Mycoviruses in Biological Control: From Basic Research to Field Implementation

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Accepted for publication 7 August 2019.

ABSTRACT

Mycoviruses from plant pathogens can induce hypovirulence (reduced virulence) in their host fungi and have gained considerable attention as potential biocontrol tools. An increasing number of mycoviruses that induce fungal hypovirulence, from a wide variety of taxonomic groups, are currently being reported. Successful application of these viruses in disease management is greatly dependent on their ability to spread in the natural populations of the pathogen. Mycoviruses generally lack extracellular routes of transmission. Hyphal anastomosis is the main route of horizontal mycovirus transmission to other isolates, and conidia of vertical transmission to the progeny. Transmission efficiencies are influenced by both the fungal host and the infecting virus. Interestingly, artificial transfection methods have shown that potential biocontrol mycoviruses often have the ability to infect a variety of fungi. This expands their possible use to the control of pathogens others than those where they were identified. Mycovirus research is also focused on gaining insights into their complex molecular biology and the molecular bases of fungus–virus interactions. This knowledge could be exploited to manipulate the mycovirus and/or the host and generate combinations with enhanced properties in biological control. Finally, when exploring the use of mycoviruses in field conditions, the pathogen life style and the characteristics of the disease and crops affected will deeply impact the specific challenges to overcome, and the development of biocontrol formulations and delivery methods.

MYCOVIRUSES EXHIBIT HIGH TAXONOMIC DIVERSITY

A characteristic feature of mycoviruses is that they generally lack an extracellular phase in their life cycles, depending exclusively on intracellular mechanisms for their transmission. The main routes of mycovirus transmission are through spores (vertical transmission to the progeny) and by hyphal anastomosis (horizontal transmission between different fungal isolates) (Pearson et al. 2009; Xie and Jiang 2014). They are therefore considered intrinsic genetic elements of the fungal isolates that host them and it was thought they had evolved to cause mainly cryptic infections. That notion changed with the identification of *Cryphonectria parasitica* hypovirus 1 (CHV1) as a mycovirus that had entered the European

populations of *Cryphonectria parasitica* and spread spontaneously, slowing the epidemic of chestnut blight (Heiniger and Rigling 1994). The recognition that mycoviruses can induce hypovirulence (reduced virulence) in their hosts elicited great interest in characterization of viruses from phytopathogenic fungi because of their potential as biocontrol tools. Scores of mycoviruses are now reported. The majority of viruses from filamentous fungi possess either double-stranded RNA (dsRNA) genomes or positive sense (+) single-stranded RNA (ssRNA) genomes with dsRNA replicative intermediates, and can possess a capsid forming true virions or be capsidless. CHV1 is the founding member of the (+) ssRNA mycovirus family *Hypoviridae*, the first one described whose members lack a capsid (Dawe and Nuss 2013; Hillman and Suzuki 2004). Three other hypoviruses have been identified in *C. parasitica*, named CHV2, CHV3, and CHV4 (Hillman and Suzuki 2004). The isolation of *Sclerotinia sclerotiorum* hypovirus 1 (SsHV1) and *Valsa ceratosperma* hypovirus 1 (VcHV1) (Xie et al. 2011; Yaegashi et al. 2012) provided the first evidence that the family *Hypoviridae* was not restricted to *Cryphonectria* species as hosts. Two additional families, *Narnaviridae* and *Endornaviridae*, comprise capsidless (+) ssRNA mycoviruses. Recently, it has been proposed that unclassified yadokariviruses may conform a new family of capsidless (+) ssRNA mycoviruses, family

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary file is published online.

The author(s) declare no conflict of interest.

Yadokariviridae (Hisano et al. 2018). Encapsidated (+) ssRNA mycoviruses include families *Alphaflexiviridae*, *Gammaflexiviridae*, and *Barnaviridae*. Mycoviruses with encapsidated dsRNA genomes form the larger group and belong to a wide variety of viral families, including *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Reoviridae*, *Megabimaviridae*, and *Quadriviridae*. In recent years, fungal viruses with (–) ssRNA genomes and with (+) ssRNA genomes possessing genes encoding reverse transcriptase have been described (Ghabrial et al. 2015). A very unusual case is that of *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1), which possesses a circular ssDNA genome (Yu et al. 2010). Current metagenomics approaches are rapidly increasing the identification of new mycoviruses and providing compelling evidence of their high abundance and taxonomic complexity.

MYCOVIRUS POTENTIAL ASSOCIATION WITH HYPOVIRULENCE CANNOT BE INFERRED BY TAXONOMIC RELATIONSHIPS

Major steps in the exploration of mycovirus-based control strategies are illustrated in Figure 1. A well-established hypovirulent effect on the host is the first requisite of a potential biocontrol mycovirus. However, mycoviruses are not infective in the classical sense, lacking extracellular routes of transmission. The question of how to fulfill Koch's postulates, providing definite evidence that a mycovirus is the infectious agent causing host fungus hypovirulence, therefore posed a challenge. The work performed with CHV1 and *C. parasitica* led the way to solve this problem with the development of infectious cDNA clones for the artificial extracellular infection by prototypical strain CHV1-EP713 (reviewed by Dawe and Nuss 2013). Two different approaches were used. First, a CHV1 full-length cDNA clone was placed under transcriptional regulation of *C. parasitica* sequences to promote synthesis of the virus coding strand upon integration of the infectious clone into the fungal genome. In the second approach, protoplasts of *C. parasitica* were directly transformed with an in vitro-transcribed synthetic copy of the coding RNA strand (Chen et al. 1994). Moleleki et al. (2003) transfected *Diaporthe perijunca* protoplasts with a synthetic transcript of *Diaporthe* RNA virus (DaRV1), confirming the feasibility of using this approach with other mycoviruses and hosts. Also using a synthetic transcript, Marzano et al. (2015) successfully transfected *Sclerotinia sclerotiorum* with a recombinant strain of hypovirus SsHV2. Infectious full-length synthetic clones, in addition to allowing fulfillment of Koch's postulates, has been critical to hypovirus molecular analysis (Dawe and Nuss 2013).

The development of functional infectious clones is nevertheless not a simple task, especially in the case of multisegmented mycoviruses. For encapsidated mycoviruses, inoculation of fungal protoplasts with purified virions, using standard polyethylene glycol-mediated transformation protocols, have been found to work well to initiate infection (Castro et al. 2003; Hillman et al. 2004; Sasaki et al. 2006) and is now an approach of common use. There are two reports of successful infection by transfecting protoplasts directly with purified viral dsRNAs (Jia et al. 2017; Kanhayuwa et al. 2015). Additional work will be required to determine if this very simple method to artificially infect fungal strains could be of more general use.

Natural intracellular transmission mechanisms are also amply used to generate isogenic virus-free and infected fungal strains. For instance, virus-free versions of naturally infected isolates, the so-called “cured” isolates, can be obtained by techniques that rely on selecting progeny or fungal tissue without the virus. The success of these approaches in curing infection appears to be highly variable. As an alternative, mycoviruses can be transmitted to uninfected isolates using the main route of horizontal mycovirus transmission, hyphal anastomosis.

