



Review

Greetings from virologists to mycologists: A review outlining viruses that live in fungi

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ABSTRACT

Viruses are genetic elements that parasitize self-replicating cells. Therefore, organisms parasitized by viruses are not limited to animals and plants but also include microorganisms. Among these, viruses that parasitize fungi are known as mycoviruses. Mycoviruses with an RNA genome persistently replicate inside fungal cells and coevolve with their host cells, similar to a cellular organelle. Within host cells, mycoviruses can modulate various fungal characteristics and activities, including pathogenicity and the production of enzymes and secondary metabolites. In this review, we provide an overview of the mycovirus research field as introduction to fungal researchers. Recognition of all genetic elements in fungi aids towards better understanding and control of fungi, and makes fungi a significant model system for studying microorganisms containing multiple genetic elements.

Keywords: Ecology, Mycovirus, RNA virus, Transmission

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Introduction

Various microbes with different growth mechanisms, such as viruses, bacteria, and fungi, coexist under natural conditions. These microbes interact with each other to form ecosystems and continually adapt to their environment to optimize their configurations. These interactions involve external factors such as resources and competitors, and internal factors such as non-Mendelian genetic elements and endogenous symbiosis/parasitism. Additionally, these interactions, especially those involving internal factors, may be overlooked if the relevant information is insufficient. By exploiting recent advances in virus detection techniques, researchers have determined that many fungal cells harbor more viruses (mycoviruses) than previously thought (Kondo et al., 2022; Villan Larios et al., 2023). These findings suggest that mycoviruses can manipulate certain fungal characteristics. A thorough understanding of mycoviruses is required for the comprehensive characterization of their fungal behaviors.

The first mycovirus identified was a pathogenic virus in edible

mushrooms (Romaine & Goodin, 2002). In 1962, virus-like particles were reported in diseased mushrooms (Hollings, 1962), resulting in a new branch of virology known as mycovirology. Until then, the existence of viruses within fungi was unknown. This groundbreaking discovery highlights the potential use of mycoviruses in controlling fungi, such as plant pathogenic fungi. Extensive research on mycoviruses in plant pathogens has gained momentum, ultimately evolving into a leading field in the realm of mycovirus research.

Definition of a virus

Viruses are a class of (noncellular) parasites that target self-replicating cellular systems. Traditionally, viruses are defined as “minute infectious agents that pass through filters that retain even the smallest cells” (Koonin et al., 2021); however, viruses larger than a cell or lacking a protein shell have been identified (Dolja & Koonin, 2012; Schulz et al., 2022). As available information regarding viruses accumulates, the traditional definition of viruses should be extended. The three important characteristic properties of these viruses are described below.

All cellular organisms, including fungi, store genomic information in double-stranded DNA (dsDNA). Viruses encode their ge-

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omic information in dsDNA, single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), or single-stranded RNA (ssRNA). These genomic differences forms the basis for one of the highest-level classifications of viruses (i.e., DNA or RNA viruses). Interestingly, according to the identified viruses for which the hosts have been clearly determined, most bacterial and archaeal viruses are DNA viruses, whereas both DNA and RNA viruses are roughly equally represented in eukaryotes (Nasir et al., 2014).

Many viruses are so small that they cannot be observed under light microscopy. Additionally, they are obligate parasites and thus cannot replicate without a host cell. Owing to these properties, virus identification has slowed down significantly compared to cellular organisms. The viruses initially identified are associated with infectious diseases in both plants and animals. The subsequently identified viruses were revealed to be causative agents of various infectious diseases.

Because viruses coevolve with their host cells, each virus can only be maintained in specific host cells. This host specificity explains why influenza viruses infect humans but not plants, and why the tobacco mosaic virus infects only plants. Therefore, the phylogenetic tree of viruses is expected to resemble that of cellular organisms (Wessner, 2010).

Viruses in fungi

Categorizing viruses according to their host organisms is useful. In addition to those infecting plants and animals (including humans), viruses also infect fungi (mycoviruses). Research on mycoviruses began in the 1970s, but the number of relevant publications in scientific journals has rapidly increased since 2000. In 2022, approximately 300 articles describing research on mycoviruses were published (according to a search of the [PubMed database](#) using

“mycovirus” as a search term). Several principal investigators worldwide are currently conducting research on mycoviruses. Review papers have been recently published (Table 1).

The presence of viruses in fungi has compelled researchers to explore whether mycoviruses can control fungi. The utility of mycoviruses in managing plant diseases caused by fungi has been confirmed (Nuss, 2005). Although there is currently only one report describing the successful control of fungi using mycoviruses, ongoing studies are aimed at identifying mycoviruses that are applicable for controlling fungal pathogens in plants and humans. The associated research has generated scientific knowledge that has greatly contributed to our understanding of viruses, including the elucidation of unbiased RNA virus diversity (Kondo et al., 2022) and characterization of RNA viruses lacking extracellular infection routes (Kotta-Loizou, 2021; Roossinck, 2011; 2012). As mentioned earlier, only a few decades ago, viruses were discovered by researchers investigating the causes of certain “abnormalities” (often disease symptoms) in living organisms. However, recognizing abnormalities in microorganisms such as fungi is difficult because they are small compared to animals and plants. Therefore, phenotype-independent methods for analyzing viruses have been established and are widely used (Ghabrial & Suzuki, 2009; Kotta-Loizou & Coutts, 2017). Consequently, in non-fungal organisms, only viruses from samples with abnormalities are analyzed, whereas in fungi, viruses are examined regardless of the presence of abnormalities, thereby revealing virus diversity.

