4–C18 alone or co-infected with both viruses. Colonies were grown on PDA for six days on the bench-top and photographed. Single infection by MyRV2 and co-infection by MyRV2 and CHV4-C18 resulted in phenotypic changes, whereas no such changes were observed in case of single infection by CHV4-C18 (Adopted from Aulia et al., 2019).

D. DISCUSSION

Viruses are the most common and abundant biological entities on earth (Edwards and Rohwer, 2005; Rohwer, 2003; Rohwer and Thurber, 2009), and because of this fact, viruses outnumbered the hosts. Virus co-infection is a common phenomenon in nature. In plants, co-infection of two or more viruses occurs following transmission by hemipteran vectors that can transmit more than one virus (Syller, 2012; Syller and Grupa, 2016). So far, in fungi, only one mycovirus has been reported to be transmitted by an insect vector (Liu et al., 2016), while the other mycoviruses seem to lack an extracellular transmission route. However, heterologous virus mixed infection is common in many species of fungi, suggesting that mixed infection occurs quite frequently in nature. Interaction of co-infecting viruses have been widely studied and so far, grouped into neutral, synergistic and antagonistic interactions.

The finding of co-infection of CHV4-C18 and MyRV2 in *C. parasitica* C18 strains is quite unexpected, because CHV4-C18 could not detected through via routine dsRNA extractions in either single infection or double infection (Fig. 2.2). Previously reported co-infection of CHV1 and MyRV1 that was artificially established under laboratory conditions and induces genome rearrangement of MyRV1 (Sun and Suzuki, 2008). In contrast, the co-infection of CHV4-C18 and MyRV2 occured in nature and apparently did not lead to the rearrangement of MyRV2. This is because a similar dsRNA segments pattern was observed between singly and doubly infected strains (Fig. 2.5).In the case of CHV1 and MyRV1 co-infection, suppression of *dcl2* induction was thought to be the main reason of the rearrangement (Tanaka et al., 2011). The presence of *dcl2* thought to maintain the MyRV2 genome organization from re-arrangement (Eusebio-Cope and Suzuki, 2015).

CHV4 was previously reported to be the most prevalent hypovirus found in North America. CHV4 was isolated from *C. parasitica* strain SR2 (Linder-Basso et al., 2005). Nucleotide sequence comparison between CHV4-SR2 and CHV4-C18 showed that they have 99.4% sequence identities. Only 54 SNPs were found, among which 16 induce amino acid changes while the two strains retained five predicted functional domains of the putative viral polyprotein (Fig. 2.3). It is not surprising that CHV4-C18 is more closely related to the type strain CHV4-SR2 than other CHV4 strains originated 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, 1993).

Although CHV4-C18 and CHV4-SR shared high nucleotide sequence identity, an interesting difference between CHV4-C18 and CHV4-SR2 is that SNPs found at the 5′ proximal region of CHV4-C18 introduced a stop codon, moving the predicted start codon 32 codons downstream of the start codon detected in CHV4-SR2. Moreover, the start codon in

the CHV4-SR2 genome also exists in the same position as it was spotted in CHV4-C18 genome, creating a small ORF. Whether this ORF is translated or not is still unclear.

CHAPTER 3. STABLE MAINTENANCE OF MyRV2 IN *C. parasitica* C18 IS FACILITATED BY CHV4-C18 THROUGH SUPPRESSION OF HOST ANTIVIRAL DEFENSE

A. Introduction

1. RNA interference (RNAi)

The term "RNA interference (RNAi)" was first introduced in 1998 to describe the observation that double-stranded RNA (dsRNA) can block the expression of a gene that shares a homologous system to the dsRNA when it is introduced into worms (Fire et al., 1998). RNAi starts with the production of small RNAs from the double-stranded RNA precursors that then guides an endonuclease to destroy their mRNA targets (Hutvágner and Zamore, 2002). RNAi mechanism is highly conserved in many eukaryotes, which is the central process of the cleavage of dsRNAs by an RNase III-like protein, known as Dicer-like protein (Dcl), into 21-28 nucleotide (nt) small RNA (sRNA) duplexes with a 2-nt overhang at the 3′ ends. These two strands of sRNAs are separated, probably by a helicase, and then RNA induce silencing complex (RISC) containing an Argonaute slicer/ RNA-binding protein recruits one of the two strands as the guide for degradation or suppression of protein translation of RNA target and repression of RNA transcription of DNA (Meister and Tuschl, 2004). The basic silencing components vary, for example the dsRNA and types of effector complex that recruit the sRNAs (Baulcombe, 2004).

RNAi in eukaryotes is mediated by sRNA, which is generally classified into three main classes: short interfering RNA (siRNA), micro RNA (miRNA), and Piwi-interacting RNA (piRNA). siRNA and miRNA are derived from dsRNA precursors that are processed by Dicer. siRNAs are derived from exogenous RNAs or endogenous transcripts such as repetitive sequences or transcripts that can form long hairpin and usually fully complementary with their mRNA targets. siRNAs are generally involved in genome defense and antiviral defense (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009). While miRNAs are from endogenous miRNA-encoding genes that generated miRNA precursor transcripts that usually form imperfect stem-loop structures. miRNAs can target endogenous mRNAs that are not fully complementary and cause mRNA degradation and translational repression (Bartel, 2004). piRNAs are mostly derived from repetitive elements, transposon, and large piRNA clusters in the germ cells. However, piRNAs have not yet been identified outside the animal kingdom. The biogenesis of piRNAs are also different as piRNAs are derived from processed single-stranded precursor and their amplifications that are mediated by Piwi family proteins (Ghildiyal and Zamore, 2009; Kim et al., 2009). The studies of piRNAs were mostly

performed in the fruit fly (*Drosophila melanogaster*), showing that piRNAs has been shown to involved in antiviral defense mechanisms. Neverthelsess, in mosquitos showed that piRNA pathway is a key mechanism in their antiviral strategy (Joosten et al., 2018; Varjak et al., 2017; Wang et al., 2018)

After the discovery of siRNA and miRNA, other kinds of small RNAs have been detected in plants. These includes tasiRNAs (*trans*-acting siRNAs) and nat-siRNAs (natural antisense transcript-derived siRNA), having distinct biosynthesis pathways than that of siRNAs and miRNAs. tasiRNAs are derived from non-protein-coding transcripts that are targeted by miRNAs. The miRNA cleavages determine the phase and is critical for the production of specific tasiRNA (Allen et al., 2005). natsiRNAs from overlapping transcripts. There is a considerable percentage of genes in eukaryotes existing in overlapping pairs, therefore natsiRNAs could mediate various cellular responses (Borsani et al., 2005).

RNAi acts as a natural antiviral defense in plants, insects, nematodes, and fungi (Deleris et al., 2006; Ding, 2010; Hamilton and Baulcombe, 1999; Segers et al., 2007; Wilkins et al., 2005). In plants, RNAi is commonly addressed as RNA silencing. There are at least three types of RNA silencing pathways in plants. The first pathway is cytoplasmic siRNA silencing (Hamilton and Baulcombe, 1999). This pathway is involved in antiviral defense because viral dsRNA, which is the precursor of siRNA, is produced during virus replication. The second pathway is the silencing of endogenous messenger RNAs by miRNAs. These miRNAs negatively regulate gene expression by base pairing to specific mRNAs, resulting in either RNA cleavage or arrest of protein translation. The third pathway of RNA silencing in plants is associated with DNA methylation and suppression of transcription (Jones et al., 2001).

RNAi is triggered by dsRNAs that are processed by a Dicer protein into short (~20–30 nt) fragments. Dicer cleave dsRNA precursors into distinct lengths through two domains of the RNase III domain. One class of RNase III enzymes is characterized by some domains such as DEXD/H ATPase domain, DUF283 domain, a PAZ domain, two tandem RNase III domain, and a dsRNA-binding domain (dsRBD) (Meister and Tuschl, 2004). Mammals and nematodes (*Caenorhabditis elegans*) only have a single Dicer that does double duty for the biogenesis of miRNAs and siRNAs, but in contrast, two distinct Dicers were found in *D. melanogaster*. A flowering plant *Arabidopsis thaliana* encodes four Dicers, while fungal pathogen *C. parasitica* encodes two Dicers (Dang et al., 2011; Garcia-Ruiz et al., 2010; Zhang and Nuss, 2008). Dicers usually isolated in a heterodimeric compound from their natural sources (Carthew and Sontheimer, 2009).

Argonaute proteins are RNA silencing effectors that are guided to their targets by a short single-stranded nucleic acid. Argonaute superfamily can be divided into three

separate subgroups: the Piwi clade that binds piRNA, the second clade that associates with miRNA or siRNAs, and the third clade so far only found only in the nematodes (Yigit et al., 2006). Argonaute proteins are the central, defining components of the various forms of RISC assembly. Ago effector associated with duplex unwinding and culmination stable association of one of the siRNA/miRNA to form RISC (Carthew and Sontheimer, 2009).

RNA-dependent RNA polymerase (RdRp) also plays an important role in RNAi. RdRp amplified siRNA that can amplify and sustain the response and in some organisms such as plant can trigger systemic silencing (Meister and Tuschl, 2004). RdRps have been identified in several species including plants, fungi and *C. elegans* (Dalmay et al., 2000; Sijen et al., 2001; Volpe and Kidner, 2002). RdRPs are thought to generate dsRNAs from single-stranded transcript either by second-strand synthesis or by relying on siRNAs to prime transcription. Thus, RdRP activity may initiate or dramatically enhanced RNAi response (Makeyev and Bamford, 2002; Sijen et al., 2001)

2. RNAi in fungi (RNA silencing)

RNAi in fungi also known as RNA silencing. RNA silencing pathway operates in most fungi, although the RNA silencing pathway has been lost sporadically in some fungal species and lineages (Nakayashiki, 2005). Like in other organisms, RNA silencing in fungi also provides an efficient and predictable gene silencing effect: recognition is based on the base-pairing between siRNA and target RNA. RNAi has been described in many fungal species, but the most intensive study has been conducted on two reference organisms: the ascomycete fungus *Neurospora crassa* and the opportunistic human zygomycete pathogen *Mucor circinelloides* (Torres-Martínez and Ruiz-Vázquez, 2017a).

RNA silencing phenomena in fungi were first demonstrated in the ascomycete fungus *N. crassa* (Romano and Macino, 1992). In *N. crassa*, siRNA and miRNA-like small RNAs (milRNAs) function in genome defense and gene regulation (Li et al., 2010). RNA silencing in *Neurospora* functions during both vegetative and sexual stages. In both vegetative and sexual stages, RNA silencing suppresses transposon invasion. During vegetative stages, the introduction of repetitive DNA sequences triggers posttranscriptional gene silencing of all homologous genes. During the sexual cycle two distinct mechanisms of RNA silencing operate: repeat-induced point mutation and meiotic silencing by unpaired DNA (MSUD) (Romano and Macino, 1992; Selker et al., 1987).

The post-transcriptional gene silencing in *N. crassa* during vegetative growth is called quelling, and it is essential for suppressing transposon replication. Quelling-like RNAi pathways in other ascomycete fungi such as *Aspergillus nidullans, Magnaporthe oryzae, Cryptococcus neoformans, Trichoderma atroviridae, Fusarium graminearum*,

and in a basidiomycete yeast *Cryptococcus neoformans* (an opportunistic human pathogen) have also been described and showing the activity to limit the transposition of transposons and retrotransposons so that the genome integrity during vegetative growth can be maintained. Similarly, as in the vegetative growth, RNAi in fungi also active during the sexual growth of *N. crassa* to protect the genome form unpaired DNA segments including integrated viruses and transposons (Torres-Martínez and Ruiz-Vázquez, 2017b). In *C. neoformans*, RNAi-mediated gene silencing is highly induced during sexual reproduction and leads to the rise of transposon-derived siRNAs (Wang et al., 2010).

3. RNAi as an antiviral defense in fungi

The defensive role of the fungal RNAi machinery against virus infection was demonstrated in the ascomycete *C. parasitica* and provided the first example of RNAi as an antiviral defense in fungi (Segers et al., 2007). *C. parasitica* has the set of keys of RNAi key genes, *dcl2* and *agl2*, are required for antiviral defense. The presence of *dcl2* and *agl2* is required for the action of antiviral defense in *C. parasitica* suggesting that this fungus follows canonical RNAi mechanism. In *C. parasitica* the expression of *dcl2* is induced by viral infections. When the fungus was infected with by a mutant of CHV1 without p29, *agl2* and *dcl2* transcript accumulated to very high levels. The *dcl2* mutant in *C. parasitica* showed impaired growth after virus infection, indicating that DCL2 is important in antiviral defense(Sun et al., 2009; Zhang and Nuss, 2008). Four *rdrp* genes have been identified in *C. parasitica* genome. Single or multiple knockouts of *rdrp* genes showed no differences when compared with the wild type strain after virus infection. This suggests that RdRPs do not play any role in the antiviral defense mechanism in *C. parasitica* (Zhang et al., 2014b).

In the phytopathogenic ascomycete fungus *Colletrotrichum higginsianum, dcl1* and *ago1* are crucial components of the antiviral response. The loss of these two genes resulting in increased accumulation of dsRNA virus (Campo et al., 2016). In addition to *C. parasitica* and *C. higginsianum*, RNAi-based antiviral defense has been found in the ascomycete *A. nidulans*, where infecting viruses can be both targets and suppressors of RNAi pathway. *A. nidulans* expresseses one Dicer and one Argonaute protein, needed for the RNA silencing (Hammond et al., 2008a).

4. Mycovirus transmission

The known modes for mycovirus spread are through horizontal transmission (hyphal anastomosis) and vertical transmission (sporulation). Transmission of mycoviruses occurs when the mycelia are genetically compatible, while incompatible strain rarely allows such transmissions. The hyphal anastomosis regulated by the

self/nonself recognition system or vegetative incompatibility (heterokaryon incompatibility) that developed by fungal species. Hyphal anastomosis occurring between somatically incompatible isolates that usually trigger programmed cell death in fungi. In *C. parasitica* this process was is controlled by a series of genetic loci called *vic*. Transmission studies showed that the differences at the *vic* loci negatively affect the horizontal virus transmission (Choi et al., 2012; Zhang et al., 2014a). Vegetative incompatibility is considered as a barrier for mycovirus transmission, but there was also report about mycovirus that affect host *vic* reaction upon infection such as suppressing the non-self-recognition that allows virus transmission between fungus with different *vic* alleles (Hamid et al., 2018; Wu et al., 2017; Yang et al., 2018).

Transmission of mycoviruses also occurs through spore (vertical transmission). The efficiency of mycoviruses transmission through spores differs between viruses. The transmission rate of hypoviruses into conidia in *C. parasitica* varies considerably among isolates rates of 0–100% (Enebak et al., 1994a; Melzer et al., 1997). Similar fashion was also observed in other mycoviruses in ascomycetes. In a pathogenic ascomycete *Ophiostoma ulmi* (the causative agent of Dutch elm disease) horizontal transmission of dsRNA is restricted by vegetative compatibility and most conidia inherit one or several dsRNA elements (Rogers et al., 1986). In *Magnaporthe grisea* (the rice blast fungus), 10% of ascospore progeny showed containing dsRNA (Chun and Lee, 1997). In *Neosartorya hiratsukae* (*Aspergillus hiratsukae*) the virus transmission into ascospore is very efficient (Varga et al., 1998).

B. Material Methods

1. Fungal strain and culturing

The virus-free and single infectant of strain C18 were obtained through single sporulation. Fungal cultures were grown at 22–27^{o}C on potato dextrose agar (PDA) plates (Difco) on the bench-top for maintenance and colony phenotypic observations and in PDB liquid media (Difco) or on PDA plates layered with cellophane for RNA preparation. The fungus and virus strain used in this chapter are listed in Table 3.1. Fungal plates were subcultured in 10 days intervals to observed virus stability. Primers used in this chapter were listed in Table 3.2.