These procedures that exploit natural intracellular transmission pathways, or rely on artificial infection methods, are identifying an increasing number of hypovirulence-associated mycoviruses and show that the taxonomic relationship of a virus cannot be taken as indicative of its effect on the host fungus. Firstly, in addition to the family *Hypoviridae*, many taxonomic groups with both dsRNA and (+) ssRNA genomes possess members associated with hypovirulence, including families *Alphaflexiviridae*, *Botybirnaviridae*, *Chrysoviridae*, *Endornaviridae*, *Megabimaviridae*, *Narnaviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae* (Ghabrial et al. 2015). Hypovirulence inducing mycoviruses also include *Sclerotinia sclerotiorum* negative-stranded RNA virus 1 (SsNSRV-1) that possesses a (–) ssRNA genome (Liu et al. 2014), and the gemini-like ssDNA virus SsHADV-1 (Yu et al. 2010) (Table 1). Secondly, viruses that are closely related phylogenetically might have very different impacts on the host. There are no mycovirus taxonomic groups that can be considered inextricably linked to hypovirulence, including the family *Hypoviridae* where species that do not impact virulence have been reported (Hillman and Suzuki 2004; Velasco et al. 2018; Wang et al. 2013). Furthermore, mycovirus effects have been shown to be strain-specific. Thus, different viral strains might behave quite differently and their effects can be further modulated by the particular genetic background of the host fungus strain (Hillman and Suzuki 2004; Hyder et al. 2013; Lin et al. 2007; Vainio and Hantula 2016).

MYCOVIRUS HORIZONTAL AND VERTICAL TRANSMISSION IN THE HOST FUNGUS

In addition to inducing hypovirulence, mycoviruses requires the ability to infect a large proportion of the pathogen populations in order to be suitable biological control tools. This is strongly dependent on how efficiently they are transmitted to uninfected isolates through hyphal anastomosis, converting them from virulent to hypovirulent, and on how efficiently they are transmitted to the progeny, that defines viral dispersion within the same genetic background. The ability of two isolates to undergo hyphal anastomosis is determined by the complex self/nonself recognition systems or “vegetative incompatibility” (*vic*) developed by fungal species. Whereas vegetative compatible isolates are able to establish successful hyphal anastomosis, the interaction of vegetative incompatible isolates triggers localized programmed cell death (PCD) which hampers exchange of genetic material (Biella et al. 2002). In the Ascomycota, this process is governed at the molecular level by a series of gene loci collectively known as *vic* (vegetative incompatibility) or *het* (heterokaryon incompatibility) (Glass and Dementhon 2006). In *C. parasitica*, *vic* loci have been characterized in detail. Transmission studies clearly established that allelic differences at the *vic* loci negatively affects horizontal mycovirus transmission in this species (Cortesi et al. 2001; Zhang et al. 2014). Disruption analysis of *vic* genes has confirmed that allelic differences at five of the six *vic* loci identified in *C. parasitica* contribute to a variable degree to restrict mycovirus transmission (Choi et al. 2012; Zhang et al. 2014).

The transmission rate of mycoviruses in the natural populations of the pathogen to be controlled is expected to depend basically on the host fungus genetics which regulates vegetative incompatibility. This is a concern in the application of mycovirus-based control strategies in plant pathogens with complex *vic* systems that results in high diversity of vegetative compatibility groups (VCGs) (Jiang et al. 2013; Pearson and Bailey 2013). However, it is becoming increasingly clear that the question of how efficiently a mycovirus can spread to isolates in the natural populations of the fungus is complex. For instance, spread of CHV1 in natural conditions was often found to be more efficient than expected by the complexity of VCGs in field isolates. Specifically addressing this question,

FIGURE 1

Schematic representation of major steps in the implementation of control strategies of fungal diseases based on the use of mycoviruses. **A**, Identification of viruses that reduce the virulence of fungal plant pathogens. Different methods have been developed to produce isogenic infected and virus-free strains to clearly establish the association of the mycovirus under study with hypovirulence. Some of these methods are also used to transfer mycoviruses to other fungal hosts and explore the potential of control strategies based on heterologous mycoviruses. **B**, Mycoviruses are generally transmitted only by intracellular mechanisms whose characterization is of great importance; efficient horizontal and vertical transmission of the hypovirulence-inducing mycoviruses will determine their ability to spread in the natural populations of the pathogen and therefore their potential as biocontrol agents. **C**, The increasing availability of tools to molecularly manipulate mycoviruses and their hosts, and the knowledge of the virus–fungus interaction these approaches are generating, is making it possible to genetically manipulate natural mycoviruses and/or their host to produce combinations with improved biological control properties. **D**, However, even when suitable combinations of mycovirus and fungal pathogen strains for the control of a plant disease become available, their successful deployment in field conditions poses a great challenge. The many problems to overcome, that are yet little addressed in the literature, are related to the life and disease cycle of the pathogen and the characteristics of the disease and the crop(s) affected.

Brusini and Robin (2013) found that transmission rates were higher on chestnut stems than in the commonly used in vitro assay on synthetic medium. These results suggest that there are environmental factors that favor CHV1 horizontal transmission. In plant pathogenic fungi, other than *C. parasitica*, *vic* alleles are poorly characterized at the molecular level. Nonetheless, there are rather straightforward approaches to characterize VCG structures in

fungus species even in the absence of detail genetic information; pairing of easily obtainable auxotrophic mutants is the most commonly used. Information about VCGs is therefore widely available and it has been exploited to determine the effect of vegetative compatibility/incompatibility on mycovirus transmission in several different host/virus systems. Taken together these studies confirm that, although vegetative incompatibility can be

TABLE 1
Mycoviruses associated with the induction of hypovirulence in phytopathogenic fungi