Second, before mycoviruses became universally recognized, viruses were thought to infect and replicate within cells, and then exit cells to infect new cells. Most mycoviruses do not have such extracellular infection routes, suggesting that viruses have two major life cycles: infection and persistence. Some plant viruses possess similar characteristics, and some of which have advanced research.

Table 1. Major review papers published in this field.

Key words	Published year	The first author	Title
Mycoviruses of plant pathogens;	1974	Lemke, P.A.	Fungal viruses.
Hypovirulence;	1980	Ghabrial, S.A.	Effects of fungal viruses on their hosts.
Hypoviruses;	1990	Nuss, D.L.	Significance of dsRNA genetic elements in plant pathogenic fungi.
Virulence;	2005	Nuss, D.L.	Hypovirulence: mycoviruses at the fungal-plant interface.
Transmission;	2009	Ghabrial, S.A.	Viruses of plant pathogenic fungi.
Biological control;	2009	Pearson, M.N.	Mycoviruses of filamentous fungi and their relevance to plant pathology.
Virus-fungus molecular interaction;	2014	Xie, J.	New insights into mycoviruses and exploration for the biological control of crop fungal diseases.
	2015	Ghabrial, S.A.	50-plus years of fungal viruses.
	2019	García-Pedrajas, M.D.	Mycoviruses in biological control: From basic research to field implementation.
	2022	Tonka, T.	Biological control of pathogenic fungi: Can mycoviruses play an important role?
	2022	Bocos-Asenjo, I.T.	New insights on the integrated management of plant diseases by RNA strategies: Mycoviruses and RNA interference.
	2023	Hough, B.	Fungal viruses unveiled: a comprehensive review of mycoviruses.
	2023	Raza, A.	Overview of diverse universe of mycoviruses and their role in pathogenesis of fungi.
Mycoviruses of human pathogenic fungi;	2010	Van De Sande, W.W.J.	Mycoviruses: future therapeutic agents of invasive fungal infections in humans?
Forest virome;	2016	Muñoz-Adalia, E.J.	The use of mycoviruses in the control of forest diseases.
Forest protection;	2021	Rumbou, A.	Towards the forest virome: high-throughput sequencing drastically expands our understanding on virosphere in temperate forest ecosystems.
Biological control of forest diseases;			
Mycoviruses of edible fungi;	2022	Zhang, Y.	Diversity of mycoviruses in edible fungi.
Ecosystem-level impact of mycoviruses;	2022	Kondo, H.	Mycovirus diversity and evolution revealed/inferred from recent studies.
Mycoviral transmission;	2022	Myers, J.M.	Mycoviruses.
Virus evolution;	2023	Andika, I.B.	Cross-Kingdom interactions between plant and fungal viruses.
Cross-kingdom infection;	2023	Ayllón, M.A.	Mycoviruses as a part of the global virome: diversity, evolutionary links and lifestyle.
Extracellular transfer;			
Mycoviral curing strategies;	2023	Khan, H.A.	An overview of mycoviral curing strategies used in evaluating fungal host fitness.
Technologies of mycovirus identification;	2023	Villan Larios, D.C.	Exploring the mycovirus universe: identification, diversity, and biotechnological applications.
Virus-fungi interactions;			

A few mycoviruses with extracellular routes of infection have been identified (Yu et al., 2013).

Mycoviruses generally have the following characteristics: (1) they do not kill the host (many are asymptomatic), (2) they have a persistent life cycle, and (3) they have an RNA genome. Furthermore, the frequency of RNA mycovirus detection in the isolated fungal strains ranges from 7% to 80% (Ghabrial et al., 2015; Kotta-Loizou & Coutts, 2017). The genome of a typical RNA mycovirus (2–15 kb) encodes 1–12 proteins. Apart from the genes encoding the enzymes (RNA-dependent RNA polymerase; RdRP) responsible for replicating viral RNA and those encoding the capsid proteins (CP) that form particles, most genes remain unannotated. In other words, functional analysis of mycovirus genes has slowed down compared to that in RNA viruses that infect animals and plants.

Several model systems have been developed for mycovirus research. The most well-known system involves the chestnut blight fungus, *Cryphonectria parasitica* and its persistent RNA virus *Cryphonectria hypovirus 1* (CHV1; +ssRNA). This system is the only one used commercially for the control of plant-pathogenic fungi, and has enabled reverse genetics approaches to reconstruct viruses from cDNA. Genetic modifications are also possible in the host fungus, and the RNAi pathway is revealed to be involved in viral defense in fungi (Segers et al., 2007). Extensive mycoviral research has been conducted on *Fusarium graminearum* (Chu et al., 2004; Yu & Kim, 2020). Notably, a new strategy has been conceptually demonstrated using mycoviruses as silencing vectors to suppress pathogenicity-related genes in *F. graminearum* (Zhang et al., 2023a). Furthermore, red bread mold, the model filamentous fungus, can serve as a platform for mycovirus research (Honda et al., 2020). Recently, attention has been drawn to a DNA virus reported in China that is capable of extracellular infection of host fungi (Li et al., 2020; Yu et al., 2010; 2013). This discovery is expected to lead

to the development of new models for mycoviral research.