Hyphal fusion or hyphal anastomosis was performed through co-culturing virus-infected donor strain with a virus-free recipient strains at a distance of 1 cm on PDA plate and incubated at room temperature for 10 days. After 10 days of hyphal contact, mycelial plugs were taken from several positions in the recipient side and subcultured in PDA.

Table 3.1 Fungal strains used in this study

Strain name	Description	References
C18/CHV1-Δp69	C18 strain infected with CHV1-Δp69	This study
C18 Δ*dcl2*	C18 strain with disruption in *dcl2* gene	This study
C18/IR-CpMK-1	C18 transformed with hairpin construct to induce *dcl2* expression. The same hairpin construct from Chiba et al. (2015)	This study
EP155/IR-CpMK-1	EP155 transformed with hairpin construct to induce *dcl2* expression	Chiba et al., 2015
C18 Δ*dcl2*/C18::dcl2C18	C18 Δ*dcl2* was complemented with *dcl2* driven by cryparin promoter	This study

2. Northern blot analysis

Total RNA was transferred onto membrane, followed by exposing the membrane in UV cross-link (120 mJ/cm^2) using UVP UV Crosslinkers (Analytik Jena, California, USA). Membrane was then prehybridize at room temperature for at least 30 min with DIG Easy Hyb (Roche Diagnostic, Basel, Switzerland). Dioxigenin (DIG)-11-dUTP-labeled probe was amplified DNA fragments through PCR following the methods recommended by the manufacturer (Roche). The probe was then denatured at 95^{o}C for 10 mins. The probe was mixed into the hybridize buffer and then hybridize at 42^{o}C for 16 h.

The membrane was washed twice with low stringency buffer (2×SSC, 0.1% SDS) at room temperature for 5 mins each and followed with high stringency buffer 0.1×SSC, 0.1% SDS) at 68^{o}C for 15 mins each. The membrane was soak in blocking buffer (Roche) at room temperature for 30 mins to reduce the nonspecific binding. The membrane was incubated with Anti-DIG monoclonal antibody (Roche). The membrane was washed twice with wash buffer (0.1M Maleic acid, 0.15M NaCl, 0.3% (w/v) Tween 20, adjust pH to 7.5 with solid NaOH) for 15 mins each and excess buffer was removed by wiping. The membrane was then incubated in detection buffer followed by CDP Star (Roche) and seal it in a plastic bag. Expose the membrane with ImageQuant LAS 4000 imaging system (GE Health system, Chicago, IL, US).

3. The *dcl2* knockout assay

A plasmid construct used for gene disruption through homologous recombination was prepared using a pGEMT-easy vector system (Promega). The disruption construct consisting of a neomycin phosphotransferase II resistance gene (NPT II) cassette flanked by 2000 bp upstream and downstream genomic sequences of the C18 *dcl2* gene

(Fig 3.1). The NPT II coding sequence was first ligated to the vector and then both upstream and downstream DNA segments were inserted into the vector at the *Sph*I and *Sal*I sites, respectively using the in-fusion cloning system (Takara Bio, Shiga, Japan). To disrupt the host *dcl2* gene through homologous recombination, C18 protoplasts were then transformed with this construct following the methods described by Faruk et al. (2008). The gene will be disrupted. The resulting transformants were selected on PDA containing hygromycin (40 µg/ml). The positive disruptants were screened by PCR using specific primers to determine whether the insertion was in the targeted region. The genomic regions from where specific primers were designed have been shown in Fig 3.2. The primers used for validating the mutants are DCL2Pro_2060up_F and Neo-inv_r; and for control, are Neomycin1_f and Neomycin1_r. To confirm that the *dcl2* gene has been completely disrupted, a set of primers were designed and used (DCL2_4025_F and DCL2_4545_R). All the primers used in this study are listed in Table 3.2.

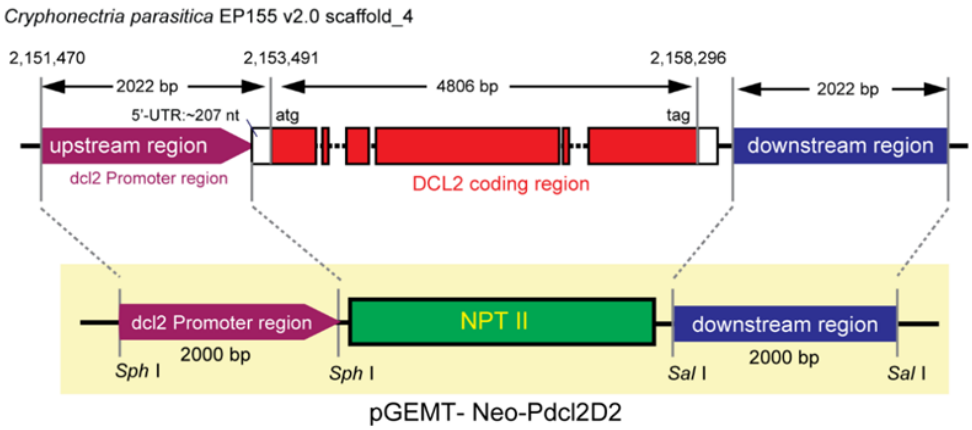

Fig. 3.1 Schematic diagram of a plasmid construct used for *dcl2* gene knock out through homologous recombination in *C. parasitica* strain C18. Coding region of NPT II (neomycin resistance gene) is flanked by 2.0kb upstream and downstream regions (from the start and stop codons) of C18 *dcl2*. These flanking regions were inserted in the vector at the *Sph*I and *Sal*I restriction sites, respectively. This figure is adopted from recent publication by Aulia et al. (2019).

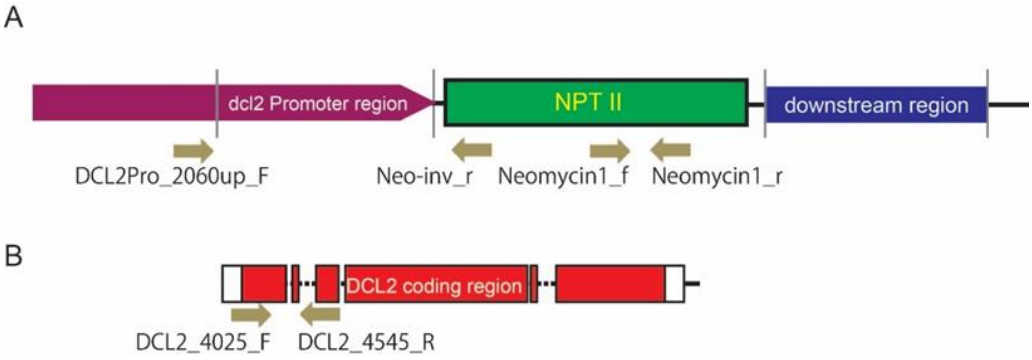

Fig. 3.2 Position of primers designed for screening *dcl2* gene disruption mutants. (A) Diagram showing primer positions for screening the *dcl2* disruption mutants. (B) Diagram showing the primer positions used for confirming the *dcl2* coding region through genomic PCR in the mutants

4. Virus particle purification

The fungal strain C18 was grown on PDB for 10 days. Mycelia (approximately 30 g, wet weight, for PDB cultures) were harvested and ground to powder in the presence of liquid nitrogen. The homogenates were mixed with 150 ml of 0.1 M sodium-phosphate, pH 7.0 using a Waring blender and clarified twice with CCl_4. NaCl and PEG 6000 were added to final concentration of 1% and 8%, respectively. After being stirred for 3h at 4°C, the suspension was centrifuged at 16,000×*g* for 20 minutes. Resultants pellets suspended in 10 ml of 0.05 M sodium-phosphate buffer, pH 7.0 were centrifuged at 7,000×*g* for 20 minutes. The supernatant was re-centrifuged through a 20% sucrose cushion (3 ml) in a Beckman SW4lTi rotor at 80,000×*g* for 2h. The pellet was suspended in a 1 ml 0.05 M Na-phosphate. After centrifugation at 7,000 rpm for 5 mins, the supernatant was fractionated through a 10–40% sucrose gradient by centrifugation at 70,000×*g* for 2 hours. Recovered virus particles were suspended in 100 µl of 0.05 M sodium phosphate buffer, pH 7.0.

5. Virus Transfection

Transfection of *C. parasitica* was performed using spheroplasts and purified virus particles in the presence of PEG (similarly with transformation method but without doing overlay). After the regeneration of spheroplasts, mycelial plugs from multiple positions were transferred onto new PDA plates and propagated. Virus infection was detected through dsRNA profiling.

Table 3.2 List of primers used to analyze MyRV2 virus stability in wildtype and *dcl2* knockout mutant of C18 strain

Primer Name	Sequence (5`-3`)	Direction	Remarks
MyRV2_S2_3294_F	AGGAGTTTATGAGCCTTACGAG	Forward	MyRV2 RT-PCR and S2 probe
MyRV2_S2_3828_R	AGTATCAGCGAGAATAGGGC	Reverse	MyRV2 RT-PCR and S2 probe
MyRV2_S10_300_F	AATTCAATTCCGCGCGAAGGGG	Forward	MyRV2 RT-PCR and S10 probe
MyRV2_S10_1200_R	TTCATTTTCACGTTGTTAAAAC	Reverse	MyRV2 RT-PCR and S10 probe
CHV4_1180_F	TGAAGGAACTGCGTCGTCT	Forward	CHV4 RT-PCR
CHV4_1632_R	CTTGGTGCTTATCGTCCATT	Reverse	CHV4 RT-PCR
5'UTR_CHV1_F	GATAATTTTGGTTGCTGCAC	Forward	CHV1-Δp69 RT-PCR and probe
5'UTR_CHV1_R	GACTCATGTGGCGACGTGCC	Reverse	CHV1-Δp69 RT-PCR and probe
DCL2_4025_F	CCTGCCCTGTTCAGTATCA	Forward	dcl2 probe
DCL2_4545_R	GTGGTAGCCCTCTCTTTGAC	Reverse	dcl2 probe
KO_SphI_C18DCL2Pro_F	TTGGGCCCGACGTCGGTTTTGCTCGACGTCGATGCGC	Forward	C18 dcl2 KO
KO_SphI_C18DCL2Pro_R	CATGGCGGCCGGGAGCTTGCAGCGTCGTACGACAGAT	Reverse	C18 dcl2 KO
KO_SalI_C18DCL2Ter_F	CCGCCTGCAGGTCGAAAGCAGACACAGTCCCAGAAAG	Forward	C18 dcl2 KO
KO_SalI_C18DCL2Ter_R	CTCCCATATGGTCGATTTCAAAAGTATTGACTAGCAC	Reverse	C18 dcl2 KO
DCL2Pro_2060up_F	GCGGAATTTGGAGTACTTGTTG	Forward	C18 dcl2 KO
Neo-inv_r	GTTTTAGTAGTTCTTTTTCTGC	Reverse	C18 dcl2 KO
DCL2_4025_F	CCTGCCCTGTTCAGTATCA	Forward	C18 dcl2 KO
DCL2_4545_R	GTGGTAGCCCTCTCTTTGAC	Reverse	C18 dcl2 KO
Neomycin1_r	GAAGAACTCGTCAAGAAGGCGA	Reverse	C18 dcl2 KO
Neomycin1_f	GCAGAAAAAGAACTACTAAAAC	Forward	C18 dcl2 KO

C. Results

1. CHV4-C18 facilitates stable maintenance of MyRV2 infection during subculturing

While maintaining MyRV2-infected C18 without coinfecting CHV4-C18, we noticed that MyRV2 often got lost during subculturing of the *C. parasitica* strain. We systematically analyzed this phenomenon by subculturing five replicates of each of the above fungal strains and monitoring for virus infection during subculturing at one-week intervals.

Interestingly, MyRV2 single infectants lost the virus as subculturing proceeded. After the 10th subculture, MyRV2 was retained in only one or zero subculture (Fig. 3.3, red column). By contrast, MyRV2 was stably maintained in doubly-infected fungal isolates during subculturing (Fig. 3.3, 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. 3.3). 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 (Fig. 3.3) was further confirmed by RT-PCR analyses (Fig. 3.4).

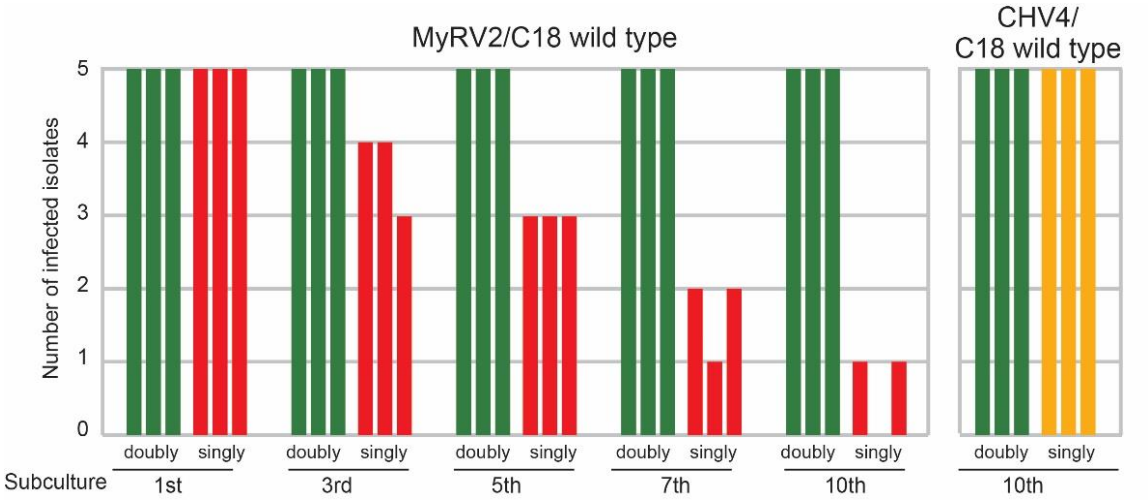

Fig. 3.3 Stability of MyRV2 and CHV4-C18 in the wildtype and *dcl2* knockout mutants of *C. parasitica* C18 strain after successive fungal subculturing. A total of five independent lines were tested with three replicates for each line (Adopted from Aulia et al., 2019).

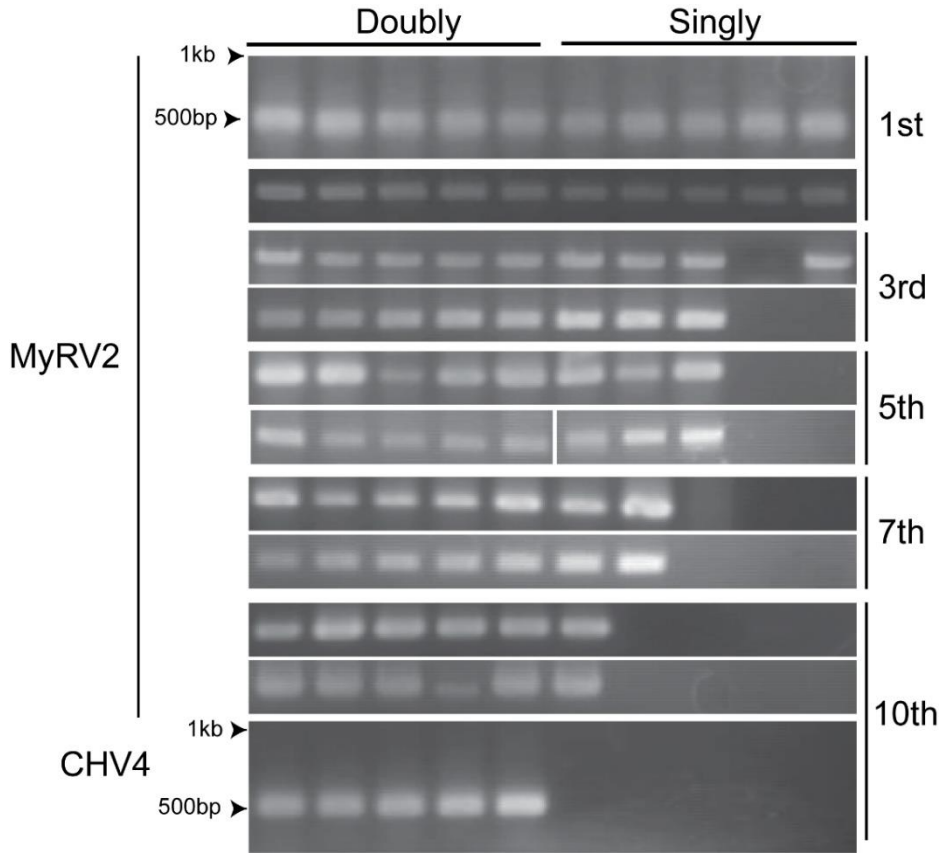

Fig. 3.4 Virus infection during subculturing. The absence of MyRV2 in the first and third sets of the subcultures shown in Fig. 3.3 was validated with RT-PCR, in which, a primer set, MyRV2_S2_3294_F and MyRV2_S2_3828_R was used. The amplified fragments were electrophoresed in 1.0% agarose gel. RT-PCR was also carried out to detect CHV4 on the first set of the 10th subcultures using a primer set, CHV4_1180_F and CHV4_1632_R (Adopted from Aulia et al., 2019)

2. MyRV2 vertical transmission via asexual spores

Vertical transmission of viruses through asexual spores is common, however the rate of transmission varies among viruses. Vertical transmission via asexual sporulation was compared among single and double infections. A total of 198 spores were screened for each condition. Consequently, a tendency similar to that observed for horizontal transmission via subculturing was detected. Co-infection with CHV4-C18 led to a higher transmission rate of MyRV2 compared to that for single MyRV2 infection. 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) (Fig. 3.5). 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. 3.5).

