LIÈGE université
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Agro-Bio Tech

Virus and entomofungus interaction management to improve plant protection

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Year: 2024

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Virus and entomofungus interaction management to improve plant protection

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs: Prof. Frédéric Francis
Prof. Xifeng Wang
Année civile: 2024

Résumé

Jiashu GUO. (2024). "Interactions virus - champignons entomopathogènes et gestion de la phytoprotection" | (Thèse de doctorat en anglais).

Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.

116 pages, 9 tableaux, 31 figures.

Les plantes vivent en association avec divers micro- et macro-organismes, y compris des insectes, qui jouent des rôles importants dans l'écologie et la physiologie des plantes. Ces dernières doivent continuellement faire face à des ravageurs et des agents phytopathogènes, seuls ou selon diverses interactions. Cette étude se concentre sur les virus associés à d'autres stress biotiques.

Bien que la plupart des virus connus infectant les champignons pathogènes des eucaryotes supérieurs soient asymptomatiques ou réduisent la virulence de leurs champignons hôtes, ceux qui confèrent une hypervirulence à des champignons entomopathogènes (CEP) sont peu explorés. Un nouveau mycovirus a été isolé à partir de la cicadelle *Laodelphax striatellus*. L *Metarhizium flavoviride* partitivirus 1 (MfPV1) est une espèce du genre *Gammapartitivirus*, famille *Partitiviridae*. MfPV1 a deux ARN double brin comme génome, de tailles respectives de 1 775 et 1 575 pb, encapsidés dans des particules isométriques. Des souches commerciales de *M. anisopliae* et *M. pingshaense* ont été transfectées avec MfPV1. La sprorulation a été considérablement améliorée et les taux de mortalité significativement plus élevés pour les larves de la pyrale du chou (*Plutella xylostella*) et la chenille légionnaire (*Spodoptera frugiperda*). Aussi, les niveaux de transcription des gènes liés à la pathogénie de *M. anisopliae* infecté par MfPV1 étaient manifestement altérés, suggérant une augmentation de la production de protéine-like d'adhésine de *Metarhizium*, de protéine hydrolysée et de synthétase de destruxine.

Ensuite, un nouveau virus à ARN positif à brin unique a été identifié à partir de larves de la mouche du blé (*Dolerus tritici*) collectée dans le nord de la Chine. Le génome complet mesure 9 594 pb pour coder une polyprotéine de 326,3 kD. L'analyse de l'arbre phylogénétique de la polyprotéine déduite a révélé que ce virus à ARN était regroupé dans le clade avec le virus des ailes déformées du genre *Iflavirus*, famille *Iflaviridae*. Le génome complet de ce virus à ARN montre des identités de séquence de 38,9 à 50,0 % avec d'autres iflavirus. Ce virus a la plus haute identité avec le virus de la paralysie lente des abeilles à 33,6 %, ce qui ne dépasse pas 90 %, suggérant qu'il s'agit d'un nouveau membre du genre Ifavirus désigné comme « Dolerus tritici iflavirus 1 » (DtIV1).

En conclusion, notre étude a examiné l'interaction entre virus et champignons et explore des stratégies de biocontrôle environnementales. De plus, la transfection de CEP par un mycovirus peut conférer une hypervirulence significative et fournit un bon exemple d'agents synergiques pour améliorer l'activité de biocontrôle des CEP.

Mots-clés: mycovirus, virus insecte, RNA-seq, champignon entomopathogène, hypervirulence, Metarhizium, partitivirus, iflavirus.

Abstract

Jiashu GUO. (2024). "Virus and entomofungus interaction management to improve plant protection" (PhD Dissertation in English).

Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège.

116 pages, 9 tables, 31 figures.

Plants live in association with diverse micro- and macro-organisms, which play important roles in the ecology and physiology of plants. Throughout their entire life, plants continuously have to cope with challenges associated with pests and pathogens. Not only do plants interact with these organisms but also these organisms are under various interactions leading to unintended outcomes for plant protection. This study focuses on the viruses associated with other biotic stressors.

Although most known viruses infecting fungi pathogenic to higher eukaryotes are asymptomatic or reduce the virulence of their host fungi, those that confer hypervirulence to entomopathogenic fungus (EPF) still need to be explored. We identified and studied a novel mycovirus in *Metarhizium flavoviride*, isolated from small brown planthopper (*Laodelphax striatellus*). Based on molecular analysis, we designated it Metarhizium flavoviride partitivirus 1 (MfPV1), a species in genus *Gammapartitivirus*, family *Partitiviridae*. MfPV1 has two double-stranded RNAs as its genome, 1,775 and 1,575 bp in size respectively, encapsidated in isometric particles. When we transfected commercial strains of *M. anisopliae* and *M. pingshaense* with MfPV1, conidiation was significantly enhanced with further higher mortality rates of the larvae of diamondback moth (*Plutella xylostella*) and fall armyworm (*Spodoptera frugiperda*). Transcript levels of pathogenesis-related genes in MfPV1-infected *M. anisopliae* were obviously found to be altered, suggesting increased production of Metarhizium adhesin-like protein, hydrolyzed protein and destruxin synthetase.

Unlike the indirect beneficial role of MfPV1 in plant protection, a novel insect-iflavirus seems to exhibit subtle effects.

A novel single positive-strand RNA virus from the larvae of wheat sawfly (*Dolerus tritici*) collected in northern China through RNA sequencing, and then determined its complete genome sequence by rapid amplification of cDNA ends. The complete genome consists of 9,594 bp in size including a poly A tail at 3′ terminus, which is predicted to encode a 326.3 kD polyprotein. The phylogenetic tree analysis of deduced polyprotein revealed that this RNA virus was clustered in the clade with deformed wing virus in genus *Iflavirus*, family *Iflaviridae*. The full genome of this RNA virus shows 42.0-50.0% sequence identities with other iflaviruses. Sequence identity at the amino acid level of coat proteins shows this RNA virus has the highest identity with slow bee paralysis virus at 33.6%, which no more than 90% suggesting that it is a new member in the genus *Ifavirus*. Thus, we tentatively designated as "Dolerus tritici iflavirus 1" (DtIV1).

In conclusion, experimental evidence was brought to show that transfection of other entomopathogenic fungal species with a mycovirus can confer significant

hypervirulence and provides a good example that mycovirus could be used as a synergistic agent to enhance the biocontrol activity of entomopathogenic fungi.

Keywords: mycovirus, insect virus, RNA-seq, entomopathogenic fungus, hypervirulence, Metarhizium, partitivirus, iflavirus.

Acknowledgments

Four years ago, I came to Beijing to begin my doctoral studies, and now I am about to graduate. I can still recall many events that have occurred during this time. Throughout these four years of study, I have gained a wealth of knowledge and received much help, I have also encountered many remarkable experiences and grown personally. As the thesis is about to be completed, I would like to express my sincerest thanks to all those who have supported me.

The deepest and sincerest gratitude goes to my supervisors, Prof. Frédéric Francis and Prof. Xifeng Wang, for offering me the opportunity to study in the Laboratory of Functional and Evolutionary Entomology and the Crop Virus Disease Lab. First of all, I would like to sincerely thank my supervisor Prof. Xifeng Wang. During the four years since I started working in the laboratory, he has guided my research conscientiously, pointed out the problems in my experiment carefully and provided me with guiding advice. Not only has Prof. Wang supported my research, but he has also shown great concern for our life. The past few years of studying with Prof. Wang have been both fulfilling and enjoyable. Not only have I gained a lot of knowledge in research, but more importantly, I have also learned many truths about life. These valuable insights are things that Prof. Wang has shared with me. Here, I would like to extend my heartfelt thanks to Prof. Wang and wish him all the best in both his personal and professional life in the future. I would also like to express my heartfelt thanks to another supervisor, Prof. Frédéric Francis. He is a friendly, humorous and open-minded person with innovative spirit which is the direction of my future life. His guidance and insights are reflected in many parts of this thesis. I thank Prof. Francis for his guidance, encouragement and academic advice throughout the experimental process and thesis writing.

I would like to thank all members of my thesis committee and defense jury, Prof. Prof. Philippe Jacques, Prof. Wenwen Liu, Prof. Rudy Caparros Megido, Prof. Frank Delvigne and Prof. Pierre Delaplace, for their guidance and suggestion for my thesis.

Many thanks to all the laboratory members of the Crop Virus Disease Group for accompanying me during these years and bringing me happiness, encouragement and support. My particular thanks go to Prof. Wenwen Liu, Prof. Huaibing Jin and Prof. Yan Liu for their selfless help in both my experiments and daily life. They put forward scientific research ideas and valuable suggestions on numerous occasions. They also helped me check and revise the articles meticulously. In addition, they are very approachable in daily life. I have great respect for each professor for their invaluable and commendable qualities.

It has been a pleasure to spend one year working at Gembloux Agro-bio Tech and to form friendships here. I would like to convey my heartfelt gratitude to Mr. Jean Victor Dernelle for his kind encouragement and heartwarming support. I truly cherish the time I have spent in Gembloux, Belgium.

I would like to thank my beloved family for their persistent support, understanding

and encouragement as well as their care and consideration in my life, which have helped me complete my doctoral studies. They always stand by my side every step of the way.

In the end, I would like to thank the Gembloux Agro-Bio Tech, University of Liège, and the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, for providing me with the precious opportunity to complete my doctoral studies.

Hereby, I extend my sincere thanks to all the professors, friends, colleagues and relatives who have helped and cared about me. I sincerely wish all of you lasting happiness and well-being!

Jiashu Guo

October 2024 in Gembloux, Belgium

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List of Abbreviations

aa	amino acid
CDD	Conserved Domain Database
CP	coat protein
CRPV	cricket paralysis virus
DEG	differentially expressed genes
dpi	days post incubation
dsRNA	double-stranded RNA
DtIV1	Dolerus tritici iflavirus 1
EPF	entomopathogenic fungus
FPKM	fragments per kilobase per million mapped fragments
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
Hel	helicase
ICTV	International Committee on Taxonomy of Viruses
ITS	internal transcribed spacer
kb	kilobases
LSD	least significant difference
LT_{50}	median lethal time
Mad1	Metarhizium adhesin-like protein
MCL1	collagen-like protein
MfPV1	Metarhizium flavoviride partitivirus 1
NGS	next generation sequencing
nt	nucleotides
ORF	open reading frame
PCA	principal component analysis
PDA	potato dextrose agar
PDB	potato dextrose broth
PEG-4000	polyethylene glycol 4000
Pr1	Metarhizium proteinase 1
Pr2	Metarhizium proteinase 2
RACE	rapid amplification of cDNA ends
RdRp	RNA-dependent RNA polymerase
Rhv	rhinovirus
RNA-seq	RNA sequencing
RPB1	RNA polymerase II first subunit

RPB2	RNA polymerase II second subunit
SBPH	small brown planthopper
SDAY	Sabouraud dextrose agar with yeast extract medium
TEF1	translation elongation factor 1-α
TEM	transmission electron microscopy
UTR	untranslated region
VPg	viral genome-linked protein

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Chapter I

General introduction

1.1 Context and objectives

1.1.1 Context

The ecological environment in which plants grow is a complex system that includes both biotic and abiotic factors, all of which play critical roles in plant health, growth, and reproduction (Liu et al., 2024). The relationship between plants and surrounding organisms is subtle and multitrophic. Sometimes they compete; sometimes they exhibit antagonistic parasitism; and occasionally, they coexist in a mutually beneficial symbiosis. Plants are engaged in a continuous co-evolutionary struggle for dominance with other organisms.

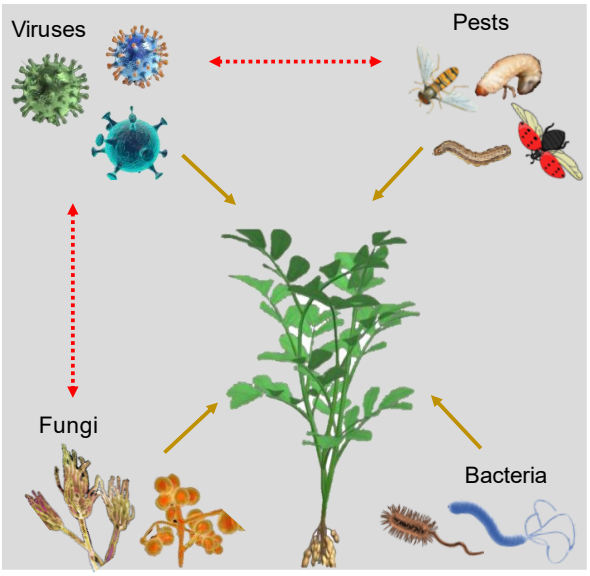


Figure 1-1 Coexistence of plants and surrounding micro- and macro-organisms.

The arrows indicate the interrelationship researched in this study.

Plant natural enemies mainly include pests and microbial pathogens such as bacteria, fungi and viruses. Insect pests are a major challenge to global agriculture, causing directly damage to crops, forests, and ornamental plants (Panizzi et al., 2021; Murray et al., 2010). Pests can cause significant harm to plants through feeding. The fall armyworm (*Spodoptera frugiperda* J. E. Smith), which has become a major threat to maize production across Africa, Asia, and the Americas (Mendesil et al., 2023). They can destroy entire maize fields, leading to yield losses of up to 50% in heavily infested areas. Annual economic losses have been estimated at 9.4 billion dollars in Africa.

(Kansiime et al., 2023). Bacterial and fungal pathogens cause visible damage such as spots, blight or necrotic lesions on leaves, stems, fruits and roots. These diseases can interfere with normal physiological processes in plant and weaken their health, ultimately causing death. Fire blight in apple and pear trees, caused by *Erwinia amylovora* (Burrill) Winslow et al., shows striking symptoms, such as blackened, scorched-looking leaves and blossoms, as if they have been burned by fire (Zwet and Keil 1979). The bacteria spread rapidly, causing cankers on branches and trunks, and the fruit turns black and becomes unusable. In severe cases, fire blight can kill entire trees, leading to heavy economic losses for orchards. Fungal disease, such as tomato late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is notorious for its catastrophic loss, which can devastate crops almost overnight. In 1845, the pathogen spread to Europe, causing a destructive potato disease (Ristaino et al., 2002). It begins subtly with dark, water-soaked lesions that quickly develop into large patches, creating a grim tapestry of decay on the leaves and stems. In the blink of an eye, lush green fields can turn into a wasteland, as the disease spreads at alarming speed under humid conditions (de Bary, 1876). Infected fruits rot rapidly, becoming inedible and leading to devastating economic losses for farmers. Unlike fungal or bacterial pathogens, plant viruses do not directly kill plants but cause stunted growth, a decline in photosynthesis and a reduction in both quality and quantity of yields. Potato virus Y (PVY) is a virus that poses a serious threat to potato crops. PVY causes symptoms like yellowing, leaf curling, and mottling, which ultimately lead to reduced tuber quality and yield. Infected plants may become more susceptible to secondary infections and stress, making them less resilient overall (Quenouille et al., 2013). As PVY can be transmitted by aphids and through infected seed potatoes, it poses a significant challenge for potato farmers, potentially resulting in severe economic losses and reduced the food supply (Bhoi et al., 2022). Even worse, diseases often predispose plants to secondary infections, worsening the plant's condition. As many ongoing studies revealed, plants have evolved a range of sophisticated responses to cope with these threats. Some evolve thicker physical barriers or produce secondary metabolites to prevent pathogen entry and deter herbivores (Divekar et al., 2022; Dangl et al., 2001). Once infected, plants activate a defense response throughout the entire organism (Yang et al., 1997). While pathogens have developed mechanisms to evade plant defenses, plants also continuously adapt by enhancing their resistance strategies. This dynamic communication shapes the evolution of both plants and pathogens leading to an arms race between the two protagonists (Wininger and Rank, 2017). However, not all microbes are hostile to plants. One of the most well-known examples of beneficial plant-bacteria collaboration is the symbiotic relationship between leguminous plants and *Rhizobium* Frank (Oldroyd et al., 2011). These bacteria colonize plant roots, forming nodules where they convert atmospheric nitrogen (N_2) into ammonia (NH_3) for plant growth. In return, the plants provide the bacteria with carbohydrates. This mutualistic interaction enhances soil fertility and reduces the need for synthetic nitrogen fertilizers in agriculture. Studying the outcomes of interactions between plants and surrounding organisms is of particular

importance for breeding and biotechnology, as these interactions can have dramatic effects on agricultural systems.

Plants not only interact with surrounding organisms, but these organisms also engage in a range of interactions with one another. Microbial interactions sometimes play a crucial role in plant growth and health. For example, beneficial microbes can collaborate with other microorganisms to enhance nutrient availability in the soil, improving plant nutrient uptake and promoting faster growth. Although harmful microorganisms are typically associated with plant diseases, in certain contexts, their interactions can unexpectedly benefit plants. For example, some harmful microbes may outcompete other more harmful pathogens, reducing the overall disease pressure on the plant. In other cases, the presence of certain harmful microorganisms may trigger the plant's immune responses, leading to enhanced resistance against future infections. These complex microbial interactions highlight the complex role that even harmful microbes can play in shaping plant health, sometimes creating conditions that inadvertently support the plant's growth and resilience. Thus, the interrelations among organisms are worth researching. Insects can serve as hosts for fungi, bacteria, and viruses, which can result in significant health issues and mortality. Viruses, as unique non-living entities, can infect fungi and bacteria. An adversary's foe can become an ally. Entomopathogenic fungi, such as *Beauveria bassiana* (Balsamo) Vuillemin, have been widely used as biopesticides around the world. A native isolate of *B. bassiana* TGS2.3 resulted in pupal and adult deformities as well as decreased adult emergence of *Spodoptera litura* Fabricius, which is a potential biocontrol agent against the destructive insect pest *S. litura* (Islam et al., 2023). Recent research reviews two entomopathogenic fungi, *B. bassiana* and *Metarhizium anisopliae* (Metsch.) Sorokin, played positive roles in promoting the growth of maize seedlings after hydroponic rhizosphere inoculation. The two fungi could establish systematic colonization in tissues of all maize organs through maize roots and established within one week. This suggests entomopathogenic fungi has great application potential in eco-agricultural fields as both biopesticides and biofertilizers (Liu et al., 2022). Those approaches promise to enhance sustainable agricultural practices and promote ecological stability by utilizing these natural pathogens while minimizing environmental impact.

Our study focuses on viruses associated with fungi and insects. Mycoviruses, also called fungal viruses or mycophages, are viruses that infect and replicate in fungal cells (Xie and Jiang, 2024). With the development of virus detection and sequencing technologies, more novel mycoviruses have been discovered across all major fungal taxa over the past ten years, which highlights a previously overlooked area of virology and mycology (Ayllón and Vainio, 2023). According to the ICTV (https://ictv.global/taxonomy), mycoviruses have been assigned to 16 orders, 42 families, 83 genera, and 534 approved species. However, many newly identified mycoviruses are not determined to classify at the family or genus level. Several studies indicate that fungi and mycoviruses have remarkably complex relationships.

Mycoviruses have gained considerable attention because of their potential to control fungal diseases. For example, the virus *Curvularia* thermal tolerance virus (CThTV) had a positive effect on the plant-fungal symbiosis between tropical panic grass *Dichanthelium lanuginosum* (Wild.) Gould and the fungus *Curvularia protuberata* (Cooke) Boedijn, enabling both organisms to grow at high soil temperatures. Individually, neither the fungus nor the plant can survive at temperatures above 38°C, but when in a symbiotic relationship, they are able to withstand higher temperatures. The cured strain is unable to provide heat tolerance, but this tolerance is regained once the virus is reintroduced into fungi. CThTV confers heat tolerance not only to its native monocot host but also to a eudicot host (Márquez et al., 2007). Although most fungi do not exhibit obvious symptoms after being infected with viruses, some mycovirus infections lead to changes in host phenotype or virulence, causing host fungi slower growth, abnormal pigment formation, reduced or even absent spore production, and reduced pathogenicity. Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) is a mycovirus that infects the *Sclerotinia sclerotiorum* (Lib.) de Bary, the pathogen of oilseed rape sclerotinia disease. The presence of SsHADV-1 in *S. sclerotiorum* not only present slower growth and smaller sclerotia, but also attenuated virulence of the host, resulting in milder disease symptoms in oilseed rape (Fu et al., 2024; Yu et al., 2010). Phytopathogenic fungi usually has a negative effect on plant health in agricultural ecosystems, so mycoviruses which cause fungal attenuation, are often applied in biocontrol. The best-known example is the hypovirus, which attenuates the virulence of the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, Cryphonectria hypovirus 1 (CHV1), was successfully used to control chestnut blight in the 1960s (Sun et al., 2006; Bian et al., 2024). To understand the effects of viral infection of fungi and the mechanisms by which viruses alter the phenotype of their fungal hosts is important and may also contribute to the development of new biological control strategies to combat fungal diseases.

Many insect viruses naturally infect and replicate in insect hosts. In recent years, the advancement of high-throughput sequencing technology has resulted in the identification of many new RNA viruses in arthropod insects. Most of them belong to the viral family: *Dicistroviridae*, *Iflaviridae*, *Picornaviridae*, *Virgaviridae*, *Chuviridae*, *Rhabdoviridae*, *Reoviridae* and *Bunyavirales* (Wang et al., 2024). On some cases, insect viruses lead to mutually beneficial interactions between the virus and the insect host. For instance, the infection of Rondani's wasp virus 1 (RoWV1), identified from parasitoid wasp (*Pachycrepoideus vindemmiae* Rossi) that parasite in fruit flies (*Drosophila melanogaster* Meigen), increased the developmental duration of *D. melanogaster* by increasing the pupal duration. Such an effect may be beneficial for the wasps, because extended pupal period of *D. melanogaster* provides a longer time for the wasps to find their pupal hosts (Zhang et al., 2021). On the other hand, some insect viruses can influence the development, productivity, survival rates, or sex ratios of their hosts. Pteromalus puparum negative-strand RNA virus 1 (PpNSRV-1) is also a virus identified from parasitoid wasps. Researches showed that PpNSRV-1

had no influence on successful parasitism and increased the longevity of adult wasps. However, PpNSRV-1 mediated the offspring sex ratio by decreasing female offspring numbers (Wang et al., 2007). Infected insect populations can experience high mortality rates, resulting in ecological effects on both pest populations and their natural predators. Thus, insect viruses are recognized for their potential as biological control agents. Not only do they reduce the risk to non-target species, but also decrease reliance on chemical pesticides. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) is an important pathogen of silkworms belonging to the family *Baculoviridae* that have been widely used in biological control. BmNPV has complex interactions with its hosts by inducing host cell autophagy to benefit its infection. Autophagy triggered by BmNPV is critical to its replication indicating that the autophagy pathway in this process may be utilized to sustain viral replication (Wang et al., 2017). Besides that, insects play a crucial role as vectors for a variety of arthropod-borne pathogenic viruses. Over 70% of plant viruses are spread by specific arthropods, such as planthoppers, leafhoppers, aphids, whiteflies, and mosquitoes (Hogenhout et al., 2008). The population dynamics of insects and the efficiency of viral transmission by insect vectors are important determinants of viral disease epidemics. Thus, identifying viruses in insects is crucial for diagnosing insect-borne viruses and confirming their role as vectors.

1.1.2 Objectives

Agricultural pests and pathogens not only severely damage plant growth and reduce yields but also create wounds that easily allow other pathogens to infect the plants resulting in even greater losses in agricultural production (Schmitz et al., 2022; Kulabhusan et al., 2022). Current control measures mainly rely on chemical insecticides and their extensive use causes long-term pollution of the environment and adverse effects on non-target organisms (Tudi et al., 2021). Frequent application can also lead to resistance in pests and pathogens. An increasing amount of research suggests the interaction between micro- and macro-organisms may yield unexpected results that are beneficial for plant growth. The objective of my research is to investigate the interactions between viruses and other organisms, with the aim of identifying viruses that can be used as biological control agents alternative to chemical insecticides.

Three specific objectives are targeted as follows:

(1) To obtain a cured strain of *M. flavoviride* or to get isogenic isolated strains by virus transfection for biological research and experimentation.

(2) To clarify the mechanism by which the novel virus confers hypervirulence to *Metarhizium*.

(3) To explore the species of viruses in wheat sawflies that damage wheat fields and to establish the foundation for the study of insect viruses in wheat sawfly.

1.2 Thesis structure

This document begins with an introduction to the general context of the thesis. In chapter I, we introduce plants growing alongside a list of other components of their biotic environment, including other plants, animal consumers and detritivores, pollinators, but also beneficial and pathogenic microorganisms. Significant progress has been made in understanding the ecosystem consequences from the interaction of plants with these organisms. In our study, we place particular emphasis on the interactions between viruses and other organisms that damage plants, as well as their effects on plant growth.

In Chapter II, we present two fungal strains infected with a novel mycovirus, which exhibit hypervirulence against pests that cause severe reductions in crop production worldwide.

In Chapter III, we identified a novel virus in insects that damages wheat leaves, affecting photosynthesis and causing a severe decline in wheat production.

Finally, in Chapter IV, Conclusions and perspectives of the study results, along with the prospects and potential application for the above research, were proposed.

1.3 Research background

1.3.1 High throughput sequencing in virus research

In the early days, virus identification was a highly challenging task that not only required expensive electron microscopes but also skilled personnel, which significantly blocks our understanding of viral diversity. The development of molecular biology and application of PCR technique have greatly benefited us to perform virus research. However, PCR technique has an important prerequisite: prior knowledge of the biological characteristics, genomic properties, or serological characteristics of the virus is required. Despite this, there are still many barriers to exploring unknown viruses (Yang and Rothman 2004). High throughput sequencing (HTS), also called next-generation sequencing (NGS), is a new method for detecting viruses that are unidentified in various ecosystems, and its advancements allow us to obtain complete viral genomes of novel viruses (Lee 2023). With the widespread utilization of high throughput sequencing and the advancement of bioinformatics tools, a new era of viral discovery has commenced. The evolution of sequencing technologies over the past four decades can be divided into three generations. The first generation, Sanger sequencing, established the groundwork for DNA sequencing. The second generation introduced massively parallel sequencing with platforms like Illumina and Ion Torrent, enabling high-throughput sequencing. Now, the third generation, which includes PacBio and Nanopore technologies, offers long-read and single-molecule sequencing capabilities (Satam et al., 2023).

In our study, we used the second-generation sequencing technology, it offers high-throughput and cost-effective methods for profiling and analyzing RNA molecules,

allowing researchers to gain deep insights into gene expression, alternative splicing, non-coding RNA regulation, and various biological processes. Total RNA and small RNA sequencing have emerged as the most widely used techniques for virus detection. Small RNA sequencing depends on activating virus-induced gene silencing to specifically target viral sequences. Total RNA sequencing generates large amounts of data, the majority of which are non-viral sequences, and often involves depleting host rRNA to enrich the viral content within the dataset. Several widely used second-generation sequencing platforms have emerged, one of which is Roche's 454 sequencing method, which relies on pyrosequencing, where the sequence is determined by detecting the release of pyrophosphate when nucleotides are added to the DNA template. Another widely used Illumina sequencing platform utilizes a sequencing-by-synthesis method based on reversible dye terminators. These second-generation sequencing technologies have significantly increased the throughput and speed of DNA sequencing, enabling a wide range of applications in genomics research and diagnostics. An upcoming technology, SOLiD sequencing (Sequencing by Oligonucleotide Ligation and Detection), employs a ligation-based approach using reversible terminators to determine the DNA sequence.

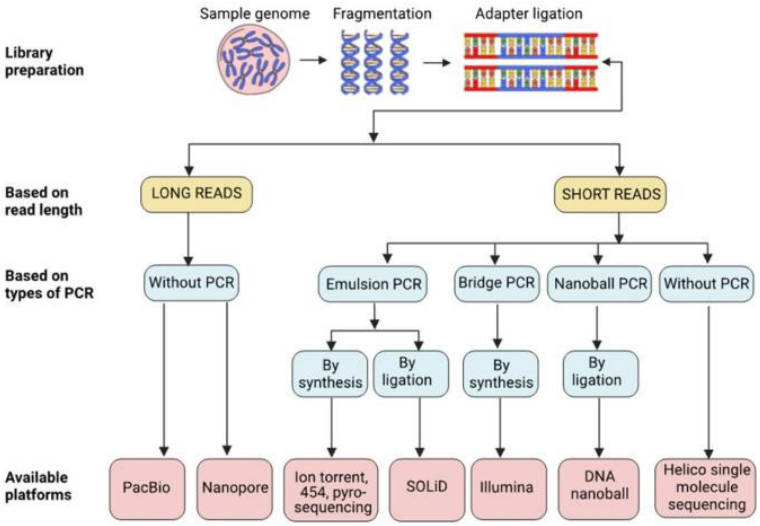


Figure 1-2 Various NGS technologies with different platforms and principles.

Complex virus disease research requires data integration from multiple omics techniques such as genomics, transcriptomics, epigenomics, and proteomics. RNA-seq is a widely used NGS application in transcriptomics that involves sequencing and measuring mRNA molecules to capture a detailed profile of gene expression in a biological sample (Satam et al., 2023). By generating millions of short sequencing reads, NGS enables precise identification and quantification of gene expression levels.

The data from RNA-seq can be used to explore differential gene expressions across conditions, uncover novel transcripts, investigate alternative splicing events, and examine changes in gene expression over time or in various tissues and cell types.