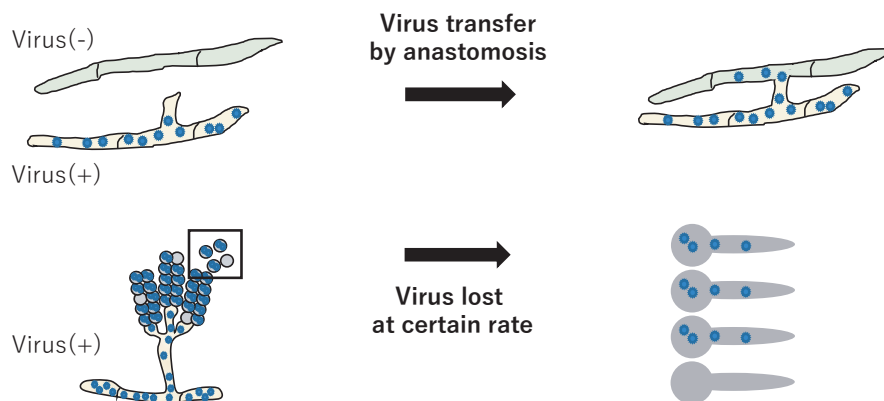
Mycoviral transmission

The transmission of mycoviruses is an important process that must be characterized to fully understand the viral ecology and fungal evolution. Unlike bacteriophages and many other eukaryotic viruses, mycoviruses do not lyse host cells. Thus, mycoviruses are generally persistent and may be transmitted via host cells (Fig. 1).

Mycoviruses are transmitted vertically among fungi via host asexual or sexual spores; however, transmission efficiency may vary (Pearson et al., 2009). In many ascomycetous fungi, such as *Aspergillus nidulans*, *Penicillium stoloniferum*, *Ustilagoidea virens*, and *Ophiostoma ulmi*, viruses are efficiently (90–100%) transferred to asexual conidia (Sutela et al., 2019). In the wheat pathogen *Fusarium graminearum*, RNA viruses can be transmitted through conidia and ascospores, with an incidence of 30–100% (Chu et al., 2004).

Mycoviruses may be horizontally disseminated via hyphal anastomosis and heterokaryosis, with the efficiency of transmission apparently dependent on the viral strain, host genotype such as the vegetative compatibility group (VCG), and interactions among these factors. For example, *Sclerotinia sclerotiorum* partitivirus 1 (SsPV1) can spread via donor and receptor hyphal fusion, and is transmitted to *Botrytis cinerea* (Xiao et al., 2014). However, CHV1 is frequently transmitted (transmission rate >50%) to vegetatively incompatible (*vic*) *Cryphonectria parasitica* strains (with two or four different *vic* loci) on chestnut stem (*in planta*) (Brusini et al., 2011). In a previous study, a mycovirus-like dsRNA element (called N10) was transmitted between vegetatively incompatible strains of *Rosellinia necatrix* when an N10-infected strain and a virus-free strain were co-inoculated to apple tree roots (Yaegashi et al., 2013).

In environments



In laboratory

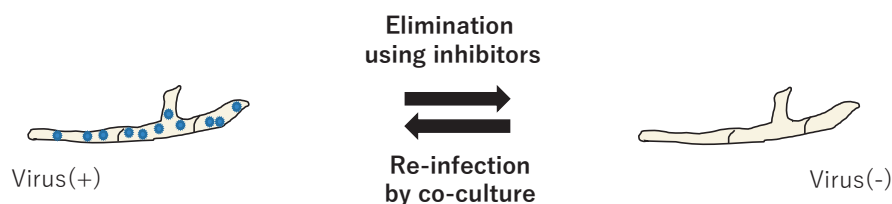


Fig. 1: Transmission/lost of mycoviruses in environment or laboratory.

Similarly, *Sclerotinia sclerotiorum* mycoreovirus 4 (SsMYRV4) can suppress host non-self-recognition, resulting in an SsMYRV4-infected strain that can easily accept other viruses through hyphal contact and serve as a reservoir to facilitate the horizontal transmission of heterologous viruses between different *Sclerotinia sclerotiorum* VCGs (Wu et al., 2017). These findings indicate that weakening fungal non-self-recognition under certain conditions may be a potential strategy for promoting mycovirus transmission. Although this is an artificial system, even capsid-less viruses (mitoviruses) can be transmitted horizontally by heterologous protoplast fusion technology (Shahi et al., 2019).

Little is known about the natural vectors for most mycoviruses, but the encapsulated single-stranded circular DNA mycovirus *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1) may be transmitted as a viral particle via an extracellular route with the assistance of a mycophagous insect as vector (Liu et al., 2016; Yu et al., 2013). Yaegashi and Kanematsu (2016) hypothesized that microarthropods or nematodes may mediate the interspecies transmission of RNA viruses because of the appearance of novel mycoviruses in *R. necatrix* mycelia after incubation in the soil. Their hypothesis was supported by the detection of a ‘phlegivirus’ of the ectomycorrhizal fungus *Thelephora terrestris* in ticks (Petrzik et al., 2016).