The vertical transmission rate for MyRV2 single infection is only 9%, whereas under co-infection condition with CHV4-C18, the transmission rate of MyRV2 jumped to 25.6%, which represents the sum of singly-infected single-conidial isolates (4.3%, red area) and doubly infected ones (21.3%, green area) (Fig. 3.5). Compared to MyRV2, CHV4-C18 vertical transmission rates were much higher in singly-infected isolates (98.9%, yellow area) and doubly-infected isolates (93.6% total, = 72.3% singly-infected isolates [yellow area] plus 21.3% doubly-infected progenies [green area]) (Fig. 3.5).

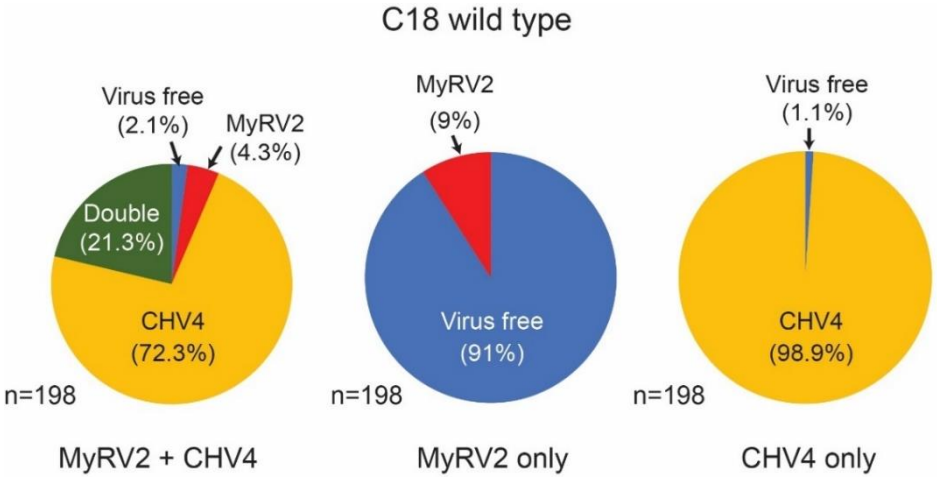

Fig. 3.5 Virus transmission rate through asexual sporulation in strain C18 wildtype. MyRV2 was highly transmitted through asexual spore in double infection condition. CHV4 transmission rate was high in both single and double condition

3. Development of *dcl2* knock-out strain in C18 strain (C18 Δ*dcl2*)

We anticipated that antiviral RNA silencing contributed to the reduced stability of MyRV2 in host. To test this possibility, we first prepared a deletion mutant of an antiviral silencing key gene dicer-like 2 (*dcl2*) in the virus-free *C. parasitica* C18 strain. RNA silencing is the primary antiviral defense mechanism in *C. parasitica*, and *dcl2* is one of the key components in this pathway. Deletion of this gene in the model host *C. parasitica* strain EP155 resulted in hyper-susceptibility toward virus infections (Chang et al., 2012; Chiba and Suzuki, 2015; Li et al., 2010; Segers et al., 2007), but it was not known whether *dcl2* deletion (Δ*dcl2*) would have a similar effect on other *C. parasitica* strains. Homologous recombination using a *dcl2* disruption construct (Fig. 3.1) was carried out to knock out the *dcl2* gene in the C18 strain. Around 300 independent transformants were obtained and screened for *dcl2* disruption by using PCR-based genotyping using three primer sets (Fig. 3.2) (Table 3.2). The first primer set (DCL2_4025_F and DCL2_4545_R)

was used to validate whether the *dcl2* coding region has been replaced or not. The second primer set (DCL2Pro_2060up_F and Neo-inv_r) was used to validate whether the homologous recombination took place at the intended site, and for control primer set (Neomycin1_f and Neomycin1_r) was used to validate if the mutants acquired the neomycin resistant gene. The *dcl2* knock out mutant lines were then screened using these three primers set. Mutants that showed no band with *dcl2* coding region primer set but showed a band for the second and third primer sets were selected (Fig. 3.6). Only two lines out of ~300 lines of mutants were obtained. The low success rate of this method was also predicted earlier in the previous studies (Choi et al., 2005; Gao et al., 1996; Park et al., 2004; Segers et al., 2004; Turina et al., 2003).

To further validated *dcl2* gene disruption in the selected mutants, CHV1-Δp69 was inoculated into those mutants to induce the transcriptional upregulation of the *dcl2* gene. C18 mutants with successful *dcl2* disruption in C18 strain (C18Δ*dcl2*), showed severe growth debilitation similar to that of CHV1-Δp69-infected EP155 Δ*dcl2* (Fig. 3.7). Furthermore, RNA blot showed an increase in CHV1-p69 viral titer and no *dcl2* transcript upregulation in *dcl2* disrupted mutants of strain C18 (Fig. 3.8).

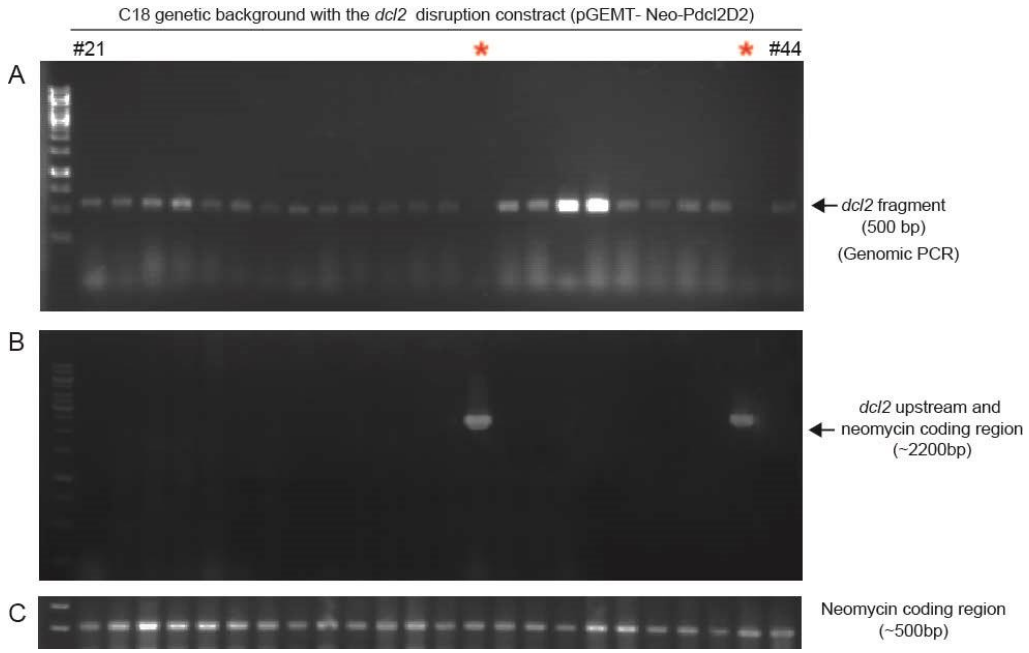

Fig. 3.6 Screening of *dcl2* knockout mutants with three different sets of primers. (A) Gel showing the PCR results using primers set specific for *dcl2* coding region. (B) Gel showing the PCR results using primers set to amplify the upstream region of *dcl2* paired with Neomycin resistant gene primer. (C) Gel picture showing the PCR results using primers set to amplify Neomycin resistance gene coding region that also acted as a control. The primers' position and their sequences were shown in Fig. 3.2 and Table 3.2.

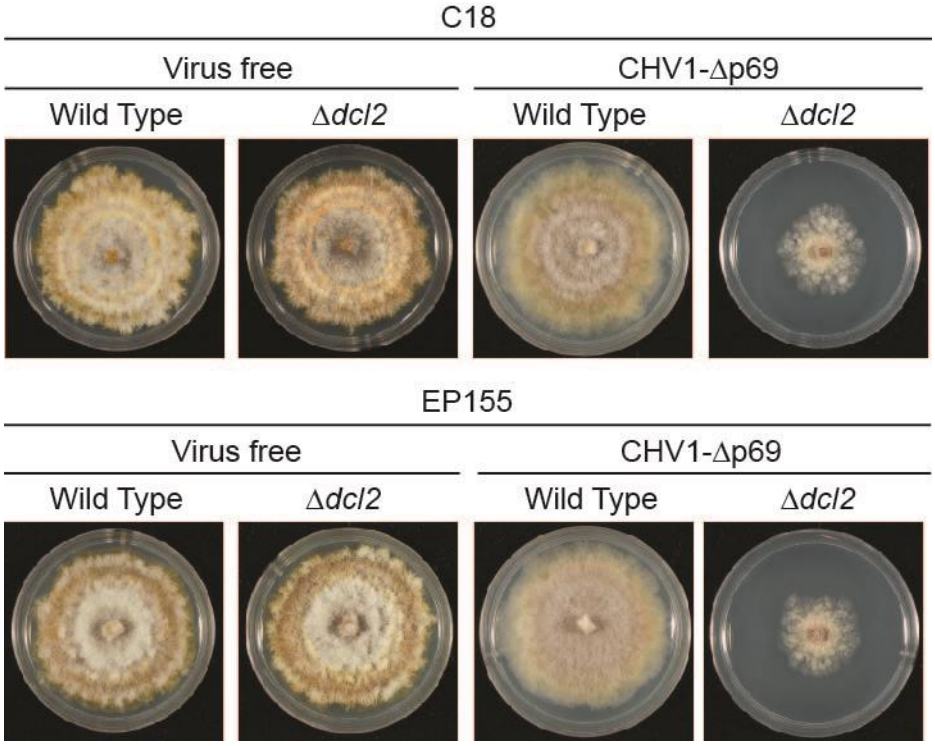

Fig. 3.7 Wild type and Δ*dcl*2 phenotypic growth of *C. parasitica* EP155 and C18 strains. Deletion of *dcl2* (C18Δ*dcl2*) did not phenotypically affect the growth of C18 strain, similarly as observed in the model fungal strain EP155. Effect of CHV1-Δp69 infection in Δ*dcl*2 led to severe growth debilitation. Colonies were grown on PDA for 6 days on the bench-top and photographed.

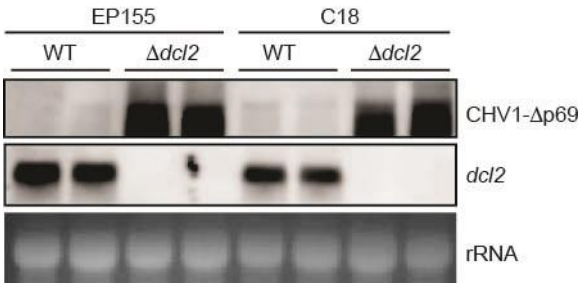

Fig. 3.8 RNA blot analysis of *dcl2* transcript and CHV1-Δp69 genome RNA in wild type and Δ*dcl2* mutant of *C. parasitica* EP155 and C18 strains. The EtBr-stained rRNAs are shown as loading control.

4. Antiviral RNA silencing target MyRV2 and reduces its stability

RNAi-mediated antiviral defense in *C. parasitica* may affect the infection of MyRV2. MyRV2 and CHV4-C18 were introduced into *dcl2* knockout mutant of C18 background in single and double infection through hyphal anastomosis. 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., 2013a; Salaipeth, 2014; Segers et al., 2007), whereas CHV4–C18-infected Δ*dcl2* was indistinguishable from CHV4–C18-infected wild-type C18 (Fig. 3.9).

Northern blotting showed great enhancement of accumulation of MyRV2 transcripts (S2 and S10 segments) in Δ*dcl2* compared to in wild-type C18. CHV4-C18 accumulation was also analyzed using northern blotting and showed that CHV4-C18 accumulation was a similar level between Δ*dcl2* mutant and wildtype. These results indicate that MyRV2, but probably not CHV4–C18, is susceptible to host RNA silencing (Fig. 3.910).

Persistence of CHV4-C18 and MyRV2 infections in Δ*dcl2* was also analyzed by multiple subculture of singly and doubly infected fungal isolates as described above. The 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. That is, MyRV2 was retained as well in Δ*dcl2* as it was in the CHV4–C18/MyRV2 co-infections. CHV4-C18 infection also showed 100% maintenance in C18 Δ*dcl2* (Fig. 3.11A).

The transmission rate of the virus into asexual spore also analyzed Δ*dcl2* mutant of the C18 strain. The transmission rate of singly infected MyRV2 was increased from 9% in the wildtype background (Fig. 3.5, red area) to 33% in the Δ*dcl2* mutant background of C18 (Fig. 3.11B, 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. 3.11B). The MyRV2 frequency 52.1% was higher in double infection than that in singly infected (33%) strains. Possible tripartite interactions among the host Δ*dcl2* and the two viruses may lead to enhanced vertical transmission.

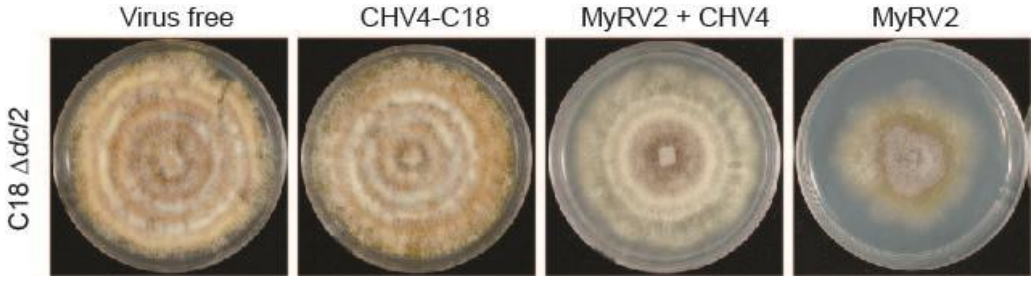

Fig. 3.11 Colony morphology of Δ*dcl2* mutant of *C. parasitica* C18 unifected or infected with MyRV2, CHV4-C18 alone or together. Colonies were grown on PDA for six days on bench top and photographed (Adopted from Aulia et al., 2019).