Table 1 Steps and tools used for NGS data analysis

	Analyze	Commonly Used Tools
Common Analysis	Quality check of sequences	FastQC, FASTX-toolkit, MultiQC
	Trimming of adaptors and low-quality bases	Trimmomatic, Cutadapt, fastp
	Alignment of sequence reads to reference genome	BWA, Bowtie, dragMAP
	Reports visualization	MultiQC
Transcriptomics	Alignment of reads to reference	Splice-aware aligner such as TopHat2, HISAT2, and STAR
	Transcript quantification	featureCounts, HTSeq-count, Salmon, Kallisto
	Differential gene expression analysis enrichment of gene categories	DESeq2, EdgeR, DAVID, clusterProfiler, Enrichr

1.3.2 Metarhizium spp. and mycovirus

Organisms that parasitize insects, leading to their death or serious impairment, are known as entomopathogens. *Metarhizium* Sorokin is the second-most common entomopathogenic fungus after *Beauveria*, known for its ability to infect and kill various soil-inhabiting insects hosts (Patel 2020). *Metarhizium* was introduced by Metchnikoff and typified by *M. anisopliae*. Currently, more than 110 species are available for the genus. The infection process of *Metarhizium* species is typically like that of other entomopathogenic fungi involving six steps: attachment to the host, spore germination, formation of the appressorium, penetration of the host's cuticle, colonization of the haemocoel, and finally, the production and release of new spores (Aw and Hue, 2017). Recent studies have identified many molecules involved in the infection process of *Metarhizium* (Yan et al., 2011; Wang and Wang, 2017). Adhesion is pivotal to the initiation of the infection through a combination of passive hydrophobic forces (Bernardo et al. 2018), electrostatic interactions (Butt et al. 2013), and protein bonding between the conidia and the host's epicuticle (Tseng et al. 2014). G-protein signal regulator Cag8 are verified to promote the expression of the hydrophobin, a specialized protein shaping the fungal cell wall and contributing to the hydrophobicity of spore surfaces (Fang et al. 2007). Following the conidia adhesion to the host cuticle, *M. anisopliae*'s germination process is triggered by the presence of suitable humidity, temperature, and various nutritional. Then, the spores swell to

develop germ tubes. At the same time, Mad1, which is an important factor replacing hydrophobins resulting in a stronger attachment of the fungus to the cuticle, facilitating conidial germination and subsequent appressorium formation. Then, conidia germinate and differentiate into appressoria to penetrate insect cuticle. To achieve this, *Metarhizium* secretes subtilisin-like serine protease, trypsin, chitinolytic and lipolytic enzymes for breaking down major constituents of the cuticle (Singh and Mazumdar, 2022). As the fungus invading the host, it transforms into yeast-like blastospores to proliferate and depleting nutrients in the host's haemocoel. When it enters, it triggers the production of phenoloxidases to activate insects' innate immune response. Fungus secrets some substance to overcome the defensive reactions, such as producing a collagen-like protein (MCL1), an evasion mechanism in response to insect immune system. *Metarhizium* generates insecticidal and other bioactive secondary metabolites, such as destruxins. These destruxins play a pivotal role in pathogenesis, inducing flaccid paralysis by altering the cellular composition of the midgut, malpighian tubules, and muscular tissues (Dumas et al. 1994). At last, as the colonization inside host proceeds the nutrients become depleted, the fungus emerges followed by conidia on the surface of the cadaver of dead insects. A new infection will occur under proper conditions of humidity and temperature.

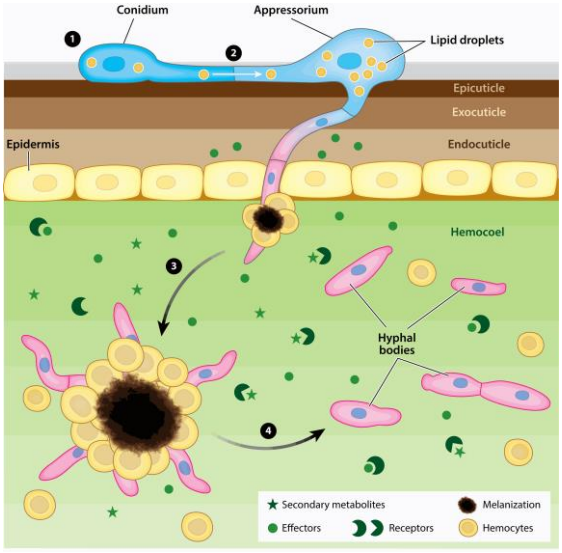


Figure 1-3 Schematic of the infection process of entomopathogenic fungi.

Metarhizium, like many other fungi, is often associated with the presence of viruses. Recently, many *Metarhizium spp.* mycoviruses have been reported and characterized while the effects of mycovirus infection on the host development and stress tolerance are limited. The first viral dsRNA was detected in *M. anisopliae*. Isogenic strains (with

or without dsRNA) did not cause hypovirulence to *M. anisopliae* against aphid *Myzus persicae* Sulzer (Leal et al., 1994). Four mycovirus including two partitiviruses Metarhizium brunneum partitivirus 1 (MbPV1) and Metarhizium brunneum partitivirus 2 (MbPV2), one polymycovirus Metarhizium brunneum polymycovirus 1 (MbPmV1), and one unassigned mycovirus Metarhizium brunneum bipartite mycovirus 1 (MbBV1) has been identified in *M. brunneum.* However, their effects on the *M. brunneum* are not reported (Wang et al., 2020; Wang et al., 2021; He et al., 2022; Wang et al., 2022). In *M. anisopliae*, the polymycovirus Metarhizium anisopliae polymycovirus 1 (MaPmV1) significantly enhanced the growth rate and conidiation of the host fungus, increased its sensitivity to UV-B irradiation, but had no significant impact on the virulence of *M. anisopliae* (Wang et al., 2023). There is evidence that *Metarhizium* spp. harbor large numbers of mycoviruses, which may not be entirely latent, and their effects on the host are varied. Therefore, in-depth knowledge of *Metarhizium* mycoviruses and their impacts is still worthy. Partitiviruses are a group of double-stranded RNA (dsRNA) viruses that belong to the family *Partitiviridae*, which includes five genera with characteristic hosts for members of each genus, including plants or fungi for both *Alphapartitivirus* and *Betapartitivirus*; fungi for the genus *Gammapartitivirus*; plants for the genus *Deltapartitivirus* and protozoa for the genus *Cryspovirus*. Recent studies have indicated gammapartitiviruses only infect ascomycetous fungi (Vainio 2018). Partitiviruses possess two essential dsRNA genome segments, dsRNA1 (or S1) and dsRNA2 (or S2), each 1300–2500 bp in length and containing one long open reading frame (ORF) on one of the RNA strands. The longer segment encoding the RNA-dependent RNA polymerase (RdRp) protein, and the shorter one encoding the capsid/coat protein (CP). Partitiviruses consistently mediate persistent infections of their hosts and are classically considered to have few effects on the host. They have hence sometimes been called latent infection virus. In more recent years, however, several examples have emerged in which host effects are seen. Metarhizium majus partitivirus 1 (MmPV1) identified from *M. majus* attenuated fungal virulence by reducing conidiation, triterpenoids production, and the levels of metarhizins A and B (Wang et al., 2023). It also decreased hydrophobicity, adhesion capacity, and cuticular penetration. Therefore, the subtle interaction between partitiviruses with their fungal hosts makes them worthy of further research.

1.3.3 Iflavirus and wheat sawfly

Iflaviruses, named after the type species "infectious flacherie virus," belong to order *Picornavirales*, the family *Iflaviridae*. According to the International Committee on Taxonomy of Viruses (ICTV), it is a small group of viruses represented by a single genus, *Iflavirus*, which contains 16 identified species. Iflaviruses possess small non-enveloped virions, measuring 22 nm and 30 nm in diameter. Their genome is monopartite and consists of a single-stranded, positive-sense RNA molecule (9 to 11 kb) containing a single open reading frame (ORF) that encodes a polyprotein of approximately 3,000 amino acids (Valles et al., 2017). At present, most known hosts

of iflaviruses are arthropods, especially members of Hemiptera, Lepidoptera, Hymenoptera, Diptera, Coleoptera and Orthoptera. The viral infection may be asymptomatic, as seen in Nilaparvata lugens honeydew virus 1 (NlHV-1) doesn't cause observable pathology to its host brown planthopper. (Murakami et al., 2013). However, some infested hosts exhibit abnormal growth and development and have a high mortality rate such as Sacbrood virus (SBV) and deformed wing virus (DWV) (Wei et al., 2022; Lanzi et al., 2006). DWV is a typical iflavirus of honey bees that can be transmitted both vertically (from queen to egg) and horizontally (from drone to queen), or is vectored by the parasitic *Varroa* mite (Martin and Brettell 2019). For beneficial insects like honeybees, infection with iflavirus can shorten their lifespan and weaken the colony.

Wheat sawfly (*Dolerus tritici* Chu), belonging to the order Hymenoptera, the family Tenthredinidae, is an important global pest of common wheat (*Triticum aestivum* L.). It has one generation per year in the northern China. The larvae damage wheat leaves by feeding on them from the edges, causing notches, and in severe cases, they can consume the entire leaf tip. This affects the photosynthesis of wheat, leading to a decrease in yield. The adults hide at the base of the wheat seedlings or in shallow soil at night, and are active, mating, and laying eggs during the day. They exhibit a form of playing dead when disturbed. Research about wheat sawfly is very limited. There have been no reports of viruses or other pathogens in the wheat sawfly. The control methods for the wheat sawfly are mainly chemical control, manual capture and killing and agricultural control measures.

2

Chapter II

Transfection of entomopathogenic *Metarhizium* species with a mycovirus confers hypervirulence against two lepidopteran pests

Abstract

Although most known viruses infecting fungi pathogenic to higher eukaryotes are asymptomatic or reduce the virulence of their host fungi, those that confer hypervirulence to entomopathogenic fungi still need to be explored. Here, we identified and studied a novel mycovirus in *Metarhizium flavoviride*, isolated from small brown planthopper (*Laodelphax striatellus*). Based on molecular analysis, we tentatively designated the mycovirus as Metarhizium flavoviride partitivirus 1 (MfPV1), a species in genus *Gammapartitivirus*, family *Partitiviridae*. MfPV1 has two double-stranded RNAs as its genome, 1,775 and 1,575 bp in size respectively, encapsidated in isometric particles. When we transfected commercial strains of *Metarhizium anisopliae* and *Metarhizium pingshaense* with MfPV1, conidiation was significantly enhanced (*t* test; *P*-value < 0. 01), and the significantly higher mortality rates of the larvae of diamondback moth (*Plutella xylostella*) and fall armyworm (*Spodoptera frugiperda*), two important lepidopteran pests were found in virus-transfected strains (ANOVA; *P*-value < 0.05). Transcriptomic analysis showed that transcript levels of pathogenesis-related genes in MfPV1-infected *M. anisopliae* were obviously altered, suggesting increased production of metarhizium adhesin-like protein, hydrolyzed protein, and destruxin synthetase. Further studies are required to elucidate the mechanism whereby MfPV1 enhances the expression of pathogenesis-related genes and virulence of *Metarhizium* to lepidopteran pests. This study presents experimental evidence that the transfection of other entomopathogenic fungal species with a mycovirus can confer significant hypervirulence and provides a good example that mycoviruses could be used as a synergistic agent to enhance the biocontrol activity of entomopathogenic fungi.

Keyword: Metarhizium, entomopathogenic fungus, partitivirus, hypervirulence

2.1 Introduction

Chemical pesticides have been used to control insect pests since the 1940s; however, their adverse effects on nontarget organisms, residues on food crops and groundwater, and development of insect resistance to chemicals have forced scientists to develop alternative ecofriendly measures such as biocontrol, the exploitation of living agents to combat harmful organisms (Stehle and Schulz, 2015; Tang et al., 2021). Biocontrol agents are an important component of a sustainable pest management program because effective agents can be chosen to persist in the environment with low ecological impact (Stenberg et al., 2021). Entomopathogenic fungi have been widely used to control agricultural, grassland, medical, and veterinary insect pests (Islam et al., 2021; Moraga 2020). Species of *Metarhizium* (Clavicipitaceae: Hypocreales) can infect more than 200 species of arthropods in over 50 families (Mongkolsamrit et al., 2020). They produce spores (conidia) that can germinate on the surface of the insect, penetrate its cuticle, and grow inside the insect. Then, they produce toxins, which presumably aid in suppressing host immune defenses, eventually killing the host, and fending off potential microbial competitors (Brunner-Mendoza et al., 2018; Sheng et al., 2020). After death of the host, the fungus will grow out of the integument and produce aerial conidia, which are disseminated into the environment and may infect more insects (Sheng et al., 2020). Pesticides developed from species of *Metarhizium* remain active for a long period, are not harmful to the environment and nontarget organisms, but they can take a long time to kill the insect, are unstable, and their control efficacy is low (Brancini et al., 2021; Braga et al., 2015). Mycoviruses have been discovered in a wide range of fungal species, and some are usually associated with symptomless infections or with reduced growth rate or virulence to higher organisms of the pathogenic fungus (Kondo et al., 2022; Li et al., 2022; Yu et al., 2021). This reduction in virulence led to the idea that mycoviruses can serve as a biocontrol measure against fungal pathogens as in the case of chestnut blight caused by the fungus *Cryphonectria parasitica* in North America (Milgroom and Cortesi, 2004; Van Alfen et al., 1975). Some pathogenic fungi can live as a beneficial or nonpathogenic endophyte when infected with a mycovirus as do *Sclerotinia sclerotiorum* or *Pestalotiopsis theae* (Hara) Steyaert (Zhang et al., 2020; Zhou et al., 2021). Few known mycoviruses, however, promote fungal growth and are associated with hypervirulent or other extreme phenotypes. The presence of a viral 6.0-kbp dsRNA in *Nectria radicicola* (Guss.) Fr., the fungus that causes ginseng nectria root rot, was found to be associated with high levels of virulence, sporulation, laccase activity, and pigmentation in this fungus (Ahn and Lee, 2001). A newly discovered example is a novel ambigrammatic mycovirus that enhances the virulence of *Puccinia striiformis* f. sp. *tritici* (Weston & Holmes) Young, which causes wheat stripe rust (Zhang et al., 2023). Many mycoviruses have also been discovered in entomopathogenic fungi. About 21.3% of a worldwide collection of isolates of the entomopathogenic fungus *Beauveria bassiana* were found to harbor various mycoviruses and other dsRNA elements (Kotta-Loizou and Coutts, 2017). Some of

the isolates infected with polymycoviruses were found to be mildly hypervirulent against the greater wax moth, *Galleria mellonella* (Linnaeus). There is also evidence that species of *Metarhizium* harbor large numbers of mycoviruses, which generally reduce mycelial growth, conidial production, and virulence of the host fungus by affecting its ability to tolerate heat and UV-B radiation and to penetrate insect cuticles (Melzer and Bidochka, 1998; Wang et al., 2023). However, information on mycoviruses in entomopathogenic fungi and their effects on the host phenotype remains limited. In the present study, we isolated and characterized a novel mycovirus Metarhizium flavoviride partitivirus 1 (MfPV1) from *Metarhizium flavoviride* (Gams & Rozsnyai) Kepler isolated from small brown planthopper (SBPH, *Laodelphax striatellus* Fallén), an important insect vector for several plant viruses (Wu et al., 2020, Liu et al., 2023). When we used MfPV1 to transfect virus-free commercial strains of two other *Metarhizium* species, conidiation and virulence of the transfected isolates were enhanced. This study reports a mycovirus in an entomopathogenic fungus that can endow significant hyper- virulence to other fungi and opens the possibility that mycoviruses can elevate the activity of biocontrol agents.

2.2 Method Details

2.2.1 Insects and Fungal Isolates.

Small brown planthopper (SBPH, *L. striatellus* Fallén) (Hemiptera: Delphacidae) were collected from Kaifeng, Henan Province, China, in June 2016 and maintained on rice seedlings (cv. Wuyujing 3) in insect-proof cages at 27°C with 16 h light/8 h dark. *P. xylostella* L. (Lepidoptera: Plutellidae) and *S. frugiperda* J. E. Smith (Lepidoptera: Noctuidae), both economically important agricultural pests, were provided by the laboratory of entomology in our institute. Strain Mf_KF18 of *M. flavoviride* sensu lato was isolated from dead SBPHs. Commercial strain Ma_114445 of *M. anisopliae* (Metschnikoff) Sorokinand Mp_336563 of *M. pingshaense* Q.T. Chen and H.L. Guo were purchased from BNCC (BeNa Culture Collection, China). Strains Ma_114445/MfPV1 and Mp_336563/MfPV1 were virus-harboring strains transformed by polyethylene glycol 4000 (PEG-4000) in this study.

2.2.2 RNA Sequencing, Assembly, and Analysis.

In 2016, we identified numerous dead adult SBPHs that were covered with a green fungus during their feeding period in insect-proof cages in our laboratory. We randomly selected 10 living adults for RNA sequencing (RNA-seq). Total RNA was extracted from the sample using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The Ribo-Zero Magnetic Kit (Epicentre, Madison, WI) was used to deplete ribosomal RNA from transcriptomes for RNA-seq; then, a library was constructed with a TruSeq total RNA Sample Prep Kit (Illumina, San Diego, CA). The Illumina HiSeq X Ten platform with PE150 bp was used for the RNA-seq. The CLC Genomics Workbench 9.5 was used for de novo assembly of

RNA-seq data. The assembled contigs were subsequently screened against the NCBI databases using BLASTn and BLASTx searches with default options. Two contigs caught our attention because of their distinctness, so we selected them for further analysis.

2.2.3 Sequencing and Analyzing the Viral Genome.

To further characterize any viruses represented by the distinct contigs identified from the RNA sequencing of the RNA from the 10 SBPHs, we designed specific primers using Primer Premier 5 (Premier Biosoft Interpairs, Palo Alto, CA) for RT-PCR and RACE-PCR (Table S2-6) to acquire the full genomic sequence of the virus(es). The sequences of the extreme ends of the genomic RNAs were determined employing the 5′-and 3′- RACE System for Rapid Amplification of cDNA Ends kits (Thermo Fisher Scientific, Waltham, MA). The PCR products were separated by electrophoresis in 1.2% agarose gels and visualized by ethidium bromide staining, and then purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). The PCR products were cloned into the PEASY-T5 cloning vector (TransGen Biotech, Beijing, China) and then inserted into Trans-T1 Chemically Competent Cells (TransGen). The positive clones were sequenced by Sanger sequencing at Sangon Biotech Co., Ltd (Shanghai, China). Contigs were assembled using the DNAMAN (v6) program (Lynnon Biosoft, San Ramon, CA). Open reading frames (ORFs) were deduced using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence identities were computed using LALIGN program of EMBL's EBI European Bioinformatics Institute (EMBL-EBI) (Madeira et al., 2022) with the default settings. Terminal sequences were aligned using MEGA 11 (v11.0.13) and the Muscle method (Tamura et al., 2021). The UNA Fold Web Server (http://www.unafold.org) was used to predict the secondary structure (Zuker, 2003). For phylogenetic analysis, sequences were aligned using the Muscle program in MEGA 11, and then phylogenetic trees were generated using the neighbor-joining (NJ) method with a P- distance model and 1,000 bootstrap (Tamura et al., 2021).

2.2.4 Isolation and Identification of Fungi from Dead SBPHs.

For isolating the sporulating fungus, the dead insects were surface-sterilized with 0.2% v/v sodium hypochlorite, then placed on PDA amended with 100 μg/ mL ampicillin and 50 μg/ mL kanamycin, and incubated at 28°C with 12 h light/12 h dark for7 to 14 d. Single colonies were transferred to a new dish of PDA and cultured for 7 d. Total DNA was extracted from each fungal culture using a genomic DNA purification kit (Promega). Partial regions of four nuclear loci, including internal transcribed spacer (ITS), translation elongation factor 1-α (*TEF1*), RNA polymerase II first, and second subunit (*RPB1* and *RPB2*), were amplified using corresponding primer pairs (Curran et al.,1994, Villamizar et al.,2021) (Table S2-6). The amplicons were purified and sequenced at Sangon Biotech Co., Ltd. The obtained sequences were analyzed using DNAMAN (v6, Lynnon Biosoft).

2.2.5 Purification and Observation of Virus Particles by TEM.

Mycelial plugs of strain Mf_KF18 were incubated in PDB on a shaking incubator at 28°C for 7d. After harvest, 30 g of mycelia was ground to a fine powder in liquid nitrogen; virions were purified from the crude extract using sucrose gradient centrifugation as previously described (Li et al., 2020). The virus-containing zone (20 to 30% sucrose) was then centrifuged at 35,000 rpm for 3 h. The pellets were then suspended in0.01 M PB (pH 7.4), pipetted on carbon-coated 200-mesh copper grids, and negatively stained with 1% w/v uranyl acetate in 0.01 M PB, and then observed by an H- 7700 transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV.

2.2.6 dsRNA Extraction and Enzymatic Treatments.

A mycelial plug of the respective fungal culture was grown in PDB with shaking at 150 rpm at 28°C for 7 d. Mycelia were harvested and ground to a fine powder in liquid nitrogen. A previously described procedure for dsRNA extraction was referred (DePaulo and Powell, 1995). dsRNA pellet was washed three times, then suspended in 100 μL DEPC-H2O, and treated with DNase I and S1 nuclease (TaKaRa, Dalian, China) according to the instructions. The digestion products were separated using 1.2% agarose gel and visualized by ethidium bromide staining, then purified with the Wizard SV Gel and PCR Clean-Up System (Promega).

2.2.7 Elimination of the Mycovirus from Strain Mf_KF18.

Virus-free isogenic fungal isolates are needed to test the effects of mycoviruses on a host fungus. Four treatments were used to eliminate RNA viruses from Mf_KF18: 1) Hyphal tips were cultured as described by Kamaruzzaman et al. (Kamaruzzaman et al., 2019). 2) Conidia were serially diluted and plated to produce single-spore colonies as described by Li et al. (Li et al., 2022). 3) Protoplasts were prepared and regenerated as described by Kamaruzzaman et al. (Kamaruzzaman et al., 2019). 4) Conidia were plated on PDA amended with 500 μM ribavirin (1- β- d- ribofuranosyl-1,2,4- triazole-3-carboxamide), a guanine nucleoside analog that is a broad-spectrum inhibitor of RNA virus replication (Hajano et al., 2020). RNA was extracted from each putative virus-free colony and tested for the virus by RT-PCR using specific primers (Table S2-6).

2.2.8 Protoplast Preparation and Transfection with the Virus.

Conidia (1×10^8/mL) of virus-free strains Ma_114445 of *M. anisopliae* and Mp_336563of *M. pingshaense* were incubated overnight in PDB to generate hyphae. Protoplasts were prepared by incubating the hyphae in a solution of 0.02%w/v driselase and w/v 0.02% snailase (Solarbio, Beijing, China) in 0.7 M NaCl for 30 min at 28°C on a shaker at 100 rpm to release protoplasts. The mixture was filtered through Miracloth (Merck, Billerica, MA) and then centrifuged at 5,000 rpm for 10 min; the resulting protoplast pellet was washed twice with 0.7 M NaCl and resuspended in 0.5 mL of STC buffer (1 M sorbitol, 50 mM Tris pH 8.0, and 50 mM $CaCl_2 \cdot 2H_2O$).

Purified VLPs (5 μL) were added to 100μL of protoplasts (about 1×10^8/mL) for a transfection assay; PEG-mediated transfection tests were conducted according to previous reports (Milgroom and Cortesi, 2004, Kamaruzzaman et al., 2019). All transfected, regenerated isolates were subcultured at least three times to confirm the stability of the mycovirus in the host.

2.2.9 Biological Characterization of MfPV1-Infected Strains of M. anisopliae and M. pingshaense.

To compare virus-infected and virus-free strains of *M. anisopliae* and *M. pingshaense* for any phenotypic differences, we evaluated colony morphology, growth, conidiation, biomass, and virulence in *P. xylostella* and *S. frugiperda*. A mycelial plug approximately 0.5 cm in diameter was removed from the edge of actively growing colonies and placed in the center of a PDA plate. Colony diameter was measured every 24 h. Colonies were also photographed at 5, 7, 9, and 21 dpi to compare colony characteristics of the colonies. Conidia were harvested in sterile water containing 0.05% Tween-80 and counted using a hemocytometer (Takahashi-Nakaguchi et al., 2020). For weighing mycelia, a mycelial plug of the respective strains was incubated in PDB at 150 rpm at 28°C for 5 d, then the mycelium was harvested, dried, and weighed using a precision balance (the accuracy to 1 mg). To evaluate chemical stress tolerance of the isolates, 2 μL of a conidial suspension (1×10^7/mL) was spotted onto 1/4 Sabouraud dextrose agar with yeast extract medium (1/4 SDAY: glucose 10 g, tryptone 2.5 g, yeast extract 5 g) amended with MNB (10 μg/mL), Congo red (500 μg/mL), NaCl (1 M), sorbitol (1M), and SDS (400 μg/mL), and then incubated for 5 d as above. Colony diameters were measured to calculate the mycelial growth (Wu et al., 2020). Five replicates were used for each strain. Means for a variable for the virus-infected and virus-free strains were compared for significant differences at $P < 0.05$ using a paired *t* test in SPSS v22.0 (IBM, Armonk, NY).Virulence of virus-infected and virus-free strains was bio assayed using second-instar larvae of *P. xylostella* that had been immersed in a conidial suspension of the fungus (5×10^7 conidia/mL 0.05% Tween-80) for 60 s then incubated at 20°C with 16 h light/8 h dark or second-day larvae of *S. frugiperda* that were allowed to feed on corn leaves that had been immersed in conidial suspension (5×10^8 conidial/mL 0.05% Tween-80) for 60 s then incubated at 28°C with 16 h light/8 h dark. Larvae were treated with 0.05% Tween-80 as the negative control. Any dead larvae were counted every 24 h, and the median lethal time (LT_{50}) was estimated. Each fungal strain was tested using 30 larvae of *P. xylostella* and 24 larvae of *S. frugiperda*, and the experiment was done three times. Means for a variable for the negative control, virus-infected, and virus-free strains were compared for significant differences at $P < 0.05$ using ANOVA and post hoc least significant difference (LSD) test for multiple comparisons in SPSS v22.0 (IBM).

2.2.10 RNA-seq of MfPV1- Infected and Virus- Free Strains of M. anisopliae and RT-qPCR Validation of Selected DEGs.

Total RNA was extracted from *M. anisopliae* strains Ma_114445 and

Ma_114445/MfPV1 that had been cultured for 3 d in cicada induced medium (PDB supplemented with 0.2% cicada slough). Total RNA from three biological replicates was pooled for each RNA-seq library. cDNA library was constructed and then sequenced on an Illumina Novaseq platform according to the manufacturer's instructions, with 150 bp paired-end reads generated (Novogene, Beijing, China). Raw data in the FASTQ format were processed using the open-source software fastp (HaploX Biotechnology, Shenzhen, China) (Chen et al., 2018); clean reads were obtained by removing reads containing adapter, ploy-N, and low-quality reads from raw data. Pearson correlation coefficient (r) and principal component analysis (PCA) were performed using ggplot2 packages in R based on the fragments per kilobase per million mapped fragments (FPKM) of all genes in each RNA-seq sample. The heatmap was performed using ggplot2 and pheatmap packages in R package. An index of the reference genome was built and paired-end clean reads aligned to the reference genome using Hisat2 v2.0.5 (Zhang et al., 2021). Genes with an adjusted *P*-value ≤0.05 and fold-change ≥1.2 found by DESeq2 were assigned as DEGs. A gene ontology (GO) enrichment analysis of the DEGs was implemented using the R package cluster Profiler (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html), in which gene length bias was corrected (Young et al., 2010). The fold-change in the expression of the DEGs based on the RNA-seq data was then validated by RT-qPCR using gene-specific primers designed using the software Oligo 7.60 (OLIGO, Colorado Springs, CO) (Table S2-6). Total RNA was used to construct template cDNA for the RT-qPCR using Hifair III first Strand cDNA Synthesis Super Mix (Yeasen, Shanghai, China) according to the manufacturer's instructions. The Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (Yeasen) was used for the RT-qPCR in a Quant Studio 6 Flex Real Time PCR system (Thermo Fisher Scientific). Transcript levels of the selected genes were normalized against. The expression of the gene for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The relative quantitative method ($2^{-\Delta\Delta CT}$) was used to infer the fold-change in expression of the selected genes (Livak and Schmittgen, 2001).

2.2.11 RT-qPCR Validation of Sporulation- Related Genes.

Total RNA was extracted from *M. anisopliae* strains Ma_114445 and Ma_114445/MfPV1 that had been cultured for 12 d on PDA medium. Sporulation-related genes were validated by RT-qPCR performed as described above. Gene-specific primers were designed using the software Oligo 7.60 (OLIGO) (Table S2-6).

2.3 Results

2.3.1 Identification of a Partitivirus by RNA-seq and Characteristics of the Virus Genome.

Of 35,443,732 total raw reads obtained from RNA- seq of the RNA library

constructed with SBPH samples, 32,766,442 clean reads of 150 nucleotides (nt) were obtained after removing adaptor sequences and low-quality reads (Table S2-1). A total of 6,690 clean reads, which accounted for 0.02% of the total reads, were mapped to the RNA1 and RNA2 of partitiviruses (Figure S2-1A). After de novo assembly of the reads into large contigs by Velvet 1.2.10, a Blastx search of the NCBI nonredundant protein sequences databases using the contigs as queries, two unknown viral contigs (1,730 and 1,553nt in length, respectively) having high identities with partitiviruses were found. Then, we confirmed the validity of the two unknown contigs by RT-PCR and determined the terminal sequences by 5′and 3′ RACE for obtaining full-length viral genome sequences (Figure S2-1B). The complete viral genome contained two segments of double-stranded RNA (dsRNA), 1,775 nt for dsRNA1 (GenBank accession MH143600), and 1,575 nt for dsRNA 2 (GenBank accession MH143601) in length. Each dsRNA segment was predicted to have a single ORF that encodes a 62.6-kD putative RNA-dependent RNA polymerase (RdRp) comprising 538 amino acids (aa) and a 47.4-kD putative capsid protein (CP) comprising 440 aa (Figure 2-1).