Although the underlying mechanisms need to be clarified, unknown environmental factors are believed to influence mycovirus transmission. For example, analyses of CHV1 and the other three viruses indicated that mycovirus transmission rates are higher on natural substrates and in the field than under *in vitro* conditions and among isolates of the same species (García-Pedrajas et al., 2019). Yaegashi et al. (2013) inoculated healthy apple trees with two incompatible *R. necatrix* strains (i.e., virus-free and virus-infected). Three years after inoculation, the mycoviruses within the two retrieved strains were analyzed, and several novel mycoviruses were detected (Yaegashi et al., 2013). Under natural conditions, the transmission of mycoviruses may involve diverse processes and can be facilitated by multiple environmental factors; however, this needs to be experimentally verified.

It is noteworthy that cross-infection of plant viruses with plant-associated fungi commonly occurs in nature. For example, tobacco mosaic virus (TMV) has been reported to be associated with rust and powdery mildew (Nienhaus, 1971; Yarwood & Hecht-Poinar, 1973), and cucumber mosaic virus (CMV) has been detected in a phytopathogenic basidiomycetous fungus strain (*Rhizoctonia solani*) (Andika et al., 2017). Thereafter, systematic viral screening of fungal strains revealed that approximately half of the detected strains harbored plant viruses (Cao et al., 2022). Viroids are also transmitted from plants to fungi under natural conditions (Wei et al., 2019). Cross-kingdom viral infections may depend on effector proteins and RNAs, which requires further research (Andika et al., 2023).

Ecological functions and dynamics of mycoviruses

Many questions remain regarding the functions and ecological significance of mycoviruses. Mycoviruses that infect endophytic fungi may play mutualistic roles in multiplex symbiosis under specific environmental conditions. For example, *Curvularia thermal tolerance virus* (CThTV) (belonging to the genus *Orthocurvulavirus* of the family *Curvulaviridae*) isolated from an endophytic fungus can enhance the heat tolerance of plants via plant–fungal symbiosis (Marquez et al., 2007). CThTV vertically transmitted in *Curvularia protuberata* that can infect several plants and enhance the heat tolerance of tomatoes (Rodriguez et al., 2008). Several CTh-

TV-like viruses have been identified in ectomycorrhizal fungi (Sutela & Vainio, 2020; Zhao et al., 2023b). Because ectomycorrhizal fungi affect the survival of woody plants and help plants tolerate harsh environmental conditions, CThTV-like viruses may positively modulate symbiotic systems.

Mycoviruses may be mobile genetic elements that increase the flexibility of their hosts, enabling rapid adaptation while also enhancing their survival during environmental changes (Ghabrial et al., 2015). Several studies have revealed that mycoviruses occur independently in environmental populations, such as some basidiomycetous tree root pathogens, including *Heterobasidion* species (Hyder et al., 2018; Vainio et al., 2013; 2015), *Rosellinia necatrix* (Yaegashi et al., 2013), and *Helicobasidium mompa* (Ikeda et al., 2005). Jia et al. (2021) continuously monitored the interannual dynamics and abundance of mycoviruses infecting *Sclerotinia sclerotiorum* in a rapeseed field for three years and discovered that the *S. sclerotiorum* virome consists of a stably maintained core population as well as dynamic mycovirus compositions. Changes in mycoviral populations under natural conditions may have beneficial effects on the survival of mycoviruses and their hosts.

Ecology-based research on most known mycovirus–host combinations are relatively limited. Schoebel et al. (2017) analyzed the genetic population structure of *Hymenoscyphus fraxineus* mitovirus 1 (HfMV1) in *Hymenoscyphus fraxineus* at small and large spatial scales and detected HfMV1 in more than 80% of European isolates, but in only approximately 1% of Japanese isolates. Because mitoviruses comprise a segment of extrachromosomal RNA and exist in fungal mitochondria, HfMV1 may provide relevant insights into the invasion history of *H. fraxineus* (native to East Asia and most likely introduced to Europe in the early 1990s) (McMullan et al., 2018). Additional ecological research on mycoviruses and their hosts needs to be conducted to thoroughly characterize the coevolution of mycoviruses, their hosts, and viral ecological functions.

Artificial infection and removal of mycoviruses

A classical method for elucidating the effects of viruses on hosts involves the generation of virus-free strains by removing viruses from infected isolates, followed by phenotypic comparison of the infected and virus-free strains (Kotta-Loizou & Coutts, 2017). Furthermore, to gain a general understanding of the effects of viruses on other strains of the same species, the virus must be introduced into other strains of the original host species, and infection-induced phenotypic changes must be determined. This artificial introduction of viruses is also useful for analyzing viral functions in cases where eliminating the virus from its original host is difficult. In mycovirus research, the development, improvement, and application of techniques for removing and introducing viruses are critical for elucidating viral functions.

Mycovirus removal

The removal of viruses typically involves the following processes: (1) isolation of host cells, (2) culturing of the isolated cells to obtain sufficient mycelia, and (3) determination of the presence or absence of viral infection using electrophoresis or reverse transcription PCR. The primary methods used to isolate cells from filamentous fungi include single-spore isolation (Fuke et al., 2011; Higashiura et al., 2019; Hillman et al., 2004; Ikeda et al., 2022; Suharto et al., 2022; Urayama et al., 2014b; Wang et al., 2022), hyphal tip isolation (Chiba et al., 2013; Kanematsu et al., 2004; Liu et al., 2022; Urayama et al., 2010), and protoplast preparation (Liu et al., 2019; Niu et al., 2016). Single-spore isolation involves inducing co-

nidiation of the filamentous fungus and isolating the spores, which is relatively easy if the ideal sporulation conditions are known for a particular fungus. Alternatively, hyphal tip isolation, which involves removing hyphal tip cells under a microscope, is appropriate for fungi that do not produce spores. Additionally, if conditions conducive to protoplast formation are known for the target fungus, protoplast preparation is a viable option.