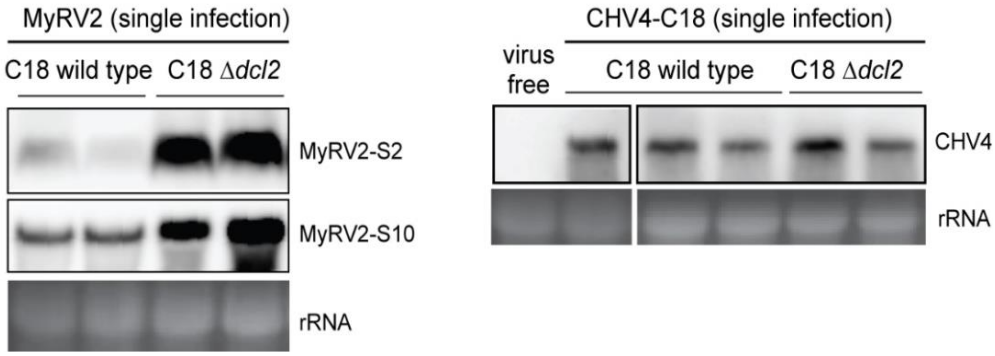

Fig. 3.10 Viral accumulation in Δ*dcl2* of *C. parasitica* C18. RNA blot analysis of the MyRV2 (S2 and S10 mRNAs, left panel) and CHV4-C18 (right panel) accumulation in wild type and Δ*dcl2* mutant. The EtBr-stained rRNAs are shown as loading control (Adopted from Aulia et al., 2019.

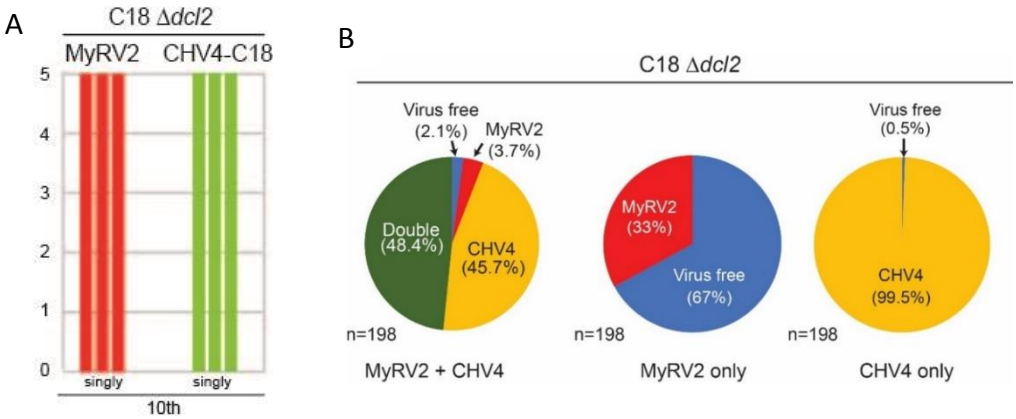

Fig. 3.9 Stability of viruses and their rate of transmission in Δ*dcl2* mutant of *C. parasitica* C18. (A) Virus stability of MyRV2 and CHV4-C18 in C18 Δ*dcl2* background. (B) Virus transmission rate through asexual spores in C18 Δ*dcl2*. MyRV2 was highly transmitted through asexual spores in double infection condition. Higher transmission level of single infection of MyRV2 is also observed. CHV4-C18 transmission rate was high under both single and double infection conditions (Adopted from Aulia et al., 2019.

5. CHV4-C18 suppresses host antiviral defense mechanism and leads to stable accumulation level of MyRV2

The aforementioned observations showed that during subculturing, MyRV2 single infection was less stable with low virus transmission efficiency via asexual spores compared to when it was co-infected with CHV4-C18. These results suggest that CHV4–C18 inhibits RNA silencing and leads to stable infection and enhanced vertical transmission of MyRV2. Only a limited number of mycoviruses have been shown experimentally to have RNA silencing suppression 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 had similar activities, northern blotting was performed to examine the *dcl2* transcripts level in the host.

Similar to the CHV1-Δp69 (a mutant devoid of RNA silencing suppressor activity) and MyRV1 (Chiba and Suzuki, 2015; Zhang and Nuss, 2008), MyRV2 alone greatly induced expression of *dcl2*, relative to virus-free C18, for which, *dcl2* expression could not be detected in the blot. Interestingly, co-infection with CHV4-C18 and MyRV2 showed a weak induction of *dcl2* transcripts, although not to the level of virus-free C18 strain (Fig. 3.12). The reduced *dcl2* transcript accumulation in doubly infected fungi strongly suggests that CHV4-C18 has the ability to suppress host antiviral defense through suppression of *dcl2* transcript upregulation.

To anticipate the 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. In contrast, CHV4-C18 accumulation was also not affected by co-infecting MyRV2 (Fig. 3.13).

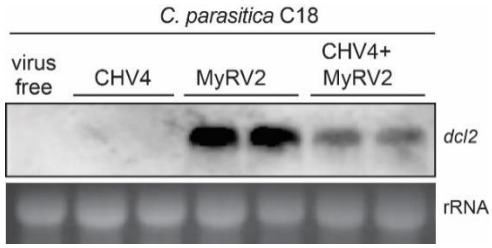

Fig. 3.12 Transcription upregulation of *dcl2* upregulation after virus infection. Single infection of MyRV2 highly upregulated the expression of *dcl2*, whereas co-infection by MyRV2 and CHV4-C18 resulted in decreased accumulation of *dcl2* transcript in C18. The EtBr-stained rRNAs are shown as loading control (Adopted from Aulia et al., 2019).

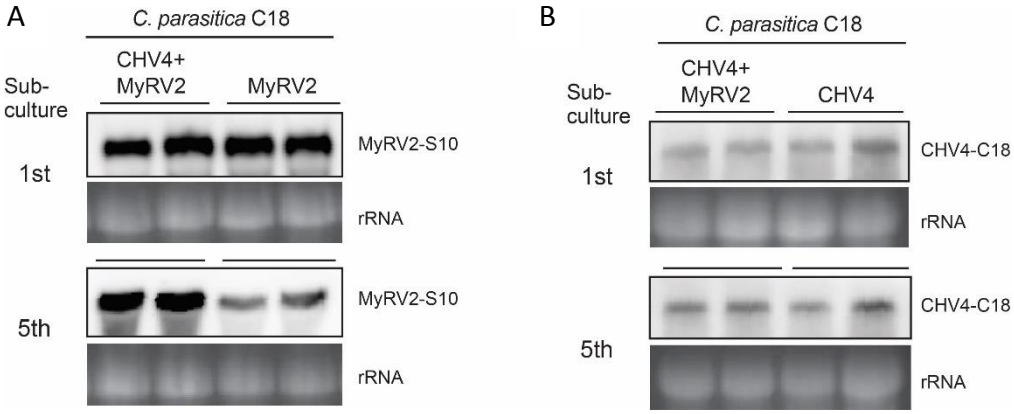

Fig. 3.13 RNA blotting analyses of the MyRV2 (S10 mRNA) and CHV4–C18. Accumulation in singly and doubly infected strains with 1st (top row) and 5th (bottom row) subcultured samples. The EtBr-stained rRNAs are shown as loading control. (A) MyRV2 viral accumulation in singly and doubly infected strain. (B) CHV4-C18 viral accumulation in singly and doubly infected strain (Adopted from Aulia et al., 2019).

6. MyRV2 is susceptible toward host antiviral defense and its induced state impairs replication and horizontal transmission.

My results suggest that the host RNA silencing mechanism contributes to the instability of MyRV2 infection. Induction of *dcl2* transcript highly reduces the fitness of MyRV2 suggesting that MyRV2 is susceptible to antiviral silencing in the fungus. To further investigate this hypothesis, horizontal transmission assays were carried out using fungal strains as recipients in which the *dcl2* expression was induced by either introducing another virus or transgenic expression of dsRNA of an endogenous gene.

In the first assay, MyRV2-infected strain was paired with CHV1-Δp69- infected strain. In CHV1-Δp69-infected strain *dcl2* induction was slightly higher than in MyRV2-infected strain. Upregulation of dcl2 in C18 strain infected with CHV1-Δp69 showed the same level to that observed in the standard model host fungus strain EP155 (Fig. 3.14A). These two fungal strains were grown side by side to allow hyphal anastomosis (Fig. 3.14B). After seven days of co-culturing, the subculture obtained from the MyRV2 side was analyzed for the presence of both viruses by RT-PCR and northern blotting. Subcultures obtained from the MyRV2-infected strain that received CHV1-Δp69 showed reduced levels of MyRV2 accumulation (Fig. 3.14B, MyRV2-side). However, subcultures obtained from the CHV1-Δp69-infected strain failed to support the introduction of MyRV2 but retained CHV1-Δp69 (Fig. 3.14B, CHV1-Δp69-side).

In the second horizontal transfer assay, *dcl2* transcript expression was induced by transgenic expression of hairpin RNA through an inverted repeat construct. RNA silencing-induced strain by transgenic expression of the hairpin RNAs of an endogenous gene *CpMK1* was reported previously. The expression of hairpin RNAs that trigger the *dcl2* induction without virus infection was used in the second system (Chiba and Suzuki, 2015). Virus-free C18 was transformed 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 observed in the standard strain EP155 with CHV1-Δp69, *dcl2* was highly induced in the C18/IR-CpMK1 similar to that of EP155 (EP155/CpMK1-IR) (Fig. 3.15A).

This transformant (C18-CpMK1-IR) was used as recipient in the co-culture assay. After seven days of co-culturing, the C18-CpMK1-IR side was subcultured (Fig. 3.15B) and analyzed for MyRV2 accumulation. Although the donor strain still retained MyRV2 infection, no MyRV2 accumulation was not detected in the subcultures derived from the C18-CpMK1-IR strain. Virus infection was confirmed by northern blot (S10 mRNA) and RT-PCR (Fig. 3.15B).

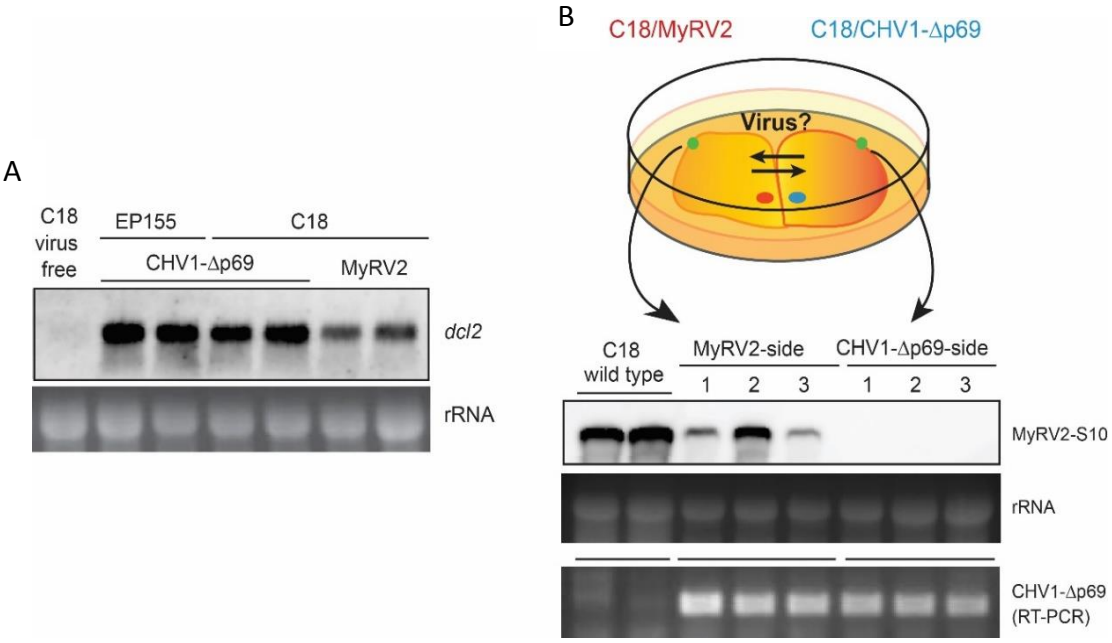

Fig. 3.14 High induction of *dcl2* by another virus infections inhibits MyRV2 horizontal transmission through hyphal fusion in the C18 strain. (A) RNA blot analysis of *dcl2* transcripts in EP155 (standard host strain) and C18 strain infected with CHV1-Δp69 or MyRV2. The *dcl2* upregulation by CHV1-Δp69 was slightly higher than by MyRV2. (B) Co-culturing 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 seven days and the mycelial plugs of the growing edge (shown as green spot regions in the cartoon) were cultured. The EtBr-stained rRNAs are shown as loading control (Adopted from Aulia et al., 2019).

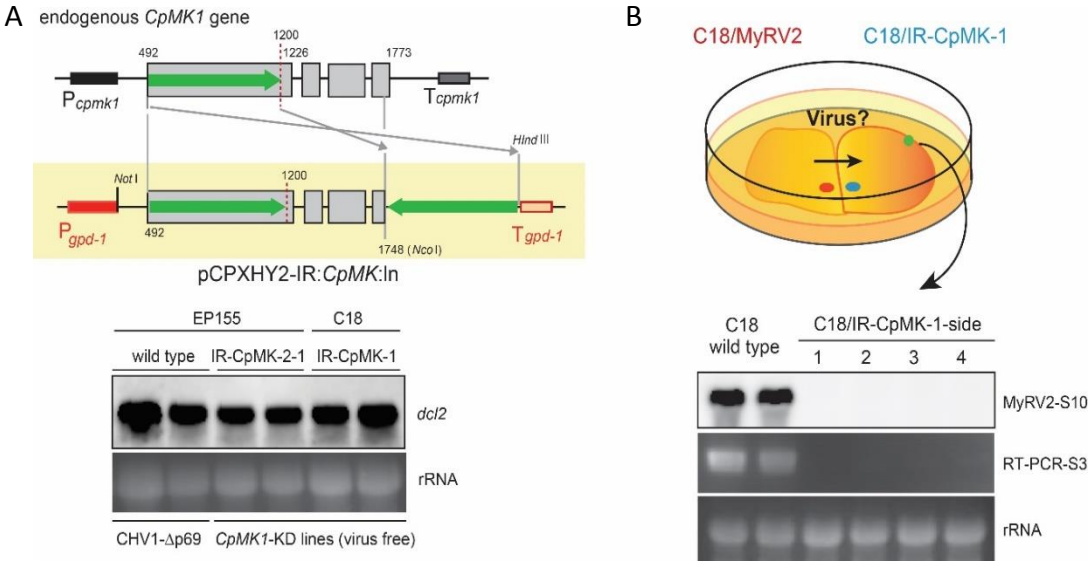

Fig. 3.15 High level induction of *dcl2* by endogenous gene transcript inhibits MyRV2 horizontal transmission through hyphal fusion in the C18 strain. (A) Upregulation of *dcl2* by inverted repeat (IR) dsRNA expression. Schematic representation of the transgene construct to obtain IR-dsRNA of mitogen activated protein kinase (CpMK1) (Chiba and Suzuki et al., 2015) (upper). The RNA blot of *dcl2* upregulation in EP155 and C18 transformant carrying *CpMK1* construct. Strain EP155 infected with CHV1-Δp69 was used as control for *dcl2* transcription level (below). The transformants were later subjected for virus horizontal transmission assay. 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 edges were cultured. The EtBr-stained rRNAs are shown as loading control (Adopted from Aulia et al., 2019).

7. Constitutive expression of *dcl2* transcript limits MyRV2 replication

To further explore the susceptibility of MyRV2 to host antiviral RNA silencing, I developed a constitutive expression construct for *dcl2* driven by the *cryparin* promoter. Cryparin is the most abundant cell-surface protein produced by *C. parasitica* (Kwon et al., 2009). The designing strategy of the constitutive *dcl2* expression construct has been designed as shown in Fig. 3.16A. Although the *dcl2* transcript level derived from this construct was too low to be detected by northern blot, but the amount of *dcl2* was sufficient to act against viral infections in *C. parasitica* (Andika et al., 2017).