Mycovirus acronym/source fungus	Phenotypic effects on the fungal host ^a	References ^b
Family <i>Hypoviridae</i> : unencapsidated and monosegmented (+) ssRNA genome		
CHV1 ^c / <i>Cryphonectria parasitica</i>	Strain CHV1/EP713: strong hypovirulence, ^d reduced growth and pigmentation, drastically reduced conidiation Strain CHV1/Euro7: moderate hypovirulence, ^d reduced conidiation and pigmentation Strain CHV1/EP721: moderate hypovirulence, ^d reduced conidiation and pigmentation	Hillman and Suzuki 2004; Lin et al. 2007
CHV2/ <i>Cryphonectria parasitica</i>	Strain CHV2/NB58: strong hypovirulence, ^e marked debilitating effect on the fungus, slow growth, isolates brown in color, thin mycelium, reduced conidiation	Hillman and Suzuki 2004; Smart et al. 1999
CHV3/ <i>Cryphonectria parasitica</i>	Strain CHV3/GH2: strong hypovirulence, ^e colonies often exhibit lobed margins, normal sporulation, orange pigment production	Hillman and Suzuki 2004; Smart et al. 1999
SsHV1/ <i>Sclerotinia sclerotiorum</i>	Strong hypovirulence, ^e abnormal colony morphology, enhanced pigmentation; phenotypic alterations are only observed in isolates coinfecting with an SsHV1-associated sat-like RNA	Xie et al. 2011
SsHV2/ <i>Sclerotinia sclerotiorum</i>	Strain SsHV2/5472: hypovirulence ^e Strain SsHV2/SX247: hypovirulence, ^f reduced growth, abnormal colony morphology Strain SsHV2L (potential recombinant of SsHV2 and an unknown hypovirus): hypovirulence, ^d delayed maturation of sclerotia	Hu et al. 2014; Khalifa and Pearson 2014; Marzano et al. 2015
FgHV2/ <i>Fusarium graminearum</i>	Hypovirulence, ^e reduced growth and conidiation, reduced DON production	Li et al. 2015
AsHV1 ^c / <i>Alternaria alternata</i>	Hypovirulence, ^{e,f} reduced growth	Li et al. 2019
Family <i>Alphaflexiviridae</i> : encapsidated and monosegmented (+) ssRNA genome		
SsDRV ^g / <i>Sclerotinia sclerotiorum</i>	Hypovirulence, ^{e,f} reduced growth, abnormal colony morphology	Xie et al. 2006
Family <i>Botybirnaviridae</i> : encapsidated and bisegmented dsRNA genome		
SsBRV2/ <i>Sclerotinia sclerotiorum</i>	Hypovirulence, ^h reduced growth, absence of sclerotia	Ran et al. 2016
BpRV1/ <i>Botrytis porri</i>	Hypovirulence, ^h reduced growth	Wu et al. 2012
BmBRV1-BdEW220 (strain of <i>Bipolaris maydis</i> botybirnavirus 1)/ <i>Botryosphaeria dothidea</i>	Hypovirulence, ^{e,f} reduced growth, abnormal colony morphology	Zhai et al. 2019
Family <i>Chrysoviridae</i> : encapsidated and multisegmented dsRNA genome		
FgV-ch9/ <i>Fusarium graminearum</i>	Hypovirulence, ^h reduced growth and conidiation, reduced perithecia formation	Darissa et al. 2012
BdCV1/ <i>Botryosphaeria dothidea</i>	Hypovirulence, ^{e,f} reduced growth	Wang et al. 2014
FodV1/ <i>Fusarium oxysporum</i> f. sp. <i>dianthi</i>	Hypovirulence, ^f reduced growth and conidiation	Lemus-Minor et al. 2018, 2019; Torres-Trenas et al. 2019

(Continued on next page)

^a The different phenotypic effects of viral strains are indicated in cases where they are well established.

^b A reference list with full details can be found in the Supplementary File S1.

^c Mycoviruses found to induce hypovirulence also in heterologous hosts upon artificial transfection.

^d Effect on virulence confirmed by use of infective cDNA clones.

^e Effect on virulence confirmed by curing of virus infection (hyphal tip isolation, single-spore isolation, protoplast regeneration, etc.).

^f Effect on virulence confirmed by transmission by hyphal anastomosis to virus free isolate.

^g The reported genome sequence of SsDRV does not encode a capsid protein.

^h Effect on virulence confirmed by transfection of protoplasts with virus particles.

ⁱ Effect on virulence confirmed by mycovirus with the ability of extracellular transmission.

generally regarded as a barrier to mycovirus horizontal transmission, the strength of this barrier can vary considerably. For example, *Heterobasidion* mycoviruses are readily transmitted even between different *Heterobasidion* species (Vainio and Hantula 2016; Vainio et al. 2018). Moreover, viruses that seemingly influence their hosts *vic* reactions have been characterized (Hamid

et al. 2018; Wu et al. 2017; Yang et al. 2018). Vegetative incompatibility functions restricting the exchange of genetic material through the induction of localized PCD (Biella et al. 2002). It is plausible therefore that higher mycovirus transmission efficiency in certain fungal species, environmental conditions or with certain mycoviruses might be related to a weakening of PCD responses.

TABLE 1
(Continued from previous page)

Mycovirus acronym/source fungus	Phenotypic effects on the fungal host ^a	References ^b
MoCV1-A/ <i>Magnaporthe oryzae</i>	Hypovirulence, ^f infection promotes host race conversion	Aihara et al. 2018
Family <i>Endornaviridae</i> : unencapsidated and monosegmented (+) ssRNA genome		
HmEV1-670/ <i>Helicobasidium mompa</i>	Hypovirulence, ^{e,f} alterations in growth not reported	Ikeda et al. 2003; Osaki et al. 2006
RsEV1/ <i>Rhizoctonia solani</i>	Hypovirulence, ^f thin mycelium, small sclerotia, dark pigmentation	Zheng et al. 2019
SmEV1/ <i>Sclerotinia minor</i>	Hypovirulence, ^f reduced growth and sclerotial production	Yang et al. 2018
Family <i>Megabirnaviridae</i> : encapsidated and multisegmented dsRNA genome		
RnMBV1 ^c / <i>Rosellinia necatrix</i>	Strong hypovirulence, ^h reduced growth. A variant with genome reorganization lost the ability to induce hypovirulence	Chiba et al. 2009; Kanematsu et al. 2014
RnMBV2/ <i>Rosellinia necatrix</i>	Hypovirulence, ^h reduce growth and irregular colony morphology, when coinfecting with partitivirus RnPV1	Sasaki et al. 2016
Family <i>Narnaviridae</i> : unencapsidated and monosegmented (+) ssRNA genome		
BcMV1/ <i>Botrytis cinerea</i>	Hypovirulence, ^{e,f} reduced growth, greatly reduced conidial production, absence of sclerotia	Wu et al. 2007; 2010
SsMV1/ <i>Sclerotinia sclerotiorum</i>	Hypovirulence, ^f reduced growth, reduced sclerotia production	Xu et al. 2015
Family <i>Partitiviridae</i> : encapsidated and bisegmented dsRNA genome		
SsPV1 ^c / <i>Sclerotinia sclerotiorum</i>	Hypovirulence, ^{f,h} reduced growth, reduced sclerotia production, cytoplasm leaks at hyphal tips	Xiao et al. 2014
RsPV2/ <i>Rhizoctonia solani</i>	Hypovirulence, ^h reduced growth	Zheng et al. 2014
HetPV13-an1/ <i>Heterobasidion annosum</i> complex	Hypovirulence (reduces the ability of <i>H. parviporum</i> to colonize wood), ^{e,f} reduced growth, increased hyphal branching	Vainio et al. 2018
BcPV2/ <i>Botrytis cinerea</i>	Hypovirulence, ^f absence of conidia and sclerotia	Kamaruzzaman et al. 2019
Family <i>Reoviridae</i> : encapsidated and multisegmented dsRNA genome		
RnMYRV-3(W370) ^c / <i>Rosellinia necatrix</i>	Hypovirulence, ^{e,f,h} reduced growth, virus-transfected strains exhibit "mosaic" colonies due to areas with different growth rate	Kanematsu et al. 2004; Sasaki et al. 2007; Wei et al. 2003
Family <i>Totiviridae</i> : encapsidated and monosegmented dsRNA genome		
HmTV1-17/ <i>Helicobasidium mompa</i>	Hypovirulence, ^f effects on growth not reported	Suzaki et al. 2005
HvV190S ^c / <i>Helminthosporium victoriae</i>	Hypovirulence, ^h reduced growth, abundant white aerial mycelium, sectorial colonies	Xie et al. 2016
PdV1/ <i>Penicillium digitatum</i>	Hypovirulence, ^{e,h} reduced growth	Niu et al. 2016
dsRNA and (+) ssRNA mycoviruses not assigned to a known family		
FgV1 ^c / <i>Fusarium boothii</i> (formerly <i>F. graminearum</i> strain DK21)	Hypovirulence, ^f reduced growth, increased pigmentation, decreased production of trichothecene mycotoxins	Cho et al. 2013
BcRV1, monopartite dsRNA virus/ <i>Botrytis cinerea</i>	Hypovirulence, ^f reduced growth	Yu et al. 2015
BdRV1, dsRNA virus 5 segment genome/ <i>Botryosphaeria dothidea</i>	Hypovirulence, ^f reduced growth, sectorial colony edges, absence of picnidia	Zhai et al. 2016
Family <i>Myonnaviridae</i> : encapsidated and monosegmented (–) ssRNA genome		
SsNSRV-1/ <i>Sclerotinia sclerotiorum</i>	Hypovirulence, ^h reduced growth, absence of sclerotia	Liu et al. 2014
DNA mycoviruses: encapsidated and monosegmented circular ssDNA genome		
SsHADV-1/ <i>Sclerotinia sclerotiorum</i>	Hypovirulence, ⁱ reduced growth, small sclerotia, abnormal colony morphology	Yu et al. 2010