Drugs may be used to promote the generation of virus-cured cells during steps (1) and (2). For example, adding nucleoside analogs such as ribavirin, which inhibits the replication of RNA viruses, to the medium often enhances the generation of virus-cured cells (Ikeda et al., 2022; Liu et al., 2019; Niu et al., 2016). A recent study demonstrated the utility of nucleoside analogs for removing major mycoviruses from *Aspergillus* fungi (Ikeda et al., 2022). Although this method is highly convenient, further research is necessary to determine the extent to which it can be applied to diverse filamentous fungi and mycoviruses. Translation inhibitors, such as cycloheximide, have also been used (Liu et al., 2022; Urayama et al., 2010), but their effectiveness has not been quantitatively evaluated.

Virus introduction via mycelial fusion

Mycoviruses are believed to primarily spread horizontally through hyphal fusion because there is no major extracellular infection route. Viruses are transmitted when an infected cell fuses with an uninfected cell. Methods for mediating viral infections via hyphal fusion are among the simplest techniques for treating artificial viral infections. By positioning the viral donor and recipient strains next to each other on the same plate, hyphal fusion and viral transmission occur naturally if the strains are compatible. Although this method is simple, it can only be used for closely-related recipient strains. Combinations of compatible strains are limited to genetically similar strains (often within the same species). Therefore, the use of this method to transmit viruses to distantly related recipient strains is challenging. In practice, this method is predominantly used to infect mycovirus to strains that share the genetic background (Higashiura et al., 2019; Kanematsu et al., 2004; Niomiya et al., 2020; Wang et al., 2022; Yaegashi et al., 2011), with a few exceptions (Liu et al., 2022). Ikeda et al. (2013) reported that zinc salts facilitate hyphal fusion between incompatible *Rosellinia necatrix* strains. Although the universality of this phenomenon has not been verified, it suggests that the virus transmission rate may be increased using drugs that promote hyphal fusion. In a more recent study, a botybirnavirus derived from *Leptosphaeria biglobosa* (Dothideomycetes) was transmitted to *Botrytis cinerea* (Leotiomy-

etes) by mixing the spores of the two fungal species and then inoculating them on agar medium (Deng et al., 2022). Although the molecular mechanism underlying the transmission remains unknown, this method represents a new option that can overcome the limitations of the hyphal fusion method. Another limitation of the hyphal fusion method is that, when the donor and recipient are the same fungal species, genetic markers are required to distinguish them after co-culture. Hence, the utility of this method is restricted to situations in which the marker sequences that can differentiate between the two strains are known. In practice, introducing drug resistance genes into recipient fungi and selecting recipient-derived cells using drugs are more common (Higashiura et al., 2019; Kanematsu et al., 2004; Yaegashi et al., 2011).

Artificial introduction

Hyphal fusion is a naturally-occurring process. An artificial method for introducing viruses into cells using the protoplast PEG method has been established (Castro et al., 2003; Chiba et al., 2013; Ejmal et al., 2018; Hillman et al., 2004; Kanematsu et al., 2010; Niu et al., 2016; Suharto et al., 2022; Zheng et al., 2014). Specifically, protoplasts of the recipient fungus are prepared, after which purified virus particles from the donor are introduced directly into the protoplasts. The main advantage of this method is that as long as recipient protoplasts and virus particles are available, even viruses obtained from distantly related donors can be introduced into cells. Whether the virus is maintained within cells depends on the virus and host combination. This approach was used to introduce a partitivirus derived from *Rosellinia necatrix* (Sordariomycetidae) into *Glomerella cingulata* (Hypocreomycetidae) (Kanematsu et al., 2010). In addition, *Aspergillus fumigatus*, *A. nidulans*, and *A. oryzae*, which are closely related species, were previously infected with a chrysovirus derived from *A. thermomutatus* (Ejmal et al., 2018). Furthermore, *Rhizoctonia solani* can be artificially infected with the cucumber mosaic virus derived from plants (Andika et al., 2017). Therefore, this approach is applicable even when the donors and recipients are genetically distant. However, to introduce viruses using the protoplast PEG method, the virus must form particles and recipient protoplasts must be prepared.