The construct that constitutively expressed *dcl2* was transformed into the *dcl2* knockout mutant of *C. parasitica* C18 strain (C18Δ*dcl2*+Crp:*dcl2*) and followed by a horizontal virus transmission assay. Interestingly, the construct that constitutively express *dcl2* in the *dcl2* knockout mutant background could restore the function of the *dcl2* as a viral defense mechanism (Fig 3.16). In other words, the constitutive *dcl2* expression limited the transmission of MyRV2, suggesting that MyRV2 is susceptible to host antiviral defense mechanism.

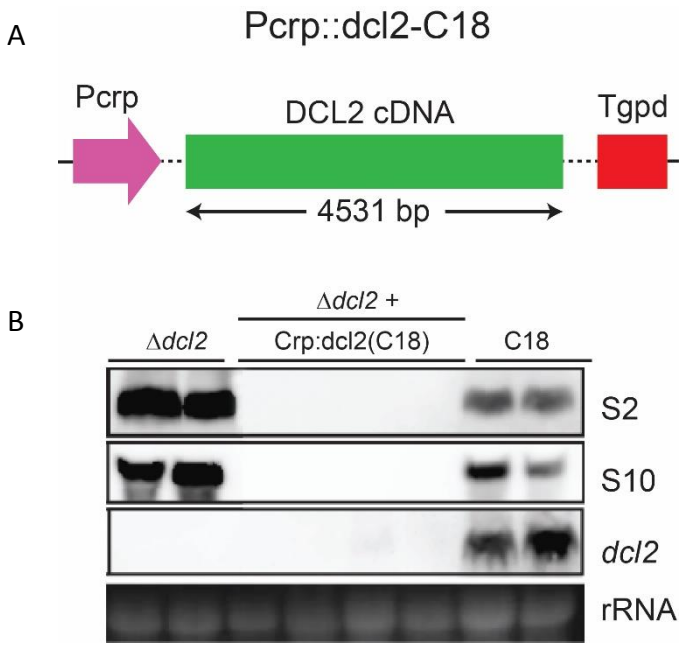

Fig. 3.16 Constitutive *dcl2* expression limiting MyRV2 transmission in C18 strain. (A) Schematic representation of the expression construct (driven by cryparin promoter, Pcrp) for constitutive expression of *dcl2* in C18. (B) RNA blot to examine MyRV2 accumulation after horizontal virus transmission assay.

D. Discussion

Mixed viral infection by heterologous viruses are frequently observed in different organism under natural conditions (DaPalma et al., 2010). The diversity of viruses that infecting the same cell can fundamentally change the effects of virus infection on the host cell. Thus, the outcome of mixed infection is often complex and difficult to predict. In this study, I discovered that *C. parasitica* C18 strain is naturally co-infected with two heterologous viruses, CHV4-C18 and MyRV2. These viruses showed one-way synergism in which the co-infection of CHV4-C18 facilitates stability and efficient vertical transmission of MyRV2.

The vertical transmission efficiency of MyRV2 into the spores of C18 strain increased by approx. three times higher when accompanied by CHV4-C18, but the co-infection did not affect the maintenance and transmission rate of CHV4-C18. This is an interesting example showing a virus that depends on another unrelated virus for its stable infection. This is clearly different from the interaction between helper and dependent viruses. A helper-dependent virus is a virus that is incapable to replicate on its own, therefore requires the presence of another virus for its multiplication. Both MyRV2 and CHV4-C18 can replicate on their own without needing the presence of other viruses in order to complete the replication.

RNA silencing is the primary antiviral defense in fungi. Inactivation of the key players in the RNA silencing pathway can increase virus accumulation compared with that in wild-type. Which RNA silencing-related genes will be upregulated for antiviral defense actually depends on the type of virus 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 additional genes appear to take part in antiviral RNA silencing in other filamentous fungi like *Sclerotinia sclerotiorum* and *Fusarium graminearum* (Mochama et al., 2018; Yu et al., 2018).

In *C. parasitica*, many 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). The observation that deletion of *dcl2* restored maintenance stability and increased transmission frequency of MyRV2 (Fig. 3.11) indicates that RNA silencing is most likely the cause of the low stability of MyRV2 in C18. Therefore, the ability of CHV4-C18 to boost stable maintenance of MyRV2 stability is likely to be associated with its suppressor activity against antiviral RNA silencing.

Single infection by CHV4-C18 did not induce *dcl2* gene, whereas coinfection by CHV4-C18 suppressed, to some extent, the upregulation of *dcl2* being induced by MyRV2 (Fig. 3.12) suggests the presence of an RNA silencing suppressor encoded by CHV4-C18.

However, note that co-infection 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. 3.13). The mechanism by which MyRV2 reproducibly accumulates less in the 5th subculture than in the first subculture in single infections has remained elusive. Host antiviral RNA silencing and CHV4-C18 countermeasures are likely involved in this phenomenon.

Similarly, deletion of *dcl2* gene in C18 strain increased MyRV2 accumulation. Furthermore, deletion of *dcl2* gene also increased the stability and vertical transmission rate of MyRV2. In contrast, the deletion of *dcl2* gene did not affect CHV4-C18 viral titer. These observations suggest that MyRV2 and CHV4-C18 are differently targeted by RNAi-mediated antiviral defense in *C. parasitica.* Restriction of MyRV2 transmission into strains constitutively expressing the *dcl2* gene suggests that MyRV2 is susceptible toward host RNA silencing.

CHAPTER 4. INVESTIGATION OF THE HOST ANTIVIRAL SUPPRESSOR ENCODED BY CHV4-C18

A. Introduction

1. Viral antiviral RNA silencing suppressor

Cellular organisms develop many types of antiviral defenses, and one of them is RNA silencing. To counteract or escape RNA silencing-mediated antiviral defense, viruses evolved various strategies. The expression of RNA silencing suppressor is the most common and widespread strategy used by plant viruses. Virus-encoded RNA silencing suppressor (RSS) was first identified from the synergistic interaction between a potyvirus and a potexvirus (bance, 1991). The study on virus RSS was first conducted to understand viral pathogenicity and virulence. Later, the potyviral protein, helper-componentprotease (HCPro) was shown to suppress host defense mechanisms (Pruss et al., 1997). After the discovery of HCPro, several others viral RSSs have also been discovered. The fact that many viruses having RSS strongly supporting the notion that RNA silencing is an important antiviral host defensive barrier against viruses.

Viral RSSs are highly diverse without having obvious sequence homology. The vast and diverse RSSs have different target within RNA silencing pathways. The increasing numbers of well-characterized RSSs facilitate a better understanding of the molecular basis of RNA silencing suppression. Nearly all plant virus families encode RSS that independently target RNA silencing through diverse mechanisms. In many cases, besides being an RSS, this protein also serves as an essential factor in virus replication or viral pathogenicity. The multifunctional nature of RSS makes it more difficult to explore the suppression mechanism, because inactivation of RSS often resulted in the loss of viability of the virus (Burgyán and Havelda, 2011).

Viral suppression of RNA silencing often poses adverse effects on the host. RSSs that also act as pathogenicity determinants are largely responsible for virus-induced symptoms. In addition to deliberately hijacking host silencing pathways to established optimal infection conditions, viruses can also evade RNA silencing through a range of means including sub-cellular compartmentalization and loss of silencing-target due to high mutation rates (Voinnet, 2005).

In the plant system, there are three common approaches to study viral RSS activities; transient expression assays, a reversal of silencing assay and stable expression assay (Roth et al., 2004). Transient expression assay is the fastest and easiest method for RSS analysis. As the method uses *Agrobacterium* co-infiltration, it enables a large number of protein variants to be tested for suppression activity. Furthermore, this method can also be used to

investigate the effect of RSS on the mobile silencing signal (Kasschau and Carrington, 2001; Ruiz et al., 1998).

Reversal of silencing assay can be used to first identify the candidate virus that may suppress RNA silencing as a major line of counter-defense (Brigneti et al., 1998). This assay usually involves a cross between a silenced transgenic plant and a second transgenic plant expressing candidate viral protein so that the expression of viral RSS could restore the expression of the silenced transgene (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998). Stable expression assay has advantages in that it offers the opportunity to examine the effects of the suppressor on different well-defined types of transgene-induced RNA silencing so that it can elucidate the mechanism of suppression.

2. Mechanism of antiviral suppressor

The lack of sequence similarities among RSS across viral families is one of the issues to fully understanding the molecular mechanism underlying the RNA silencing. In the case of plant viruses, a common feature shared by many viral RSS is they often also function as pathogenicity or host range determinant factors. Many unrelated viral proteins have evolved as silencing-suppressor in addition to their other functions, contributing to the complexity of the character of these types of viral proteins. Moreover, a single virus may encode several distinct suppressors (Lu et al., 2004). Thus, the modes of action of RSS vary depending on the virus, and one suppressor may have more than one mode of action during suppressing RNA silencing.

The most common mechanism of host silencing suppression by viruses is directly interfering with the components of the RNA silencing pathway. Many RSSs either directly bind siRNAs to inhibit RNAi-mediated degradation or block the dicer to process dsRNA elements. A well-known example is p19 RSS protein of tombusvirus that binds siRNA (Silhavy et al., 2002). Because siRNAs are ubiquitous in the RNA silencing mechanism, this finding suggested that p19 would be effective in a broad range of organisms (Voinnet, 2005). HC-Pro RSS of potyvirus is a suppressor that affects the accumulation of miRNA, a small molecular RNA that is implicated in plant development (Kasschau et al., 2003; Mallory et al., 2002).

Another suppression mechanism is the recruitment of endogenous negative regulators of RNA silencing. HC-Pro-interacting factor, a calmodulin-related protein, when is overexpressed, this protein mimics the silencing suppression by HC-Pro RSS, but the affected pathway still unknown (Anandalakshmi et al., 2000). This kind of silencing suppression also identified in a nematode *C. elegans* (Kennedy et al., 2004).

Another mechanism involves modification of the host transcripts. Geminivirus transcriptional-activator proteins (TrAPs) have been identified as RSSs that work at the host DNA level. TrAPs induce over-accumulation of host proteins and result in dominant-

negative effects that interfere through competition of host factors required for the normal function of a protein (Trinks et al., 2005). The well-characterized plant RSSs encoded by plant and animal viruses are listed in the Table 4.1.

Unlike the products of plant virus genes, only a few mycovirus proteins are known to be RNA silencing suppressors and the mechanism of such suppressions are still unclear. However, the extent to which this occurs between fungal RNA silencing and mycoviruses is unclear. In mycoviruses, suppression of *dcl2* was studied mostly in CHV1 through expression of an RSS, papain-like protease p29 (Segers et al., 2006; Segers et al., 2007). There are some examples of RNA silencing suppressions where RSSs, by interfering with the host *dcl2* activity, downregulated the production of siRNAs. In *A.nidulans*, siRNAs derived from the inverted repeat transgene could not be detected after virus infection (Hammond et al., 2008b). Another report of RNA silencing suppressor in fungi was showed in the infection of MyRV3 in *R. necatrix* also suppressed dsRNA-induce transgene RNA silencing and reduced the accumulation of green fluorescent protein (GFP)-siRNA. The S10 gene of MyRV3 has RSS activity via a conventional *in plant* RSS assay (Yaegashi et al., 2013).

The reduction of siRNA is not always the effect of suppression of RNA silencing pathways. For example, Magnaporthe oryzae virus 2 (MoV2, a victorivirus) without directly suppressing the host RNA silencing, evaded host RNA silencing by lowering the level of MoV2-derived siRNA accumulation by an as yet unknown novel mechanism (Himeno et al., 2010). A recent report showed that suppression of *Fusarium graminearum DICER2* and *AGO1* expression by Fusarium graminearum virus 1 (FgV1, a fusarivirus). The suppression of *FgDICER2* and *FgAGO1* was mediated by FgV1 ORF2-encoded product that binds to the upstream region of *FgDICER2* and *FgAGO1*. This example showed that FgV1 interfering with the host RNA silencing induction in a promoter-dependent manner (Yu et al., 2019). Although several examples of mycovirus-derived RSSs have been reported, the mechanisms of how mycoviral RSSs interfere with the host RNA silencing pathway still poorly understood (Hammond et al., 2008b).

Recent studies also provided pieces of evidence for other pathosystem (outside of viruses) that produce effectors with the ability to suppress host RNA silencing pathways. These effectors reduce the maturation of host miRNAs, which are activated by basal defense pathways. A well-characterized nonviral example of an effector was reported from a bacterial pathogen *Pseudomonas syringae* DC3000 (Navarro et al., 2008; Wang et al., 2011). Non-viral eukaryotic plant pathogen that generates silencing suppressor was an oomycete *Phytophthora sojae*, that generates two effectors, one that reduces the abundance of siRNAs, and the other that affects tasiRNAs (Qiao et al., 2013).

Table 4.1 RNA-silencing suppressor encoded by plants, insects and vertebrate viruses (Adapted from Voinnet, 2005)

Viral Genera	Virus Species	Suppressor	Other functions
Positive-strand RNA viruses in plants			
Carmovirus	Turnip Crinkle virus	P38	Coat protein
Cucumovirus	Cucumber mosaic virus; Tomato aspermy virus	2b	Host-specific movement
Closterovirus	Beet yellows virus Citrus tristeza virus	P21 P20 P23 CP	Replication enhancer Replication enhancer Nucleic acid binding Coat protein
Comovirus	Cowpea mosaic virus	S protein	Small coat protein
Hordeivirus	Barley yellow mosaic virus	ɣb	Replication enhancer, movement, seed transmission, pathogenicity determinant
Pecluvirus	Peanut clump virus	P15	Movement
Polerovirus	Beet western yellows virus Cucurbit aphid-borne yellows virus	P0	Pathogenicity determinant
Potexvirus	Potato virus X	P25	Movement
Potyvirus	Potato virus Y Tobacco etch virus Turnip yellow virus	HcPro	Movement, polyprotein processing, aphid transmission, pathogenicity determinant
Sobemovirus	Rice yellow mottle virus	P1	Movement, pathogenicity determinant
Tombusvirus	Tomato bushy stunt virus Cymbidium ringspot virus Carnation Italian ringspot virus	P19	Movement, pathogenicity determinant
Tobamovirus	Tobacco mosaic virus Tomato mosaic virus	P30	Replication
Tymovirus	Turnip yellow mosaic virus	P69	Movement, pathogenicity determinant
Negative-strand RNA viruses in plants			
Tospovirus	Tomato spotted wilt virus	NSs	Pathogenicity determinant
Tenuivirus	Rice hoja blanca virus	NS3	Unknown
DNA viruses in plants			
Geminivirus	African cassava mosaic virus Tomato yellow leaf curl virus Mungbean yellow mosaic virus	AC2 C2 C2	Transcriptional activator protein (TrAP)
Positive-strand RNA viruses in animals			
Nodavirus	Flock house virus, Nodamura virus	B2	Plaque formation
Negative-strand RNA viruses in animals			
Orthomyxovirus	Influenza virus A	NS1	Poly(A) binding, inhibitor of mRNA export, PKR inhibitor
DNA viruses in animals			
Adenovirus	Adenovirus	VA1 RNA	PKR inhibitor
Poxvirus	Vaccinia virus	E3L	PKR inhibitor

B. Material Methods

1. Fungal strain and culturing

Fungal cultures were grown at 22–27ºC on PDA plates with an appropriate antibiotic (Difco) on the bench-top for maintenance or colony phenotypic observations. Fungi were grown on PDB (Difco) or on cellophane-overlaid PDA plates for RNA preparation. The fungal strains, virus strains and primers used in this chapter are listed in Table 4.2 and Table 4.3

Hyphal anastomosis was performed through co-culturing virus-infected donor strain with a virus-free recipient strains at a distance of 1 cm on PDA plate and incubated at room temperature for 10 days. Mycelial plugs were then taken from several positions in the recipient side and subcultured on PDA.