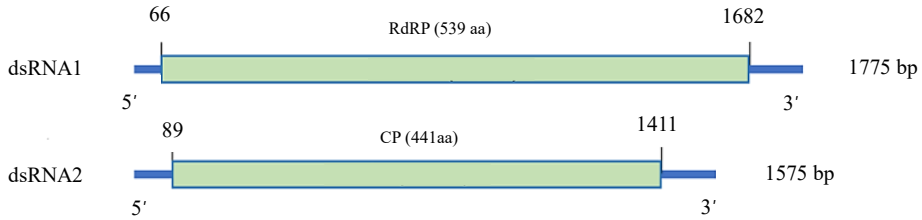


Figure 2-1 A diagram shown the two proteins, RNA-dependent RNA polymerase (RdRP) and coat protein (CP), encoded by the two dsRNAs, respectively, in genome of MfPV1.

The identity of deduced aa sequences of dsRNA1 and dsRNA2 with those of approved species in family *Partitiviridae* was 28.6 to 70.9% for RdRp and 20.2 to 59.5% for CP, with the highest identities to the species in genus *Gammapartitivirus* (Table S2-2). All the identities were much lower than the species demarcation thresholds in the family *Partitiviridae* (Vainio et al., 2018). Conserved nucleotide sequences at the 5′ and the 3′ terminal of the genomic RNAs were identical to that of gammapartitiviruses (Figure 2-2A and Figure S2-1C). In addition, a 65-nt and an 88 nt stem-loop structure was present in the 5′-untranslated region (5′ UTR) of dsRNA1 and dsRNA2, respectively (Figure 2-2B).

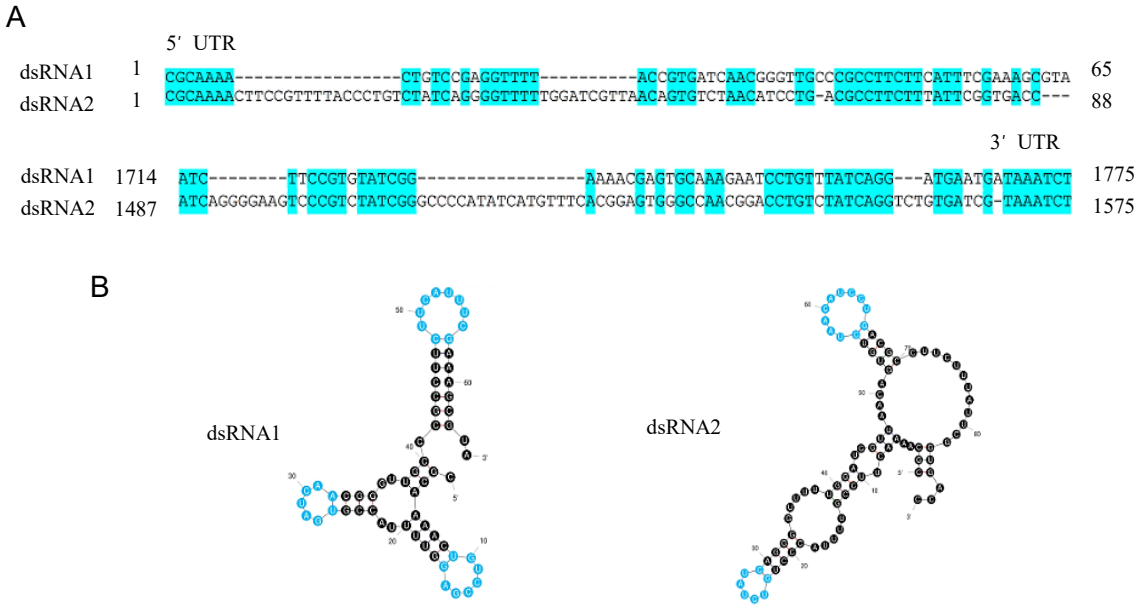


Figure 2-2 (A) Sequence alignment of 5′ untranslated regions (UTR) or 3′ UTR between dsRNA1 and dsRNA2 of MfPV1 genome. (B) Prediction of the structure of 5′ UTR in dsRNA1 or dsRNA2 using the UNAFold web server (www.unafold.org).

Phylogenetic trees based on the deduced aa sequences of both RdRp and CP were constructed using MEGA 11. In both trees, the sequences from the virus that we identified were clustered in a clade of gammapartitiviruses (Figure 2-3A). At the aa level, the RdRp encoded by dsRNA1 has six conserved motifs (III to VII) within the genus *Gammapartitivirus* (Figure 2-3B). The results presented above suggest that this virus belongs to a separate species in the genus *Gammapartitivirus*.

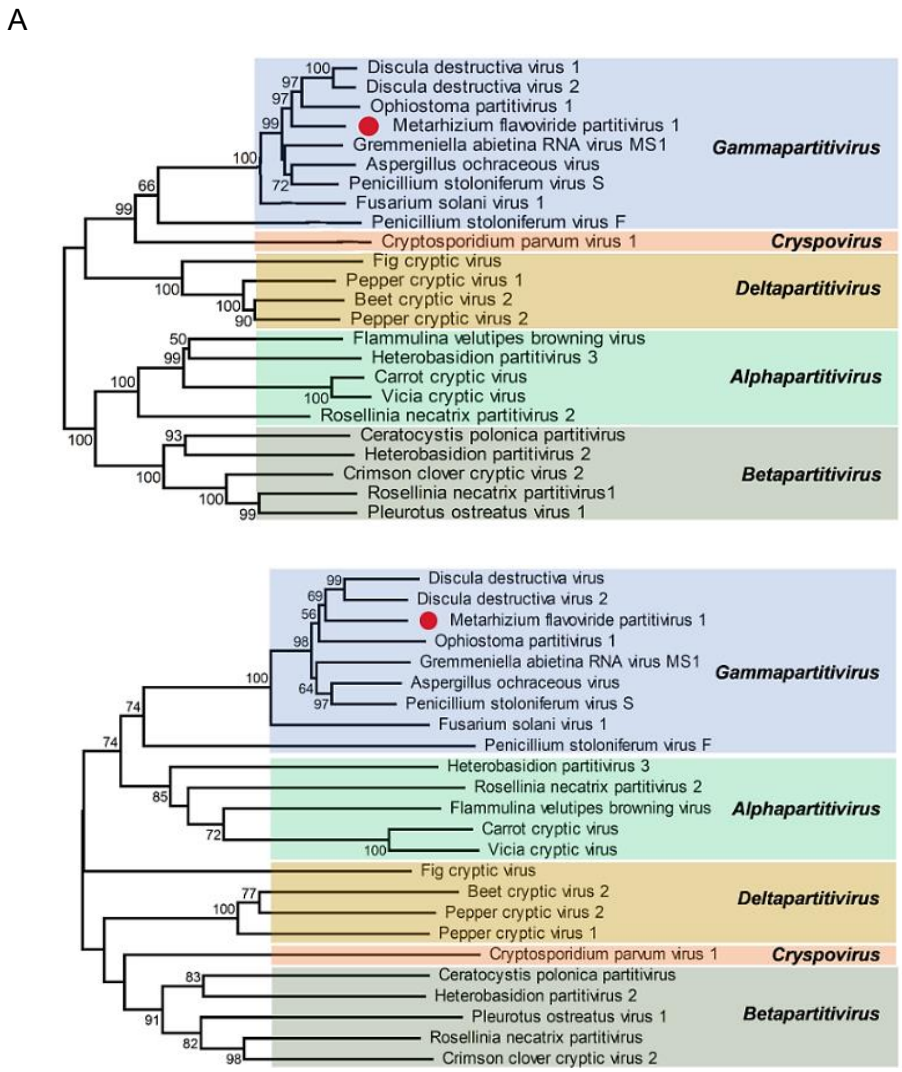


Figure 2-3 (A) Phylogenetic analysis among the species in genus *Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus*, *Deltapartitivirus*, and *Cryspovirus* using the deduced amino acids of RdRP and CP. (B) Analysis of six conserved domains in RdRP among the nine species in genus *Gammapartitivirus* using the deduced amino acids.

2.3.2 Identification of the Virus Host and Purification of Viral Particles.

Species in genus *Gammapartitivirus* so far comprise only viruses isolated from various fungal species (Vainio et al., 2018). Fungal isolate KF18 which was isolated from dead SBPHs (Figure 2-4A) exhibited globose colony with irregularly brown mycelia and green conidia at the edge of the colony on potato dextrose agar (PDA) medium (Figure 2-4B). Phylogenetic analysis based on sequences of ITS, *TEF1*, *RPB1*, and *RPB2* amplified from strain KF18 with other related fungi showed that the isolate has been clustered together with *M. flavoviride* var. minus ARSEF 1764, minus ARSEF 2037, minus ARSEF1099 (Figure S2-2), suggesting isolate KF18 belongs to *M. flavoviride*.

Figure 2-4 (A) The pictures show the dead small brown planthoppers (SBPHs) (Left and Middle panels), or the cadavers (Right panel), with the green fungi observed around the dead SBPHs. (B) Morphology of *M. flavoviride*, KF18 (the 18th clone isolated from the SBPH cadavers obtained from Kaifeng, Henan province). KF18 was found to grow slowly and generate the green spore in the reproductive phase.

In addition, isometric, nonenveloped, spherical viral particles of about 28- 30 nm in diameter were observed from purified virus particles prepared from the mycelia of Mf_KF18 by TEM (Figure 2-5A), similar to the particles as reported for other species in family *Partitiviridae* (Ochoa et al., 2008). Besides that, the result of RT-PCR using the primer set ORF1-F/R and ORF2-F/R showed that the existences of RNA1 and RNA2 of the virus both in strain Mf_KF18 and purified viral fractions (Figure 2-5B and C). Thus, we propose this virus as “Metarhizium flavoviride partitivirus 1” (MfPV1).

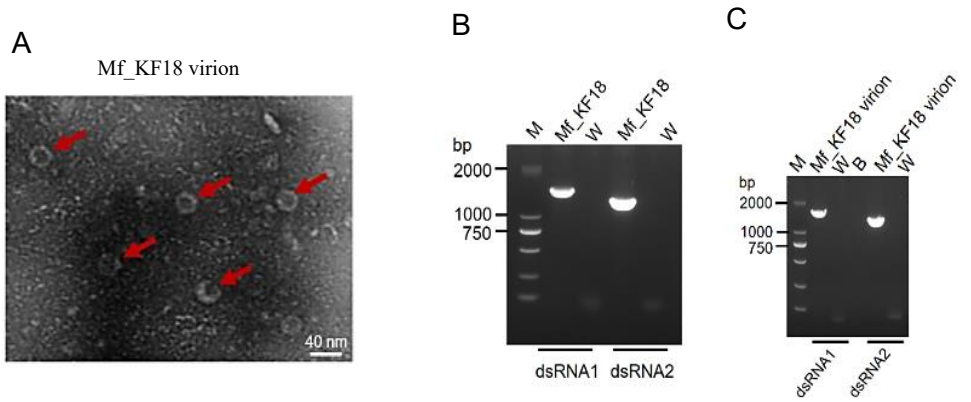


Figure 2-5 (A) The isometric, nonenveloped, spherical particles of 28 to 30 nm in diameter were observed using transmission electron microscopy (TEM) in the purified viral fractions. (B) RT-PCR detection of MfPV1 in KF18 (Mf_KF18) using the specifical primers derived from the dsRNA1 and dsRNA2, respectively. The sizes of PCR productions from dsRNA1 and dsRNA were 1,717 and 1,382, respectively. Water was used for the PCR templets as the negative control. (C) RT- PCR determination of MfPV1 in Mf_KF18 virions.

2.3.3 dsRNAs in Mf_KF18 and Transfection of MfPV1 to Other Metarhizium spp.

A total of seven bands with corresponding sizes between 900 and 1,900 bp as estimated by dsDNA markers were obtained in strain KF18 dsRNA preparations (Figure 2-6A: line 2), suggesting Mf_KF18 might host several virus species. To elucidate any biological impacts of MfPV1 and other potential viruses in Mf_KF18, we first used several treatments to eliminate viruses from Mf_KF18, including single-conidium subculture, hyphal-tip transfer, and treatment with ribavirin or cycloheximide, but they failed to eliminate the viruses (Figure S2-3). Then, we transfected the purified MfPV1 particles into protoplasts of virus-free *M. anisopliae* Ma_114445 and *Metarhizium pingshaense* Mp_336563 to obtain MfPV1-infected isogenic isolates. Colonies were regenerated from selected protoplasts on PDA and confirmed to be positive for MfPV1 RdRp and CP by RT- PCR (Figure 2-6B and 2-7A).

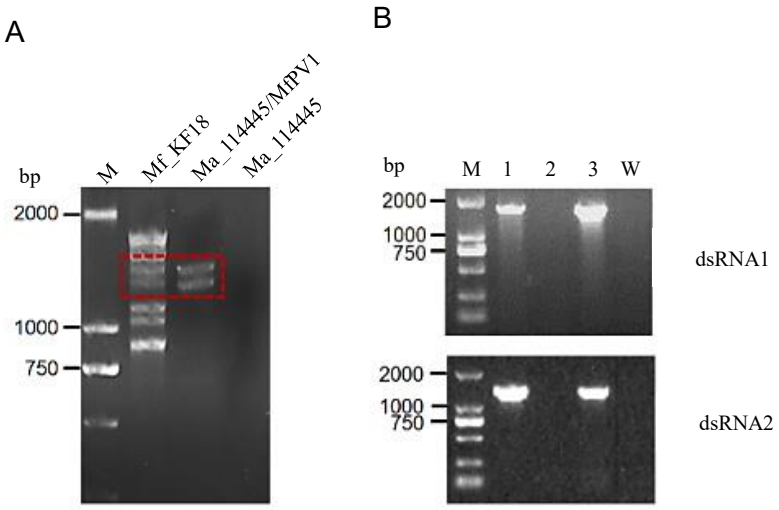


Figure 2-6 (A) Separation of dsRNAs from the Mf_KF18 and Ma_114445/MfPV1 isolates (see below) in the agarose gel. Several bands were detected in the Mf_KF18 but only two bands found in the Ma_114445/MfPV1 isolates. dsRNAs extracted from the Ma_114445 were used as the negative control. (B) Screen of positive culture in virion-transfected protoplasts of *Metarhizium anisopliae* by RT-PCR using specifical primers. The RNAs extracted from different samples were used for cDNA synthesis as the PCR templets. M, marker; line 1, positive culture; line 2, virus- free strain Ma_114445; line 3, Mf_KF18.

The transfection rates were 55.0% for *M. anisopliae* and 61.5% for *M. pingshaense*. After the colonies were subcultured several times, RT- PCR showed that the MfPV1 was stably maintained in the virus-infected isolates (Ma_114445/MfPV1 and Mp_336563/MfPV1). Alignments of CDS of MfPV1 genomes in Ma_114445/MfPV1 and those from RNA-seq revealed only one base- pair difference in RNA1 and three in RNA2, but no difference in aa sequences both in RNA1 and RNA2 (Figure S2-4 A-D). As expected, the dsRNA from Ma_114445/MfPV1 or Mp_336563/MfPV1 yielded two dsRNA bands (Figure 2-6A: line3, 2-7B: line 2), and no dsRNA bands were found for Ma_114445 or Mp_336563 (Figure 2-6A: line 4, 2-7B: line 3). The targeted dsRNA bands were then recovered and amplified, confirming the presence of the virus (Figure S2-4E).

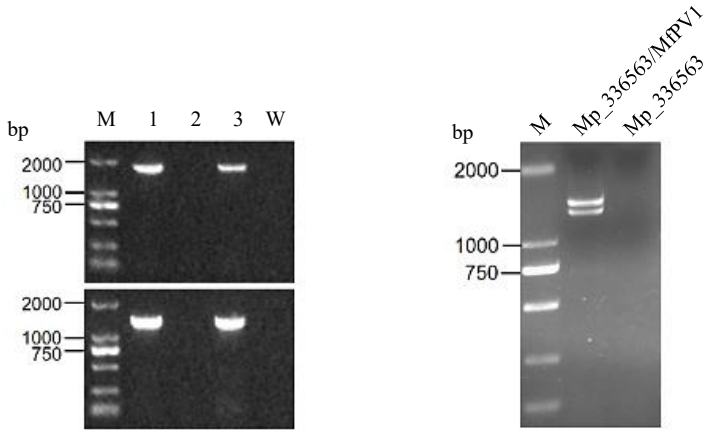


Figure 2-7 (A) Screen of positive culture in virion-transfected protoplasts of *M. pingshaense* (Mp_336563) by RT-PCR using specifical primers of MfPV1. The RNAs extracted from different samples were used for cDNA synthesis as the PCR templets. M, marker; line 1, positive culture; line 2, virus-free strain Mp_336563; line 3, Mf_KF18; W, water. (B) Examination of dsRNAs from the Mp_336563/MfPV1 isolates in the agarose gel. Two bands, representing two dsRNAs, were observed in the Mp_336563/MfPV1 strain. dsRNAs extracted from the Mp_336563 strain were used as the negative control.

2.3.4 Changes in Biological Characteristics of MfPV1-Transfected Isolates of M. anisopliae and M. pingshaense.

For *M. anisopliae*, the colony diameter of the virus-free strain Ma_114445 and the transfected strain Ma_114445/MfPV1 only had a slight difference during a culture period over 11 d (Figure S2-5A). However, by day 5, their colony morphologies differed; Ma_114445 had a yellow and white colony with a wavy margin, in contrast to the smooth-edged, green and gray colony of the transfected strain Ma_114445/MfPV1 (Figure 2-8A). Both colonies retained these features until they conidiated, when the mycelia of strain Ma_114445/MfPV1 became denser and formed a thicker layer of conidia than that of Ma_114445 with sparse conidia (Figure 2-8B).

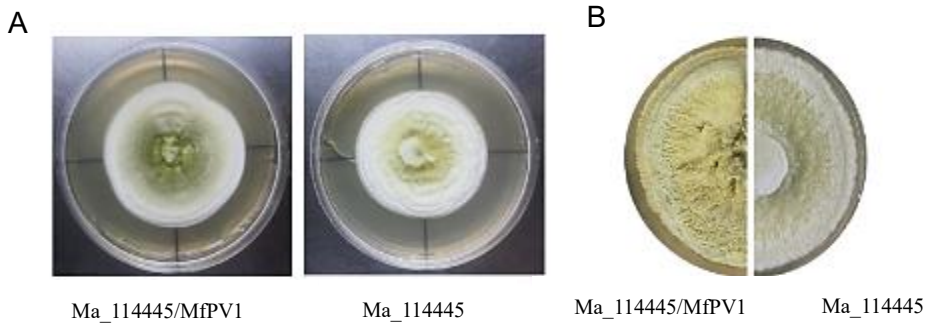


Figure 2-8 (A) Morphological difference of Ma_114445 (a strain of *M. anisopliae*) with or without MfPV1. Compared with the Ma_114445, Ma_114445/MfPV1 showed a more obvious green and gray colony with smooth edge. The picture was taken on the fifth day of clone growth.

On day 21, significantly more conidia were produced by Ma_114445/MfPV1 than by Ma_114445 (approximately 5.4 times more, Figure 2-9A; *t*-test: $P = 0.0024$). However, the mycelial biomass of the virus-infected strain after 5 d was significantly lower than that of the virus-frees train (approximately 11.45% less, Figure 2-9B, *t* test: $P = 0.0019$). On the hypertonic medium containing 1/4 SDAY with either1 M NaCl or 1 M sorbitol, the mean diameter of the virus-infected colonies on day 5 was larger than that of the virus-free strain. Similarly, when the conidia were placed on 1/4 SDAY with 400 μg/mL SDS, the virus-infected colonies were larger than those of the virus-free strain, but the colony diameters of the virus-free strain were larger on 1/4 SDAY amended with 500 μg/mL Congo red (Figure S2-5 B and C), suggesting that chemical tolerance of the virus-infected strain was enhanced.

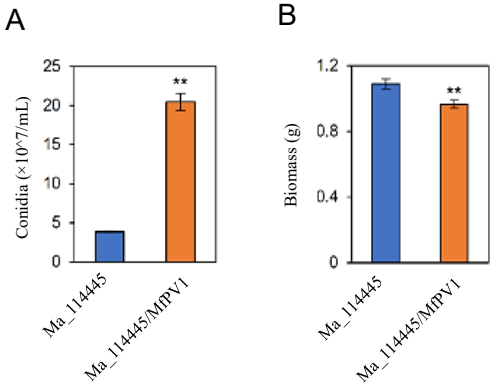


Figure 2-9 Statistical analysis of the spore yield between the Ma_114445 and Ma_114445/MfPV1 strains (A). (B) Statistical analysis of the mycelium biomass between the Ma_114445 and Ma_114445/MfPV1 strains on the fifth day of clone growth. In (A and B), data are means ± SD of three biological replications. $^{**}P < 0.01$ (Student's *t* test).

In virulence bioassay using diamondback moth (*P. xylostella*), the highest mortality rate for the larvae of the negative control was 13.3% by day 5, but that of the larvae treated with conidia of Ma_114445/MfPV1 on days 1 to 5 was 2.2%, 4.4%, 22.2%, 41.1%, and 54.4%, respectively, compared with 0%, 0%, 10.0%, 24.4%, and 41.1%, respectively, for Ma_114445 (Figure 2-10A). The mortality rates of larvae treated with conidia of Ma_114445/MfPV1 were significantly higher than those of Ma_114445 at 2, 3, 4, 5 dpi (32.4% higher at 5 dpi; ANOVA, $P < 0.05$, Figure 2-10A). In addition, white mycelia appeared on the dead larvae after inoculation with either Ma_114445 or Ma_114445/MfPV1 in high humidity (Figure 2-10B), suggesting the larvae had been killed by the fungal strains. When each strain on the dead larvae was tested by RT- PCR, the results showed that the virus was still present inMa_114445/MfPV1 strain but not in Ma_114445 strain (Figure 2-10C), again suggesting that MfPV1 was responsible for the higher mortality rates of the larvae.

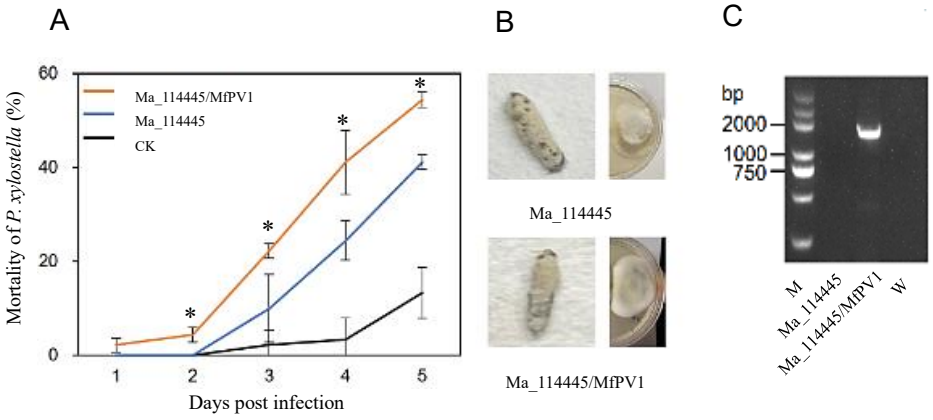


Figure 2-10 (A–C) Virulence assay demonstrated the enhancement of insecticidal ability to *P. xylostella* larvae by Ma_114445/MfPV1 strain. Measurement of the mortality rates of *P. xylostella* larvae treated by 0.05% Tween-80 combined with the spores of Ma_114445 or Ma_114445/MfPV1 at 1 to 5 d postinoculation. The mortality rates of *P. xylostella* larvae treated by 0.05% Tween-80 were used as the negative control (A). The dead *P. xylostella* larvae were used to isolate the Ma_114445 strains in PDA medium (B), which were further exanimated the MfPV1 by RT-PCR with specifical primers (C). In (A), data are means ± SD of three biological replications.

When we evaluated the mortality of larvae of fall armyworm (*Spodoptera frugiperda*) after feeding on corn leaves that had been soaked in the conidial suspension of either Ma_114445/MfPV1 or Ma_114445, the mortality rates of larvae on Ma_114445/MfPV1-treated corn leaves were significantly higher (ANOVA, $P < 0.05$) than those on Ma_114445-treated leaves at 3 dpi (68.9% and 48.3% respectively), 4 dpi (83.3% and 63.3% respectively), 6 dpi (93.3% and 78.3% respectively), and 7 dpi (95.0% and 81.67% respectively). The mortality rate of the

negative control was only12.2% by day 7 (Figure 2-11). The corresponding LT_{50} was 3.85 d for Ma_114445 and 2.99 d for Ma_114445/MfPV1.

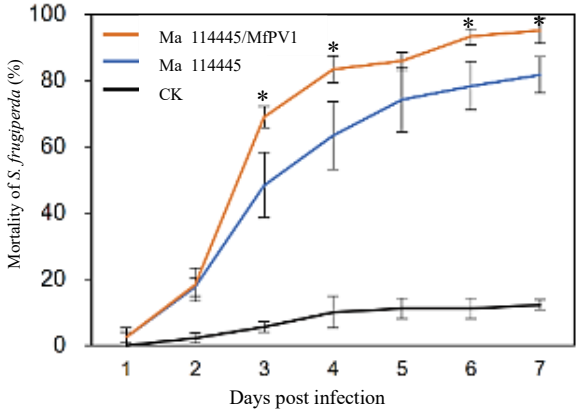


Figure 2-11 Virulence assay demonstrated the enhancement of insecticidal ability to *S. frugiperda* larvae by Ma_114445/MfPV1 strain. Data are means ± SD of three biological replications.

Similarly, the transfection of the virus-free strain Mp_336563 of *M. pingshaense* with MfPV1 did not affect the host growth rate (Figure S2-6). The white colonies of the transfected and virus-free strains were similar in morphology before day 3 but differed by day 5; the infected strain remained white, but a yellow center had developed in the white virus-free colony (Figure 2-12).

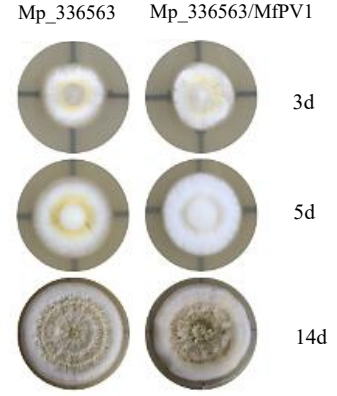


Figure 2-12 Morphological difference of Mp_336563 (a strain of *M. pingshaense*) with or without MfPV1. Compared with the Mp_336563, Mp_336563/ MfPV1 showed more stronger of white colony and displayed more mycelium. The picture was taken at 3, 5, and 14 d of clone growth, respectively.

Conidial and biomass production by Mp_336563/MfPV1 were also significantly greater than in the virus-free strain (Figure 2-13A and B). In the virulence bioassay using *P. xylostella*, the mortality rate for the larvae in the negative control was 14.2% by day 5; the mortality rates of larvae treated with conidia of Mp_336563/MfPV1 were significantly higher (ANOVA, $P < 0.05$) than those of Mp_336563 at 2 dpi (35.6% vs 19.2%, respectively), 3 dpi (55.6% vs 39.2%), 4 dpi (70.0% vs 50.0%), and 5 dpi (77.5%vs 63.3%; 32.4% higher, Figure 2-13C). The corresponding LT_{50} was 4.43 d for Mp_336563 and 3.22 d for Mp_336563/MfPV1.

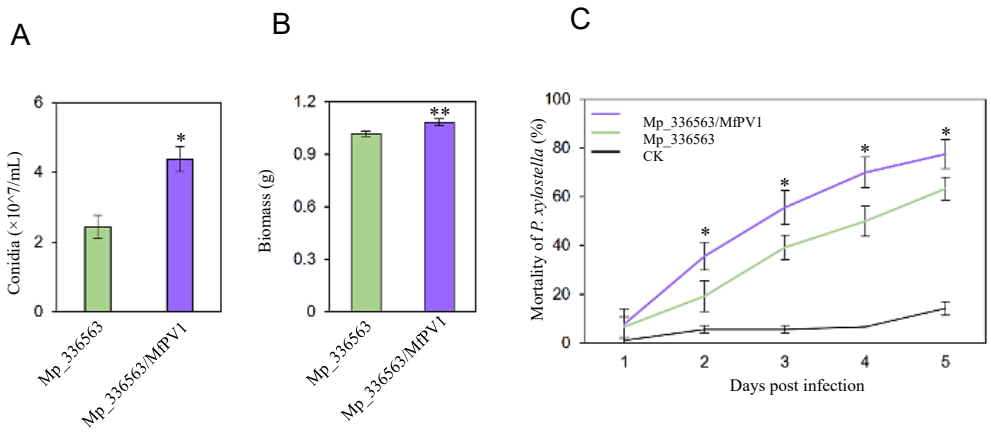


Figure 2-13 (A and B) Effects of MfVP1 on the spore yield (A) and (B) biomass of *M. anisopliae*. (C) Measurement of the mortality rates of *Plutella xylostella* larvae treated by 0.05% Tween-80 combined with the spores of Mp_336563 or Mp_336563/MfPV1 at 1 to 5 d postinoculation. The mortality rates of *P. xylostella* larvae treated by 0.05% Tween-80 were used as the negative control. In (A and B), data are means ± SD of three biological replications. *$P < 0.05$; **$P < 0.01$ (Student's *t* test). In (C), data are means ± SD of three biological replications.

2.3.5 Identification of Differentially Expressed Genes (DEGs) in M. anisopliae in Response to MfPV1 Infection and Validation of Selected Genes Using RT-qPCR.