Effects of viruses on host individuals

Mycoviruses are currently being functionally characterized based on comparisons between virus-free strains obtained using the aforementioned methods and corresponding virus-infected

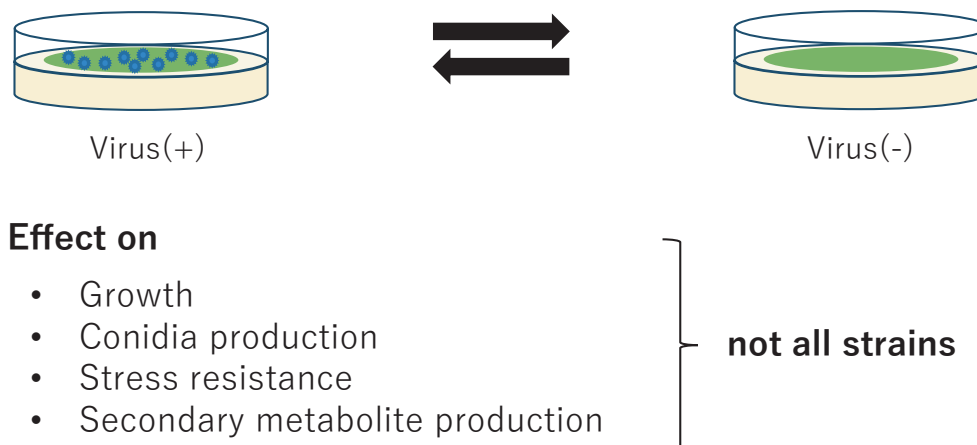


Fig. 2: Effects of viruses on host individuals

strains (Fig. 2). The relationship between mycoviruses and pathogens, such as *Cryphonectria* and *Fusarium* species, has been extensively investigated, and the effects of mycoviral infections on various characteristics, such as colony morphology, hyphal growth, conidiation, pathogenicity, and secondary metabolite production have been reported (García-Pedrajas et al., 2019; Heiniger & Rigling, 1994; Li et al., 2019; Milgroom & Cortesi, 2004; Nuss, 2005). Importantly, many mycoviruses are associated with decreased host virulence (hypovirulence) and enhanced host virulence (hypervirulence). Here, we focus on examples of intriguing phenotypes induced by mycoviruses.

Viral effects on host production

Recently, research on the relationship between specific fungal activities, including the production of secondary metabolites, enzymes, and mycoviruses, is rapidly increasing. For example, *Alternaria alternata* chrysovirus 1 (AaCV1) from *Alternaria alternata* can disrupt host growth while promoting toxin production and increasing host pathogenicity in plants (Okada et al., 2018). Moreover, the magnitude of the phenotypic changes varies depending on the viral copy number. Several other recent reports have described mycovirus-induced changes in the production of secondary metabolites, including the enhanced production of ochratoxin in *Aspergillus ochraceus* and tenuazonic acid in *Magnaporthe oryzae*, as well as the decreased production of altersolanol A in *Stemphylium lycopersici* (Liu et al., 2022; Nerva et al., 2019a; Ninomiya et al., 2020). Furthermore, infection of the basidiomycete *Pleurotus ostreatus* with *Pleurotus ostreatus* virus 1 (PoV) inhibited the production of various enzymes, leading to restricted fruiting body development (Song et al., 2020).

Viral effects on fungal pathogens of humans

Recently, research on mycoviruses infecting human pathogens has increased. A comprehensive exploration of mycoviruses has been conducted using clinical isolates from 27 genera, including *Aspergillus*, *Microsporum*, and *Cladosporium*, resulting in the detection of 11 viral species, reflecting the ubiquitous presence of mycoviruses (Kinsella et al., 2022). In the extensively studied pathogen *A. fumigatus*, infection with *Aspergillus fumigatus* chrysovirus 41362 (AfuCV41362) or *Aspergillus fumigatus* polymycovirus-1 (AfuPmV-1) leads to a decrease in pathogenicity in certain strains, whereas an infection with *Aspergillus fumigatus* chrysovirus (AfuCV) or *Aspergillus fumigatus* partitivirus (AfuPV-1) does not affect pathogenicity, but an infection with an uncharacterized A78 virus increases pathogenicity (Özkan & Coutts, 2015; Takahashi-Nakaguchi et al., 2020; 2019). Controlling the pathogenicity of human pathogens using mycoviruses may lead to new treatment strategies that do not require drugs. However, because of the diversity in phenotypes among different fungal strains or even among different viruses, further research is required to develop viable methods.

Viral effects on host stress tolerance

Viruses can influence the stress tolerance of their hosts, and changes in stress tolerance often affect pathogenicity. Accordingly, interest on this research area is increasing. In *Aspergillus fumigatus*, strains infected with AfuCV41362 exhibited decreased tolerance to low oxygen, oxidative, and osmotic stress, and decreased pathogenicity in mice (Takahashi-Nakaguchi et al., 2019). In *Colletotrichum fructicola*, infection with *Colletotrichum fructicola* our-

mia-like virus 1 (CfOLV1) leads to decreased osmotic stress tolerance and pathogenicity (Guo et al., 2022). In *C. higginsianum*, infection with *Colletotrichum higginsianum* non-segmented virus (ChNRV1) results in decreased high-temperature stress tolerance and increased pathogenicity, but no changes in osmotic stress tolerance (Olivé & Campo, 2021). In *Alternaria tenuissima*, infection with the *Alternaria alternata* botybirnavirus 1 AT strain (AaBRV1-AT1) enhances growth and increases resistance to difenoconazole, leading to increased pathogenicity in plants; however, these phenotypes are attenuated in non-natural host strains (Liang et al., 2022). Previous analyses of *Penicillium crustosum* infected with *Penicillium crustosum* chrysovirus 1 (PcCV1), as well as *P. digitatum* co-infected with *Penicillium digitatum* polymycovirus 1 (PdPmV1) and *Penicillium digitatum* Narna-like virus 1 (PdNLV1) showed decreased resistance to prochloraz, suggesting a common effect among *Penicillium* species (Niu et al., 2018; Wang et al., 2019).