Very interesting in this regard is the work of Wu et al. (2017), which showed that *Sclerotinia sclerotiorum* mycoreovirus 4 (SsMYR4) infection downregulated critical cellular activities and signaling pathways associated with vegetative incompatibility-mediated PCD, facilitating horizontal transmission of other coinfecting viruses. Mycoviruses with this property can be of great interest for their exploitation in biological control. Analysis of the molecular mechanisms underlying enhanced horizontal transmission will generate knowledge that could be broadly exploited to improve mycovirus-based control strategies.

Mycovirus dispersion is also greatly impacted by transmission rates to the progeny (vertical transmission), which determines the stability of the infection within a genetic background. In *C. parasitica*, hypoviruses are excluded from ascospores. Analysis of their transmission rates to conidia has provided evidence that poor vertical transmission correlates with limited dispersion of the virus in the host populations. Sparse transmission to the progeny is thought to play an important role in the lack of ecological fitness of CHV2. This hypovirus induces strong hypovirulence in *C. parasitica* but is transmitted in only 2 to 5% of the conidia and has a very limited geographical distribution (Hillman and Suzuki 2004). There are systematic studies of mycovirus vertical transmission in other species. Lee et al. (2014) analyzed transmission rates to conidia of four *Fusarium graminearum* viruses from different viral families. Interestingly, this work revealed that two mycoviruses that induced hypovirulence and growth alterations were more efficiently transmitted than those that caused symptomless infections. Attempts to cure mycovirus infection through the analysis of single-spore cultures have also produced abundant data that confirm that mycovirus transmission rates to conidia can vary considerably among species, and that less impact on the host is not necessarily associated with more efficient vertical transmission. A recent paper by Aulia et al. (2019) has shown that the presence of a coinfecting mycovirus can greatly impact the efficiency of vertical transmission, likely due to suppression of RNA silencing. Exclusion from sexual spores was considered a common feature of mycovirus transmission to the progeny. However, mitoviruses and later on two mycoreoviruses from *C. parasitica* were found to be vertically transmitted through ascospores (Deng et al. 2007b; Polashock et al. 1997). Mycovirus BVX from *Botrytis cinerea* is also present in ascospores (Pearson and Bailey 2013), and *Heterobasidion* mycoviruses are transmitted via basidiospores (Vainio and Hantula 2016). It would be of interest to perform more systematic analyses of mycovirus transmission into sexual spores. Sexual reproduction in nature appears to be infrequent but it would be an avenue of mycovirus exchange between different VCGs (Pearson and Bailey 2013). Furthermore, there are fungal species that do not conidiate or in which conidia play a small role in the life cycle. Hence, it would also be of interest to expand current studies of mycovirus vertical transmission to other asexual structures. For example, the sclerotia produced by *S. sclerotiorum* might play an important role in the vertical transmission of mycovirus in this fungal species lacking conidia (Jiang et al. 2013). Recently, transmission of an endornavirus to sclerotia was reported in the related species *S. minor* (Yang et al. 2018).

POTENTIAL CONTROL OF PLANT PATHOGENS WITH MYCOVIRUSES IDENTIFIED IN OTHER FUNGAL SPECIES

The search of mycoviruses with potential as biological control agents has focused mainly on identifying and characterizing those viral species naturally infecting the fungi to be controlled. However, mycovirus of interest have not been identified in a number of phytopathogenic species with great economic impact and genetic resources available to characterize mycovirus–host interactions.

The use of heterologous mycoviruses could be an alternative in such fungal pathogens. There are a few reports of different fungal species, even phylogenetically distant, naturally infected with strains of the same mycovirus (reviewed by Xie and Jiang 2014). This was an indication that there are viruses with the ability to parasitize a variety of fungi. Natural transmission of mycoviruses between unrelated species is, however, not often encountered. Hence, the artificial transfection methods above described have been exploited to provide clear evidence that mycoviruses can have broad host ranges. For example, the synthetic transcript of CHV1-EP713 has been used to artificially infect species closely related phylogenetically to *C. parasitica*, including *C. radicalis*, *C. havanensis*, *C. cubensis*, and *Endothia gyrosa* (Chen et al. 1996; van Heerden et al. 2001). In all these fungi, CHV1 was able to replicate and induce hypovirulence and other phenotypic alterations. Furthermore, upon infection, CHV1 was readily transmitted to other isolates of the same species by hyphal anastomosis whereas its effect on sporulation and its vertical transmission to conidia was more variable. Sasaki et al. (2002) used the full-length cDNA clone under fungal regulatory sequences to expand CHV1 infection to other *Diaporthe* species, like *Valsa ceratosperma* and *Phomopsis* G-type (teleomorph *Diaporthe* Nitschke). CHV1 was also able to infect these more distantly related species causing alteration in growth and reduced virulence.

Transfection of protoplasts with viral particles is the method commonly applied to infect new species with encapsidated mycoviruses. Kanematsu et al. (2010) used purified virus particles of *Rosellinia necatrix* partitivirus 1 (RnPV1) and *Rosellinia necatrix* mycoreovirus 3 (RnMyR3) to transfect protoplast of *Diaporthe* sp., *C. parasitica*, *V. ceratosperma*, and *Glomerella cingulata*. Partitivirus RnPV1 was able to establish infection in all four new species, whereas RnMyR3 infected all except *G. cingulata*. Furthermore, RnMyR3 induced attenuated virulence in *Diaporthe* sp., *C. parasitica*, and *V. ceratosperma*, as in its own natural host. Both mycoviruses were readily transmitted by hyphal anastomosis in all new species but vertical transmission to spores was poor. Salaipeth et al. (2014) employed the same approach to transfer *Rosellinia necatrix* megabirnavirus 1 (RnMBV1) to *C. parasitica*. Again, RnMBV1 persistently infected *C. parasitica*, inducing similar phenotypic alterations in growth and virulence as in *R. necatrix*. Transfection of protoplasts of *B. cinerea* with purified virions of mycovirus *Sclerotinia sclerotiorum* partitivirus 1 (SsPV1) also resulted in stable persistent infection and induction of reduced virulence and growth alterations (Xiao et al. 2014). It is worth mentioning here that attempts to infect other species with SsHADV-1 showed that this unusual mycovirus, which can be replicated in and transmitted by mycophagous flies, by contrast, has a rather narrow fungal host range (Yu et al. 2013; Liu et al. 2016).