To learn more about potential molecular mechanisms underlying the virus-induced hypervirulence in the host fungus, we analyzed the RNA-seq data from MfPV1-infected and virus-free strains of *M. anisopliae* cultured in potato dextrose broth (PDB) medium with cicada slough addition. The relative percentage of reads was similar in all samples (Table S2-3). Results of the read alignment revealed that >80% of the reads were mapped to the reference genome (Table S2-4). The square of Pearson correlation coefficient (r) was 0.928, 0.978, and 0.956 for three replicates of Ma_114445, and 0.972, 0.955, 0.93 for Ma_114445/MfPV1 (Figure S2-7A). Three samples of Ma_114445 were clustered together with those of Ma_114445/MfPV1, whereas the intergroup was dispersed in the PCA (Figure S2-7B). All the above

analyses of RNA-seq data indicated that the data were reliable. Using *P*-value < 0.05 and fold-change >1.2 as criteria for differential expression, we identified 1,872 DEGs for strain Ma_114445/MfPV1, with1,136 of these (60.68%) up-regulated and 736 (39.32%) down-regulated (Figure 2-14). Hierarchical clustering revealed similar expression patterns among the three biological replicates of strains Ma_114445/MfPV1 and Ma_114445 (Figure S2-7C). In the GO analysis, the annotations for 1,388 of the DEGs were from three major functional ontologies (biological process, cellular component, and molecular function); 415 were annotated as biological process with 392 up-regulated and 23 down-regulated; 637 were annotated as molecular function with 372 up-regulated and 265 down-regulated; and 336 were annotated as cellular component with 235 up-regulated and 101 down-regulated. Based on low to high of *P*-value, the top four enriched terms were cofactor binding (GO:0048037, molecular function), heme binding (GO:0020037, molecular function), and tetrapyrrole binding (GO:0046906, molecular function) (Figure S2-7D).

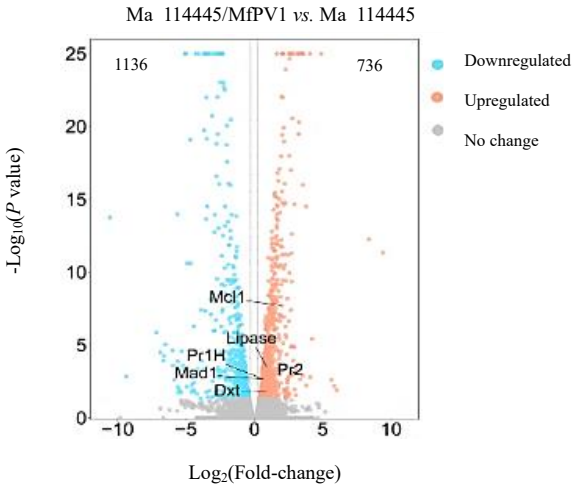


Figure 2-14 Volcano plot shows the upregulation and downregulation of genes in *M. anisopliae* stimulated by the MfPV1. Upregulation of the genes functioning relative to the pathogenicity of *M. anisopliae* are marked by black font.

The expression of several genes related to *Metarhizium* pathogenicity, including genes involved in destruxin synthesis and the genes encoding adhesin-like protein (Mad1), a protease that hydrolyzes the insect cuticle, and a collagen-like protein (MCL1), was up-regulated (Table S2-5). Six of the up-regulated DEGs (MAN_02447, MAN_05588, MAN_06794, MAN_07719, MAN_03711, MAN_10464) were selected to assess their expression by RT-qPCR using gene-specific primers. In agreement with the RNA-seq results, the RT-qPCR results showed that all six

pathogenesis-related genes were up-regulated significantly inMa_114445/MfPV1 cultured in cicada induced medium at 3 dpi. Especially high was the expression of Mad1, approximately 7-120 times higher than the other selected DEGs (Figure 2-15A). Thus, we validated its expression in the MfPV1-infected and MfPV1-free strains cultured in PDB from 6 to 36 h after transfection as described above. The results confirmed that Mad1 expression was higher in the MfPV1-infected strain than in the virus-free strain from 12 to 36 h (Figure 2-15B).

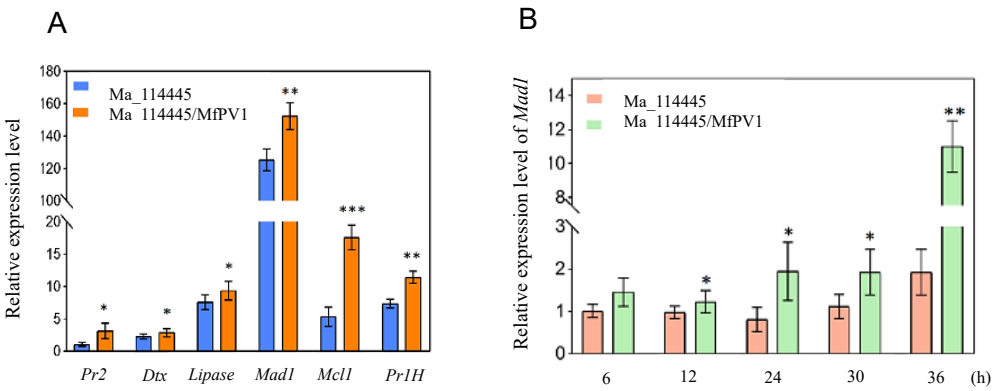


Figure 2-15 (A and B) Validation of the six pathogenesis- related genes by RT-qPCR at 3 d postinoculation (A). The Mad1 gene, a virulence factor induced to express highly in early phase of *Metarhizium* infection, was confirmed by RT-qPCR at 6, 12, 24, 30, and 36 h, respectively (B). In (A and B), the data are means ± SD of at least three biological replications. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test).

We also validated the expressions of the three central regulators (*BrlA*, *AbaA*, and *WetA*) of the conidiation pathway in filamentous fungi by RT-qPCR using gene-specific primers in *M. anisopliae*, which were cultured on PDA medium for 12 d. The results showed significantly up-regulated expressions of them inMfPV1-infected strain (Figure 2-16).

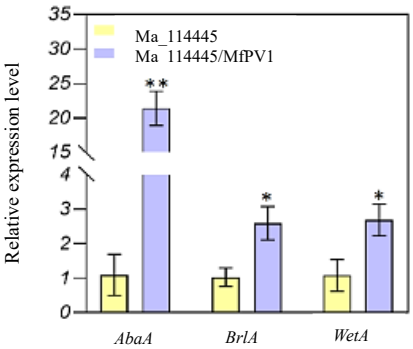


Figure 2-16 Detection of the three conidiation- related genes by RT-qPCR in Ma_114445 and Ma_114445/MfPV1 strains cultured on PDA medium for 12 d. The data are means ± SD of at least three biological replications. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test).

2.4 Discussion

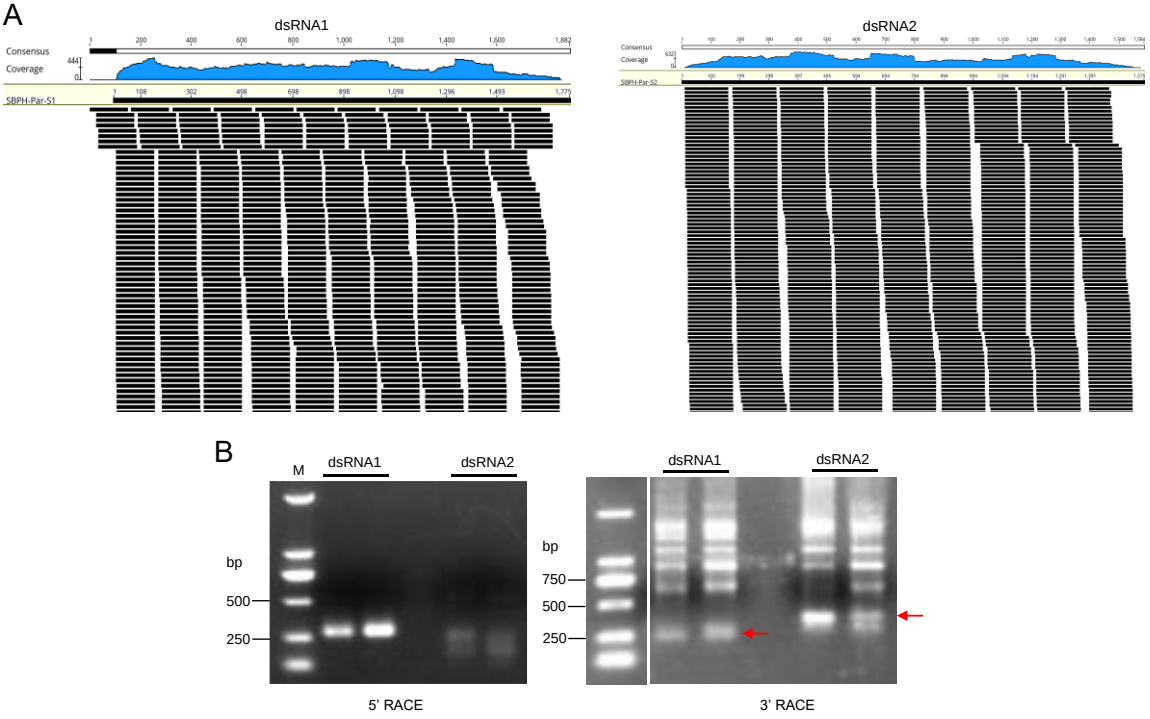
The mycovirus MfPV1 that we isolated from *M. flavoviride* in *L. striatellus* enhanced the conidiation and virulence of *M. anisopliae* and *M. pingshaense* after they were transfected with purified virions. The virus is phylogenetically most closely related to species in the genus *Gammapartitivirus*, family *Partitiviridae* (Figure 2-3A),and shares the highest aa identity for RdRp with Penicillium stoloniferum virus S (70.9%) and for CP with Discula destructivavirus 2 (59.5%) (Table S2-2), both of them are lower than the species demarcation criteria for family *Partitiviridae* (≤90% aa identity for RdRp and/or ≤80% for CP) (Vainio et al., 2018, http://ictvonline.org/), suggesting that MfPV1 should be classified as a species in the genus *Gammapartitivirus*. Partitiviruses are transmitted intracellularly by seeds in plants, by oocysts in protozoa, or by hyphal anastomosis, cell division, and sporogenesis in fungi (Vainio et al., 2018). All the tested conidia of *Metarhizium* KF_18 remained positive for MfPV1 after we tried to eliminate the mycovirus via single-conidium isolation (Figure S2-3). Because of the ineffectiveness of hyphal tip isolation to eliminate partitivirus particles or dsRNA (Chen et al., 2019; Peyambari et al., 2014), it was hard to remove MfPV1 from its native host *M. flavoviride* by single-conidium or hyphal-tip subculture, and ribavirin or cycloheximide treatments (Figure S2-3). Transfection of other hosts with purified partitivirus particles to obtain isogenic isolates has been reported (Chiba et al., 2013; Bhatti et al., 2011; Xiao et al., 2014), so here we transfected virus-free protoplasts of commercial strains Ma_114445 of *M. anisopliae* and Mp_336563 of *M. pingshaense* with MfPV1 using PEG (Figure 2-6B and 2-7A). After subculturing the virus-transfected strains for at least 10 generations, the morphologies of colonies and conidial production were consistent with the previous generation, suggesting stable presence of MfPV1 in the transfected fungal strains. In future studies, we will transfect other entomopathogenic fungi with MfPV1 in efforts

to improve their virulence. In general, partitiviruses seem to be associated with symptomless infections of their fungal hosts (Chen et al., 2019; Velasco et al., 2020). However, some of the viruses have deleterious effects on the host fungus, resulting in slower growth, reduced conidiation, altered morphology, and disrupted sexual reproduction, which can lead to hypovirulence in some fungal phytopathogens (Bhatti et al., 2011, Mahillon et al., 2021, Sun et al., 2023). In contrast, one gammapartitivirus, Talaromyces marneffei partitivirus 1 can enhance the virulence of its native host *T. marneffei*, an opportunistic pathogen of mammals, presumably by upregulating virulence factors and suppressing host RNAi machinery (Lau et al., 2018). B. bassiana polymycovirus-1(BbPmV-1, genus *Polymycovirus*, family *Polymycoviridae*) and B. bassiana nonsegmented virus 1 (BbNV-1, genus *Unirnavirus*, family *Unirnaviridae*) reduce the growth of their host *B. bassiana*, but have a mild hypervirulent effect against the greater wax moth (*Galleria mellonella)* (Melzer and Bidochka, 1998). However, purified particles of BbPmV-1and BbNV-1 could not be introduced by transfection into protoplasts of the commercial, virus-free strain *B. bassiana* ATCC704040, a biocontrol agent for various arthropod pests. In this study, mycelial growth of the MfPV1-transfected *M. anisopliae* strain did not differ clearly from that of the strain without the virus, but its culture morphology differed, and it produced more conidia (Figure 2-8, Figure 2-9A) and had significantly higher virulence against larvae of two important agricultural pests, *P. xylostella* (Figure 2-10A) and *S. frugiperda* (Figure 2-11). Similar results were also obtained for the MfPV1-transfected strain of *M. pingshaense* (Figure 2-13C). Thus, the present study demonstrates that a mycovirus can confer substantial hypervirulence to other species of an entomopathogenic fungus and is an important finding in the field of biological control. Thus, mycoviruses might be used to enhance the virulence of commercially available *Metarhizium* strains and provide a viable alternative to using genetic engineering to improve the biocontrol efficacy of entomopathogenic fungi. In our transcriptomic analysis, mRNA levels for diverse genes differed in MfPV1-infected Ma_114445/MfPV1 compared to those in the virus-free isolate. Among 1,136 up-regulated genes, 11 are involved in infection processes (Table S2-5). We selected six of these 11, encoding Mad1, Pr1H, Pr2, Mcl1, lipase, and destruxin synthetase and validated their upregulation using RT-qPCR (Figure 2-15A). Adhesin protein MAD1 is important for the attachment of conidia to the host surface; disruption of Mad1 blocks adhesion of conidia of *M. anisopliae* to insect surfaces and greatly reduces its virulence in caterpillars, suggesting that MAD1 is a potential virulence factor (Wang and St Leger, 2007). In our transcriptomics results, the expression of Mad1 was up-regulated in virus-infected strain by 1.66 times (Table S2-5) and was always higher than in virus-free strain by 12 h after culturing (Figure 2-15B). Later in the infection process, *Metarhizium* species secrete proteolytic, chitinolytic, and lipolytic enzymes that degrade major constituents of the cuticle (Beys da Silva et al., 2010; Junges et al., 2014; Schrank and Vainstein, 2010). Overexpression of the gene encoding subtilisin-like proteases (Pr1) from *M. anisopliae* in the hemolymph of Manduca sexta activates the prophenoloxidase system, which is involved in proteolytic cleavage during a

cascade of trypsin-like enzymatic reactions. The combined toxic effects of Pr1 and the reaction products of phenoloxidase cause larvae inoculated with a Pr1-overexpressed fungus to die 25% faster and reduce their food consumption by 40% compared to rates after inoculation with the wild-type fungus (St Leger et al., 1996). The expression of Pr1H, Pr2, and a gene for lipase up-regulated in the virus-infected strain by 1.39,11.80, and 1.87 times, respectively (Table S2-5). *Metarhizium* can synthesize a collagen-like protein (Mcl1), an immune modulator that suppresses the insect host defense system (Wang and St Leger, 2006). In this study, the mRNA level of Mcl1 was 4.07 times higher in the virus-infected strain than in the virus-free strain. The gene encoding destruxin synthetase in the virus-infected strain was also up-regulated (1.87 times higher than in the virus-free strain). Destrucxin produced by *Metarhizium* is a bioactive secondary metabolite that accelerates death of the infected insect by weakening its immune defense, damaging its muscular system and the malpighian tubules, affecting excretion, and eventually impairing feeding and mobility (Pal et al., 2007). All of these results suggest that the up-regulated expression of numerous virulence-related genes in the virus-infected strain is responsible for the hypervirulence to the target pests. Further studies are required to determine the mechanism for enhancing the expression of virulence-related genes in *Metarhizium*. *Metarhizium* species may be introduced in the field as conidia or mycelia fragments in aqueous solutions, and they then enter the host either directly or indirectly (Zeng et al., 2017). The complex process of conidiation in filamentous fungi involves many aspects such as the differentiation of specialized structures, regulation of gene expression, and responses to environmental conditions (Park and Yu, 2012). A central regulatory pathway (*BrlA- AbaA-WetA*), which is involved in conidiogenesis and other processes, is functionally conserved in phyto- and entomopathogenic fungi (Lau and Hamer, 1998; Ohara et al., 2004; Wang et al., 2015). In *B. bassiana*, *BrlA*, *AbaA*, and *WetA* are important regulators of conidiation, conidial maturation, and virulence (Li et al., 2015, Zhang et al., 2019). Mycoviruses can affect the fitness of the fungi by enhancing or reducing the sporulation. The L1 dsRNA of *N. radicicola* could stimulate sporulation and increasing virulence of *N. radicicola* (Zhang et al., 2032). The mycovirus Phytophthora infestans RNA virus 2 stimulates sporangia production in *Phytophthora infestans*, which causes potato late blight (Cai et al., 2019). In the present study, MfPV1-infected *M. anisopliae* produced five times more conidia than the virus-free strain (Figure 2-9A). Our transcriptomic experiment used *M. anisopliae* strains cultured in cicada-amended PDB medium. The cicada slough can induce the expression of virulence genes in *M. anisopliae* because it serves as a substitute for insect cuticle to stimulate the expression of pathogenicity genes in entomopathogenic fungi (St Leger et al., 1994). Transcriptomic data analysis showed that sporulation-related genes were not up-regulated because the sporulation of fungi cultured in the liquid medium needs air as a necessary factor (Adams et al., 1998). When we detected the expressions of the three central regulators (*BrlA*, *AbaA*, and *WetA*) of conidiation by RT-qPCR, using the fungal cultures on PDA medium for 12 d, all three genes are significantly up-regulated (Figure 2-16), which indicates that MfPV1 achieved

sporulation stimulation likely through upregulation the central regulatory pathway of sporulation in *M. anisoplia*. In conclusion, we have shown here that the virulence of two species of *Metarhizium* from commercial biological control products is enhanced after they were transfected with the mycovirus MfPV1 that we isolated from *M. flavoviride*. The transfected species were hypervirulent against two lepidopteran pests and produced higher levels of conidia and expression of pathogenesis-related genes. The mycovirus thus has the potential to enhance the bio-control activity of entomopathogenic fungi as an environmentally safe alternative to chemical insecticides.

Supplementary information



C

```
                5' UTR
MfPV1-dsRNA1    -CGCAAAACT-GTCCGAGGTTTTACCGTGA
MfPV1-dsRNA2    -CGCAAAACT------TCCGTTTTACCCTG
AoV-dsRNA1      -CGCAAAACT---ACCAGTTTTACTCCCTG
AoV-dsRNA2      -CGCAAAACT----TCTAGTTTTACTTCTG
DdV1-dsRNA1     GCGCAAAAGCCTCGCGCTTTTCT----CTG
DdV1-dsRNA2     -CGCAAAATTCTGGCTGAATTTTGACCCTG
DdV2-dsRNA1     GCGCAAAACG--ACCGAGTTTTACCTGTGA
DdV2-dsRNA1     GCGCAAAAGT----GGTACTTTTGGCACAG
FsV-dsRNA1      -GGCGATGCT-------------------
FsV-dsRNA2      GC---------------------------
GaV-MS1-dsRNA1  -CGCAAAATA-TACGATATTTTTATTCACG
GaV-MS1-dsRNA2  -CGCAAAATT-TGACAAATTTTGGCCTGTG
OPV1-dsRNA1     -ACCGAAAATAGTCTATCTGTTTACCACTG
OPV1-dsRNA2     CTGGAGATTTACGGTAGACCTGATAGACAG
PsV-F-dsRNA1    -CGTAAAAATAATGGAAACTACAACACCCG
PsV-F-dsRNA2    -CGTAAAATATCAACATACTTACAAACAAG
PsV-S-dsRNA1    GCGCAAAATC--TCTAAGATTTTACCTGTG
PsV-S-dsRNA2    GCGCAAAATT----CGTGTTTTGACTGCTG

                                                3' UTR
MfPV1-dsRNA1    AATCCTGTTTATCAGGATGAATGA---TAAATCT--
MfPV1-dsRNA2    ----------------------------TAAATCT--
AoV-dsRNA1      -TTATCTTTTAGATGAGTGAACTTTAAATAAACTCC
AoV-dsRNA2      ----------------------------CATATCTCC
DdV1-dsRNA1     CGATGTGTGTAAAACATGTCGTGCAGTAAAAACTCC
DdV1-dsRNA2     ----------------------------TAAACCTCC
DdV2-dsRNA1     CGTTATGTGAAACATAATGTGCAG---TAAAACTCA
DdV2-dsRNA1     ----------------------------ATAAAACTCC
PsV-F-dsRNA1    TAACCATTTCAAATATTTAAAATTAAAAAAAAAT--
PsV-F-dsRNA2    CAGTATAATCAAAAAAATAAA------AAAAACT--
PsV-S-dsRNA1    CAGTCTATCTGTTGTGTTAACAA----TAAATCTCT
PsV-S-dsRNA2    ----------------------------TAAATCTCT
```

Figure S2-1 RNA-seq analysis and terminal sequence characteristic of MfPV1. (A) Mapping of 6,690 clean reads (0.02% total reads) into the two dsRNAs of MfPV1 genome. (B) Determination of the terminal sequences of MfPV1 genome using 5' and 3' RACE assays. Arrowheads represent the objective bands in right panel. (C) Sequence alignment of 5' untranslated regions (UTR) or 3' UTR among the species in genus *Gammapartitivirus*.

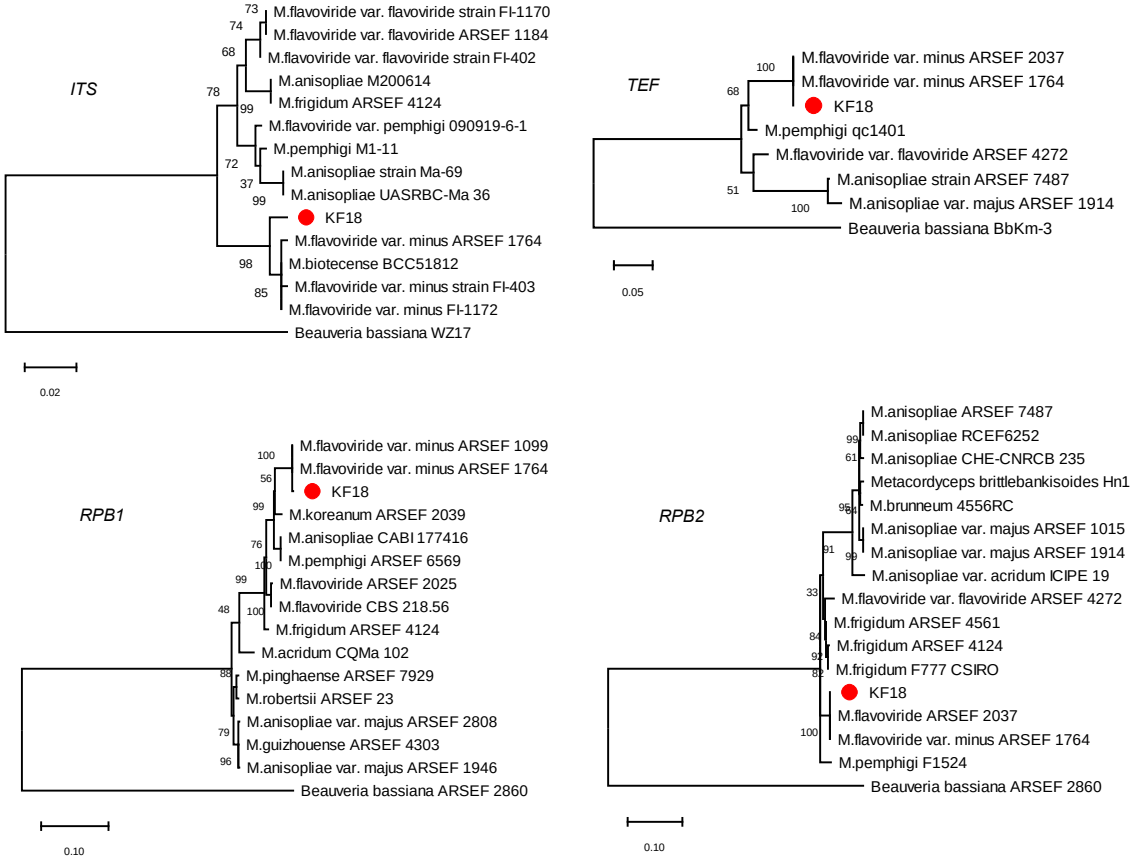


Figure S2-2 Phylogenetic analysis of four conserved genes (*ITS*, *TEF*, *RPB1* and *RPB2*) for identification of the species of KF18 strain.

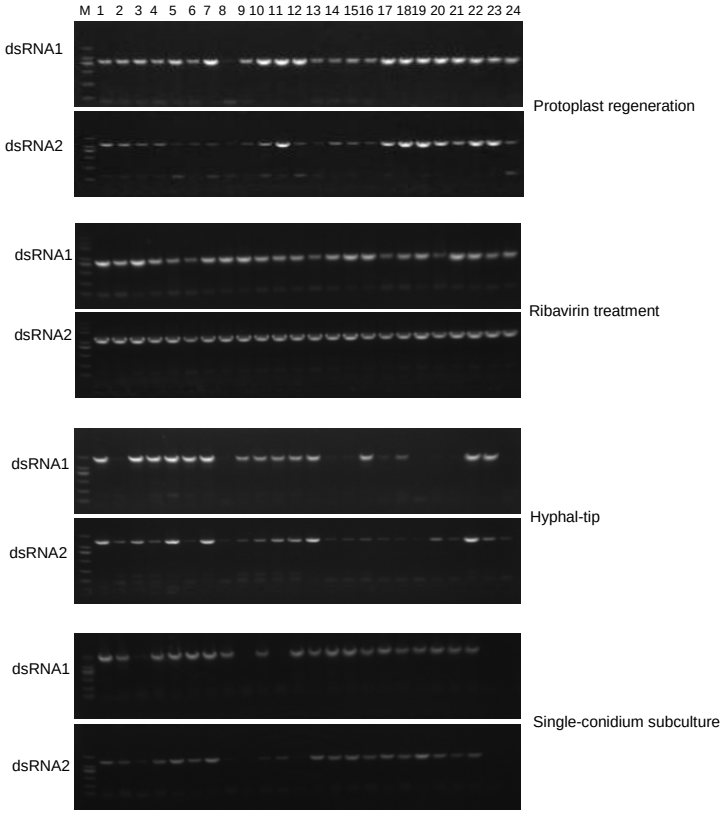
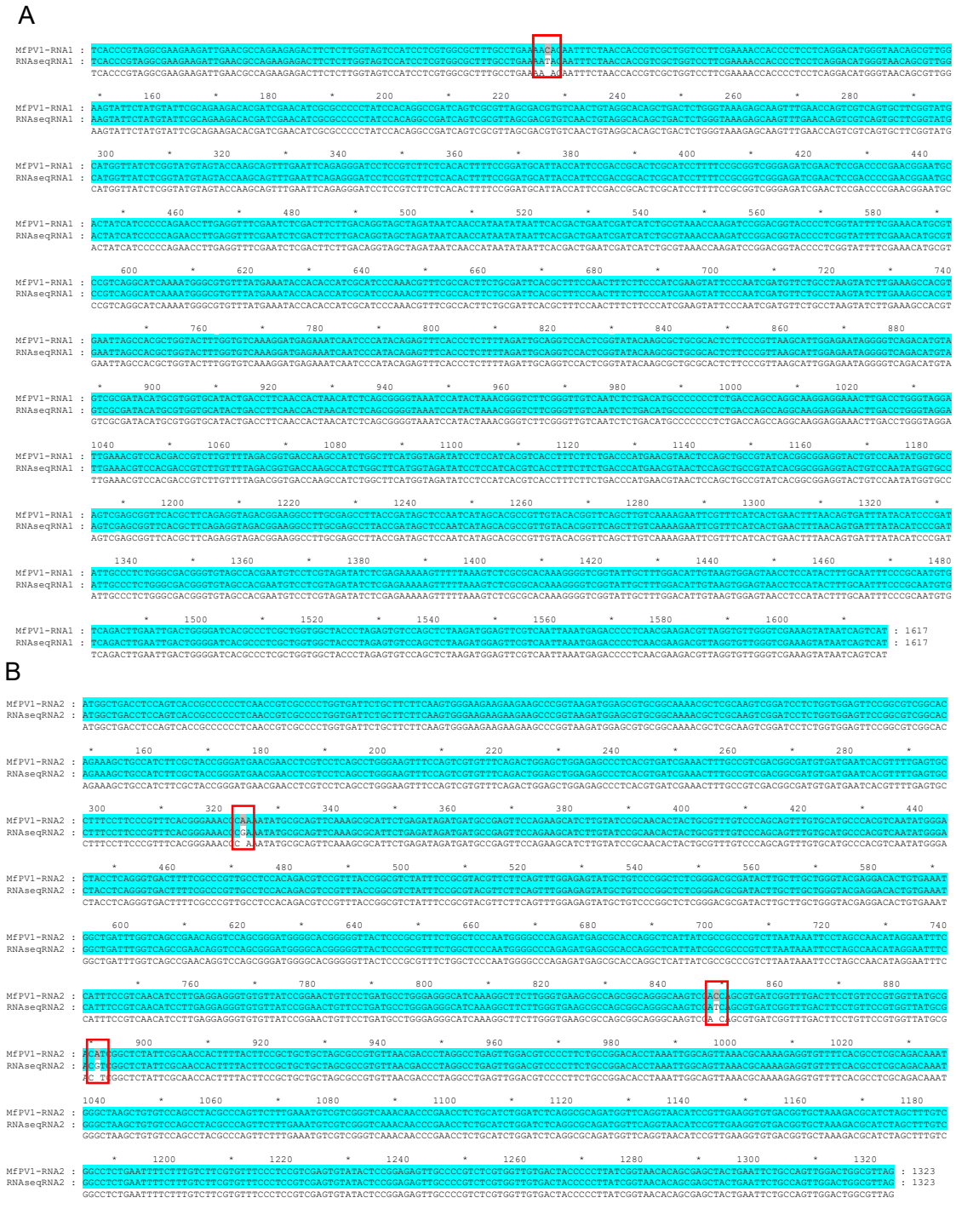


Figure S2-3 Mycovirus elimination in Mf_KF18. RT-PCR detection of MfPV1 in Mf_KF18 treated by protoplast regeneration, ribavirin, hyphal-tip and single-conidium subculture, respectively, using specific primer as previous described in Figure 2-5B.