Table 4.2 Fungal strains used for CHV4-C18 RNA silencing suppressor analysis

Strain	Description	References
C18 *dcl2pro*::*egfp*	C18 transformant expressing *egfp* that driven by C18 *dcl2* promoter	This study
C18p24	C18 transformant expressing C18 p24	This study

2. Green fluorescent protein observation

To prepare fungal culture, a micro cover glass (22 mm × 22 mm, thickness 0.12~0.17 mm) (Matsunami, Japan) was placed on the top of cellophane-overlaid PDA and at the edge of micro cover glass, a small plug of fungal culture was placed and allowed to grow for three days. After the mycelia grew through the micro cover glass, the clover glass was detached and placed on top of a micro slide glass (Matsunami). GFP expression was observed using Olympus Fluoview FV1000 confocal laser scanning, microscope (Olympus, Japan)

Table 4.3 List of primers used for analyzing CHV4-C18 RNA silencing suppression

Primer Name	Sequence (5`-3`)	Direction	Remarks
NotI_C18DCL2Pro_R	GCATGCGCGGCCGCCTTGCAGCGTCGTACGACAGAT	Reverse	C18 dcl2pro::egfp
SalI_C18DCL2Pro_F	CCCCCTGTCGACTGAGGAGGGTGGGGACAAAGGT	Forward	
NotI_eGFP_F	GTTAACGCGGCCGCATGGTGAGCAAGGGCGAGGAGC	Forward	C18 dcl2pro::egfp and egfp probe
SphI_eGFP_R	AGGTCAGCATGCTTACTTGTACAGCTCGTCCATG	Reverse	
CHV4-287_F	ATGTCTGAGCAACAACTCATCT	Forward	CHV4 RT-PCR
CHV4-853_R	TGCCATCCACCAGATGCCAGTT	Reverse	CHV4 RT-PCR
DCL2_4025_F	CCTGCCCTGTTCAGTATCA	Forward	C18 dcl2 KO
DCL2_4545_R	GTGGTAGCCCTCTCTTTGAC	Reverse	C18 dcl2 KO
pCold-I-KpnI-CHV4P52_F	CCCATATGGAGCTCGGTACCATGTCTGAGCAACAACTCATCTA	Forward	CHV4 protease cleavage assay
pCold_NdeI-CHV4-p52_F	ATATCGAAGGTAGGCATATGTCTGAGCAACAACTCATCTA	Reverse	CHV4 protease cleavage assay
pCPXHY3-HpaI-CHV4p24_F	ACGCGGCCAAGCTTGTTAACATGTCTGAGCAACAACTCATCTA	Reverse	C18 p24
pCPXHY3-HpaI-CHV4P24_R	ATGCGCGGCCGCGTTAACCCAAGGCGTGCACGCTTTGTC	Reverse	C18 p24
MyRV2-E2-10_300_F	AATTCAATTCCGCGCGAAGGGG	Forward	MyRV2 S10 probe
MyRV2-E2-10_1200_R	TTCATTTTCACGTTGTTAAAAC	Reverse	MyRV2 S10 probe
5'UTR_CHV1_F	GATAATTTTGGTTGCTGCAC	Forward	CHV1-Δp69 RT-PCR and probe
5'UTR_CHV1_R	GACTCATGTGGCGACGTGCC	Reverse	CHV1-Δp69 RT-PCR and probe

3. Protein expression and purification

The fusion protein was expressed from pColdI (Takara Bio). Sequence of interest derived from PCR amplicon was inserted into the vector following the In-Fusion cloning method (Takara Bio). Individual expression plasmid was transformed into *Escherichia coli* BL21 strain and grown overnight. Precultured bacteria (2ml) were added into 100 ml of fresh LB amended with ampicillin and incubated at 37ºC for one hour. Cultured media was then transferred to a shaking incubator and incubated at 15ºC with shaking at 150 RPM for 30 mins. The cultured was then induced with 0.3 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and incubate at 15ºC for 4 h. Harvested cells were centrifuged at 2,800 *g* for 5 mins at 4ºC. Pelleted cells were vortexed with 1.5 ml extraction buffer (100 mM Hepes-KOH, 300 mM NaCl, 0.5% Triton X-100, and 1× proteinase inhibitor (Toyobo)) followed by sonication to lyse the cells. Lysed-cells were centrifuged at 25,000 *g* for 10 mins at 4ºC. The supernatants were collected and incubated with the anti-HA beads (Thermo Scientific, Waltham, MA, US) in a rotating machine for 1 h at 4ºC, followed by three times washing with wash buffer (100 mM Hepes-KOH, 300 mM NaCl, 0.5% Triton X-100). The purified protein was eluted with required amount of water.

4. SDS-PAGE

Purified protein was dissolved in equal volume of 2× PAGE loading buffer (0.1 M Tris-HCl pH 6.8; 4% SDS; 12% β-mercaptoethanol, 20% Glycerol; 0.005% Bromophenol blue) for SDS-polyacrylamide gel electrophoresis (PAGE), and boil the sample for 5 mins followed by centrifugation at 25,000 *g* speed for five minutes. Sample was then run on a denaturing polyacrylamide gel consisting of stacking gel (4.75% Acrylamide/Bis (29:1); 125 mM Tris HCl pH 6.8; 0.1% SDS; 0.1% APS (Ammonium persulfate); 0.1% TEMED (Tetramethylethylenediamine)) and resolving gel (10% Acrylamide/Bis (29:1); 375 mM Tris HCl pH 8.8; 0.1% SDS; 0.1% APS; 0.05% TEMED) in 1× running buffer (25 mM Tris; 192 mM Glycine; 0.1% SDS). Transfer the protein from the gel into the membrane after gel running finished. Protein were subsequently transferred onto polyvinylidene diflouride (PVDF) membrane using 10mM CAPS buffer containing 10% methanol and followed with amido black staining (0.1% amido black, 10% acetic acid, 40% methanol).

5. Amino acid sequence

The specific band obtained from amido black staining was then excised from the membrane and processed for amino acid sequences on a gas-phase protein sequencer Shimadzu Model PPSQ-31A (Kyoto, Japan) at the Department of Instrumental Analysis and Cryogenics, Advanced Science Research Center, Okayama University.

6. Small RNA analysis

Total RNA extracted from virus-infected *C. parasitica* C18 was subjected to small RNA sequencing analysis. Small RNA cDNA library preparation and subsequent deep sequencing with Illumina platform (HiSeq 2500; 50-bp single-ends reads) were conducted by Macrogen Inc. (Tokyo, Japan). After trimming adapters with low-quality base and size filtering (15 to 30 nt in length), the retained read sequences in each library were mapped into each constitutive virus genome using CLC Genomic Workbench (version 11; CLC Bio-Qiagen). The virus-derived small RNA reads were used for in-depth analysis with the program MISIS-2 (Seguin et al., 2016).

C. Result

1. Development of a method for assessing RNA silencing suppressor activities

The previous chapter showed that CHV4-C18 allowed stable maintenance of co-infecting MyRV2 in *C. parasitica* C18 strain likely through suppression of antiviral RNA silencing. This observation strongly suggests that CHV4-C18 has the ability to suppress RNA silencing similar to the case of a multifunctional protein, CHV1 p29 that suppresses *dcl2* upregulation upon virus infection. To examine the RNA silencing suppression activities of CHV4-C18, I developed a reporter construct containing an *egfp* gene whose transcription is driven by the *dcl2* promoter derived from C18 strain (Fig. 4.1A). This construct was transformed to the C18 strain and positive transformants were selected on PDA containing hygromycin B (C18 *dcl2pro*::*egfp*).

First, I examined the transformants for their ability as reporter strains for *dcl2* induction. MyRV2, CHV4-C18, CHV1, and CHV1-Δp69 were introduced to induce the *dcl2* gene upregulation in the reporter strains. Virus infection was validated through dsRNA or RT-PCR (Fig. 4.1B). Note that CHV1-Δp69 and MyRV2 are known to highly elevate the *dcl2* transcript level. As per expectation, CHV1-Δp69 and MyRV2 highly upregulated *egfp* transcription in these reporter strains, whereas CHV1 and CHV4-C18, did not (Fig. 4.1C). The *dcl2* and *egfp* transcript upregulation was correlated well; viruses that highly induced *dcl2* also highly induced *egfp* transcripts (Fig. 4.1B). Importantly, *dcl2* and *egfp* transcription results were consistent with the fluorescent image observed through confocal microscopy (Fig. 4.2), validating that the transformant successfully serves as a reporter strain.

To test the suppression activities of CHV4-C18, reporter fungal strains, infected with CHV1-Δp69 and MyRV2, were then co-infected with CHV4-C18 through hyphal anastomosis. The infection of CHV4-C18 was confirmed with RT-PCR (Fig. 4.3, right-side

panel). Interestingly, the co-infection of CHV4-C18 reduced the *dcl2* and *egfp* transcript upregulation that was analyzed through the northern blot (Fig. 4.3, right-side panel). Confocal laser microscopy also confirmed the reduction of green fluorescent intensity after co-infection with CHV4-C18 (Fig. 4.3, left panel). These results strongly suggest that CHV4-C18 has RNA silencing suppressor activities, and the developed reporter fungal strain is useful for assessing RSS activities.

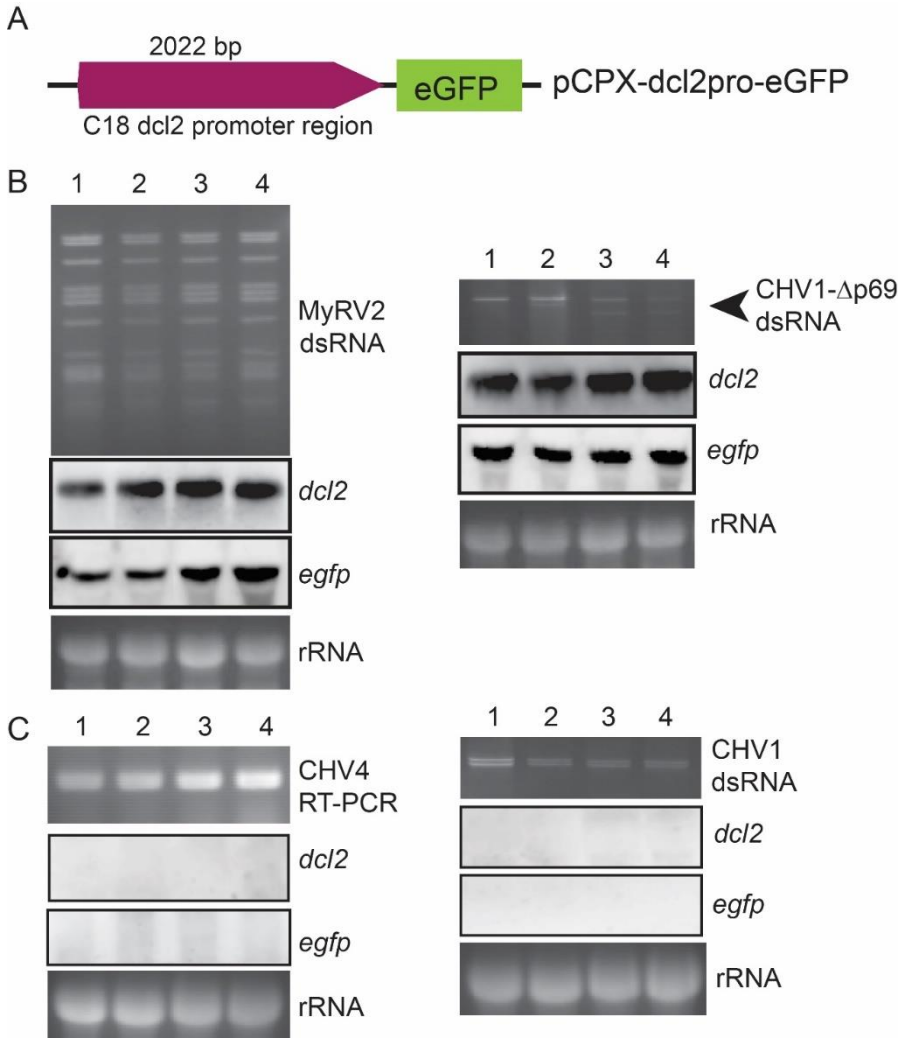

Fig. 4.1 *eGFP* reporter and expression assays after virus infections. (A) The *egfp* reporter construct. Expression of *egfp* was driven by *dcl2* promoter derived from *C. parasitica* C18 strain. (B) High level of *egfp* expression was observed after the host strain infected with MyRV2 and CHV1-Δp69. This expression pattern is correlated with *dcl2* expression. (C) CHV1 and CHV4-C18 infection did not induce expression of *egfp* and *dcl2*.

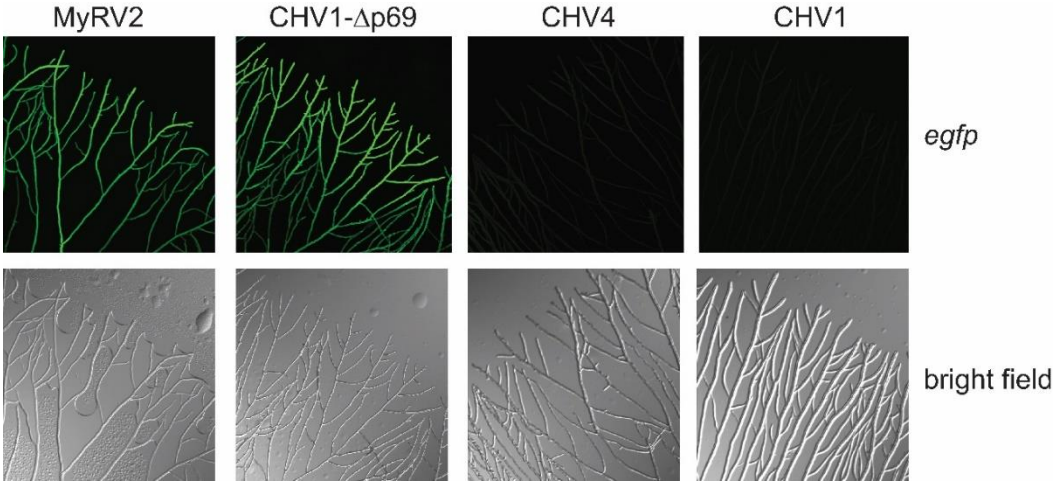

Fig. 4.2 Confocal laser microscopy imaging showing high level of fluorescence in MyRV2 and CHV1-Δp69-infected reporter strains (C18 *dcl2*pro::*egfp*). Strains infected with either CHV1 or CHV4-C18 showed no fluorescence.

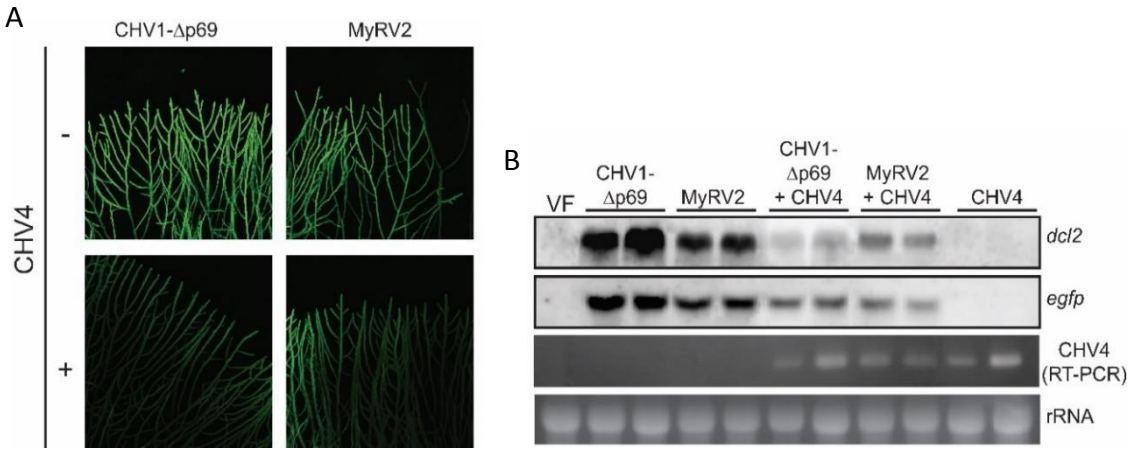

Fig. 4.3 Effect of CHV4-C18 coinfection to *egfp* expression. (A) Fluorescence imaging of CHV1-Δp69 and MyRV2-infected reporter (C18 *dcl2*pro::*egfp*) strain with or without CHV4-C18 co-infection. (B) Gene expression assays of *dcl2* and *egfp* through northern blotting. *dcl2* expression was correlated with *egfp* expression. Co-infection of MyRV2 and CHV1-Δp69 with CHV4-C18 reduces upregulation of *egfp* expression.