Protoplast fusion is another approach used to transfer mycoviruses to new hosts. It has the advantage that it does not require any manipulation of the virus and can be used with encapsidated and capsidless species. Lee et al. (2011) successfully transferred mycovirus FgV1-DK21 to *F. graminearum*, *F. asiaticum*, *F. oxysporum* f. sp. *lycopercisi*, and *C. parasitica* via protoplast fusion. FgV1-DK21 was able to replicate in all these hosts, even the distantly related *C. parasitica* where it caused reduced virulence and growth alterations that differed from those observed in CHV1 infections.

The works above described, although not numerous, provide evidence that the heterologous virus approach to control plant pathogens could be feasible and worth exploring. First of all, viruses able to replicate in a variety of fungal species appear to be common. Secondly, although mycovirus effects vary with the fungus involved, they tend to retain the ability to induce hypovirulence when transfer to new hosts. Hence, the main factor restricting mycoviruses usually to a single host, or a few closely related hosts,

could be mainly their dependence on intracellular transmission routes of exchange of genetic material that only take place between individuals of the same species. Furthermore, the ability to reduce fungal virulence is a feature that, although it can be modulated by the host fungus, appears to strongly depend on the viral strain. It should be noted that the inability of SsHADV-1 virions to infect fungal species others than those closely related to its natural host suggests that, when mycovirus spread is not restricted by lack of extracellular transmission routes, other factors may contribute to limit the fungal host range.

THE MYCOVIRUS–FUNGUS INTERACTION: HOST AND VIRAL DEFENSE MECHANISMS

In the virus–fungus molecular interaction there are two broad aspects to be examined that although linked can be considered separate questions. Firstly, what makes the virus efficiently replicate and persist in the fungal tissues and secondly, which cellular processes and/or signaling pathways are altered in the host resulting in hypovirulence and associated traits. Mycoviral accumulation levels are expected to depend primarily on the host antiviral defenses and how the virus counteracts them. The analysis of these processes might be of particular interest in the case of mycoviruses that, as they lack an extracellular phase in their life cycles, can be considered intrinsic genetic elements of their hosts. RNA silencing (RNAi) is a prominent posttranscriptional gene silencing (PTGS) system that has an ample spectrum of functions in eukaryotes. The key components and general mechanism of RNAi are broadly conserved (Meister and Tuschl 2004; Tomari and Zamore 2005). The process is triggered by the presence of dsRNA that is processed by Dicer or Dicer-like (DCL) proteins into small interfering RNAs (siRNAs). One strand of the siRNA is loaded into an RNA-induced silencing complex (RISC), where the Argonaute or Argonaute-like (AGL) proteins play a central role. The siRNAs guide the RISC to the target RNAs, cleaving them. RNA-dependent RNA polymerases (RDRs) convert single-stranded RNA (ssRNA) into dsRNA thus amplifying the siRNA signals.

In plants and invertebrates RNAi is known to play a critical role in antiviral defense. This PTGS machinery was naturally the first process to focus on when antiviral responses were examined in *C. parasitica*. This fungal species possesses two Dicer genes and four Argonaute genes. Functional analysis of these genes by production of disruption mutants provided strong evidence that a single Dicer and a single Argonaute were effectively involved in antiviral defense in *C. parasitica*. Mutants of the Dicer gene *dcl2* or the Argonaute gene *agl2* challenged with CHV1-EP713 exhibited a severely debilitated phenotype and increased viral RNA levels (Segers et al. 2007; Sun et al. 2009). A later study extended the analysis to additional CHV1 viral strains and highlighted the complexity of RNAi antiviral responses (Zhang et al. 2012). Hypovirus strains CHV1-Euro7 and CHV1-EP721 are highly similar in nucleotide sequence to CHV1-EP713 but cause noticeably milder effects. Additionally, CHV1-EP721 exhibits significantly lower accumulation levels. Zhang et al. (2012) found that both CHV1-Euro7 and CHV1-EP721 showed an increase of the viral accumulation levels in the *dcl2* mutant strains but, in contrast with CHV1-EP713, neither of these viral strains significantly induced *dcl2* expression. Furthermore, in CHV1-EP721 increased viral levels was not correlated with more debilitating effects on the host. Mycoviruses from other families also exhibit increased viral RNA accumulation and more dramatic phenotypic effects upon deletion of *dcl2*, including heterologous species artificially transfected to *C. parasitica* (Chiba et al. 2013a, b; Salaipeth et al. 2014; Segers et al. 2007). *C. parasitica* RNAi appears not to have a prominent defense role against certain viruses. Very recently, Shahi et al. (2019) showed that RNA levels of Cryphonectria mitovirus

1 (CpMV1) are not affected by deletion of critical components of RNAi machinery, nor does CpMV1 infection upregulate expression of these genes. Members of the genus *Mitovirus* infect mitochondria and this subcellular localization might allow them to escape RNAi defenses.

The potential antiviral role of RNAi is being examined in an increasing number of fungal pathogens. In *Colletotrichum higginsianum*, two Dicer genes and two Argonaute genes have been functionally characterized by production of deletion mutants. Phenotypic analysis of wild-type and deletion mutants infected with *Colletotrichum higginsianum* nonsegmented dsRNA virus 1 showed that, as previously described in *C. parasitica*, a single Dicer and a single Argonaute are involved in the antiviral RNAi response in this species (Campo et al. 2016). Other studies have revealed though that Dicers and Argonautes can have redundant functions in antiviral defense. For example, in *S. sclerotiorum* the debilitated phenotype observed in double mutants, lacking its two Dicer genes, infected with either a hypovirus or the DNA virus SsHADV1, was reverted by complementation with a single Dicer gene (Mochama et al. 2018). In *F. graminearum* the role of RNAi against three different mycoviruses, FgV1 and FgV2, which induce hypovirulence, and FgV3, that causes latent infections, was investigated (Yu et al. 2018). This species encodes two Dicers and two Argonautes. It was found that a single Argonaute gene, *FgAGO-1*, and a single Dicer gene, *FgDicer-2*, are preferentially upregulated during mycovirus infection but taken together the results of Yu and collaborators point to some functional redundancy of Dicers and Argonautes in *F. graminearum* and virus-specific responses (Yu et al. 2018). Functional analysis of the three Argonautes of *Pyricularia oryzae* (*Magnaporthe oryzae*) has led to the intriguing result that one of them interferes with RNAi (Nguyen et al. 2018). These authors found that deletion of MoAGO2 resulted in a dramatic decrease in the accumulation levels of two mycoviral RNAs. Furthermore, this decrease in viral RNA levels did not occur in a double mutant lacking both MoAGO2 and the Dicer MoDCL2, confirming that the strong silencing of mycoviruses in the $\Delta moago2$ strain is RNAi-dependent. In *Verticillium dahliae*, we have found that viral RNA levels of a partitivirus (Cañizares et al. 2014) and an unclassified (+) ssRNA mycovirus (Cañizares et al. 2018) are not affected by deletion of Dicer or Argonaute genes. This species possesses two Dicer genes and two Argonaute genes and even in double mutants fully lacking Dicer or Argonaute activity, accumulation levels of both mycoviruses were very similar to those exhibited by the wild-type strain (M. C. Cañizares and M. D. García-Pedrajas, unpublished data).