A
B

C

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MfPV1-aa1  : MTDYTFDPTPNVFVEGSHLIDELHLRAGHSRVATSEGVIPSQFKSDTLREIAKYGGYSTYNVQSNTDPFVRETLKTFSRDIYEDIRGYTRRPEGNIGMYKSLLKFSDETNSFDKLNR
RNAseq-aa1 : MTDYTFDPTPNVFVEGSHLIDELHLRAGHSRVATSEGVIPSQFKSDTLREIAKYGGYSTYNVQSNTDPFVRETLKTFSRDIYEDIRGYTRRPEGNIGMYKSLLKFSDETNSFDKLNR
             MTDYTFDPTPNVFVEGSHLIDELHLRAGHSRVATSEGVIPSQFKSDTLREIAKYGGYSTYNVQSNTDPFVRETLKTFSRDIYEDIRGYTRRPEGNIGMYKSLLKFSDETNSFDKLNR

             120         *       140         *       160         *       180         *       200         *       220         *
MfPV1-aa1  : VQRRAMIGAIGKARKAFRLPLKREPLDWHHIGQYLRRDTAAGVTFMGQKKGDVMEDIYHEARWLGHRLKQDGRGRFNPTQVKFPPCLAGQRGGMSEIDNPKTRLVWIYPAEMLVVEG
RNAseq-aa1 : VQRRAMIGAIGKARKAFRLPLKREPLDWHHIGQYLRRDTAAGVTFMGQKKGDVMEDIYHEARWLGHRLKQDGRGRFNPTQVKFPPCLAGQRGGMSEIDNPKTRLVWIYPAEMLVVEG
             VQRRAMIGAIGKARKAFRLPLKREPLDWHHIGQYLRRDTAAGVTFMGQKKGDVMEDIYHEARWLGHRLKQDGRGRFNPTQVKFPPCLAGQRGGMSEIDNPKTRLVWIYPAEMLVVEG

                240         *       260         *       280         *       300         *       320         *       340         *
MfPV1-aa1  : QYAPRMYRDYMSDPYSPMLNGKSAQRLYTEWTCNLKEGETLYGIDFSSFDTKVPAWLIHVAFKILRQNIDWEYFDGKKVGKRESQKWRNVWDAMVWYFINTPILMPDGRMFRKYRGV
RNAseq-aa1 : QYAPRMYRDYMSDPYSPMLNGKSAQRLYTEWTCNLKEGETLYGIDFSSFDTKVPAWLIHVAFKILRQNIDWEYFDGKKVGKRESQKWRNVWDAMVWYFINTPILMPDGRMFRKYRGV
             QYAPRMYRDYMSDPYSPMLNGKSAQRLYTEWTCNLKEGETLYGIDFSSFDTKVPAWLIHVAFKILRQNIDWEYFDGKKVGKRESQKWRNVWDAMVWYFINTPILMPDGRMFRKYRGV

                   360         *       380         *       400         *       420         *       440         *       460
MfPV1-aa1  : PSGSWFTQMIDSVVNYIMVDYLATCQEVEIRNLKVLGDDSAFRSGSEFDLPTAEKDASAVGMVMHPEKCEKTEDPSEFKLLGTTYRDNHAYRSTDDWFKLALYPESAVPTVDTSLTR
RNAseq-aa1 : PSGSWFTQMIDSVVNYIMVDYLATCQEVEIRNLKVLGDDSAFRSGSEFDLPTAEKDASAVGMVMHPEKCEKTEDPSEFKLLGTTYRDNHAYRSTDDWFKLALYPESAVPTVDTSLTR
             PSGSWFTQMIDSVVNYIMVDYLATCQEVEIRNLKVLGDDSAFRSGSEFDLPTAEKDASAVGMVMHPEKCEKTEDPSEFKLLGTTYRDNHAYRSTDDWFKLALYPESAVPTVDTSLTR

              *       480         *       500         *       520         *
MfPV1-aa1  : LIGLWIGGAMFDRVFCEYIEYFQRCYPCPEEGWFSKDQRRWLEIVFSGKAPRGWTTKRSLFWRSIFFAYG : 538
RNAseq-aa1 : LIGLWIGGAMFDRVFCEYIEYFQRCYPCPEEGWFSKDQRRWLEIVFSGKAPRGWTTKRSLFWRSIFFAYG : 538
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D

```
MfPV1-aa2  : MADLQSPPPSTVAPGDSASSSGKKKKPGKMERAAKRSQVGSSGGVPASAQKAAIFATGMNEPRPQPGKFPVVFQTGAGEPSRDRNFAVDGDVMNHVLSAFPSRFTGNPKYAQFKAHS
RNAseq-aa2 : MADLQSPPPSTVAPGDSASSSGKKKKPGKMERAAKRSQVGSSGGVPASAQKAAIFATGMNEPRPQPGKFPVVFQTGAGEPSRDRNFAVDGDVMNHVLSAFPSRFTGNPKYAQFKAHS
             MADLQSPPPSTVAPGDSASSSGKKKKPGKMERAAKRSQVGSSGGVPASAQKAAIFATGMNEPRPQPGKFPVVFQTGAGEPSRDRNFAVDGDVMNHVLSAFPSRFTGNPKYAQFKAHS

             120         *       140         *       160         *       180         *       200         *       220         *
MfPV1-aa2  : EIDDAEFQKHLVSATLLRLSQQFVHAHVNMGLPQGDFSPVASTDVRLPASISAYVLQFGEYAVPALGTRYLLAGYEDTVKWLIWSAEQVQRDGARGLLPRFWLPMGPRDERTRLIIA
RNAseq-aa2 : EIDDAEFQKHLVSATLLRLSQQFVHAHVNMGLPQGDFSPVASTDVRLPASISAYVLQFGEYAVPALGTRYLLAGYEDTVKWLIWSAEQVQRDGARGLLPRFWLPMGPRDERTRLIIA
             EIDDAEFQKHLVSATLLRLSQQFVHAHVNMGLPQGDFSPVASTDVRLPASISAYVLQFGEYAVPALGTRYLLAGYEDTVKWLIWSAEQVQRDGARGLLPRFWLPMGPRDERTRLIIA

                240         *       260         *       280         *       300         *       320         *       340         *
MfPV1-aa2  : ARLNKFLANIGISISVNILEEGVLSGTVPDAWEGIKGFLGEAPAAGQVDQRDFDFLFRGYATSALFATTFTSAAASAVLTTLGLSWTSPSAGHLNWQLNAKEVFSRLADKWAKLCP
RNAseq-aa2 : ARLNKFLANIGISISVNILEEGVLSGTVPDAWEGIKGFLGEAPAAGQVDQRDFDFLFRGYATSALFATTFTSAAASAVLTTLGLSWTSPSAGHLNWQLNAKEVFSRLADKWAKLCP
             ARLNKFLANIGISISVNILEEGVLSGTVPDAWEGIKGFLGEAPAAGQVDQRDFDFLFRGYATSALFATTFTSAAASAVLTTLGLSWTSPSAGHLNWQLNAKEVFSRLADKWAKLCP

                   360         *       380         *       400         *       420         *       440
MfPV1-aa2  : AYAQFFEMSSGQTTRTSASGSQAQMVQVTSVEGVTVLKTHLALSASEFSLSSCFPPSSVYSGELPRLVVVTTPLSVTQRATEFCQLDWR : 440
RNAseq-aa2 : AYAQFFEMSSGQTTRTSASGSQAQMVQVTSVEGVTVLKTHLALSASEFSLSSCFPPSSVYSGELPRLVVVTTPLSVTQRATEFCQLDWR : 440
             AYAQFFEMSSGQTTRTSASGSQAQMVQVTSVEGVTVLKTHLALSASEFSLSSCFPPSSVYSGELPRLVVVTTPLSVTQRATEFCQLDWR
```

E

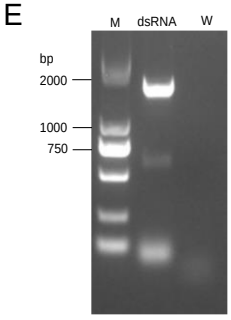


Figure S2-4 Sequence analysis in transfected strain of *Metarhizium anisopliae*. Sequence alignments of CDS in dsRNA1 and dsRNA2 from *M. anisopliae* Ma_114445/MfPV1 and from those obtained by RNA-seq. Nucleotide variations in CDS in dsRNA1 (A) and dsRNA2 (B), deduced amino acid variations in dsRNA1 (C) and dsRNA2 (D). Differences are framed in red. RT-PCR detection of dsRNA extracted from Ma_114445/MfPV1 (E).

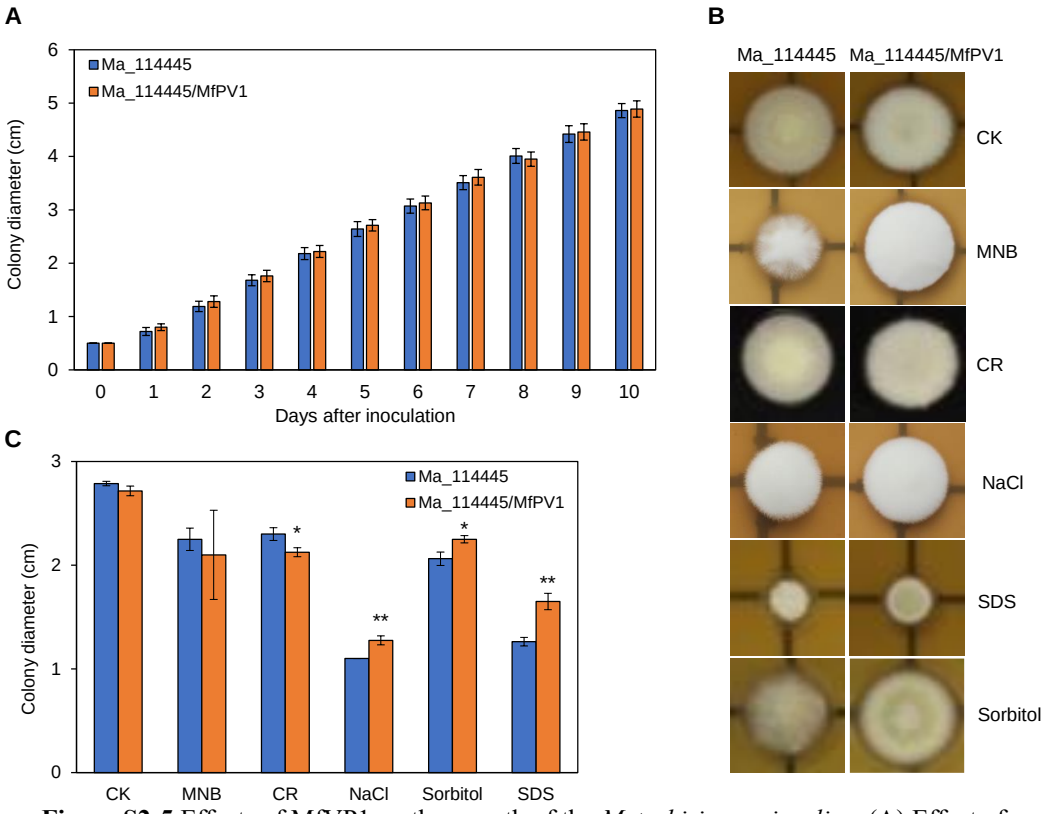


Figure S2-5 Effects of MfVP1 on the growth of the *Metarhizium anisopliae*. (A) Effect of MfPV1 on the growth rate of *M. anisopliae* Ma_114445. Colony diameters of Ma_114445 and Ma_114445/MfPV1 showed no significant difference during eleven days, with culturing on PDA. (B and C) Effect of MfPV1 on the tolerance of Ma_114445 to chemicals. Morphological difference of Ma_114445 with or without MfPV1 cultured in medium supplement with five chemical substances, menadione nicotinamide bisulfite (MNB), congo red (CR), NaCl, Sorbitol, and sodium dodecyl sulfate (SDS) (B). Statistical analysis of the colony diameters of Ma_114445 and Ma_114445/MfPV1 under the stresses induced by five different chemicals (C).

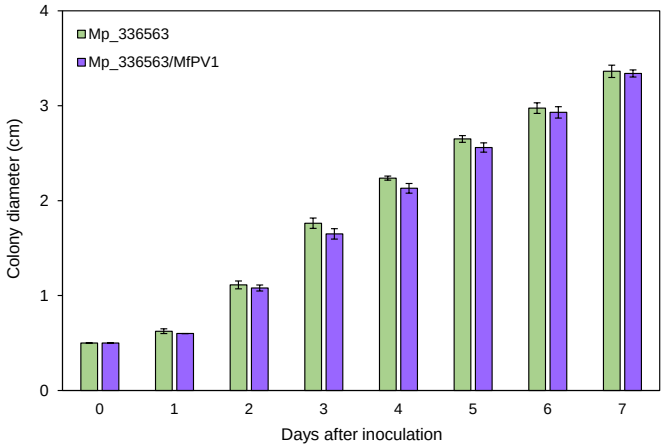
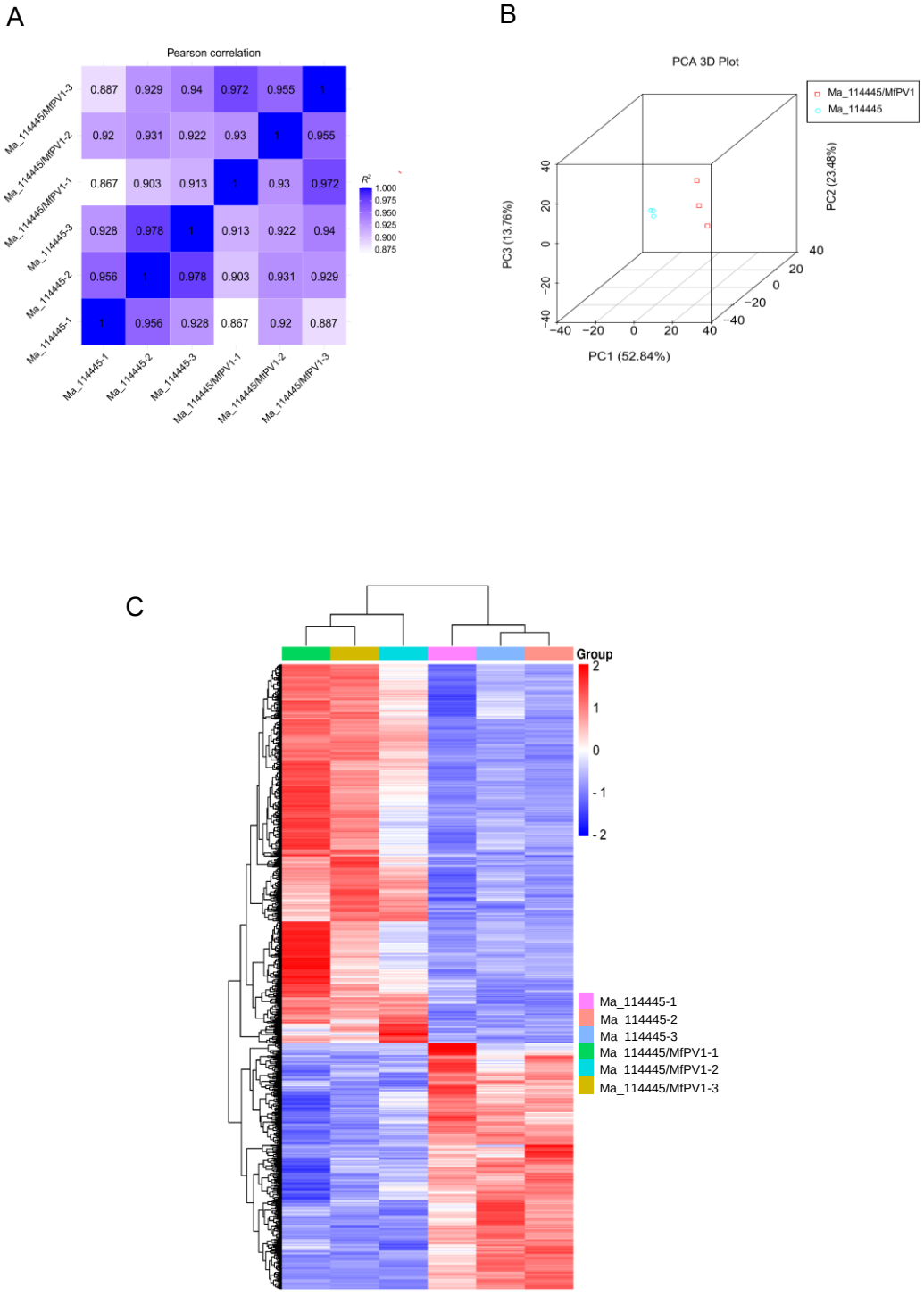


Figure S2-6 Growth rate difference of *Metarhizium pingshaense* Mp_336563 with or without MfPV1. Colony diameters of Mp_336563 and Mp_336563/MfPV1 showed no significant difference over eleven day's culturing.


A
Pearson correlation
Ma_114445/MfPV1-3
Ma_114445/MfPV1-2
Ma_114445/MfPV1-1
Ma_114445-3
Ma_114445-2
Ma_114445-1
0.887 0.929 0.94 0.972 0.955 1
0.92 0.931 0.922 0.93 1 0.955
0.867 0.903 0.913 1 0.93 0.972
0.928 0.978 1 0.913 0.922 0.94
0.956 1 0.978 0.903 0.931 0.929
1 0.956 0.928 0.867 0.92 0.887
R^2
1.000
0.975
0.950
0.925
0.900
0.875
B
PCA 3D Plot
Ma_114445/MfPV1
Ma_114445
PC3 (13.76%)
PC2 (23.48%)
PC1 (52.84%)
C
Group
2
1
0
- 1
- 2
Ma_114445-1
Ma_114445-2
Ma_114445-3
Ma_114445/MfPV1-1
Ma_114445/MfPV1-2
Ma_114445/MfPV1-3

D

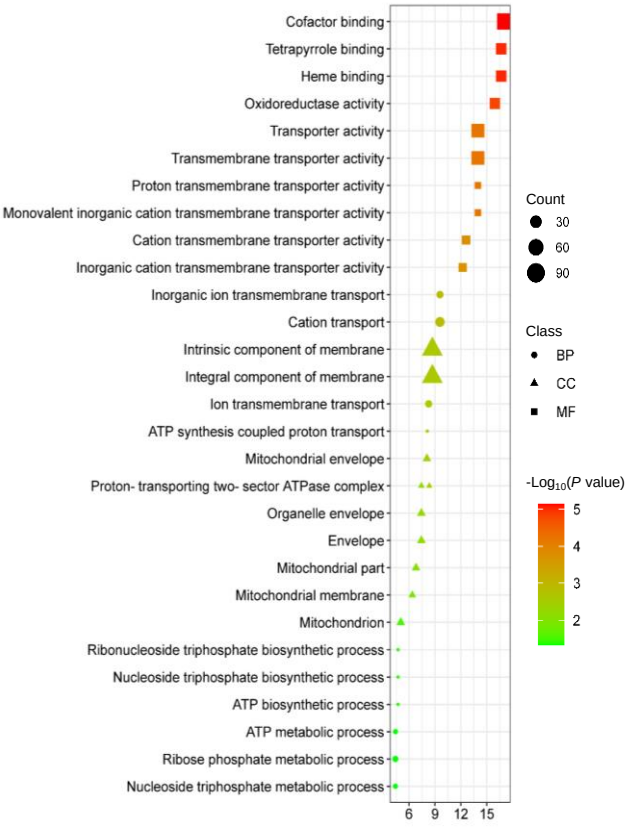


Figure S2-7 Transcriptomic analysis of the *Metarhizium anisopliae* samples with or without MfPV1 (Ma_114445, Ma_114445/MfPV1). Each two group of samples include three biological repeats (Ma_114445-1, Ma_114445-2, and Ma_114445-3, and Ma_114445/MfPV1-1, Ma_114445/MfPV1-2, and Ma_114445/MfPV1-3). (A) Pearson's correlation coefficient (R^2) between each two samples. (B) Principal component analysis (PCA) analysis shown two clusters, with each cluster representing the group that from the samples with or without MfPV1. (C) The hierarchical analysis shown the expression patterns of DEGs in six samples. (D) GO analysis of the biological process, cellular component, and molecular function using the DEGs shown in Figure 5A.

Table S2-1 Statistical data of RNA sequencing from small brown planthopper (SBPH)

Sample	Raw reads (bp)	Raw bases (bp)	Clean reads (bp)	Clean bases (bp)	Read length (bp)
SBPH-DL	35,443,732	10,633,116,096	32,766,442	9,829,929,336	150;150

Table S2-2. Alignments of amino acids (aa) using RdRP and CP of MfPV1 with other species in family *Partitiviridae*

Genus	Virus	Abbreviation	dsRNA1 (RdRp)			dsRNA2 (CP)		
			Accession No.	Size (nt)	Identity of RdRp (%)	Accession No.	Size (nt)	Identity of CP (%)
Alpha-partitivirus	Beet cryptic virus 1	BCV1	NC_011556	2008	15.1	NC_011557	1783	14.3
	Carrot cryptic virus	CarCV	FJ550604	1971	17.1	FJ550605	1776	15.8
	Cherry chlorotic rusty spot associated partitivirus	CCRSAPV	AJ781401	2021	15.7	AJ781402	1841	17.9
	Chondrostereum purpureum cryptic virus 1	CpCV1	AM999771	1920	22.2	AM999772	1757	17.0
	Flammulina velutipes browning virus	FvBV	AB465308	1915	15.8	AB465309	1730	18.4
	Helicobasidium mompa partitivirus V1	HmPV1	AB110979	2247	17.5	AB110980	1776	14.1
	Heterobasidion partitivirus 1-ab1	HetPV1-ab1	HQ541323	2017	15.7	HQ541324	1866	15.3
	Heterobasidion partitivirus 3	HetPV3	FJ816271	1885	18.6	FJ816272	1826	17.8
	Heterobasidion partitivirus 12	HetPV12	KF963175	1884	18.4	KF963176	1806	18.4
	Heterobasidion partitivirus 13-an2	HetPV13-an2	KF963179	1872	19.8	KF963180	1775	17.9
	Heterobasidion partitivirus 15-pa1	HetPV15-pa1	KF963186	1882	20.0	KF963187	1791	12.0
	Rosellinia necatrix partitivirus 2	RnPV2	NC_020234	1985	22.6	NC_020235	1828	15.2

	Vicia cryptic virus	VCV	NC_007241	2012	16.2	NC_007242	1779	16.2
	White clover cryptic virus 1	WCCV1	NC_006275	1955	15.2	NC_006276	1708	17.0
Beta-partitivirus	Atkinsonella hypoxylon virus	AhV	NC_003470	2135	20.2	NC_003471	1790	12.0
	Cannabis cryptic virus	CanCV	JN196536	2420	17.6	JN196537	2290	11.1
	Ceratocystis resinifera partitivirus 1	CrPV1	NC_010755	2305	20.1	NC_010756	2207	13.7
	Crimson clover cryptic virus 2	CCCV2	JX971982	2444	17.6	JX971983	2354	5.7
	Dill cryptic virus 2	DCV2	NC_021147	2430	14.5	NC_021148	2354	10.3
	Fusarium poae virus 1	FpV1	LC150606	2431	17.9	LC150607	2231	15.9
	Heterobasidion partitivirus 2	HetPV2	HM565953	2290	14.4	HM565954	2238	13.8
	Heterobasidion partitivirus 7	HetPV7	JN606091	2297	18.5	JN606090	2231	9.6
	Heterobasidion partitivirus 8	HetPV8	JX625227	2281	18.1	JX625228	2235	11.2
	Hop trefoil cryptic virus 2	HTCV2	NC_021098	2431	16.9	NC_021099	2349	10.2
	Pleurotus ostreatus virus 1	PoV1	NC_006961	2296	18.8	NC_006960	2223	11.7
	Primula malacoides virus 1	PmV1	NC_013109	2390	17.3	NC_0131010	2344	2.7
	Red clover cryptic virus 2	RCCV2	NC_021096	2430	16.1	NC_021097	2353	8.7
	Rhizoctonia solani virus 717	RhSV717	NC_003801	2363	17.2	NC_003802	2206	7.0
	Rosellinia necatrix virus 1	RnPV1	NC_007537	2299	17.4	NC_007538	2279	11.4
	White clover cryptic virus 2	WCCV2	NC_021094	2435	17.2	NC_021095	2348	8.4

Cryspovirus	Cryptosporidium parvum virus 1	CSpV1	KY884720	1836	23.4	KY884721	1510	2.9
Delta-partitivirus	Beet cryptic virus 2	BCV2	HM560703	1575	19.3	HM560704	1522	16.3
	Fig cryptic virus	FCV	NC_015494	1696	19.3	NC_015495	1415	7.7
	Pepper cryptic virus 1	PepCV1	JN117276	1563	20.8	JN117277	1512	6.0
	Pepper cryptic virus 2	PepCV2	JN117278	1609	19.5	JN117279	1525	16.4
Gamma-partitivirus	Aspergillus ochraceous virus	AoV	EU118278	1555	65.7	EU118279	1220	54.1
	Discula destructiva virus 1	DdV1	NC_002797	1787	69.8	NC_002800	1585	57.7
	Discula destructiva virus 2	DdV2	NC_003710	1781	69.9	NC_003711	1611	59.5
	Fusarium solani virus 1	FsV1	NC_003885	1645	57.9	NC_003886	1445	35.3
	Gremmeniella abietina RNA virus MS1	GaRV-MS1	NC_004018	1782	68.5	NC_004019	1586	52.1
	Ophiostoma partitivirus 1	OPV1	AM087202	1744	69.4	AM087203	1567	52.1
	Penicillium stoloniferum virus F	PsVF	NC_007221	1677	28.6	NC_007222	1500	20.2
	Penicillium stoloniferum virus S	PsVS	NC_005976	1754	70.9	NC_005977	1582	56.2

Table S2-3 Statistical data of RNA sequencing from *Metarhizium anisopliae* with or without MfPV1

Sample	Raw reads (bp)	Raw bases (G)	Clean reads (bp)	Clean bases (G)	Error rate	Q20	Q30	Gc pet
Ma_114445-1	46030296	6.9	45101312	6.77	0.03	96.41	90.85	55.09
Ma_114445-2	46123342	6.92	44732380	6.71	0.03	96.96	92.13	55.28
Ma_114445-3	50121188	7.52	48932466	7.34	0.03	96.86	91.92	55.24
Ma_114445/MfPV1-1	50127314	7.52	48653614	7.3	0.03	96.72	91.61	55.16
Ma_114445/MfPV1-2	39538376	5.93	38822436	5.82	0.03	96.98	92.17	55.35
Ma_114445/MfPV1-3	43346986	6.5	42244526	6.34	0.03	97.25	92.77	55.4

Table S2-4 Mapping ratio using RNA sequencing data

Sample	Total reads (bp)	Total map	Unique map	Multi map	Proper map
Ma_114445-1	45101312	37920709(84.08%)	37852581(83.93%)	68128(0.15%)	34778108(77.11%)
Ma_114445-2	44732380	38059486(85.08%)	37993357(84.93%)	66129(0.15%)	35121678(78.52%)
Ma_114445-3	48932466	41642294(85.1%)	41573472(84.96%)	68822(0.14%)	38414974(78.51%)
Ma_114445/MfPV1-1	48653614	40464003(83.17%)	40380930(83.0%)	83073(0.17%)	36883088(75.81%)
Ma_114445/MfPV1-2	38822436	33515262(86.33%)	33458443(86.18%)	56819(0.15%)	30900198(79.59%)

Table S2-5 Analysis of the expressions of upregulated pathogenicity-related genes in RNA sequencing data

Description	Gene name	Gene id	Ma_114445/ MfPV1	Ma_114445	FC	regulation	P_adj	Strand	Length (bp)
Adhesin-like protein	Mad1	MAN_02447	245855.3	147837.2	1.663015	up	0.002243	+	2097
Subtilisin-like protease	Pr1H	MAN_05588	33652.55	24155.61	1.393156	up	0.001893	+	1602
	Pr1C	MAN_06309	24.3704	4.607404	5.286427	up	0.009217	-	2751
Trypsin-like protease	Pr2	MAN_06794	3929.883	333.0794	11.79714	up	0.001245	+	768
	Pr2	MAN_01927	222.1671	67.30057	3.302042	up	2.56E-05	+	771
Immunity	Mcl1	MAN_07719	21974.95	5397.792	4.071139	up	1.98E-08	+	1944
Lipase	Lipase family	MAN_03873	182.971	80.36133	2.279401	up	0.005556	-	984
	lipase	MAN_03711	1818.101	723.9079	2.511874	up	1.52E-08	-	1050
	Esterase/ Lipase	MAN_00146	4979.783	2664.588	1.868936	up	0.000323	+	987
	Lipase 3 precursor	MAN_07732	33.14767	4.66039	7.140914	up	0.001104	-	2169
Destruxin synthetase	Destruxin synthetase	MAN_10464	27219.53	14539.47	1.872117	up	0.014679	-	23739