2. Viral small RNA profiles of CHV4-C18 and MyRV2 in single and double infections

It is known from the previous result that the disruption of *dcl2* in *C. parasitica* affecting the small RNA profile of CHV1-Δp29 (Andika et al., 2019). The deletion of *dcl2* resulting in the absence of minus-strand small RNA and size distribution change of plus-strand small RNA (Andika et al., 2019). To investigate the small RNA profiles of CHV4-C18 and MyRV2 in single and double infection, small RNA sequencing analysis using deep sequencing was carried out. The standard *C. parasitica* strain EP155 was infected with CHV1, a well-known hypovirus having an RNA silencing suppressor, was used as a control. CHV1 small RNA showed less accumulation in the negative strand of small RNA of CHV1 (Fig. 4.4A, right). Small RNA size distribution showed a peak in 21 nt in both sense and antisense, a typical distribution of viral siRNA. This showed that although the upregulation of *dcl2* is being suppressed but the enzymatic activity of *dcl2* is not completely suppressed by CHV1 RSS, p29.

Interestingly, single infection of CHV4-C18 alone showed small RNA distribution profile similar to that of small RNA profile in Δ*dcl2* mutant in EP155 genetic background infected with CHV1-Δp29 (Fig. 4.4A, left) (Andika et al., 2019) in which viral small RNA pool consisted of predominantly positive-strand with wide size distributions. Unlike CHV1, CHV4-C18 could not be detected through routine dsRNA extraction. The low amount of CHV4-C18 dsRNA accumulation may be the reason for the unusual profile of small RNA distribution. If there is no high-level of dsRNA accumulation in the cells, dsRNA processing may not be happening predominantly by *dcl2*. It is possible that another type of endonuclease might function against CHV4-C18 (Andika et al., 2019). Importantly, in co-infection with MyRV2, CHV4-C18 small RNAs profile distribution showed both positive and negative strand small RNAs with the size of 21 nt as the highest peak (Fig. 4.4B). Thus, MyRV2 dsRNA maybe triggering *dcl2* activity and *dcl2* cleaves the CHV4-C18 dsRNA. Moreover, after normalization, level of siRNA in CHV4-C18 and MyRV2 doubly infected strain was reduced compared with that in MyRV2 singly infected strain, suggesting the RSS activity of CHV4-C18.

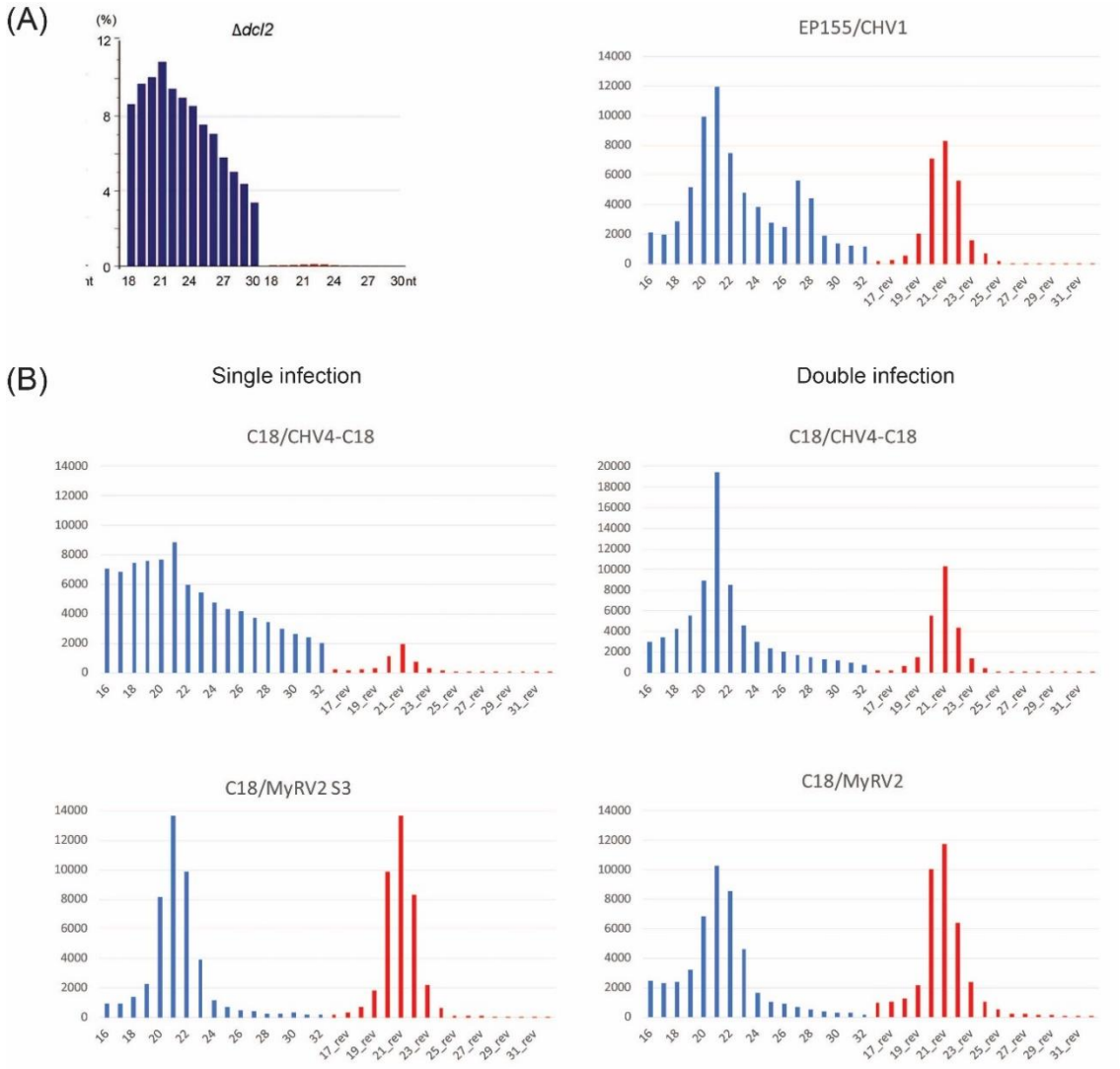

Fig. 4.4 Small RNA profiling of *C. parasitica* C18 in either singly or doubly infected by CHV4-C18 and MyRV2. (A) small RNA profile in *dcl2* knockout mutant of the standard model host EP155 infected with CHV1-Δp69 and wildtype standard model host strain EP155 infected with CHV1. (B) Small RNA profile in single and double infection of CHV4-C18 and MyRV2 after normalized.

3. Mapping of the functional domain of papain-like protease encoded by CHV4-C18

The multifunctional protein p29 encoded by the prototype hypovirus CHV1-EP713 is the first RSS that was identified from mycoviruses (Segers et al., 2006; Suzuki et al., 2003). CHV1-p29 is cleaved from ORF A-encoded polyprotein p69 by its autocatalytic protease activity in a co-translational manner and exerts RSS activities through the cancellation of the *dcl2* transcriptional upregulation. My results showed that CHV4-C18 has RSS activity.

Polyprotein encoded by CHV4-C18 and another strain CHV4-SR2 is predicted to have at least 5 functional domains such as those for protease, glycosyltransferase, and RdRp (Fig. 2.3) (Linder-Basso et al., 2005). I hypothesized that the RNA silencing suppressor of CHV4-C18 resides in the N-terminal protease coding domain of its polyprotein, similar as in the case of CHV1-p29.

To investigate this hypothesis, I first attempted to determine whether the CHV4-C18 polyprotein is cleaved by the N-terminal coding domain. The previous study has shown that the N terminal of CHV4 showed to have papain-like protease motifs as observed in CHV1 but the cleavage site of this papain-like protease is remained unclear (Linder-Basso et al., 2005). To this end, a pCold-I-based expression construct, containing the presumed cleavage site and further downstream coding region, was used to express the N-terminal portion of CHV4 polyprotein (Fig. 4.5A). The construct carried a hexa histidine-tag and a GST (glutathione S-transferase) tag at the C-terminus and an HA (hemagglutinin)-tag at the N-terminus. The CHV4-C18 derived sequence spans from nucleotide map positions 287 to 1706 encoding 473 amino acids from position 1 to 213 with the estimated molecular weight of 52 kDa. The total molecular weight of the CHV4-C18 protease and the tags was ~84 kDa.

The recombinant protein that was expressed in *E. coli* cells, and total cell extract was run on SDS-PAGE followed by Coomassie Brilliant Blue staining. Several prominent protein bands were observed after the staining in the cell cultures that induced by cold shock and IPTG treatment (Fig. 4.5B). To obtain the expected protein, cell extract was purified with anti-HA beads. The purified protein fraction was subjected to SDS-PAGE gel and stained thereafter with amido-black dye. A specific protein band with the size of ~36 kDa was detected in purified preparations after induction by cold shock and IPTG treatment (Fig. 4.5B), while many other minor proteins assumed to be derived from *E. coli*, also appeared. The 36 kDa protein was not detectable in negative controls (*E. coli* without cold shock and IPTG induction treatment).

This protein band was then transferred onto the membrane and subjected to amino acid sequencing. Table 4.4 showed a sequence located after the His and Cys residues in CHV4-C18 papain-like protease at the amino acid position 214/215. The same cleavage site motif is also present in the papain-like protease of CHV1. The similarity between these two papain-like proteases cleavage motifs being encoded by CHV4-C18 and CHV1 suggests that CHV4-C18 also encode self-cleavage papain-like protease at the N-terminal. Based on the amino acid weight calculation, the molecular weight of CHV4-C18 papain-like protease is 24 kDa, and therefore we refer this protease as p24 (Fig. 4.6).

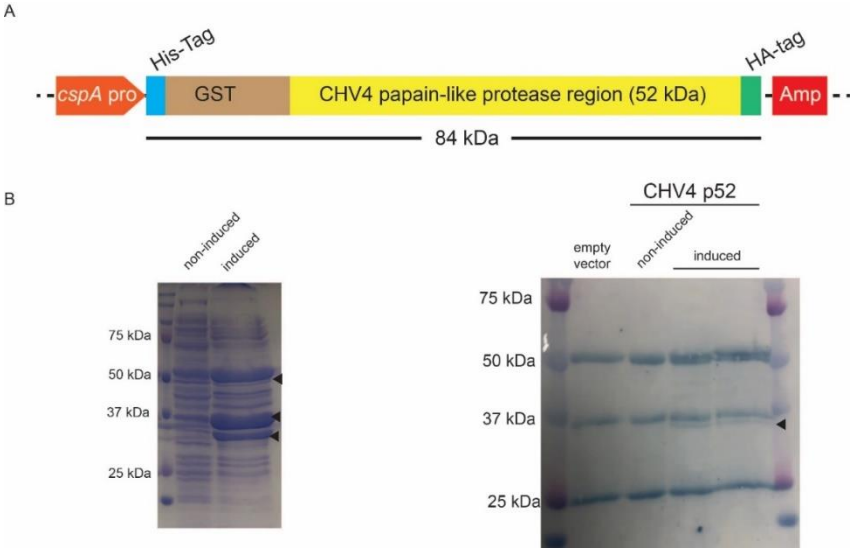

Fig. 4.5 Putative CHV4-C18 papain-like protease self-cleavage analysis. (A) Schematic diagram of the C18 p52 construct in pColdI expression vector. (B) SDS-PAGE of crude (left) and purified (right) cell extracts.

Table 4.4 Amino acid sequence of N-terminal of a cleaved product derived from CHV4-C18 p52 construct

		1	2	3	4	5	6	7	8	9	10
Automatic analysis results	1st	**G**	M	**E**	T	**D**	**A**	**D**	L	V	K
	2nd	S	**R**	V	**S**	L	G	P	**S**	**H**	**P**
	3rd	Q	D	L	Y	P	P	S	E	N	N
	4th	A	V	P	P	N	E	N	P	P	E
Reliability (%)		19.0	14.1	29.7	13.0	22.3	26.3	97.2	12.9	36.9	10.2

```
MSEQQLIYGE VGPPPIKAAP QRGSIPTKLE YHDLDQFAWL GDVWLTMCLR LTCVTRHSVV
DQRWCETILS NANLRRFLTD DGWIVPDSLS DHGAGQSAEF LFCRDSDFRR RFCTAHGLVE
AHTGLADLTP YGAEAGRDVD QCWRRLFRGP VTGHYSFPLE KWMSRAQLEE VAGQNALSSG
SFGLEVDGTN WHLVDGSMSA REVLASLTKR ARLG↓GRESDA DSHP
```

Fig. 4.6 Identification of cleavage site of CHV4-C18 papain-like protease on the polyprotein. The cleavage site is indicated by an arrow

4. The p24 papain-like protease of CHV4-C18 functions as an RSS

After determining that N-terminal polyprotein encoded by CHV4-C18 is proteolytically processed to p24, the investigation of p24 as an RSS was performed. The p24 domain encoding DNA fragment was introduced into a fungal-based expression vector. A *gpd* promoter was used to drive the expression of CHV4-C18 p24. This construct was then

transformed into the C18 virus-free wild-type strain. After getting p24-positive transformants (C18p24), *dcl2* induction assay was carried out to examine the ability of p24 to suppress *dcl2* expression. These transformants were inoculated with CHV1-Δp69 through hyphal anastomosis. Virus infection was validated by RT-PCR and *dcl2* upregulation after virus infection was analyzed by northern blotting. The transformants that showed a reduction in *dcl2* expression were selected for inoculation with MyRV2 (Fig. 4.7A).

Selected fungal transformants were co-cultured with MyRV2 to check whether p24 could facilitate the horizontal transmission of MyRV2. As expected, efficient horizontal virus transmission between fungal transformants expressing p24 with MyRV2-infected fungal strain was confirmed. As a control, virus-free wild-type C18 strain was unable to receive MyRV2 from MyRV2-infected strain (Fig. 4.7B). MyRV2 accumulation in p24 transformant background was compared with MyRV2 accumulation in CHV4-C18 co-infection background using northern blotting. MyRV2 accumulation was lower in p24 transformant than CHV4-C18 co-infected condition. Strikingly, in p24 transformant infected with MyRV2, *dcl2* transcript induction was suppressed at the level similar to the double infection condition (Fig. 4.8).