Functional characterization of RNAi components through analysis of virus-infected disruption mutants is providing evidence that antiviral RNAi responses might be more significant in some fungal species and/or against certain mycoviruses. However, it is a rather labor-intensive approach. Presence of virus-derived siRNAs (vsiRNAs) is considered the hallmark of an active antiviral RNAi mechanism. Detection of vsiRNAs can therefore provide indirect evidence of the antiviral role of RNAi in mycovirus–fungus interactions. Nowadays, next generation sequencing is increasingly used to characterize vsiRNAs populations as a simple way to show that mycoviral infection triggers an antiviral RNAi response in the corresponding fungal species. Interestingly, some of these studies have shown that vsiRNAs could target fungal host-specific genes thus inducing their silencing (Özkan et al. 2017; Wang et al. 2016a). RNAi antiviral responses could therefore modulate fungal cellular processes through this molecular mechanism contributing to the phenotypic effects of mycovirus infection.

Viruses have evolved strategies to counteract the host RNAi responses including encoding RNA silencing suppressors (RSSs). Regarding how mycoviruses defend themselves from antiviral fungal defenses, little information is yet available in mycovirus–fungus interactions. There is one exception to this, the RSS

protein p29 encoded by CHV1-EP713 which has been studied in detail. The ability of the p29 suppressor protein to enhance viral RNA accumulation of homologous and heterologous viruses has been confirmed by multiple approaches, including coinfection analyses and p29 transgenic expression studies (Chiba et al. 2013b; Segers et al. 2006; Sun et al. 2006; Suzuki et al. 2003). The mechanism of action of p29 likely involves downregulation of the RNAi antiviral response that involves increased expression of *dcl2* and *agl2*. Mycovirus RnMyRV3 from *Rosellinia necatrix* also encodes an RSS, termed VP10, but its mode of action is unclear (Yaegashi et al. 2013b). Interestingly, large-scale sequencing of vsiRNAs and bioinformatic analyses showed that the RNAi genes Dicer1 and RDR5 of *F. graminearum* could be among the potential targets of hypoviruses FgHV1 and FgHV2 derived siRNAs (Wang et al. 2016a). This result suggests that degradation of RNAi gene transcripts targeted by hypovirus siRNAs could be a strategy to suppress RNAi antiviral responses in addition to the role played by RSS proteins.

THE MYCOVIRUS–FUNGUS INTERACTION: HOST CELLULAR PROCESSES ALTERED BY VIRUS INFECTION

Comparison of the transcription patterns of isogenic virus-free and virus-infected fungal strains is an approach used in an increasing number of studies to provide insights into basic aspects of the virus–fungus interaction. Mycoviruses that induce hypovirulence often cause diverse growth alterations. The pleiomorphic nature of these alterations suggested that hypovirulence-inducing mycoviruses interfere with host signaling pathways. Using microarray technology, Dawe et al. (2004) showed that around half of the *C. parasitica* genes whose expression was altered by CHV1 infection, exhibited similar changes in expression in deletion mutants lacking G-protein α -subunit or β -subunit genes. CHV1 also alters the expression of transcription factor (TF) genes, very interestingly, it downregulates the expression of *cpst12* encoding a homolog of the *Saccharomyces cerevisiae* TF Ste12, a key target of MAPKs signaling and critical for invasive growth and mating. Moreover, the differential gene expression pattern induced by CHV1 infection is very similar to that induced by *cpst12* deletion (Deng et al. 2007a). These results uncovered a probable coordination of G-protein and MAPK signaling pathways in the regulation of TFs involved in virulence in *C. parasitica*. Nuskern et al. (2018) confirmed that CHV1 infection induces epigenetic changes. These authors analyzed the effect of six CHV1 strains on the global cytosine methylation level of three different *C. parasitica* isolates finding that hypovirus infection induces DNA hypermethylation in *C. parasitica*, and that higher methylation levels correlate with more severe phenotypic effects. Other *C. parasitica* cellular processes have been identified as altered in the presence of CHV1. For example, transcriptomic as well as proteomic approaches showed that CHV1 infection affects host secretory pathways and vesicle-mediated transport systems, and upregulates stress responses (Allen et al. 2003; Kazmierczak et al. 2012; Wang et al. 2016b). In summary, hypovirulence and associated traits caused by CHV1 infection of *C. parasitica* is likely a consequence of virus interference with multiple secretory and regulatory mechanisms.

F. graminearum is another plant pathogen in which global responses to mycovirus infection have been characterized in some detail. In the first of these studies, Kwon et al. (2009) used a proteomic approach to characterize changes in protein host profiles in response to mycovirus FgV1-DK21, which induces hypovirulence and reduced mycotoxin production. This work showed upregulation of proteins involved in differentiation processes and downregulation of proteins with antioxidant properties and involved in glycolysis in the infected strain. Cho et al.

(2012) analyzed *F. graminearum* transcriptional responses to the same hypovirulence-associated mycovirus with similar results to the above described responses of *C. parasitica* to CHV1. Thus, FgV1 caused upregulation of genes associated with cAMP-mediated signaling, the transcriptional and translational machineries as well as ABC transporters. Genes involved in transport systems, and primary and secondary metabolism were, on the other hand, downregulated by FgV1 infection. Later on, using RNA-seq technology, Lee et al. (2014) compared the transcriptomic response of *F. graminearum* to FgV1-DK21 and three additional viruses, FgV2, FgV3, and FgV4. These mycoviruses are from different families and differed too in their host effects. Similarly to FgV1-DK21, FgV2 induces reduced virulence and growth changes, whereas FgV3 and FgV4 cause symptomless infections. Unexpectedly, Lee et al. (2014) found that the mycoviruses that had higher impact on host transcription profiles were FgV1 and FgV4. FgV2, although inducing very noticeable phenotypic changes in *F. graminearum*, had clearly less impact on host gene expression. Hence, a greater impact on the host phenotype does not necessarily correlate with a bigger impact on its transcription patterns. Remarkably, each mycovirus affected the expression of a different set of fungal genes. For example, in all four interactions the expression of TF genes was significantly changed but each mycovirus regulated a different set of TFs (Lee et al. 2014). The transcriptional response of most key components of the RNAi machinery was also virus-specific.