Table S2-6 Primers used in this study

Function	Primer	Position (nt)	Sequence (5' to 3')	Tm (℃)	Product (bp)
RACE-PCR	S1-5'GSP1	463-478	AGAGGTAGACGGAAGG	51	
	S1-5'GSP2	310-329	GTAGCCACGAATGTCCTCGT	59	
	S1-5'GSP3	234-257	GCTTTGGACATTGTAAGTGGAGTA	57	
	S1-3'GSP1	1554-1573	CCTGAGGAGGGGTGGTTTTC	59	
	S1-3'GSP2	1618-1637	CGCCACGAGGATGGACTACC	61	
	S2-5'GSP1	441-456	TCGGCATCATCTATCT	47	
	S2-5'GSP2	378-397	ACGGGAAGGAAAGGCACTCA	60	
	S2-5'GSP3	171-190	TGCCGCACGCTCCATCTTAC	61	
	S2-3'GSP1	1327-1346	AGAGTTGCCCCGTCTCGTGG	64	
	S2-3'GSP2	1333-1352	GCCCCGTCTCGTGGTTGTGA	61	
Detection primer	ITS1		TCCGTAGGTGAACCTGCGG	60	750
	ITS4		TCCTCCGCTTATTGATATGC	52	
	EF1T		ATGGGTAAGGARGACAAGAC	52	717
	EF2T		GGAAGTACCAGTGATCATGTT	52	
	PRPB1-F		CAAGGTGTTGGCCGATACTGTT	58	1314
	PRPB1-R		CCAAGGACATGATCAAATGCAAGC	57	
	PRPB2-F		TTGTCCAATTATTTGCGAAGAT	49	2910
	PRPB2-R		CGCAGCAGTTCAGATACAGAGT	57	
	ORF1-F	36-56	GTTGCCCGCCTTCTTCATTTC	57	1717
	ORF1-R	1732-1752	ACAGGATTCTTTGCACTCGTT	54	
	ORF2-F	62-82	TCCTGACGCCTTCTTTATTCG	54	1382
	ORF2-R	1423-1443	ACCTCATTGGATCCTCGACCC	59	

RT-qPCR	Q-GAPDH-F		AGCCTCCTTGATGGCCTCCTTG	62	137
	Q-GAPDH-R		AGAACATCATTCCCAGCAGC	55	
	Q-Dtx-F	8253-8273	TGAGACACGCGGAAATGAAGG	58	
	Q-Dtx-R	8369-8389	GACAACCTGCTCTGAAGTCGA	57	
	Q-MCL1-F	270-290	TTGCAAGAAGACACACGCCTT	57	205
	Q-MCL1-R	454-474	GGGCAGATCCTTGGCATAGTT	57	
	Q-Pr1H-F	602-622	CCGATCATGTGGACTTTGAGG	56	173
	Q-Pr1H-R	754-774	ATTGGAGCGGAGAACCTTGAC	58	
	Q-Pr2-F	494-514	AGCTGCAAAAGGTGACGGTTC	59	173
	Q-Pr2-R	646-666	CAAGACATCGCCCTGGACAAT	58	
	Q-Mad1-F	278-298	GTGATATCCAGGGCCGCACTT	60	187
	Q- Mad1-R	444-464	CCGTCAGGCATATCGTAGTGG	58	
	Q-lipase2-F	816-836	AGATAACTGCATGAACGCGGC	59	128
	Q-lipase2-R	923-943	GATCAACAATGTCCGGCTGCT	58	
	Q-AbaA-F	1216-1236	CTCACGGGCTTTGTCGAGGTT	62	227
	Q- AbaA -R	1422-1442	ACTGCAGGGAAAGGAACGTGA	61	
	Q-BrlA-F	790-810	CGCCGAGCCATGTGCAAATGT	64	183
	Q- BrlA -R	952-972	GGCGTGTAGCTTGCGATGATT	62	
	Q-WetA-F	523-542	TCTCCAAAACCTCCCAACAC	57	106
	Q- WetA -R	609-628	CTGCTTTCCTATTTCGTCGC	56	

3

Chapter III

Complete genome sequence of a novel iflavirus from wheat sawfly (*Dolerus tritici*)

Abstract

Little is known about the insect viruses in wheat sawfly, *Dolerus tritici*, which is an important agricultural insect feeding on wheat leaves. Here, we identified a novel single positive-strand RNA virus from the larvae of wheat sawfly collected in northern China through RNA sequencing, and then determined its complete genome sequence by rapid amplification of cDNA ends. The complete genome consists of 9,594 bp in size including a poly A tail at 3′ terminus, which is predicted to encode a 326.3 kD polyprotein. The phylogenetic tree analysis of deduced polyprotein revealed that this RNA virus was clustered in the clade with deformed wing virus in genus *Iflavirus*, family *Iflaviridae*. The full genome of this RNA virus shows 42.0-50.0% sequence identities with other iflaviruses. Sequence identity at the amino acid level of coat proteins shows this RNA virus has the highest identity with slow bee paralysis virus at 33.6%, with no more than 90% suggesting that it is a new member in the genus *Iflavirus*. Thus, we tentatively designated as "Dolerus tritici iflavirus 1" (DtIV1). According to our knowledge, this is the first report of the insect virus in wheat sawfly.

3.1 Introduction

Wheat sawfly (*Dolerus tritici* Chu) is an important global pest of common wheat (*Triticum aestivum L.*). The larvae of wheat sawfly damage wheat leaves, causing a severe decline in wheat production (Chu, 1949). With the development of high-throughput sequencing technology, more insect viruses have been found in agricultural insects (Qi et al., 2023; Shi et al., 2016; Wu et al.,2020). Insects from the order Hymenoptera account for the highest proportion with nearly 300 viruses, and most of them come from beneficial insects, such as bees (Qi et al., 2023; Shi et al., 2016). As pests, the wheat sawflies have rarely been found to harbor any virus until now. In the present study, we isolated and identified a novel putative iflavirus from wheat sawfly, tentatively named Dolerus tritici iflavirus 1 (DtIV1).

The family *Iflaviridae* contains a single genus, *Iflavirus*. According to the International Committee on Taxonomy of Viruses (ICTV), the genus *Iflavirus* includes 16 approved species (Valles et al., 2017). All iflaviruses are identified from arthropod species (Wu et al., 2019; Wang et al., 2019; Wu et al., 2018). Viruses in the genus *Iflavirus* possess a positive-strand RNA genome with 9-11 kilobases (kb), with non-enveloped virions (Valles et al., 2017). Iflavirus genomes usually have an open reading frame (ORF) that encodes a polyprotein followed by proteolytic cleavage into functional structural proteins and non-structural proteins (van Oers, 2010). A genome-linked viral protein (VPg) participating in viral life cycle is covalently attached to 5′-end of the genome, and the 3′-end of the genome is polyadenylated (Hashimoto et al., 1986). Some of them are associated with symptomless infections (Murakami et al., 2013; Parry et al., 2020). While some can be harmful to host insects, such as deformed wing virus, which causes characteristic wing deformity and premature mortality of honeybees (Lanzi et al., 2006).

3.2 Materials and Methods

During a field investigation in March 2024, wheat sawflies were found feeding on wheat plants in Yuanyang, Henan province, China. We randomly pooled three larvae for RNA sequencing (RNA-seq). Total RNA was extracted from the sample using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and then a library was constructed for RNA-seq by the Illumina HiSeq X Ten platform. After removal of adaptor sequences, the CLC Genomics Workbench 9.5 was used for de novo assembly of RNA-seq data. The assembled contigs were subsequently screened against the NCBI databases using BLASTn and BLASTx searches with default options (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three viral contigs were derived from an unidentified virus that was related to members of the family *Iflaviridae*.

To obtain the full genome sequence of DtIV1, we used SMARTer RACE 5′/3′ Kit (Takara Bio, USA) for performing both 5′- and 3′-rapid amplification of cDNA ends (RACE) to get the terminal sequences. Subsequently, RT-PCR was carried out using

the total RNA extracted from the single larva and primers which was designed using the software Oligo 7.60 (OLIGO, Colorado Springs, CO) (Table S3-1). Three fragments of DtIV1 with overlap sequences were amplified using RT-PCR. Subsequently, we used SMARTer RACE 5′/3′ Kit (Takara Bio, USA) for performing both 5′- and 3'-rapid amplification of cDNA ends (RACE) to obtain the terminal sequences. Then, sequences were assembled using the DNAMAN (v6) program (Lynnon Biosoft, San Ramon, CA). Stem-loop structures were predicted in the 5′ UTR by a secondary structure prediction RNAfold Webserver (http://rna.tbi.univie.ac.at/). Sequence identities were computed using LALIGN program of EMBL's EBI European Bioinformatics Institute (EMBL-EBI) with the default settings (EMBOSS Needle < EMBL-EBI). The Conserved Domain Database (CDD) at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd) and InterProScan (https://www.ebi.ac.uk/interpro/) were used to analysis of the conserved domains of DtIV1 and showed a typical characteristic of genus *Iflavirus* (Valles et al., 2017).

3.3 Results

To obtain the full genome sequence of DtIV1, RT-PCR and RACE were carried out using the total RNA extracted from the single larva and specific primers. We amplified respective overlapping the fragments of 3071 bp, 2893 bp and 3752 bp as well as 562 bp at 5′ terminal and 1433 bp at 3′ terminal with overlap sequences with overlap sequences (Figure 3-1).

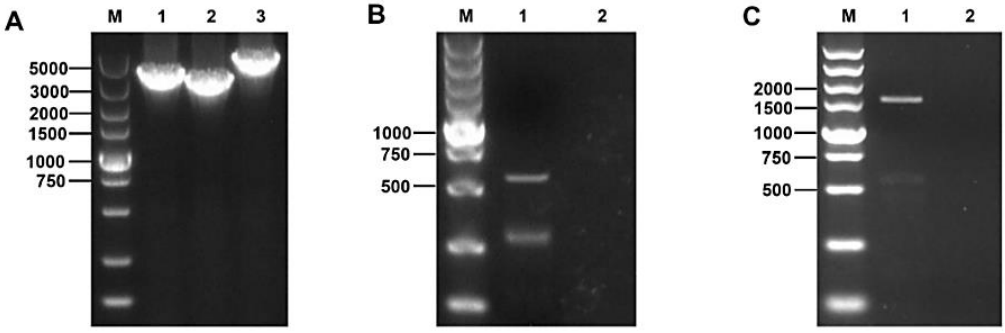


Figure 3-1 PCR determination of DtIV1 in wheat sawfly. A, three fragments of DtIV1 with overlap sequences are amplified from a single larva using RT-PCR and overlap primers (Table S3-1). Lane 1: the first amplicon will be 3100 bp; Lane 2: the second amplicon will be 2900 bp, Lane 3: the third amplicon will be 3800 bp. M: Marker. B, Amplification of the 5' terminal sequence of DtIV1 by RACE assay. Lane 1: 5' product will be 600 bp; Lane 2: negative control. C, Amplification of the 3' terminal sequence of DtIV1 by RACE assay. Lane 1: 3' product will be 1600 bp; Lane 2: negative control.

The whole viral genome was 9,594 nucleotides (nt) in length including the polyA tail (GenBank accession PQ323359), with the 5′ untranslated region (UTR) of 807 nt and 3′ UTR of 180 nt. When the clean reads were re-aligned back to the DtIV1 genome, we observed the DtIV1 genome had nearly full coverage by the sequencing reads. 985,214 reads were mapped to the DtIV1 genome, with an average sequencing depth of 14,831x (Figure 3-2). The genome was predicted to have a single ORF (808 nt-9414 nt) using Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/) that encoded a 326.3 kDa polyprotein comprising 2868 aa. Compared to the deduced amino acid (aa) sequences obtained from RNA-seq, it revealed seven aa were varied. Totally 18 ATG triplets existed in 5′ UTR and complicated stem-loop structures were predicted in the 5′ UTR. The identities of deduced amino acid sequence of coat protein (CP) with those of species in the order Picornavirales were 6.6% to 33.6%, which was much lower than the species demarcation thresholds in the genus *Iflavirus* (Valles et al., 2017) (Table S3-2).

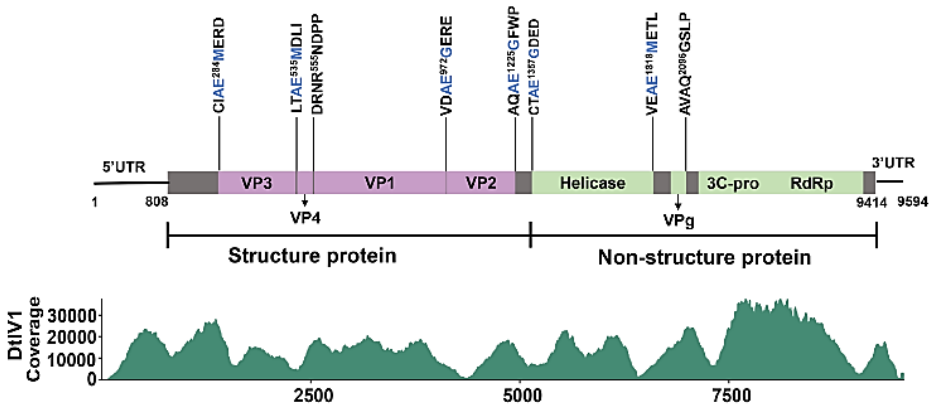


Figure 3-2 Genome organization schematic of DtIV1 and the coverage of transcriptome reads. Vertical lines indicate conserved 3C-protease cleavage sites, which are inferred by amino acid alignment and conserved protease consensus sites (conserved amino acids in blue font; variable amino acids in black font).

The deduced polyprotein of DtIV1 was predicted to be cleaved into mature and functional proteins by the putative autocatalytic and 3C cysteine protease cleavage sites according to the characteristics of iflaviruses (Parry et al., 2020; Lanzi et al., 2006; Geng et al., 2014). DtIV1 showed a typical characteristic of genus *Iflavirus* (Valles et al., 2017). At the N-terminus of polyprotein, two rhinovirus-like (Rhv) structural protein domains: Rhv1 domain (accession no. IPR033703) and Rhv2 domain (accession no. IPR033703), and a cricket paralysis virus coat domain (CRPV) (accession no. IPR014872) were identified. The three conserved domains at residues 358-524, 632-793, and 995-1207 corresponded to VP3, VP1 and VP2 of DtIV1, respectively. The predicted coat proteins of DtIV1 contained four conserved

sequences in structural protein region, as described in picorna-like viruses (Liljas et al., 2002; Chen et al., 2023) (Figure 3-3). The conserved VP4 cleavage site (Nx/DxP) was also found at aa 535 position, at the end of the C-terminus of the VP3, suggesting that a small protein VP4 was positioned right between VP3 and VP1 (Jia et al., 2021).

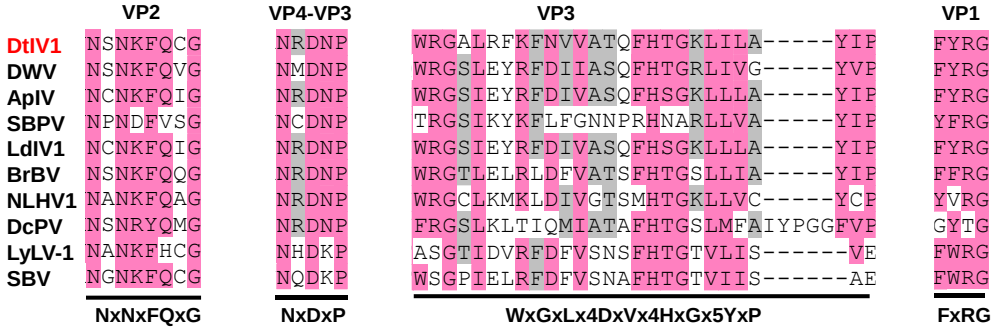


Figure 3-3 Alignment of VP1-VP4 proteins of DtIV1 among the iflaviruses using the deduced amino acids.

At the C-terminus of polyprotein, non-structural proteins appeared in the following order: RNA helicase protein (domain accession no. IPR014759) at residues 1509-1676, 3C cysteine protease protein (domain accession no. IPR009003) at residues 2125-2327, and RNA-dependent RNA polymerase (domain accession no. IPR001205) at residues 2386-2839. Three conserved helicase superfamily domains were identified in DtIV1 that were usually associated with NTP-binding, which were Hel-A (Gx2GxGKS), Hel-B (Qx5DD), and Hel-C (KKx4Px5NSN). While the motif Hel-C of DtIV1 was slightly different from the consensus sequence KGx4Sx5STN [16] (Figure 3-4A). The 3C cysteine protease domains of picornaviruses, including a cysteine-protease motif (GxCG) and a substrate binding motif (GxHx2G) were found in DtIV1(Koonin and Dolja, 1993; Gorbalenya et al., 1989) (Figure 3-3B). All eight recognized RNA-dependent RNA polymerase (RdRp) domains featured in picorna-like family were also found in DtIV1 (Figure 3-4C). The core domains (IV, V, and VI) thought to be involved in catalysis and NTP binding of virus life (Koonin and Dolja, 1993; Baker and Schroeder, 2008; et al., Koonin 1991). Besides that, a highly conserved TSxGxP domain, similar to that found in some picornaviruses, was located immediately prior to the RdRp domain I (Lanzi et al., 2006; de Miranda et al., 2010). Overall, DtIV1 possessed characteristic domains of structural proteins, RNA helicase, 3C cysteine protease, and RdRp of iflaviruses. The identity values for the genomic nucleotide sequence and amino acid sequence of the polyprotein and CP of DtIV1 with some viruses in the order Picornavirales were calculated using EMBOSS Needle. The identities were only 38.9-50.0% for total genome, 9.7-32.6% for polyprotein, and 6.6-33.6% for CP (Table S3-2).

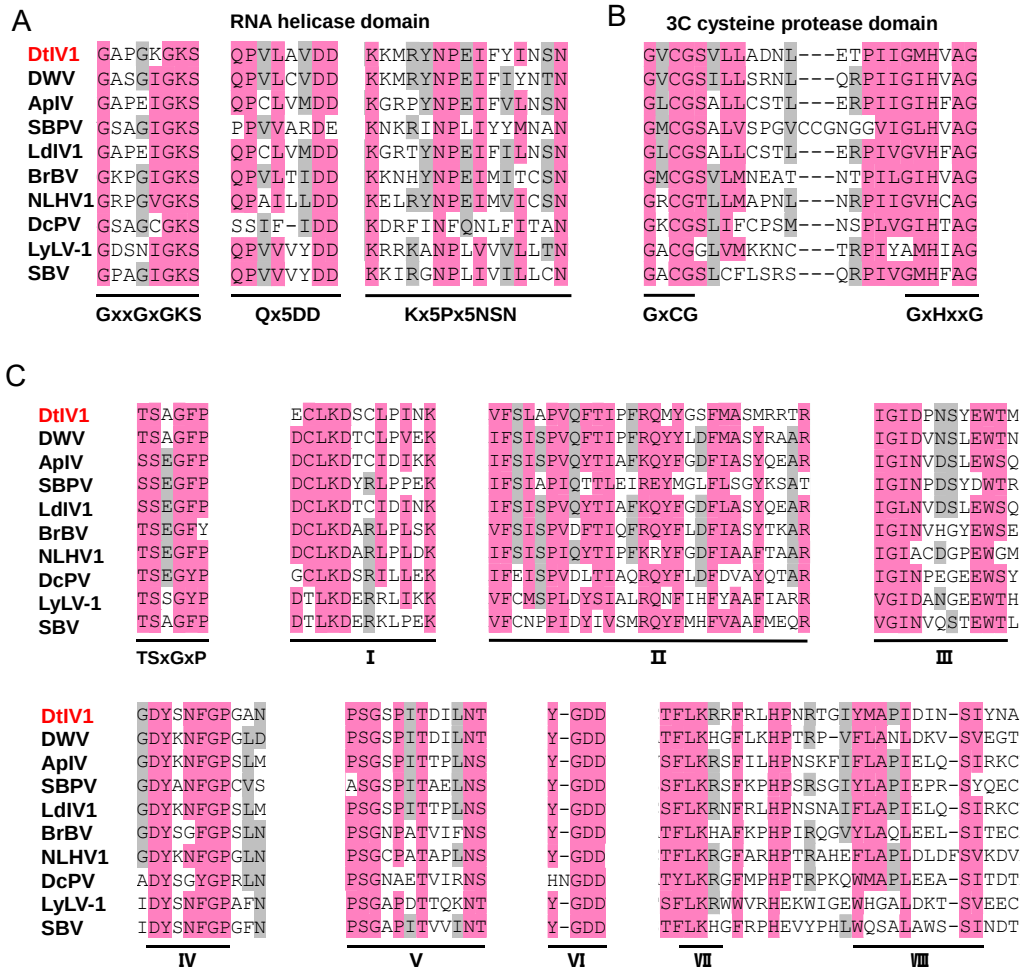


Figure 3-4 Alignment of RNA helicase (A) and 3C-protease proteins(B) and RdRp (C) of DtIV1 among the iflaviruses using the deduced amino acids. The amino acids shaded in pink indicate the conservative and similar residues. Viral information is given in Table S3-2.

Phylogenetic trees based on the deduced amino acid sequences of polyprotein (Figure 3-5A) and RdRp (Figure 3-5B) were constructed using the neighbor-joining (NJ) method with 1,000 bootstrap in MEGA 11. Foot-and-mouth disease virus A and human poliovirus 1 of picorna-like viruses belonging to family *Picornaviridae*, and Heterosigma akashiwo RNA virus of family *Marnaviridae* were used as outgroups. In both trees, the sequences of DtIV1 that we identified were clustered in a clade of genus *Iflavirus*, with the closest relationship to deformed wing virus.

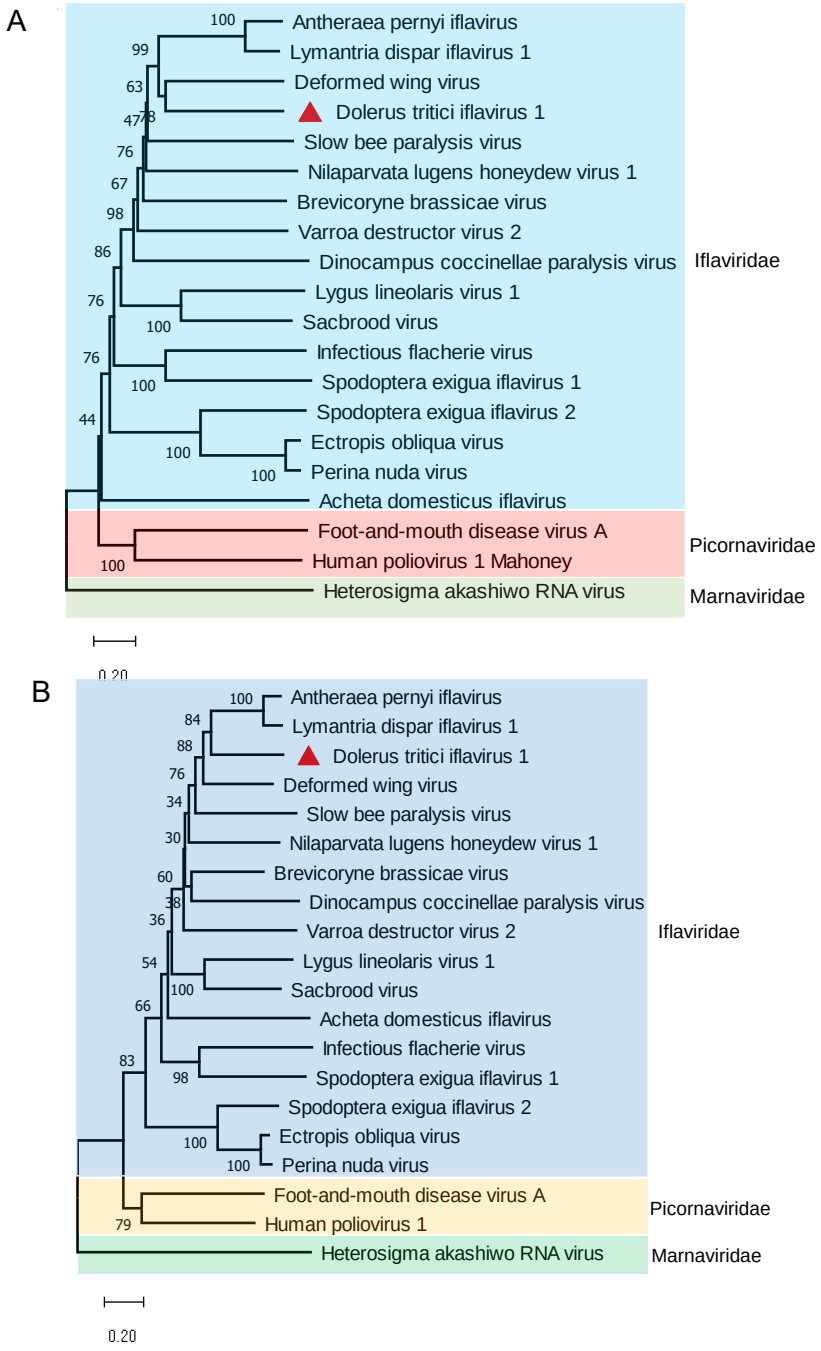


Figure 3-5 Phylogenetic analysis among the species in genus *Iflavirus* with other picorna-like viruses using deduced amino acids of polyprotein(A) and RdRp (B).

3.4 Conclusions and Discussions

The identification and genomic characterization of a new virus naturally infecting wheat sawfly (*D. tritici*) was reported. Based on its overall genome sequence, structure, and phylogenetic relationships, DtIV1 can be regarded as a member of the genus *Iflavirus*, family *Iflaviridae*.

In both phylogenetic trees, DtIV1 shows the closest evolutionary relationship with Deformed wing virus (DWV), followed by *Antheraea pernyi* iflavirus (ApIV), Lymantria dispar iflavirus 1 (LdIV1) and Slow bee paralysis virus (SBPV). Wing deformities in honeybees (*Apis mellifera* L.) have long been linked to a virus called DWV, which is spread by the ectoparasitic mite *Varroa destructor* Anderson & Trueman (2000). The virus is named after the most noticeable deformity it causes in honeybee pupae, which is shrunken and malformed wings. Research using various methods has shown a nearly 100% correlation between the wing deformities and high virus titters. In the presence of the Varroa mite, the virus appears to amplify, possibly because it can replicate within the mite. The larvae of the Chinese oak silkmoth (*Antheraea pernyi* Guérin-Méneville) are frequently infected by virus ApIV, causing Antheraea pernyi Vomit Disease (AVD). Infected larvae become lethargic, and their pygidia turn black and white liquid is vomited from the midgut. As the disease progresses, the larvae stop eating, resulting in shortened bodies that with the death of the larvae turn dark as decay sets in. ApIV could been detected in AVD-affected larvae, and injection of purified ApIV leads to AVD symptoms. SBPV is one of several honeybee (*Apis mellifera* Linnaeus) viruses linked to high mortality of colonies infested with the ectoparasitic mite *V. destructor*. SBPV induces paralysis of the front two pairs of legs about 10 days after the injection of the virus into the abdomen of adult bees. The replication of the virus is primarily accumulated in the head, the hypopharyngeal, mandibular and salivary glands, the fat body, crop, and forelegs. SBPV infects both honey bees and bumble bees. It is often found at low (<5%) prevalence in honey bee populations. It is, though, found widely in bumble bees (*Bombus* spp.), where it can reach high prevalence (35% in *B. hortorum*), presumably transmitted directly by the faecal-oralroute. The spongy moth virus LdIV1, originally identified from a *Lymantria dispar* cell line. Although high genome levels of LdIV1 were not associated with any obvious disease symptoms in spongy moth, the severity of cytopathic effect correlated with viral titter with disrupted, enlarged, misshapen or vacuolated in cell line derived from the spongy moth cells observed after 6 days post-treatment. The viruses closely related to DtIV1 almost caused symptomatic infections in their hosts. In this study, we found that there are no macroscopic symptoms in the wheat sawfly larvae infected with DtIV1, but we did not do more in-depth research.

To our knowledge, DtIV1 is the first novel insect virus in wheat sawfly. Further research is needed to investigate the origin and host range of the virus and to assess the possible impact of DtIV1 on its host insect in the wheat field ecosystem.

Supplementary information

Table S3-1 Primers used in this study to determine the sequence of DtIV1.