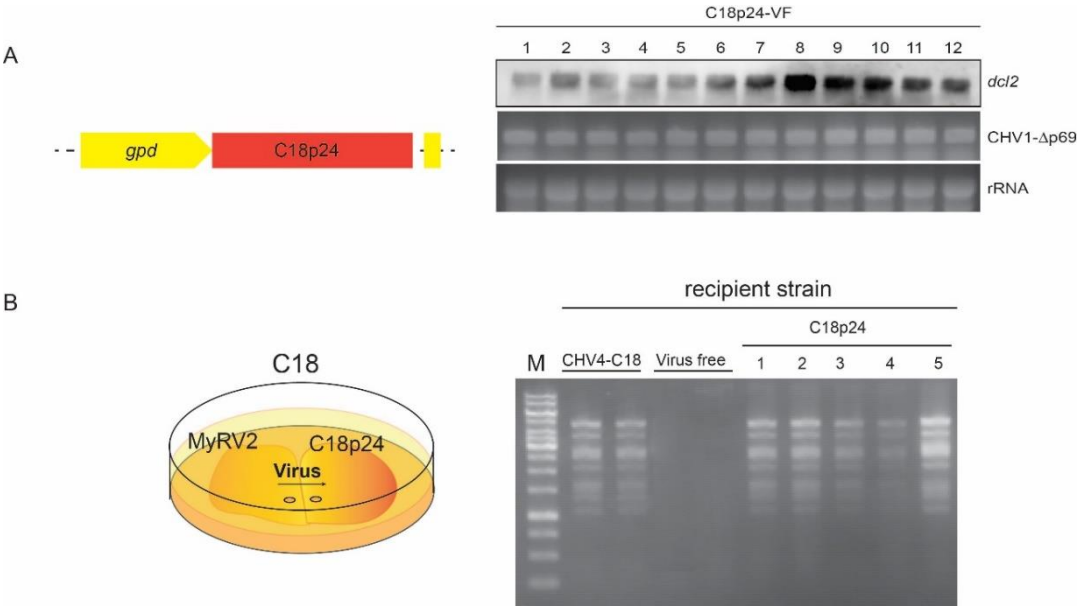

Fig. 4.7 CHV4-C18 function as an RSS in C18 strain. (A) construct of CHV4-C18 p24 expressed under *gpd* promoter in C18. p24 can suppress *dcl2* transcript upregulation after CHV1-Δp69 infection in C18 strain, and virus infection was validated with RT-PCR. (B) C18p24 that showed strong *dcl2* suppression and C18 strain that pre-infected with CHV4-C18 were allowing hyphal transmission of MyRV2. But no transmission was observed when C18 VF strain was used as recipient.

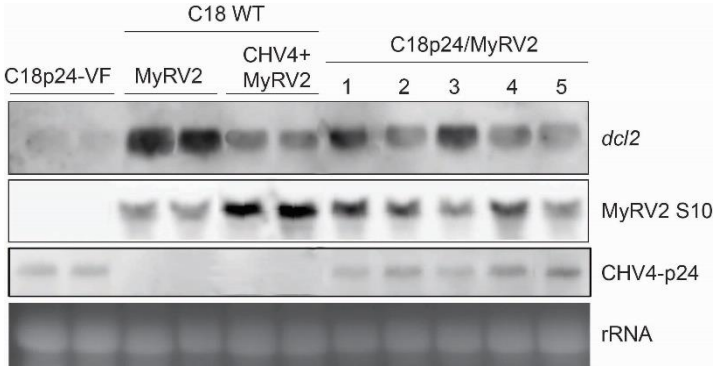

Fig. 4.8 RNA blot analysis of *dcl2* transcript and virus accumulation after MyRV2 infection in C18p24 transformant

5. CHV4-C18 p24 facilitates MyRV2 stable infection

My result showed that co-infection of MyRV2 with CHV4-C18 leads to a stable infection of MyRV2. Based on this result, I wanted to examine whether p24 expression can also facilitate stable infection of MyRV2 in the C18 strain. To address this question, a successive sub-culture experiments of MyRV2 in p24 transformant were conducted. MyRV2 co-infected with CHV-C18 was used as positive control and MyRV2 single infection was used as a negative control. Three replicated fungal isolates for each independent line were used and subcultured in 10 days interval.

Virus infection was observed at 1st, 3rd, 5th, 7th, and 10th subculture. Virus infection was validated through RT-PCR. MyRV2 infection was retained to the 10th subcultured in CHV-C18 co-infected strain and in p24 transformants, although not 100%(Fig. 4.9). This result suggests that p24 suppresses *dcl2* induction to facilitate MyRV2 stable infection in the fungal host.

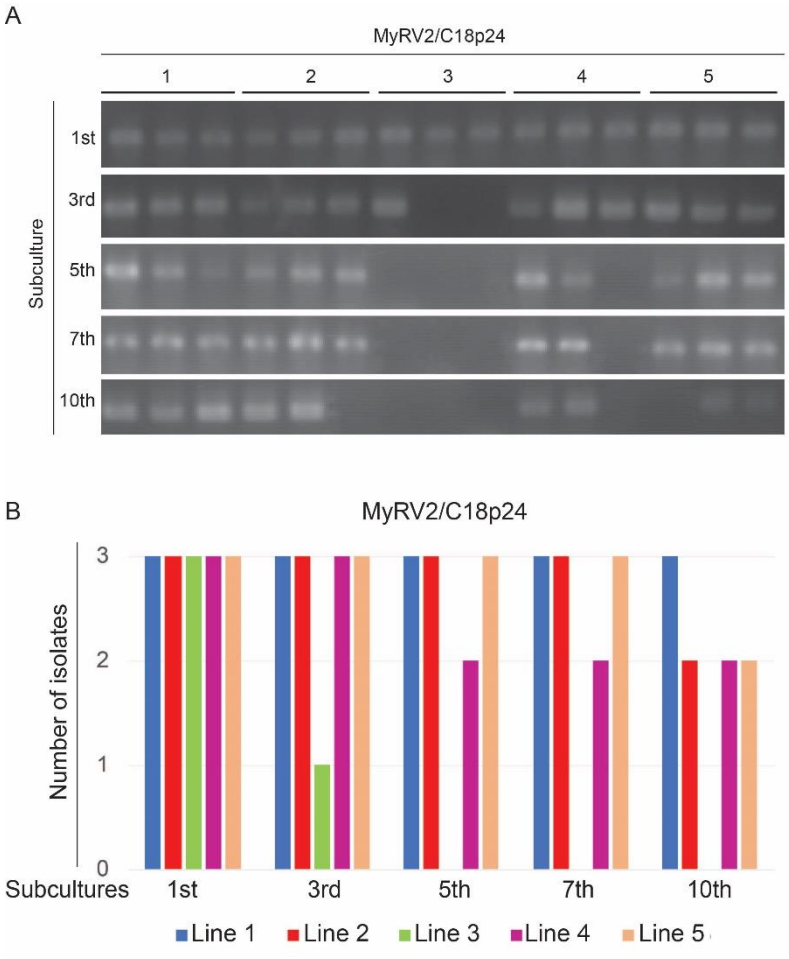

Fig. 4.9 CHV4-C18 p24 facilitates MyRV2 stable infection upon subculturing. (A) MyRV2 infection upon subculturing in five lines of C18p24 was validated through RT-PCR. (B) Graphical representation of MyRV2 infection in each line of C18p24 upon subculturing

D. Discussion

Suppression of RNA silencing is one of the strategies by virus to counteract host antiviral machinery. RNA silencing suppression mechanism in virus-infected animals and plants have been well studied. On the other hand, study of RNA silencing suppression mechanism in virus infecting fungi is still poorly understood. Only a few mycovirus proteins have been identified as RSSs; among them are VP10 of another mycoreovirus, MyRV3, p29 of the hypovirus CHV1 and ORF2 of FgV1 (Segers et al., 2006; Yaegashi et al., 2013; Yu et al., 2019). VP10 can suppress transgene (GFP) RNA silencing in plants and possibly in fungi (Yaegashi et al., 2013). The multifunctional protein p29 serves as RSS for CHV1 (Segers et al., 2006), as well as a protease that plays roles in polyprotein processing

(Choi et al., 1991a; Choi et al., 1991b), and also as a symptom determinant (Craven et al., 1993; Suzuki et al., 1999).

There are many examples of mycoviruses that manipulate host siRNAs profile to evade the host antiviral defense (Hammond et al., 2008b; Himeno et al., 2010). The siRNA profiling in *C. parasitica* C18 strain with a single or double infection showed an interesting result where CHV4-C18 single infection has an unusual small RNA size distribution. The co-infection of CHV4-C18 with MyRV2 restored the normal small RNA size distribution of CHV4-C18 most probably due to dsRNA of MyRV2 that can be recognized by the cellular antiviral system and trigger *dcl2* upregulation that cleaves dsRNA of CHV4-C18. These results suggest that CHV4-C18 does not trigger *dcl2* upregulation and CHV4-C18 may produce a low level of dsRNA so that it cannot be recognized by *dcl2* of the fungal host.

Here, I developed a rapid assay system to examine mycoviral RSS. A GFP reporter that is driven by a *dcl2* promoter was transformed into fungus and followed by virus inoculation. MyRV2 infection induced a high level of GFP fluorescent while CHV4-C18 infection did not. However, after the co-infection of these two viruses in the same fungus, change in GFP fluorescent could be detected. The reduction in GFP fluorescence intensity after co-infection is an obvious indication of the RSS activity. Thus, this is a good and rapid method for detecting RSS activity conferred by a mycovirus.

The result of reporter assay strengthens the notion that CHV4-C18 has the ability to suppress antiviral RNA silencing. Identification of RSS in CHV4–C18 is not easy based solely on sequence comparison, because no clear homolog of CHV1 p29 has been detectable on a polyprotein of CHV4–C18. However, previous research already reported several putative cleavage sites in the CHV4-SR2 polyprotein (Linder-Basso et al., 2005). Based on the reported result, functional mapping to identify a protease in CHV4–C18 or an RNA silencing suppressor that possibly potentiates the enhancement of vertical transmission and stability of MyRV2 in the host strain has been performed. Similar cleavage site motifs and self-cleavage ability of CHV4-C18 with that observed in CHV1 suggest the similarity between CHV4-C18 p24 and CHV1 p29 and thus p24 might have a similar mode of action with p29 to suppress RNA silencing mechanism.

CHV4-C18 p24 is also able to facilitate better transmission and more stable infection of MyRV2 without increasing the accumulation of MyRV2. So far, there is no apparent benefit for CHV4-C18 can be observed during co-infection conditions. Nevertheless, the change of small RNA profiles during co-infection may provide some mechanistic insights that need to be further examined.

CHAPTER 5. GENERAL DISCUSSION AND SUMMARY

The filamentous ascomycete, *C. parasitica*, causes one of the three most destructive tree diseases, chestnut blight. However, biological control using mycoviruses (so called "virocontrol") of this disease has been practiced in Europe and some areas of North America. Only hypovirulence-inducing CHV1 has been used in virocontrol thus far, despite the fact that *C. parasitica* can host a number of mycoviruses with different genome types. In fact, there are many reported mixed-infection cases in the chestnut fungus. Effects of mycovirus co-infections on host fungi are diverse, involving alterations in the host transcriptome, small RNAome, proteome, metabolome, lipidome, and epigenome. Co-infections of single fungal host strains by multiple viruses are common, but constraints on the accumulation of multiple viruses in a single host remain unclear. Interactions of co-infecting viruses are categorized into a few groups such as synergistic, mutualistic and antagonistic interactions. These interactions are of great importance from perspectives of practical virocontrol and basic virology as well. Here, I showed interesting interplays between two distinct RNA viruses, a mycovirus MyRV2 with a 11-segmented dsRNA genome and a hypovirus CHV4-C18 with a (+)RNA genome in *C. parasitica* where CHV4-C18 commensally facilitates stable infection by and trans-enhances vertical transmission of MyRV2.

A previous study reported that co-infection of CHV1 with MyRV1 under the lab condition resulted in one-way synergism where CHV1 enhances the replication and vertical transmission of more severe phenotypic change compare with single infection of either virus (Sun et al., 2006). Similarities between the CHV1/MyRV1 and CHV4-C18/MyRV2 interactions include one-way synergism between a hypovirus and a mycoreovirus. However, their synergistic effects are different; that is, the augmentation of MyRV2 accumulation by coinfecting CHV4-C18 was not observed but instead, MyRV2 accumulation, which otherwise decreased, was stably maintained by it (Chapter 3). Importantly, CHV1 reduces the hypovirulence of MyRV1-infected fungal colonies, thus negatively affecting virocontrol potential of MyRV1. Because MyRV2 was unstable in the original *C.parasitica* host strain C18 or the standard strain EP155 without CHV4-C18, I could not compare the virulence level of fungal colonies singly infected by MyRV2 and doubly infected by MyRV2 and CHV4-C18. However, the original, doubly infected fungal strain shows hypovirulence (Enebak et al., 1994b; Hillman and Suzuki, 2004), to which CHV4-C18 is now known to indirectly contribute by allowing MyRV2 stable maintenance. Differences between the two systems include symptomatology of the four player viruses. Of particular note is the symptomless nature of CHV4-C18 able to exert synergistic effect on MyRV2, which contrasts the profound symptomatic infection by CHV1. All of the other viruses show symptomatic infections in *C. parasitica*.

It was anticipated that the observed commensal interactions between CHV4-C18 and MyRV2 was associated antiviral RNA silencing and its inhibition by CHV4. This possibility was supported by a few observations. First, CHV4-C18 suppressed the transcriptional induction of an RNA silencing key gene, *dcl2* that was highly up-regulated by MyRV2 single infection as shown in Chapter 3. This notion was confirmed by directly comparing *dcl2* transcript levels via northern blot analyses between single infectants with MyRV2 and double infectants with CHV4-C18. In addition, a newly developed GFP-based reporter system provided supportive data. Second, an RNA silencing deficient mutant lacking the *dcl2* gene, which was prepared by targeted disruption via homologous recombination, allowed for stable maintenance and efficient vertical transmission of MyRV2 in the absence of CHV4. This phenotype was very similar to that of the fungal strain C18 co-infected by MyRV2 and CHV4-C18 (Chapter 3). These results suggest that MyRV2 is susceptible to host antiviral RNA silencing, and CHV4-C18 suppresses this host defense to facilitate MyRV2 stable infection and efficient transmission.

I also designed experiments to identify a possible CHV4-C18 RSS (Chapter 4). I referred to available information on a well-established RSS, CHV1 p29, which also act as a papain-like protease and a symptom determinant (Hillman and Suzuki, 2004; Segers et al., 2006; Suzuki et al., 1999; Suzuki et al., 2003). CHV1 p29 exerts its suppressor activities via inhibiting the transcriptional up-regulation of the RNA silencing key genes such as *dcl2* and *agl2* (Chiba and Suzuki, 2015; Sun et al., 2009). This transcriptional regulation requires DCL2 as a positive feed-back player and a general transcriptional co-activator, the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex (Andika et al., 2017). In fact, DCL2 is involved in transcriptional up-regulation of not only *dcl2* and *agl2* but also many other host genes and some of them play in another layer of antiviral defense to mitigate virus symptom induction (Andika et al., 2019). How CHV1 p29 inhibits the RNA silencing pathway is unknown. CHV4-C18, a member of the same virus family, *Hypoviridae* as CHV1 (Suzuki et al., 2018), has a candidate, designated as p24, of the papain-like protease encoded in the most N-terminal portion of the polyprotein (Linder-Basso et al., 2005). First, I confirmed the self-cleavage activity of CHV4-C18 p24 and identified its cleavage site in *E. coli*. The RSS activity of CHV4 -C18 was mapped to the protease p24 by combined transformation and GFP-reporter assay with different RNA silencing triggers. Namely, transgenic expression of CHV4-C18 p24 led to the reduced levels of *dcl2* in infectants with trigger viruses relative to negative controls infected with the same viruses (Chapter 4). Furthermore, transgenic *C. parasitica* strains with CHV4-C18 p24 manifested stable maintenance during repeated sub-culturing of, and efficient vertical transmission of, MyRV2 in the absence of CHV4-C18 infection (Chapter 4). These results clearly indicate that CHV4-C18 p24 is multifunctional in its polyprotein processing and suppression of RNA silencing. CHV4-C18 p24 will be among only a few established RNA silencing suppressors of fungal virus origin.

Collectively, this study shows a commensal interplay between the two naturally coinfecting mycoviruses, CHV4-C18 and MyRV2, in which CHV4-C18 facilitates stable infection by and efficient vertical transmission of MyRV2, likely through the RSS activities of multifunctional CHV4-C18 p24. The interaction between these two viruses may be associated with the natural infection of C18 by MyRV2 that otherwise would readily be lost from infectants.

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