Wang et al. (2018) recently evaluated the transcriptional response of *Botryosphaeria dothidea*, the causal agent of pear ring rot, to a chrysovirus and a partitivirus potentially associated with hypovirulence and growth alterations. Similar to the above described transcriptional responses of *C. parasitica* to CHV1 and *F. graminearum* to FgV1, infection of *B. dothidea* with either mycovirus led to upregulation of genes involved in transcription and signal transduction pathways such as MAPK signaling and stress responses. The expression of key genes of the RNAi machinery was also induced in the infected *B. dothidea* strain. In another recent study, Vainio et al. (2018) reported the effect of Heterobasidion partitivirus 13 (HetPV13-an1) infection on the phenotype and gene expression patterns of *H. parviporum*, a conifer pathogen. It was found that although HetPV13-an1 causes hypovirulence and associated phenotypic alterations, it affects host gene expression patterns in a very different manner than other hypovirulence-inducing mycoviruses. HetPV13-an1 appeared to greatly impact host metabolism; the infected strain showed altered carbohydrate and amino acid metabolism and upregulation of alternative metabolic pathways. Furthermore, HetPV13-an1 infection repressed several potential components of the RNAi machinery (Vainio et al. 2018). The DNA virus SsHADV1 has the very unusual particularity within the mycoviruses that it can infect intact fungal cells as viral particles. The transcriptional response of its host, *S. sclerotiorum*, to early stages of SsHADV1 infection has also been characterized (Ding et al. 2019). Analysis of the functional categories of genes differentially expressed in pre- and post-infection hyphae has led Ding et al. (2019) to propose that SsHADV1 breaks through the fungal cell wall and use the host Ras-small G protein signal transduction pathway to modulate changes in metabolism that provides the energy for its own invasion and proliferation.

Current knowledge of fungal transcriptional responses to viral infection allows concluding that diverse molecular mycovirus–host interactions might have a few common features. For instance, mycovirus infections, including symptomless infections, generally result in extensive host transcriptional reprogramming. Alteration of the expression of genes encoding transcription factors and components of signal transduction pathways appears to be another fairly common response to mycovirus infection. However, it is also evident that the responses observed are, to a large extent,

mycovirus-host-specific. The regulation of fungal virulence and development is a very complex process that involves the interplay of multiple signaling pathways, regulatory mechanisms and cellular processes. Hence, different mycoviruses can interfere with different sets of genes with similar phenotypic consequences for the host.

ENGINEERING MYCOVIRUSES AND/OR THEIR FUNGAL HOSTS TO ENHANCE THE POTENTIAL OF BIOLOGICAL CONTROL STRATEGIES

Our understanding of the molecular biology of mycoviruses and their hosts is rapidly increasing. In this scenario, manipulating the virus and/or the fungus with the aim of producing the best combinations for effective disease control is becoming possible. For instance, molecular approaches have been used in the extensively studied *C. parasitica*–CHV1 interaction in an attempt to increase the potential of mycovirus dispersion (recently reviewed by Rigling and Prospero 2018). The nuclear integration of a full-length cDNA copy of CHV1 provides a strategy to enhance vertical and horizontal transmission. It results in transmission of the virus to ascospores, and this in turn allows horizontal transmission through sexual crosses regardless of vegetative incompatibility. Field trials have nonetheless failed to support the better performance of *C. parasitica* strains harboring a nuclear copy of CHV1 in spreading the virus (Root et al. 2005). In a different approach, “super donor” *C. parasitica* strains able to transfer CHV1 to isolates from a variety of VCGs have been produced by disrupting *vic* loci known to restrict mycovirus transmission. These strains were expected to enhance the effective delivery of hypoviruses in *C. parasitica* populations (Zhang and Nuss 2016). A recent study provided evidence that biocontrol formulations based on “super donor” strains worked more efficiently in the transmission of hypovirus in an American chestnut forest setting with highly divergent *C. parasitica* populations (Stauder et al. 2019).

The knowledge gained through the functional analysis of virus sequences could be used, on the other hand, to produce chimeric mycoviruses with enhanced properties as biocontrol tools. For example, the potential lack of ecological fitness of the fungal strains harboring biocontrol viruses poses a major concern. Viruses that induce hypovirulence usually have a negative impact on vegetative growth. This might affect their ecological fitness and therefore the spread and prevalence of the biological control agent in natural conditions. The association of hypovirulence with alterations in vegetative growth is common to the extent that these morphological changes are referred to as hypovirulence-associated traits. It should be taken into account though that many mycoviruses that induce hypovirulence were first selected for characterization because their host strains exhibited obvious growth alterations. This might bias the screening toward mycoviruses that affect both vegetative growth and virulence, whereas viruses that only affect pathogenic development would escape notice. Furthermore, mycoviruses that induce growth changes allow virus-free from infected isolates to easily be distinguished, thus providing a simpler experimental system. Some mycoviruses could however affect signaling pathways and cellular processes exclusively associated with pathogenic development. Functional analysis of the prototypical CHV1-EP13 strain sequences has allowed mapping genome regions associated with specific effects on the host (reviewed by Dawe and Nuss 2013). These analyses reveal that it might be possible to fine tune the effect on the host through the molecular manipulation of the mycovirus, uncoupling hypovirulence from alterations in growth. Less impact on the host growth could indeed be an advantage for the dispersion of the mycovirus used as biological control agent in natural conditions (Robin et al. 2010). It is true that the debilitating effect on the host growth might be a factor contributing to disease control by reducing the ability of the virus-infected fungus to colonize plant

tissue (Vainio et al. 2018). However, this is not necessary a general scenario and in many pathogens molecular mechanisms critical for pathogenic development but with no impact on fungal growth have been elucidated. The key question in the molecular manipulation of a mycovirus would be to achieve the proper balance between the factors that makes the mycovirus successful in preventing disease, and those that contribute to its ecological fitness allowing it to become prevalent in the pathogen populations. To produce transgenic viruses with enhanced properties as biocontrol tools is doubtless a difficult task. It requires deep understanding of the complex mycovirus molecular biology and extensive field trials. However, the potential of this approach should not be disregarded.

THE BROAD IMPLEMENTATION OF MYCOVIRUS-BASED CONTROL STRATEGIES FACES MULTIPLE CHALLENGES

The confirmation that spread of CHV1 was lowering the incidence of chestnut blight in European stands led to the implementation of a program of artificial introduction of CHV1-infected strains to control *C. parasitica*, which remains active nowadays in Europe. The disease chestnut blight has features that favor mycovirus-based control strategies. *C. parasitica* affects a tree species causing necrotic lesions in the bark (cankers), which on an individual tree are usually formed from a single point of infections. Treatment of these cankers with spores of hypovirulent strains harboring the hypovirus can halt their development (Hillman and Suzuki 2004; Rigling and Prospero 2018). In biological control strategies, the ideal situation is that, once introduced, the biocontrol agent spreads independently and persists in the ecosystem exerting continuous control. Chestnut blight control offers the possibility of a therapeutic hypovirus treatment of easily identifiable localized symptoms. To improve hypovirus formulations, *vic* types in the area to be treated are determined and the hypovirus is transmitted to local fungal isolates by hyphal anastomosis using an in vitro transmission assay. The inoculum thus prepared is used to fill holes made at the margins of the cankers with a cork borer. The therapeutic use of CHV1-infected *C. parasitica* strains works well to lower the incidence of chestnut blight in Europe but mostly failed in North America. The higher VCG diversity of the *C. parasitica* North American populations might contribute to these differences (Dawe and Nuss 2013; Milgroom and Cortesi, 2004). However, it is becoming increasingly clear that the efficient spread of mycoviruses upon introduction through hypovirulent infected strains might depend on multiple factors.