Function	Primer name	Sequence (5' to 3')	Production (bp)
Viral fragments	1-F	GGTTATAGCCTCTTTATTCG	3071
	1-R	GTAATCCTATTGCTGGTTGT	
	2-F	GACGTTATGCTGGAGATTTT	2893
	2-R	GAAATAACACTCTGCGCTAT	
	3-F	TGTGTCTGCAGAAGCTTTAC	3752
	3-R	GTTTCACCTTATATCCCACC	
RACE-PCR	5'RACE-NGSP1	GATTACGCCAAGCTTCAGGCTCATCATC ATTAGTCGCAG	562
	5'RACE-GSP1	GATTACGCCAAGCTTCATATTCCAATCCA CTCAGGCGAC	
	3'RACE-NGSP1	GATTACGCCAAGCTTGTGAAGAAGTGG CTATTGTGGGTG	1433
	3'RACE-GSP1	GATTACGCCAAGCTTCCAGTTACAACAG AACCAGCTCC	

Table S3-2 Nucleotide and amino acid sequences identities between Dolerus tritici iflavirus 1 and other viruses in the order Picornavirales

Virus	Accession No. (Genome)	Nucleotide Identity (%)	Amino Acid Identity (%)	
		Full Genome	Polyprotein	CP
Acheta domesticus iflavirus (AdDNV)	NC_078647.1	42.0	17.9	14.3
Antheraea pernyi iflavirus (ApIV)	KF751885.1	50.0	31.6	30.8
Brevicoryne brassicae virus (BrBV)	EF517277.1	47.7	27.5	26.3
Deformed wing virus (DWV)	MF770715.1	50.0	32.6	31.7
Dinocampus coccinellae paralysis virus (DcPV)	KF843822.1	46.3	23.2	22.8
Ectropis obliqua virus (EoV)	AY365064.1	43.2	19.4	18.1
Infectious flacherie virus (IFV)	HM569717.1	42.8	18.7	18.1
Lygus lineolaris virus 1 (LyLV-1)	JF720348.1	43.0	19.5	17.4
Lymantria dispar iflavirus 1 (LdIV1)	OP895019.1	49.9	31.6	31.0
Nilaparvata lugens honeydew virus 1 (NLHV-1)	AB766259.1	45.6	25.9	26.2
Perina nuda virus (PnPV)	AF323747.1	43.8	19.2	17.6
Sacbrood virus (SBV)	MN082651.1	44.7	21.3	18.7
Slow bee paralysis virus (SBPV)	NC_014137.1	46.6	27.9	33.6
Spodoptera exigua iflavirus 1 (SeIV-1)	JN091707.1	43.4	17.9	16.5
Spodoptera exigua iflavirus 2 (SeIV-2)	KJ186788.1	42.7	20.1	19.9
Varroa destructor virus 2 (VDV-2)	KX578271.1	45.8	22.2	19.4
Cricket paralysis virus (CrPV)	AF218039.1	43.2	19.1	14.3
Himetobi P virus (HiPV)	KM270559.1	42.6	17.9	13.5
Solenopsis invicta virus 3 (SINV-3)	FJ528584.1	45.8	14.7	6.6

Heterosigma akashiwo RNA virus (HaRNAV)	AY337486.1	42.0	9.7	12.3
Human poliovirus 1 (HPV-1)	V01149.1	40.4	16.8	14.3
Foot-and-mouth disease virus A (FMDV)	AY593791.1	38.9	17.6	14.0

4

Chapter IV

Conclusions and perspectives

4.1 Conclusions and discussions

The study of mycoviruses and insect viruses lag far behind plant, animal and bacterial viruses. The discovery of an increasing number of new mycoviruses and insect viruses has expanded our understanding of viruses. In this thesis, we investigated two insect models, namely small brown planthopper and wheat sawfly, using RNA sequencing (RNA-seq). From samples of small brown planthopper (SBPH, *L. striatellus*), we identified a new virus, MfPV1. It is a mycovirus infecting *Metarhizium flavoviride*. Transfection of commercial strains of *M. anisopliae* and *M. pingshaense* with MfPV1 produced greater conidia and enhanced virulence of the fungi to diamondback moth (*P. xylostella*) and fall armyworm (*S. frugiperda*), two important lepidopteran pests. The biological phenomenon has been verified by transcriptome analysis and qRT-PCR, which showed that MfPV1-infected fungi up-regulated pathogenesis-related genes and conidia-related genes. This discovery opens a possibility for enhancing the insecticidal activity of entomopathogenic fungi, thereby facilitating the production and application of microbial insecticides. From the samples of wheat sawfly, we identified a new virus belonging to family *Iflaviridae*. We determined the full viral genome by performing RACE and RT-PCR using the total RNA extracted from the single larva and primers designed from RNA-seq data. We confirmed the new iflavirus by analyzing the genome characteristic and phylogenetic relationship. Our research enriched the variety of viruses in wheat sawfly.

4.2 Methodology and mechanism research in mycovirus

Viruses cause significant economic losses worldwide. Virology research involves a series of methodical steps to understand viruses, their life cycle, pathogenesis, and potential ways to combat them. There is no direct method to control viruses, except using resistant cultivars. No curative treatments are available once infection occurs. So, early and efficient diagnosis is a key strategy for managing viral disease (Adams et al., 2009). Both of our two virus studies are based on high-throughput sequencing. RNA sequencing (RNA-seq) is a highly versatile technique that serves multiple purposes in molecular biology and virology. By sequencing the entire transcriptome, RNA-seq enables researchers to detect viral RNA sequences, including those from unknown or unexpected viruses, making it a crucial tool for virus discovery and surveillance. However, samples for RNA sequencing cannot be taken for granted as host for new viruses. Initially, we discovered plenty of planthoppers had died in insect-proof cages. We selected some living adult SBPHs for RNA sequencing in hopes of discovering potential insect viruses that may be causing its death. After confirming the genome sequence and identifying its taxonomy, we found that the virus was clustered with viruses that are all infecting fungi rather than insects. In addition, we observed that death SBPHs would be covered with green mold a few days after their

death. Finally, we isolated an irregular fungus from dead SBPHs. RT-PCR detection, virus particle purification and dsRNA extraction from the fungal strain all confirmed that the host of the novel virus is *M. flavoviride*. The reason why a mycovirus could identify from SBPH is probably because the samples selected for RNA sequencing were already under the early stage of infection, which at this level is not enough to cause planthopper mortality.

In addition to virus identification, RNA-seq provides precise quantitative data on gene expression levels across different tissues, developmental stages, or environmental conditions. This allows researchers to measure changes in gene activity, identify differentially expressed genes, and better understand how biological processes are regulated at the molecular level. Furthermore, RNA-seq can detect alternative splicing events, RNA editing, and other post-transcriptional modifications, offering a more comprehensive view of gene regulation. In the mycovirus study, compared to the virus-free strain, the virus-infected fungal strain (viruliferous strain) produced more spores and showed greater virulence against lepidopteran insects. This virulence bioassay has been further confirmed by subsequent transcriptomic data. In the transcriptomic experiment, viruliferous and virus-free *M. anisopliae* strains were cultured in cicada-amended medium (PDB supplemented with 0.2% w/v cicada slough) for 3 d and then total RNA from the cultures were used for RNA-seq. The cicada slough in PDB medium can induce the expression of virulence genes in *M. anisopliae* because it serves as a substitute for insect cuticle to stimulate the expression of pathogenicity genes in entomopathogenic fungi (St Leger et al, 1994). While transcriptome data analysis showed that genes related to sporulation were not regulated, because fungi grew vegetatively in the liquid medium, and air is a necessary factor to stimulate sporulation according to the article by Adams et al. (1998): "*A. nidulans* will also grow vegetatively in liquid submerged culture, with few exceptions (see below) such a culture is aconidial. Typically, hyphae must be exposed to an air interface to stimulate conidiophore production." Park & Yu (2012) also stated, "In response to internal (e.g. genetic competence) and/or external cues (e.g. exposure to air or nutrient deficiency), hyphal cells cease normal growth and initiate conidiophore formation." Conidiation mechanisms have been extensively characterized in many filamentous fungi, especially in the model organisms *Aspergillus nidulans* (Eidam) W. Gams and *Neurospora crassa* Shear & Butler. Three genes, *BrlA*, *AbaA*, and *WetA*, have been verified to define a central regulatory pathway that coordinates conidiation-specific gene expression and determines the order of gene activation during conidiophore formation and spore maturation, which is conserved in many filamentous (Boylan et al., 1987). Subsequently, we studied the expression of *BrlA*, *AbaA*, and *WetA* in MfPV1-tranfected *M. anisopliae* that was cultured on PDA rather than PDB at 28℃ for 12 d (when sporulation starts). They were significantly upregulated at 12 dpi as we expected.

In our next study, the key subject is to research how mycovirus MfPV1 upregulates pathogenic genes. After analyzing the genome of MfPV1, it was found to consist of

two dsRNA segments, which encode the viral coat protein (CP) and the RNA-dependent RNA polymerase (RdRp). To further elucidate the viral components affecting the biological characteristics of *M. anisopliae*, the CP and RdRp were cloned into the fungal expression vector pDL2. pDL2-CP and pDL2-RdRp were separately or co-transformed into virus-free *M. anisopliae* strain by $CaCl_2$-PEG-mediated method. Incubating them at 27°C, the phenotype of colony was observed, and the conidiation was assessed after around 12 days post inoculation. The corresponding virus-free strains were used as control. Subsequently, conidia were collected to perform bioassays on pests such as the fall armyworm and the diamondback moth to determine the efficacy of pest control for different transformed strains. By analyzing the impact of different viral components on biological characteristics, we will identify the key viral components responsible for regulating the biological traits of the fungus.

After confirming the key composition of the virus, we need to determine the components in the host fungi that interact with the virus. Total RNA extracts from *M. anisopliae*, and a yeast two-hybrid cDNA library constructes using the pGADT7 vector. The viral CP and RdRp are cloned into the bait vector pGBKT7. Yeast two-hybrid screening is then performed using pGBKT7-CP and pGBKT7-RdRp as baits to identify proteins in *M. anisopliae* that interact with the viral CP and RdRp. The interactions are further verified using the swapped vectors. By combining the previously identified key viral components and transcriptomic data, the key proteins in *M. anisopliae* that interact with the virus are identified. The functions of these key proteins were further elucidated using overexpression or gene knockout methods, allowing for the identification of the key pathways and molecular mechanisms through which the virus promotes *M. anisopliae* growth, conidiation, and enhances its insecticidal activity.

4.3 Mycovirus transmission and its potential ecological applications

Mycoviruses infect fungi and replicate in fungal cells that are widespread in all major fungal groups. Some mycoviruses are asymptomatic in certain environmental conditions or specific virus–fungus interactions, but they sometimes will significantly affect fungi fitness once the surrounding environment changes, such as by new mycovirus infections, competitor appearance, ecological niches, and host range conversion. In addition, external environmental factors help mycovirus transmission between fungal species and between kingdoms. As a part of the virus world, mycoviruses may play an ecologically important role in nature.

Mycoviruses are usually transmitted horizontally via hyphal anastomosis and vertically via sexual/asexual spores. A few mycoviruses have potential transmission vectors. Current research supports the hypothesis that cross-species transmission of mycoviruses occurs frequently in nature, and the host range of mycoviruses is wider than previously recognized. But complex vegetative incompatibility (VIC) results in

fungal cell death to restrict the horizontal transmission of most mycoviruses. VIC is an innate immune mechanism to limit detrimental infections. We did experiments using dual cultures of virus-free and viruliferous strains of *M. anisopliae*, and virus-free and viruliferous strains of *M. pingshaense* on PDA for 20 days at 28℃. The results showed that there was a clear boundary between the cultures of virus-free and viruliferous strains after 12 or 20 day postinoculation (dpi). The hyphae at the junction were observed under a microscope and no fused hyphal structures with locked-like symphysis were observed. Our experiment finds no evidence that hyphal anastomosis could occur between the virus-free and viruliferous strains. Furthermore, viral RNA was not detected in the virus-free colony at 20 dpi. Therefore, it is transmitted horizontally in *M. anisopliae* and *M. pingshaense*, being restricted by incompatibility, cannot directly infect the hyphae of *Metarhizium* spp., even in the same species. This may be strong evidence for the spread limitation of MfPV1 in nature as hyphal anastomosis carries the risk of disrupting the original species in the ecosystem and causing human-induced interference to the environment.

Based on our previous experiments, we used several treatments to eliminate viruses from *M. flavoviride*, including single-conidium subculture. However, the detection result showed that the mycovirus was presented in all generations. In addition, after subculturing these strains for at least 10 generations, the morphology of the colonies remained consistent with the previous generation, indicating the stable presence of MfPV1 in both original strains and commercial *M. anisopliae* and *M. pingshaense* fungal strains. The 100% transmission rate by spore ensures the further spread of infected strains in the field.

Until now, only SsHADV1 has been experimentally confirmed to be transmitted by a mycophagous insect, *Lycoriella ingenua* (Dufour). Researchers found virus-infected isolates produced less repellent volatile substances to attract adults of *L. ingenua*. Virus acquired by larvae feeding on colonies of a virus-infected strain of *S. sclerotiorum* was replicated and retained in larvae, pupae, adults, and eggs. Larvae could transmit the virus once they acquired. Offspring larvae hatched from viruliferous eggs were virus carriers and could also successfully transmit the virus (Liu et al., 2016). For MfPV1, whether there is a vector for transmitting the virus remains to be studied. The mycovirus was also detected in the live SBPHs, in agreement with the RNA-seq result, because the samples for RNA sequencing are living adult SBPHs. This may be because SBPH were already infected with the fungus while they were alive. Due to the high lethality of the fungus to the SBPH that conferred by MfPV1, it is unlikely to be the vector of the virus. Until now, there is no evidence to suggest any transmission vectors for MfPV1. Then, we tested the rice leaves that were fed to the virus-positive SBPHs. We did not detect the virus in the rice plants. The mycovirus MfPV1 does not infect plants in nature, it is safe for plants.

The entomopathogenic members in genus *Metarhizium* have been used to control agricultural, grassland, and medical pests around the world. Mycoviruses have the potential to serve as synergistic agents, amplifying the biocontrol efficacy of

entomopathogenic fungi. In order to better apply this highly virulent strain in the field, it must comply with the relevant regulations in different regions. As WTO stated in May 2014: Genetically modified organisms (GMOs) can be defined as organisms (i.e. plants, animals or microorganisms) in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination. It allows selected individual genes to be transferred from one organism into another, also between nonrelated species. MfPV1 is a naturally virus, not a man-made one. In the experiment of transferring virus particles into fungi, no foreign genes were introduced into the host fungal genome. The virus is never inserted into the fungal genome. We transferred virus particles rather than viral dsRNA to host fungi. Moreover, the virus transfected into the fungi cell and replicated independently in it. Therefore, the virus-transferred strains are not belonging to GMOs, making them safe for global promotion.

4.4 Fermentation conditions and different formulations development of *Metarhizium* strains for practical applications

For the next research, we will more focus on the transfected *M. anisopliae*, because it produced more spores that have been measured and verified by experiments. For another reason, *M. anisopliae* is a more commonly used biological insecticide fungus, especially suitable for controlling soil pests, forest pests, and other agricultural pests. in the world for biocontrol. We will employ a liquid-solid dual-phase fermentation method. Firstly, the *M. anisopliae* (MfPV1) strain is activated and cultured on slant culture medium at 27°C. When the slants are fully covered with spores, they are washed by 5 mL of 0.05% Tween-80 and collected them up. Then, they are transferred to a liquid shake flask for further culture until the fungus forms white and viscous mycelium. Subsequently, the liquid-cultured *M. anisopliae* is inoculated onto solid medium for sporulating. The optimal fermentation conditions, such as temperature, humidity, pH, and culture medium need to be screened. During the solid fermentation, humidity is a key factor. Typically, the requirement of air humidity is between 85% and 95% to ensure the fungus growth. The composition of the culture medium is also critical. Optimizing the medium composition by adding an appropriate amount of trace element mix to a rice-based medium, the growth and spore production of *M. anisopliae* will be improved. The fermentation efficiency and spore yield of *M. anisopliae* can be enhanced, providing high-quality fungus spores for subsequent formulations.

Hypovirulence-associated mycoviruses have the ability to diminish or suppress the virulence of pathogenic fungi. When these mycoviruses are introduced into the natural environment, they can trigger a reduction in virulence across the entire fungal population, leading to effective management of fungal diseases. One of the biological control strategies with mycoviruses for managing fungal diseases is specifically

targeting phytopathogenic fungi with hypovirulence-associated mycoviruses. Chestnut blight in Europe has been successfully controlled by directly targeting *C. parasitica* by CHV1. While biological control with CHV1 has failed almost entirely in North America. *C. parasitica* in north America with more complex IC groups restrict horizontal transmission of CHV1 (Milgroom and Cortesi, 2004). The limitation of (VIC) in filamentous fungi is a significant obstacle to successfully controlling fungal diseases. VIC is an innate immune mechanism to limit detrimental infections and is usually governed by serial *vic* genes. To overcome the limitation of fungal VIC on the transmission of CHV1 and enhance the effectiveness of controlling chestnut blight, researchers created a super virus donor by systematically disrupting the multilocus *vic* genes of *C. parasitica*. They then introduced these genetically engineered strains into chestnut forests and observed that the super virus donor allowed the virus to spread to various VIC populations, whereas the virus-infected wild type could not (Zhang and Nuss).

Another biological control strategies with hypovirulence-associated mycoviruses is plant vaccine like attenuated vaccines or live-avirulent vaccines used in mammals and humans. SsHADV1, initially discovered in *S. sclerotiorum* strain DT-8, confers hypovirulence on *Sclerotinia* spp. and exhibits strong infectivity. Although strain DT-8 has lost its ability to cause disease in rapeseed, its hyphae can still penetrate plant cell walls and grow inside the plant as endophytes, acquiring nutrients through a unique structure. This suggests that strain DT-8 colonize rapeseed using an adaptive strategy without causing harm but can activate plant defense and hormone signaling genes, thereby promoting plant growth and boosting immunity. Therefore, SsHADV1-mediated plant vaccine controls diseases through enhancing the broad-spectrum resistance of treated plants (Fu et al., 2024). A vaccine composed of hyphae of the strain infected by mycovirus can be applied by inoculating seeds. During seed germination, the hyphae of strain DT-8 on the seed surface grow and colonize the seedlings. The seed coating method simplifies the application of the mycovirus-mediated plant vaccine, requiring no additional agricultural practices, just the direct sowing of the coated seeds. Another method is to introduce SsHADV1 into field populations of *S. sclerotiorum*. This vaccine can be applied by spraying the aerial parts of plants via an unmanned aerial vehicle. SsHADV1 can spread to noninoculated fields by mushroom flies (*L. ingenua*) originating from inoculated fields.

We have filed two patents for MfPV1-carrying strains. Next, we will prepare different formulations of *M. anisopliae*-MfPV1 strain insecticide for bioassay in the field, aiming to identify the most effective and stable formulation. Initially, formulations such as wettable powder, dispersible oil suspension, and granules are selected for processing. Firstly, the spores of *M. anisopliae* are mixed with fillers (such as diatomaceous earth, starch, talcum powder, etc.) and additives like UV radiation protectants, and then processed into powder. On this basis, a suitable number of surfactants is added to improve their dispersibility and suspension in water. The wettable powder is dissolved in a 0.05% Tween-80 stock solution and then diluted to

the appropriate concentration for spraying. We will also prepare dispersible oil suspension. The suspending agent and emulsifier are mixed and rapidly stirred until dissolved. The spores of *M. anisopliae* are then mixed with the suspension, and co-solvents are used for pretreatment to ensure they are uniformly dispersed in the oil suspension. Water or other additives can be added as needed to adjust the viscosity and stability of the suspension. Besides the above two formulations, we will also prepare granules formulation. The spore of *M. anisopliae*, synergist (such as talcum powder), and corn oil are mixed in a certain ratio. Salad oil or other vegetable oils are added to the mixture. Then, materials are fully blended and formed into granules through stirring and kneading. Finally, the granules are dried or treated to adjust their particle size and shape.

4.5 Field study of DtIV1 and its potential application in biological control

Wheat sawfly (*D. tritici* Chu) is an important global pest of common wheat (*T. aestivum* L.). It has one generation per year in the northern China. The third instar of wheat sawfly larvae begin to climb and damage the wheat leaves the following morning. In severe cases, they can completely consume the leaves, leaving only the main vein. Research about wheat sawfly is very limited. The control methods for the wheat sawfly are mainly chemical control and agricultural control measures. We isolated a novel insect virus DtIV1 from the wheat sawfly. In addition to recovering the full genome, it is essential to conduct biological characterizations, including assessing the incidence, transmission methods (such as vectors), host range, and symptoms of the newly identified viruses, in order to evaluate their potential agronomic, commercial, and biosecurity impacts. Koch's postulates should be applied to both plant and insect viruses. In the case of insect viruses, it is important not only to study their effects on the insect host but also to investigate how they interact with the plant viruses that the insect transmits. Insect viruses typically exhibit two patterns of vertical transmission: transovarial, where the virus is contained within the eggs and passed from mother to offspring, leading to a high transmission rate, and transovum, where the virus is present on the surface of the egg, resulting in a lower transmission rate (Wang et al., 2023). These transmission patterns may influence the virus incidence. Some insect viruses can be horizontally transmitted through vectors or by feeding on infected food sources. In this study, the transmission of DtIV1 has not been evaluated. And the virus infection rate in the wheat sawfly population remains unclear, which is another important aspect of disease investigation. We should sample more colonies of wheat sawfly from different locations to determine the prevalence of DtIV1. If both infected and healthy insects are identified in the same condition, the effects of virus on wheat sawfly and vertical/horizontal transmission pattern could be studied. The effects of the virus can be studied by silencing its expression in the insect using RNAi technology. Future research could also explore the viral localization within the cells and tissues of the host, as well as investigate potential interactions between insects

and viruses. We still need to research the origin and host range of the virus and to assess the possible impact of DtIV1 on its host insect in the wheat field ecosystem. To our knowledge, DtIV1 is the first novel insect virus identified in wheat sawfly. Pest control plays a crucial role in safeguarding plants from viral diseases. If insect viruses affect the insects themselves or interact with plant viruses, insect viruses could serve as potential targets for biological control. Gaining a deeper understanding of insect viruses could lay the groundwork for novel approaches and strategies in plant disease management.

Reference

Adams, I.P., Glover, R.H., Monger, W.A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M., Boonham, N., 2009. Next-generation sequencing and metagenomic analysis: A universal diagnostic tool in plant virology. *Mol. Plant Pathol.* 10, 537–545. https://doi.org/10.1111/j.1364-3703.2009.00545.x

Adams, T.H., Wieser, J.K., Yu, J.H., 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol Rev. 62(1):35-54. https://doi.org/10.1128/MMBR.62.1.35-54.1998

Ahn, I.P., Lee, Y.H., 2001. A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. Mol Plant Microbe Interact. 14(4):496-507. https://doi.org/10.1094/MPMI.2001.14.4.496

Aw, K.M.S., Hue, S.M., 2017. Mode of infection of *Metarhizium* spp. fungus and their potential as biological control agents. J Fungi (Basel). 3(2):30. https://doi.org/10.3390/jof3020030

Ayllón, M.A., Vainio, E.J., 2023. Mycoviruses as a part of the global virome: diversity, evolutionary links and lifestyle. Advances in Virus Research 115:1-86. https://doi.org/10.1016/bs.aivir.2023.02.002

Baker, A.C., Schroeder, D.C., 2008. The use of RNA-dependent RNA polymerase for the taxonomic assignment of Picorna-like viruses (order *Picornavirales*) infecting *Apis mellifera L.* populations. Virol J. 5:10. https://doi.org/10.1186/1743-422X-5-10

Bernardo, C.C., Barreto, L.P., E Silva, C.S.R., Luz, C., Arruda, W., Fernandes, É.K.K., 2018. Conidia and blastospores of *Metarhizium* spp. and *Beauveria bassiana* s.l.: Their development during the infection process and virulence against the tick *Rhipicephalus microplus*. Ticks Tick Borne Dis. 9(5):1334-1342. https://doi.org/10.1016/j.ttbdis.2018.06.001.

Beys da Silva, W.O., Santi, L., Schrank, A., Vainstein, M.H., 2010. *Metarhizium anisopliae* lipolytic activity plays a pivotal role in *Rhipicephalus* (*Boophilus*) *microplus* infection. Fungal Biol. 114(1):10-5. https://doi.org/10.1016/j.mycres.2009.08.003

Bhatti, M.F., Jamal, A., Petrou, M.A., Cairns, T.C., Bignell, E.M., Coutts, R.H., 2011. The effects of dsRNA mycoviruses on growth and murine virulence of *Aspergillus fumigatus*. Fungal Genet Biol. 48(11):1071-5. https://doi.org/10.1016/j.fgb.2011.07.008

Bhoi, T.K., Samal, I., Majhi, P.K., Komal, J., Mahanta, D.K., Pradhan, A.K., Saini, V., Nikhil Raj, M., Ahmad, M.A., Behera, P.P., Ashwini, M., 2022. Insight into aphid mediated Potato Virus Y transmission: A molecular to bioinformatics prospective. Front Microbiol 13:1001454. https://doi.org/10.3389/fmicb.2022.1001454

Bian, R., Ren, H., Jiang, M., Zhang, Z., Zhai, S., Pang, T., Andika, I.B., Sun, L., 2024. Cryphonectria hypovirus 1 infection suppresses the pathogenicity but increases

the mycotoxin deoxynivalenol production of *Fusarium graminearum*. Phytopathol Res 6:54. https://doi.org/10.1186/s42483-024-00272-7

Boylan, M.T., Mirabito, P.M., Willett, C.E., Zimmerman, C.R., Timberlake, W.E., 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. Mol Cell Biol. 7(9):3113-8. https://doi.org/10.1128/mcb.7.9.3113-3118.1987

Braga, G.U., Rangel, D.E., Fernandes, É.K., Flint, S.D., Roberts, D.W., 2015. Molecular and physiological effects of environmental UV radiation on fungal conidia. Curr Genet. 61(3):405-25. https://doi.org/10.1007/s00294-015-0483-0

Brancini, G.T.P., Bachmann, L., Braga, G.Ú.L., 2021. Timing and duration of light exposure during conidia development determine tolerance to ultraviolet radiation. FEMS Microbiol Lett. 26,368(19):fnab133. https://doi.org/10.1093/femsle/fnab133

Brunner-Mendoza, C., Reyes-Montes, M.D., Moonjely, S., Bidochka, M.J., Toriello, C., 2018. A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. Biocontrol Science and Technology, 29(1), 83–102. https://doi.org/10.1080/09583157.2018.1531111

Butt, T.M., Greenfield, B.P., Greig, C., Maffeis, T.G., Taylor, J.W., Piasecka, J., Dudley, E., Abdulla, A., Dubovskiy, I.M., Garrido-Jurado, I., Quesada-Moraga, E., Penny, M.W., Eastwood, D.C., 2013. *Metarhizium anisopliae* pathogenesis of mosquito larvae: a verdict of accidental death. PLoS One. 8(12):e81686. https://doi.org/10.1371/journal.pone.0081686

Cai, G., Fry, W.E., Hillman, B.I., 2019. PiRV-2 stimulates sporulation in *Phytophthora infestans*. Virus Res. 271:197674. https://doi.org/10.1016/j.virusres.2019.197674

Chen, B., Chen, Y., Chen, H., Liang, Z., Chen, J., Wu, R., Zhang, T., Zhou, G., Yang, X., 2023. Identification, characterization and prevalence in southern China of a new iflavirus in the leafhopper *Recilia dorsalis* (Hemiptera: Cicadellidae). Virus Res. 323:199005. https://doi.org/10.1016/j.virusres.2022.199005

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17):i884-i890. https://doi.org/10.1093/bioinformatics/bty560

Chen, Y., Gai X, Chen, R., Li, C., Zhao, G., Xia Z., Zou, C., Zhong, J., 2019. Characterization of three novel betapartitiviruses co-infecting the phytopathogenic fungus *Rhizoctonia solani*. Virus Res. 270:197649. https://doi.org/10.1016/j.virusres.2019.197649

Chiba, S., Lin, Y.H., Kondo, H., Kanematsu, S., Suzuki, N., 2013. Effects of defective interfering RNA on symptom induction by, and replication of, a novel partitivirus from a phytopathogenic fungus, *Rosellinia necatrix*. J Virol. 87(4):2330-41. https://doi.org/10.1128/JVI.02835-12

Chu, H, 1949. The wheat sawfly, *Dolerus tritici* new species. Contributions from141

the Institute of Zoology, National Academy of Peiping 5(3): 79-92

Curran, J., Driver, F., Ballard, J.W.O., Milner, R.J., 1994. Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. Mycological Research 98, (5):547-552. https://doi.org/10.1016/S0953-7562(09)80478-4

Dangl, J., Jones, J., 2001. Plant pathogens and integrated defence responses to infection. Nature 411, 826–833. https://doi.org/10.1038/35081161

de Bary, H.A., 1876. Researches into the nature of the potato fungus, *Phytophthora infestans*. Journal of the Royal Agricultural Society of England 12: 239-269.

de Miranda, J.R., Dainat, B., Locke, B., Cordoni, G., Berthoud, H., Gauthier, L., Neumann, P., Budge, G.E., Ball, B.V., Stoltz, D.B., 2010. Genetic characterization of slow bee paralysis virus of the honeybee (*Apis mellifera L.*). J Gen Virol. 91:2524-2530. https://doi.org/10.1099/vir.0.022434-0

DePaulo, J., Powell, C., 1995. Extraction of double-stranded RNA from plant tissues without the use of organic solvents. Plant Dis. 79 (3): 246-248. https://doi.org/10.1094/PD-79-0246

Divekar, P.A., Narayana, S., Divekar, B.A., Kumar, R., Gadratagi, B.G., Ray, A., Singh, A.K., Rani, V., Singh, V., Singh, A.K., Kumar, A., Singh, R.P., Meena, R.S., Behera, T.K., 2022. Plant secondary metabolites as defense tools against herbivores for sustainable crop protection. Int J Mol Sci. 23(5):2690. https://doi.org/10.3390/ijms23052690

Dumas, C., Robert, P., Pais, M., Vey, A., Quiot, J.M., 1994. Insecticidal and cytotoxic effects of natural and hemisynthetic destruxins. Comp Biochem Physiol Pharmacol Toxicol Endocrinol.108(2):195-203. https://doi.org/10.1016/1367-8280(94)90031-0

Fang, W., Pei, Y., Bidochka, M.J., 2007. A regulator of a G protein signalling (RGS) gene, cag8, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis. Microbiology (Reading). 153(Pt 4):1017-1025. https://doi.org/10.1099/mic.0.2006/002105-0

Fu, M., Qu, Z., Pierre-Pierre, N., Jiang, D., Souza, F.L., Miklas, P.N., Porter, L.D., Vandemark, G.J., Chen, W., 2024. Exploring the mycovirus sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 as a biocontrol agent of white mold caused by *Sclerotinia sclerotiorum*. Plant Dis. 108(3):624-634. https://doi.org/10.1094/PDIS-07-23-1458-RE

Geng, P., Li, W., Lin, L., de Miranda, J.R., Emrich, S., An, L., 2014. Genetic characterization of a novel iflavirus associated with vomiting disease in the Chinese oak silkmoth *Antheraea pernyi*. Plos One 9(3): e92107. https://doi.org/10.1371/journal.pone.0092107