Viral effects on plant–fungus interactions

The drought tolerance of *Dichantheium lanuginosum* (a rosette grass species) increases substantially through symbiosis with *Colletotrichum protuberata*. Interestingly, *C. protuberata* strains free of *Curvularia thermal tolerance virus* (CThTV) are susceptible to drought stress, implying the virus contributes to drought tolerance (Marquez et al., 2007). In addition, infection with *Pestalotiopsis theae*, a pathogen of *Camellia sinensis*, and *Pestalotiopsis theae* chrysovirus-1 (PtCV1) results in a loss of pathogenicity, leading to the absence of disease symptoms. Moreover, PtCV1 is stably maintained within plants and produces vaccine-like effects, thereby enhancing host plant resistance to pathogens (Zhou et al., 2021). These studies revealed rare instances where the infection of a host fungus with a particular virus resulted in a nonpathogenic fungus that had positive effects on plants. As these findings have significant implications for plant protection, mechanisms underlying the tripartite relationships among plants, filamentous fungi, and viruses should be precisely elucidated.

“Killer” phenomenon

A well-known effect of mycoviruses is the “killer” phenomenon in yeast-like fungi. In *Saccharomyces cerevisiae*, the presence of M dsRNA, a satellite dsRNA fragment of totiviruses, leads to the production of killer toxins. The ability to produce toxins varies because of the diversity of viral genomic sequences (Rowley, 2017). Similar toxins have been identified in many other *Saccharomyces* species (Fredericks et al., 2021). The association between the presence of a virus and production of killer toxins has been demonstrated in *Xanthophyllomyces dendrorhous*, *Cryptococcus aquaticus*, and *Cystofilobasidium infirmominatum* (Baeza et al., 2010; Golubev et al., 2003; Pfeiffer et al., 2004). Furthermore, *Torulaspota delbrueckii* and *Wickerhamomyces anomalus* strains with antimicrobial activities reportedly contain sequences corresponding to killer toxins, suggesting a potential relationship with mycoviruses (Abu-Mejdad et al., 2020).

Condition dependence

Based on available information, the relationship between mycoviruses and their hosts is complex because the type, magnitude, and direction of the effects vary among specific combinations. To reveal the overall effects of viruses on host fungi, in addition to the research conducted to date on mycoviruses that induce significant phenotypic changes, studies exploring latent mycoviruses are also

necessary (Komatsu et al., 2019; Villan Larios et al., 2023; Wang et al., 2018). Previous studies have confirmed that even in combinations that do not result in visible phenotypic changes, gene expression and secondary metabolite production can be altered (Lee et al., 2014; Zhang et al., 2023b). Additionally, although previous research on mycoviruses has primarily focused on the phenotypes of natural host strains under single conditions, recent studies have attempted to introduce viruses into different fungal strains and examine the phenotypic changes under various culture conditions (Córdoba et al., 2022; Kashif et al., 2019; Kuroki et al., 2023; Ning et al., 2022; Nuskern et al., 2021).

Detection of mycoviruses

Initially, the detection of mycoviruses required a labor-intensive process that involved purifying the viruses from fungal cells and screening for viral particles using electron microscopy. Even in situations where the presence of a pathogen was suggested (e.g., in the aforementioned case of mushrooms), it was difficult to determine whether the pathogen was a virus or another microbe. Therefore, the use of viral particles to identify mycoviruses remains challenging.

The establishment of a simple method in the late 1970s greatly facilitated mycoviral research. This method utilized long dsRNAs as molecular markers of mycoviruses (Morris & Dodds, 1979). Generally, cells lacking RNA viruses do not contain long dsRNAs; however, if dsRNA is detected, it likely originates from the dsRNA viral genome or the ssRNA viral replicative intermediate. The simplicity of purifying dsRNA from biological samples via chromatography (including spin columns) and subjecting it to electrophoresis enables detection of the presence or absence of RNA mycoviruses, ultimately leading to reports of numerous mycoviruses (Ghabrial et al., 2015; Kotta-Loizou & Coutts, 2017). However, this method is not applicable to DNA mycoviruses.

In the 2000s, the development of high-throughput RNA sequencing (RNA-seq) accelerated mycovirus identification. Mycoviruses have been serendipitously identified during analyses of fungal transcriptome data (Gilbert et al., 2019). However, detection of mycoviruses that do not possess a poly(A) fragment requires total RNA-seq analysis. In addition, the identification of RNA mycoviruses based on sequence data depends on their similarities with known RNA viruses. Therefore, a comprehensive search for RNA mycoviruses is yet to be conducted. To conduct a comprehensive search for RNA mycoviruses, we established a dsRNA-specific full-length sequencing method (Urayama et al., 2016; 2018), which was used to elucidate RNA mycovirus diversity (Chiba et al., 2021a; Kuroki et al., 2023; Takahashi-Nakaguchi et al., 2019; Zhao et al., 2023b).

After a particular mycovirus has been identified and its nucleotide sequence has been determined, techniques such as (reverse transcription) PCR amplification or antibody-based detection of antigens may be employed to detect the same or closely related mycoviruses. Relatively simple and effective detection methods have been developed for studies focused on specific mycoviruses (Urayama et al., 2014a).