There are currently a number of fungal pathogens in which mycoviruses with good potential as biocontrol tools are available. The vegetative incompatibility system of the fungal pathogen to be controlled is frequently the first concern in the potential field application of mycoviruses as a complex VCG structure might hamper spread of the mycovirus to natural fungal isolates. How to favor mycovirus horizontal transmission is therefore an active area of research (Xie and Jiang 2014). There are, however, other key factors that will determine the approach used to deliver hypovirulence-inducing mycoviruses in the field and their ability to spread upon introduction and effectively control the disease. For instance questions related to the life and disease cycle of the pathogen and the characteristics of the disease and crop(s) affected. It could be mentioned in the first place that the simplest way to implement the use of mycoviruses is probably as a therapeutic treatment of visible symptoms but this is an option only in a limited number of plant diseases. Also, in contrast with woody plant diseases, herbaceous crops that are harvested after each growing season might not offer favorable conditions to the colonization of pathogen populations by the virus in field conditions (Jiang et al. 2013). Necrotrophic and biotrophic fungal pathogens can also offer

very different contexts for the mycovirus' ability to disperse in the local fungal populations in time to effectively control the disease. The mycovirus–fungus formulations and the field delivery methods might vary considerably in soilborne and airborne pathogens and depending on the main agents of dispersal of the disease and inoculum source. Pathogens that survive as resistant structures in the absence of the host plant likely have less chance of the hypovirulence transmission. In summary, the management of *C. parasitica* with CHV1-infected hypovirulent strains does not necessarily provide a general model to devise formulations of the biological control agent and delivery methods, or to predict potential challenges to the implementation of this type of control strategies. A few examples of different scenarios are considered below to illustrate how the life cycle of the pathogen and the characteristics of the crop(s) affected can impact the practical application of mycovirus-based control strategies, a question that has been so far rarely addressed in the literature.

In the genus *Heterobasidion*, which includes very devastating conifer pathogens, horizontal virus transmission takes place readily between different species (Vainio and Hantula 2016). In this regard, *Heterobasidion* species are well suited for the use of biocontrol mycoviruses. Recently, a promising *Heterobasidion* partitivirus, HetPV3-an1, with a strong debilitating effect on the host was reported (Vainio et al. 2018). *H. parviporum* can spread from tree to tree via root contacts in the form of vegetative mycelium, whereas the basidiospores gain entry through fresh stump surfaces and stem wounds. In their analysis of HetPV3-an1, Vainio et al. (2018) confirmed that it reduces the ability of *H. parviporum* to grow in spruce trees by colonizing wood plugs with mycelium of the hypovirulent strain and inserting them into holes drilled in the root collar of large spruce trees. In a previous study, Vainio et al. (2013) introduced a virus-infected *H. parviporum* strain by spraying freshly exposed stumps exhibiting visible wood decay with liquid mycelial preparations. Interestingly, after three growing seasons the artificially introduced *H. parviporum* strain could not be detected but the mycovirus it carried had been transmitted to indigenous strains. These analyses provide clues about how mycoviruses could be deployed for *Heterobasidion* disease control.

R. necatrix is a soilborne pathogen that causes white root rot in a variety of crops including fruit trees and grapevine and in which mycoviruses of interest in biological control are available (Chiba et al. 2009; Kanematsu et al. 2004). The main source of *R. necatrix* infection is the mycelium, with the disease transmitted to neighboring trees by root contact (Pliego et al. 2012). Usually, affected orchards exhibit specific field locations where symptoms are repeatedly observed. These disease patches could provide a workable area in which to attempt mycovirus-based control strategies. Yaegashi et al. (2013a) devised a way to inoculate apple trees with infected and virus-free incompatible *R. necatrix* strains to analyze mycovirus epidemiology. For that, the fungus was cultured on apple twigs that were then tied to apple roots. Interestingly, Yaegashi et al. (2013a) found that 3 years after introducing the mycovirus in the orchard, it had been transmitted between the two incompatible strains used in the study and, what is more, these strains had become infected with other mycoviruses of unknown origin. This is an indication that horizontal virus transmission can occur between incompatible isolates of *R. necatrix* in nature.

S. sclerotiorum is another plant pathogen which has become a model system for characterization of fungus–mycovirus associations. Mycoviruses of interest have been described in *S. sclerotiorum*. Furthermore, some of them are known to be transmitted between vegetative incompatible isolates (Hamid et al. 2018; Wu et al. 2017). Yet, a major issue with the use of mycoviruses to control this pathogen is that most of its hosts are herbaceous crops that are harvested after each growth season (Jiang et al. 2013). A particular example within *S. sclerotiorum* mycoviruses is represented by

SsHADV-1, which is infectious as viral particles. Yu et al. (2013) found that spraying rapeseed plants with either virus particles or infected mycelium functioned well both as a preventive and therapeutic treatment against *S. sclerotiorum*. It is unclear whether a mycovirus with these characteristics could be used as a typical biological control agent that has the ability to persist in the ecosystem upon introduction. Nevertheless, its use as a biofungicide that could be sprayed periodically as part of a disease control program might be an interesting step in the exploitation of mycovirus to control *S. sclerotiorum*. The case of SsHADV-1 is very specific though, whereas the vast majority of mycoviruses can only be delivered in the plant pathogen natural populations through infected fungal strains.

In *F. graminearum*, the main causal agent of Fusarium head blight and a concern for its production of mycotoxins, several hypovirulence inducing mycoviruses are well characterized (Cho et al. 2012; Li et al. 2015). However, there are no thoughts yet in the literature regarding how to implement their use to control a disease that affects annual grain crops, mostly wheat and barley, and can be devastating in a short period of time. Similar challenges might pose the use of mycoviruses to control *Magnaporthe oryzae*, the most devastating pathogen of rice.

Current knowledge indicates that mycoviruses could be applied to the control of a wide variety of fungal pathogens. This includes fungi with or without natural viral infections of interest, as it has been shown that mycoviruses frequently have broad host ranges and retain the ability to induce hypovirulence when artificially transferred to heterologous hosts. Information regarding the role of *vic* genes in restricting horizontal transmission, and the characterization of mycovirus that lower vegetative incompatibility barriers, can be exploited to improve the ability of biocontrol viruses to efficiently spread in the natural populations of the pathogen. This might be of particular importance to expand their application to the control of fungal pathogens with complex *vic* systems. Exploring the use of mixed infections, for example to combine a virus that favor the horizontal and/or vertical transmission with the mycovirus inducing the desired effect on virulence, is also emerging as an interesting topic to explore. In summary, there have been great advances in the exploration of mycoviruses as potential biological agents. Currently, diverse mycoviruses with good potential to control a variety of pathogens are available, and ways to manipulate virus–fungus interactions to improve their properties are being devised. In our view, the real challenge will be to develop field implementation methods that take into account aspects such as main inoculum source and routes of infection, plant colonization modes and disease symptoms and, very importantly, the characteristic of the crops affected.

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