Diversity of mycoviruses

Following animals and plants, fungi have been thoroughly analyzed in terms of the diversity of RNA viruses (Charon et al., 2022). Herein, we provide an overview of recent advances in mycoviral diversity research. Excellent reviews are available on this topic (Ghabrial et al., 2015; Kondo et al., 2022; Kotta-Loizou & Coutts,

2017; Myers & James, 2022). These reviews are a rich source of information regarding the detected viruses (e.g., taxonomy, genome organization, and lifestyle).

Over the past decade, the application of RNA-seq technology has resulted in an exponential increase in the number of novel mycoviruses. Traditionally, mycoviruses have been investigated via electrophoretic analysis of dsRNA in infected strains, after which the virus is identified through cloning and Sanger sequencing. Conventional mycovirus research has typically focused on relatively few viruses, with most studies describing only one virus (Chiba et al., 2009; Kanhayuwa et al., 2015; Urayama et al., 2010; Xie et al., 2011). The first report of the use of RNA-Seq technology to identify mycoviruses was published in 2016 (Marzano et al., 2016; Nerva et al., 2016; Osaki et al., 2016). It subsequently became common for studies to identify 10–100 viruses (Arjona-Lopez et al., 2018; Chiba et al., 2021a; Jia et al., 2021; Marzano et al., 2016; Nerva et al., 2019b; Ruiz-Padilla et al., 2021; Sutela et al., 2020). According to the accepted list of mycoviruses from the International Committee on Taxonomy of Viruses (ICTV) Master Species List, 2020. v1 (Kondo et al., 2022), 113 virus species were identified between 1969 and 2015. However, after RNA-seq technology was broadly adopted, 93 virus species were identified in just four years (2016–2020).

The presence of numerous ssRNA viruses in fungi has been confirmed using RNA-seq. The number of ssRNA and dsRNA viruses in the aforementioned mycovirus list suggests that, prior to the widespread use of RNA-seq, there was a bias towards dsRNA viruses (74 species of dsRNA viruses and 27 species of ssRNA viruses). However, using RNA-seq, an equal number of ssRNA viruses (99 species of dsRNA viruses and 94 species of ssRNA viruses) have now been described. The application of RNA-Seq eliminated this bias. This underestimation of ssRNA virus diversity can be attributed to biases in the screening methods (Kondo et al., 2022). Several studies have indicated that traditional screening methods that detect dsRNA through electrophoresis can potentially overlook viruses with low dsRNA accumulation, including ssRNA viruses (Chiba et al., 2021a; Myers & James, 2022; Nerva et al., 2016).

With regard to DNA viruses, only a few ssDNA viruses have been identified (Li et al., 2020; Yu et al., 2013), and reports of dsDNA viruses or pararetroviruses are not yet available, although the integration of dsDNA viruses into the fungal genome has recently been reported (Zhao et al., 2023a), suggesting the existence of dsDNA viruses that infect fungi. Given that the number of reported DNA viruses has not increased remarkably even in the era of mycovirus hunting using RNA-seq data (including mRNA from DNA viruses), they may represent a minor proportion of viruses in fungi.

Unprecedented genomic structures have been reported in recent mycoviral studies. For example, the first divided RdRP was identified in some mycoviruses (Chiba et al., 2021a; 2021b; Jia et al., 2021; Ruiz-Padilla et al., 2021; Sutela et al., 2020). The finding of divided RdRP encoded by two open reading frames (ORFs) on different genomic segments altered the view that RdRP, the only universal gene for non-retro RNA viruses, must be encoded by a single ORF. Circular RNA viruses with hybrid features of RNA viruses and viroid-like elements have also been identified (Forgia et al., 2023). In addition to the genomic structures, unique lifestyles have been discovered. Yadokariviruses lacks their own capsid gene, but be encapsidated by capsid proteins encoded by other unrelated viruses (yado-kari/yado-nushi nature) (Sato et al., 2022; Zhang et al., 2016). Although hadakaviruses have 10–11 segments, they lack a capsid gene (hadaka nature) (Sato et al., 2020).

Mycovirus research has expanded our knowledge of the diversity of eukaryotic RNA viruses. However, the scope of mycovirus research has been biased towards pathogenic ascomycetes and basid-

iomycetes (Myers et al., 2020); thus, our understanding of viral diversity, even in mycoviruses alone, is far from complete. Recent studies using unexplored sources have led to the continuous discovery of novel viruses at species or higher taxonomic levels (Chiba et al., 2021a; Myers & James, 2022; Zhao et al., 2023b).

Virologists love fungi

For many fungal researchers, the fact that latent mycoviral infections of fungal isolates are common seems unfavorable. The possibility that these mycoviruses are eliminated during cultivation is also a concern. Nevertheless, entities such as mycoviruses are continually being identified in various organisms (Chiba et al., 2020; 2023; Fujita et al., 2021; Fukasawa et al., 2020; Mizutani et al., 2022; Urayama et al., 2020, 2022). Fungi may be considered advanced organisms for inclusive research. Therefore, mycovirus research may provide new insights into the relationship between cells and persistent viruses. A viroid-like replicon, previously known only as a plant pathogen, has been recently reported to exist in fungi (Dong et al., 2023). Clarifying the relationship between cells and persistent replicons, and their roles, will depend on focused fungal research.

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