Gorbalenya, A.E., Donchenko, A.P., Blinov, V.M., Koonin, E.V., 1989. Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common structural fold. FEBS Lett 243(2):103–

114. https://doi.org/10.1016/0014-5793(89)80109-7

Hajano, J.U., Raza, A., Zhang, L., Liu, W., Wang, X., 2020. Ribavirin targets sugar transporter 6 to suppress acquisition and transmission of rice stripe tenuivirus by its vector *Laodelphax striatellus*. Pest Manag Sci. 76(12):4086-4092. https://doi.org/10.1002/ps.5963

Hashimoto, Y., Watanabe, A., Kawase, S., 1986. Evidence for the presence of a genome-linked protein in infectious flacherie virus. Arch Virol 90 (3-4):301-312. https://doi.org/10.1007/BF01317378

He, L., Wang, P., Yang, G., Chen, X., Huang, B., 2022. A novel polymycovirus infecting the entomopathogenic fungus *Metarhizium brunneum*. Arch Virol. 168(1):6. https://doi.org/10.1007/s00705-022-05684-4

Hogenhout, S.A., Ammar, el-D., Whitfield, A.E., Redinbaugh, M.G., 2008. Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol. 46:327-59. https://doi.org/10.1146/annurev.phyto.022508.092135

Islam, S.M.N., Chowdhury, M.Z.H., Mim, M.F. Momtaz, M.B., Islam, T., 2023. Biocontrol potential of native isolates of *Beauveria bassiana* against cotton leafworm *Spodoptera litura* (Fabricius). Sci Rep 13, 8331. https://doi.org/10.1038/s41598-023-35415-x

Islam, W., Adnan, M., Shabbir, A., Naveed, H., Abubakar, Y.S., Qasim, M., Tayyab, M., Noman, A., Nisar, M.S., Khan, K.A., Ali, H., 2021. Insect-fungal-interactions: A detailed review on entomopathogenic fungi pathogenicity to combat insect pests. Microb Pathog. 159:105122. https://doi.org/10.1016/j.micpath.2021.105122

Jia, W., Wang, F., Li, J., Chang, X., Yang, Y., Yao, H., Bao, Y., Song, Q., Ye, G., 2021. A novel iflavirus was discovered in green rice leafhopper *Nephotettix cincticeps* and its proliferation was inhibited by infection of rice dwarf virus. Front Microbiol. 11:621141. https://doi.org/10.3389/fmicb.2020.621141

Junges, Â., Boldo, J.T., Souza, B.K., Guedes, R.L., Sbaraini, N., Kmetzsch, L., Thompson, C.E., Staats, C.C., de Almeida, L.G., de Vasconcelos, A.T., Vainstein, M.H., Schrank, A., 2014. Genomic analyses and transcriptional profiles of the glycoside hydrolase family 18 genes of the entomopathogenic fungus *Metarhizium anisopliae*. PLoS One. 9(9):e107864. https://doi.org/10.1371/journal.pone.0107864

Kamaruzzaman, M., He, G., Wu, M., Zhang, J., Yang, L., Chen, W., Li, G., 2019. A novel partitivirus in the hypovirulent isolate QT5-19 of the plant pathogenic fungus *Botrytis cinerea*. Viruses 11(1):24. https://doi.org/10.3390/v11010024

Kansiime, M.K., Rwomushana, I., Mugambi, I., 2023. Fall armyworm invasion in Sub-Saharan Africa and impacts on community sustainability in the wake of Coronavirus Disease 2019: reviewing the evidence. Current Opinion in Environmental Sustainability 62: 101279. https://doi.org/10.1016/j.cosust.2023.101279

Kondo, H., Botella, L., Suzuki, N., 2022. Mycovirus diversity and evolution

revealed/inferred from recent studies. Annu Rev Phytopathol. 60:307-336. https://doi.org/10.1146/annurev-phyto-021621-122122

Koonin, E.V., 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol. 72(9): 2197-2206. https://doi.org/10.1099/0022-1317-72-9-2197

Koonin, E.V., Dolja, V.V., 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol. 28:375–430. https://doi.org/10.3109/10409239309078440

Kotta-Loizou, I., Coutts, R.H., 2017. Studies on the virome of the entomopathogenic fungus *Beauveria bassiana* reveal novel dsRNA elements and mild hypervirulence. PLoS Pathog. 13(1):e1006183. https://doi.org/10.1371/journal.ppat.1006183

Kulabhusan, P.K., Tripathi, A., Kant, K., 2022. gold nanoparticles and plant pathogens: an overview and prospective for biosensing in forestry. Sensors 22, 1259. https://doi.org/10.3390/s22031259

Lanzi, G., de Miranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A., Capucci, L., Camazine, S.M., Rossi, C., 2006. Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera L.*). J Virol 80(10):4998-5009. https://doi.org/10.1128/JVI.80.10.4998-5009.2006

Lau, G.W., Hamer, J.E., 1998. Acropetal: a genetic locus required for conidiophore architecture and pathogenicity in the rice blast fungus. Fungal Genet Biol. 24(1-2):228-39. https://doi.org/10.1006/fgbi.1998.1053

Lau, S.K.P., Lo, G.C.S., Chow, F.W.N., Fan, R.Y.Y., Cai, J.J., Yuen, K.Y., Woo, P.C.Y., 2018. Novel partitivirus enhances virulence of and causes aberrant gene expression in *Talaromyces marneffei*. mBio. 9(3):e00947-18. https://doi.org/10.1128/mBio.00947-18

Leal, S. C. M., Bertioli, D. J., Ball, B. V., & Butt, T. M. (1994). Presence of double-stranded RNAs and virus-like particles in the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology*, *4*(1), 89–94. https://doi.org/10.1080/09583159409355316

Lee, J.Y., 2023. The Principles and applications of high-throughput sequencing technologies. Dev Reprod. 27(1):9-24. https://doi.org/10.12717/DR.2023.27.1.9

Leger, R., Joshi, L., Bidochka, M.J., Roberts, D.W., 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc Natl Acad Sci U S A 93(13):6349-54. https://doi.org/10.1073/pnas.93.13.6349

Li, B., Cao, Y., Ji, Z., Zhang, J., Meng, X., Dai, P., Hu, T., Wang, S., Cao, K., Wang, Y., 2022. Coinfection of two mycoviruses confers hypovirulence and reduces the production of mycotoxin alternariol in *Alternaria alternata f. sp. mali*. Front Microbiol. 13:910712. https://doi.org/10.3389/fmicb.2022.910712

Li, F., Shi, H.Q., Ying, S.H., Feng, M.G., 2015. *WetA* and *VosA* are distinct regulators of conidiation capacity, conidial quality, and biological control potential of

a fungal insect pathogen. Appl Microbiol Biotechnol. 99(23):10069-81. https://doi.org/10.1007/s00253-015-6823-7

Li, P., Wang, S., Zhang, L., Qiu, D., Zhou, X., Guo, L., 2020. A tripartite ssDNA mycovirus from a plant pathogenic fungus is infectious as cloned DNA and purified virions. Sci Adv. 6(14):eaay9634. https://doi.org/10.1126/sciadv.aay9634

Li, Y., Li, S., Liang, Z., Cai, Q., Zhou, T., Zhao, C., Wu, X., 2022. RNA-seq analysis of *Rhizoctonia solani* AG-4HGI Strain BJ-1H infected by a new viral strain of rhizoctonia solani partitivirus 2 reveals a potential mechanism for hypovirulence. Phytopathology 112(6):1373-1385. https://doi.org/10.1094/PHYTO-08-21-0349-R

Liljas, L., Tate, J., Lin, T., Christian, P., Johnson, J.E., 2002. Evolutionary and taxonomic implications of conserved structural motifs between picornaviruses and insect picorna-like viruses. Arch Virol. 147: 59-84. https://doi.org/10.1007/s705-002-8303-1

Liu, F., Xi, M., Liu, T., Wu, X., Ju, L., Wang, D., 2024. The central role of transcription factors in bridging biotic and abiotic stress responses for plants' resilience, New Crops 1: 100005. https://doi.org/10.1016/j.ncrops.2023.11.003

Liu, S., Xie, J., Cheng, J., Li, B., Chen, T., Fu, Y., Li, G., Wang, M., Jin, H., Wan, H., Jiang, D., 2016. Fungal DNA virus infects a mycophagous insect and utilizes it as a transmission vector. Proc Natl Acad Sci U S A. 113(45):12803-12808. https://doi.org/10.1073/pnas.1608013113

Liu, W., Wei, T., Wang, X., 2023. Plant reoviruses hijack autophagy in insect vectors. Trends Microbiol. 31(12):1251-1261. https://doi.org/10.1016/j.tim.2023.06.008

Liu, Y., Yang, Y., Wang, B., 2022. Entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* play roles of maize (*Zea mays*) growth promoter. Sci Rep 12:15706. https://doi.org/10.1038/s41598-022-19899-7

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402-8. https://doi.org/10.1006/meth.2001.1262

Madeira, F., Pearce, M., Tivey, A.R.N., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., Lopez, R., 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res. 50(W1):W276-W279. https://doi.org/10.1093/nar/gkac240

Mahillon, M., Decroës, A., Caulier, S., Tiendrebeogo, A., Legrève, A., Bragard, C., 2021. Genomic and biological characterization of a novel partitivirus infecting Fusarium equiseti. Virus Res. 297:198386. https://doi.org/10.1016/j.virusres.2021.198386

Márquez, L.M., Redman, R.S., Rodriguez, R.J., Roossinck, M.J., 2007. A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. Science. 315(5811):513-5. https://10.1126/science.1136237

Martin, S.J., Brettell, L.E., 2019. Deformed Wing Virus in Honeybees and Other

Insects. Annu Rev Virol. 6(1):49-69. https://doi.org/10.1146/annurev-virology-092818-015700.

Melzer, M.J., Bidochka, M.J., 1998. Diversity of double-stranded RNA viruses within populations of entomopathogenic fungi and potential implications for fungal growth and virulence. Mycologia 90(4):586–94. https://www.jstor.org/stable/3761217

Mendesil, E., Tefera, T., Blanco, C.A. Paula-Moraes, S., Huang, F., Viteri, D., Hutchison, W., 2023. The invasive fall armyworm, *Spodoptera frugiperda*, in Africa and Asia: responding to the food security challenge, with priorities for integrated pest management research. J Plant Dis Prot. 130:1175–1206. https://doi.org/10.1007/s41348-023-00777-x

Milgroom, M.G., Cortesi, P., 2004. Biological control of chestnut blight with hypovirulence: a critical analysis. Annu Rev Phytopathol. 42:311-38. https://doi.org/10.1146/annurev.phyto.42.040803.140325

Mongkolsamrit, S., Khonsanit, A., Thanakitpipattana, D., Tasanathai, K., Noisripoom, W., Lamlertthon, S., Himaman, W., Houbraken, J., Samson, R.A., Luangsa-Ard, J., 2020. Revisiting *Metarhizium* and the description of new species from Thailand. Stud Mycol. 5,95:171-251. https://doi.org/10.1016/j.simyco.2020.04.001

Moraga, E.Q., 2020. Entomopathogenic fungi as endophytes: their broader contribution to IPM and crop production. Biocontrol Science and Technology 30(9):864–877. https://doi.org/10.1080/09583157.2020.1771279

Murakami, R., Suetsugu, Y., Kobayashi, T., Nakashima, N., 2013. The genome sequence and transmission of an iflavirus from the brown planthopper, *Nilaparvata lugens*. Virus Res. 176:179–187. https://doi.org/10.1016/j.virusres.2013.06.005

Murray, G.M., Brennan, J.P., 2010. Estimating disease losses to the Australian barley industry. Australasian Plant Pathology 39:85-96. https://api.semanticscholar.org/CorpusID:41835098

Ochoa, W.F., Havens, W.M., Sinkovits, R.S., Nibert, M.L., Ghabrial, S.A., Baker, T.S., 2008 Partitivirus structure reveals a 120-subunit, helix-rich capsid with distinctive surface arches formed by quasisymmetric coat-protein dimers. Structure. 16(5):776-86. https://doi.org/10.1016/j.str.2008.02.014

Ohara, T., Inoue, I., Namiki, F., Kunoh, H., Tsuge, T., 2004. REN1 is required for development of microconidia and macroconidia, but not of chlamydospores, in the plant pathogenic fungus *Fusarium oxysporum*. Genetics 166(1):113-24. https://doi.org/10.1534/genetics.166.1.113

Oldroyd, G.E.D., Murray, J.D., Poole, P.S., Downie, J.A., 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Genet. 45:119–144. https://doi.org/10.1146/annurev-genet-110410-132549

Pal, S., St Leger, R.J., Wu, L.P., 2007. Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*. J Biol

Chem. 282(12):8969-77. https://doi.org/10.1074/jbc.M605927200

Panizzi, A. R., Lucini, T., Mitchell, P. L., 2021. Feeding sites of true bugs and resulting damage to plants. Electronic Monitoring of Feeding Behavior of Phytophagous True Bugs (Heteroptera) 47-64. https://doi.org/10.1007/978-3-030-64674-5_3

Park, H.S., Yu, J.H., 2012. Genetic control of asexual sporulation in filamentous fungi. Curr Opin Microbiol. 15(6):669-77. https://doi.org/10.1016/j.mib.2012.09.006

Parry, R., Naccache, F., Ndiaye, E.H., Fall, G., Castelli, I., Lühken, R., Medlock, J., Cul,l B., Hesson, J.C., Montarsi, F., Failloux, A.B., Kohl, A., Schnettler, E., Diallo, M., Asgari, S., Dietrich, I., Becker, S.C., 2020. Identification and RNAi profile of a novel iflavirus infecting senegalese *Aedes vexans arabiensis* mosquitoes. Viruses 12(4):440. https://doi.org/10.3390/v12040440

Patel, T.K., 2020. *Metarhizium*, Beneficial Microbes in Agro-Ecology. Academic Press 593-610. https://doi.org/10.1016/B978-0-12-823414-3.00029-0

Peyambari, M., Habibi, M.K., Fotouhifar, K.B., Dizadji, A., Roossinck, M.J., 2014. molecular characterization of a novel putative partitivirus infecting *cytospora sacchari*, a plant pathogenic fungus. Plant Pathol J. 30(2):151-8. https://doi.org/10.5423/PPJ.OA.01.2014.0005

Qi, Y., Ye, Z., Zhang, C., Chen J., Li, J., 2023. Diversity of RNA viruses in agricultural insects. Comput Struct Biotec 21: 4312-4321. https://doi.org/10.1016/j.csbj.2023.08.036

Quenouille, J., Vassilakos, N., Moury, B., 2013. Potato virus Y: a major crop pathogen that has provided major insights into the evolution of viral pathogenicity. Molecular Plant Pathology 14(5):439-52. https://doi.org/10.1111/mpp.12024

Ristaino, J.B., 2002. Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. Microbes and Infection 4: 1369–1377. https://doi.org/10.1016/S1286-4579(02)00010-2

Satam, H., Joshi, K., Mangrolia, U., Waghoo, S., Zaidi, G., Rawool, S., Thakare, R.P., Banday, S., Mishra, A.K., Das, G., Malonia, S.K., 2023. Next-Generation sequencing technology: current trends and advancements. Biology (Basel). 12(7):997. https://doi.org/10.3390/biology12070997.

Schmitz, A., Badgujar, C., Mansur, H., Flippo, D., McCornack, B., Sharda, A., 2022. design of a reconfigurable crop scouting vehicle for row crop navigation: a proof-of-concept study. Sensors 22(16):6203. https://doi.org/10.3390/s22166203

Schrank, A., Vainstein, M.H., 2010 *Metarhizium anisopliae* enzymes and toxins. Toxicon 56(7):1267-74. https://doi.org/10.1016/j.toxicon.2010.03.008

Sheng, H., McNamara, P.J., St Leger, R.J., 2022 *Metarhizium*: an opportunistic middleman for multitrophic lifestyles. Curr Opin Microbiol. 69:102176. https://doi.org/10.1016/j.mib.2022.102176

Shi, M., Lin, X., Tian, J., Chen, L., Chen, X., Li, C., Qin, X., Li, J., Cao, J., Eden,

J.S., Buchmann, J., Wang, W., Xu, J., Holmes, E.C., Zhang, Y., 2016. Redefining the invertebrate RNA virosphere. Nature 540(7634): 539-543. https://doi.org/10.1038/nature20167

Singh, P., Mazumdar, P., 2022. Microbial pesticides. Biopesticides. 37–71. https://doi.org/10.1016/B978-0-12-823355-9.00023-7

St Leger, R.J., Bidochka, M.J., Roberts, D.W., 1994. Characterization of a novel carboxypeptidase produced by the entomopathogenic fungus *Metarhizium anisopliae*. Arch Biochem Biophys. 314(2):392-8. https://doi.org/10.1006/abbi.1994.1458

Stehle, S., Schulz, R., 2015. Agricultural insecticides threaten surface waters at the global scale. Proc Natl Acad Sci U S A. 112(18):5750-5. https://doi.org/10.1073/pnas.1500232112

Stenberg, J. A., Sundh, I., Becher, P. G., Björkman, C., Dubey, M., Egan, P.A., Friberg, H., Gil J.F., Jensen, D.F., Jonsson, M., Magnus Karlsson, M., Khalil, S., Ninkovic, V., Rehermann, G., Vetukuri, R.R., Viketoft, M., 2021. When is it biological control? A framework of definitions, mechanisms, and classifications. Journal of Pest Science, 94(3), 665–676. https://doi.org/10.1007/s10340-021-01354-7

Sun, A., Zhao, L., Sun, Y., Chen, Y., Li, C., Dong, W., Yang, G., 2023. Horizontal and vertical transmission of a mycovirus closely related to the partitivirus RhsV717 that confers hypovirulence in *Rhizoctonia solani*. Viruses 15(10):2088. https://doi.org/10.3390/v15102088

Sun, L.Y., Nuss, D.L., Suzuki, N., 2006. Synergism between a mycoreovirus and a hypovirus mediated by the papain-like protease p29 of the prototypic hypovirus CHV1-EP713. J Gen Virol. 87:3703-14. https://doi.org/10.1099/vir.0.82213-0

Takahashi-Nakaguchi, A., Shishido, E., Yahara, M., Urayama, S.I., Sakai, K., Chibana, H., Kamei, K., Moriyama, H., Gonoi, T., 2020. Analysis of an intrinsic mycovirus associated with reduced virulence of the human pathogenic fungus *Aspergillus fumigatus*. Front Microbiol. 10:3045. https://doi.org/10.3389/fmicb.2019.03045

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol. 38(7):3022-3027. https://doi.org/10.1093/molbev/msab120

Tang, F.H.M., Lenzen, M., McBratney, A., Maggi, F., 2021. Risk of pesticide pollution at the global scale. Nat. Geosci. 14:206–210. https://doi.org/10.1038/s41561-021-00712-5

Tseng, M.N., Chung, C.L., Tzean, S.S., 2014. Mechanisms relevant to the enhanced virulence of a dihydroxynaphthalene-melanin metabolically engineered entomopathogen. PLoS One. 9(3):e90473. https://doi.org/10.1371/journal.pone.0090473

Tudi, M., Daniel Ruan, H., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., Phung, D.T., 2021. Agriculture development, pesticide application and its impact on the

environment. Int J Environ Res Public Health. 18(3):1112. https://doi.org/10.3390/ijerph18031112

Vainio, E.J., Chiba, S., Ghabrial, S.A., Maiss, E., Roossinck, M., Sabanadzovic, S., Suzuki, N., Xie, J., Nibert, M., Consortium, I.R., 2018. ICTV Virus Taxonomy Profile: *Partitiviridae*. J Gen Virol. 99(1):17-18. https://doi.org/10.1099/jgv.0.000985

Valles, S.M., Chen, Y., Firth, A.E., Dma, G., Hashimoto, Y., Herrero, S., de Miranda, J.R., Ryabov, E., Consortium, I.R., 2017. ICTV Virus Taxonomy Profile: *Iflaviridae*. J Gen Virol 98(4):527–528. https://doi.org/10.1099/jgv.0.000757

Van Alfen, N.K., Jaynes, R.A., Anagnostakis, S.L., Day, P.R., 1975. Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. Science 189(4206):890-1. https://doi.org/10.1126/science.189.4206.890

van Oers, M.M., 2010. Genomics and biology of Iflaviruses. Insect Virol 231–250. https://api.semanticscholar.org/CorpusID:81640614

Velasco, L., López-Herrera, C., Cretazzo, E., 2020. Two novel partitiviruses that accumulate differentially in *Rosellinia necatrix* and *Entoleuca sp.* infecting avocado. Virus Res. 285:198020. https://doi.org/10.1016/j.virusres.2020.198020

Villamizar, L.F., Barrera, G., Hurst, M., Glare, T.R., 2021. Characterization of a new strain of *Metarhizium novozealandicum* with potential to be developed as a biopesticide. Mycology. 12(4):261-278. https://doi.org/10.1080/21501203.2021.1935359

Wang, C., St Leger, R.J., 2006. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc Natl Acad Sci U S A 103(17):6647-52. https://doi.org/10.1073/pnas.0601951103

Wang, C., St Leger, R.J., 2007. The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryot Cell. 6(5):808-16. https://doi.org/10.1128/EC.00409-06

Wang, C., Wang, S., 2017. Insect Pathogenic Fungi: Genomics, Molecular Interactions, and Genetic Improvements. Annu Rev Entomol. 62:73-90. https://doi.org/10.1146/annurev-ento-031616-035509

Wang, F., Fang, Q., Wang, B., Yan, Z., Hong, J., Bao, Y., Kuhn, J.H., Werren, J.H., Song, Q., Ye, G., 2017. A novel negative-stranded RNA virus mediates sex ratio in its parasitoid host. PLoS Pathog. 13(3):e1006201. https://doi.org/10.1371/journal.ppat.1006201

Wang, H., Chen, Q., We,i T., 2024. Complex interactions among insect viruses-insect vector-arboviruses. Insect Sci. 31(3):683-693. https://doi.org/10.1111/1744-7917.13285

Wang, H., Liu, Y., Liu, W., Cao, M., Wang, X., 2019. Full genome sequence of a novel iflavirus from the leafhopper *Psammotettix alienus*. Arch Virol. 164(1): 309-311 https://doi.org/10.1007/s00705-018-4041-1

Wang, L., Xiao, Q., Zhou, X., Zhu, Y., Dong, Z., Chen, P., Pan, M., Lu, C., 2017. Bombyx mori nuclear polyhedrosis virus (BmNPV) induces host cell autophagy to benefit infection. Viruses 10(1):14. https://doi.org/10.3390/v10010014

Wang, M., Sun, X., Zhu, C., Xu, Q., Ruan, R., Yu, D., Li, H., 2015. PdbrlA, PdabaA and PdwetA control distinct stages of conidiogenesis in *Penicillium digitatum*. Res Microbiol. 166(1):56-65. https://doi.org/10.1016/j.resmic.2014.12.003

Wang, P., Yang, G., Lu, H., Huang, B., 2023. Infection with a novel polymycovirus enhances growth, conidiation and sensitivity to UV-B irradiation of the entomopathogenic fungus *Metarhizium anisopliae*. Front Microbiol. 14:1214133. https://doi.org/10.3389/fmicb.2023.1214133

Wang, P., Yang, G., Shi, N., Huang, B., 2020. Molecular characterization of a new partitivirus, MbPV1, isolated from the entomopathogenic fungus *Metarhizium brunneum* in China. Arch Virol. 165(3):765-769. https://doi.org/10.1007/s00705-019-04517-1

Wang, P., Yang, G., Shi, N., Huang, B., 2021. A novel gammapartitivirus from the entomopathogenic fungus *Metarhizium brunneum*. Arch Virol. 166(3):977-981. https://doi.org/10.1007/s00705-020-04939-2

Wang, P., Yang, G., Shi, N., Huang, B., 2022. Molecular characterization of a novel double-stranded RNA virus infecting the entomopathogenic fungus *Metarhizium brunneum*. Arch Microbiol. 204(10):606. https://doi.org/10.1007/s00203-022-03224-x

Wang, P., Yang, G., Shi, N., Zhao, C., Hu, F., Coutts, R.H.A., Kotta-Loizou, I., Huang, B., 2023. A novel partitivirus orchestrates conidiation, stress response, pathogenicity, and secondary metabolism of the entomopathogenic fungus *Metarhizium majus*. PLoS Pathog. 19(5):e1011397. https://doi.org/10.1371/journal.ppat.1011397

Wei, R., Cao, L., Feng, Y., Chen, Y., Chen, G., Zheng, H., 2022. Sacbrood virus: a growing threat to honeybees and wild pollinators. Viruses 14(9):1871. https://doi.org/10.3390/v14091871

Wininger, K., Rank, N., 2017. Evolutionary dynamics of interactions between plants and their enemies: comparison of herbivorous insects and pathogens. Ann N Y Acad Sci. 1408(1):46-60. https://doi.org/10.1111/nyas.13541

Wu, H., Pang, R., Cheng, T., Xue, L., Zeng, H., Lei, T., Chen, M., Wu, S., Ding, Y., Zhang, J., Shi, M., Wu, Q., 2016. Abundant and diverse RNA viruses in insects revealed by RNA-seq analysis: ecological and evolutionary implications. MSystems 5(4):e00039-20. https://doi.org/10.1128/msystems.00039-20

Wu, N., Zhang Plant reoviruses hijac, L., Ren, Y., Wang, X., 2020. Rice black-streaked dwarf virus: From multiparty interactions among plant-virus-vector to intermittent epidemics. Mol Plant Pathol. 21(8):1007-1019. https://doi.org/10.1111/mpp.12946

Wu, N., Zhang, P., Liu, W., Cao, M., Massart, S., Wang, X., 2019. Complete genome sequence and characterization of a new iflavirus from the small brown planthopper (*Laodelphax striatellus*). Virus Res. 272: 197651. https://doi.org/10.1016/j.virusres.2019.197651

Wu, N., Zhang, P., Liu, W., Cao, M., Wang, X., 2018. Sequence analysis and genomic organization of a new insect iflavirus, Sogatella furcifera honeydew virus 1. Arch Virol. 163(7): 2001-2003158. https://doi.org/10.1007/s00705-018-3817-7

Xiao, X., Cheng, J., Tang, J., Fu, Y., Jiang, D., Baker, T.S., Ghabrial, S.A., Xie, J., 2014. A novel partitivirus that confers hypovirulence on plant pathogenic fungi. J Virol. 88(17):10120-33. https://doi.org/10.1128/JVI.01036-14

Xie, J., Jiang, D., 2024. Understanding the diversity, evolution, ecology, and applications of mycoviruses. Annu Rev Microbiol. 78(1):595-620. https://doi.org/10.1146/annurev-micro-041522-105358

Yan, H., Wang, D., Zhao, X., Huang, D., Ma, X., 2011. Observation of the infection process of *Metarhizium anisopliae* on the cuticle of *Anoplophora glabripennis* larvae with scanning electron microscopy. Front. Agric. China 5, 662–665. https://doi.org/10.1007/s11703-011-1134-y

Yang, S., Rothman, R.E., 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 4(6):337-48. https://doi.org/10.1016/S1473-3099(04)01044-8

Yang, Y., Shah, J., Klessig, D.F., 1997. Signal perception and transduction in plant defense responses. Genes and Development, 11(13), 1621-1639. https://doi.org/10.1101/gad.11.13.1621

Young, M.D., Wakefield, M.J., Smyth, G.K., Oshlack, A., 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 11(2):R14. https://doi.org/10.1186/gb-2010-11-2-r14

Yu, X., Li, B., Fu, Y., Jiang, D., Ghabrial, S.A., Li, G., Peng, Y., Xie, J., Cheng, J., Huang, J., Yi, X., 2010. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. Proc Natl Acad Sci U S A. 107(18):8387-92. https://doi.org/10.1073/pnas.0913535107

Zeng, G., Chen, X., Zhang, X., Zhang, Q., Xu, C., Mi, W., Guo, N., Zhao, H., You, Y., Dryburgh, F.J., Bidochka, M.J., St Leger, R.J., Zhang, L., Fang, W., 2017. Genome-wide identification of pathogenicity, conidiation and colony sectorization genes in *Metarhizium robertsii*. Environ Microbiol. 19(10):3896-3908. https://doi.org/10.1111/1462-2920.13777

Zhang, A.X., Mouhoumed, A.Z., Tong, S.M., Ying, S.H., Feng, M.G., 2019. *BrlA* and *AbaA* govern virulence-required dimorphic switch, conidiation, and pathogenicity in a fungal insect pathogen. mSystems. 4(4):e00140-19. https://doi.org/10.1128/mSystems.00140-19

Zhang, D.X., Nuss, D.L., 2016. Engineering super mycovirus donor strains of

chestnut blight fungus by systematic disruption of multilocus vic genes. Proc Natl Acad Sci U S A. 113(8):2062-7. https://doi.org/10.1073/pnas.1522219113

Zhang, H., Xie, J., Fu, Y., Cheng, J., Qu, Z., Zhao, Z., Cheng, S., Chen, T., Li, B., Wang, Q., Liu, X., Tian, B., Collinge, D.B., Jiang, D., 2020. A 2-kb mycovirus converts a pathogenic fungus into a beneficial endophyte for brassica protection and yield enhancement. Mol Plant. 13(10):1420-1433. https://doi.org/10.1016/j.molp.2020.08.016

Zhang, J., Wang, F., Yuan, B., Yang, L., Yang, Y., Fang, Q., Kuhn, J.H., Song, Q., Ye, G., 2021. A novel cripavirus of an ectoparasitoid wasp increases pupal duration and fecundity of the wasp's *Drosophila melanogaster* host. ISME J. 15(11):3239-3257. https://doi.org/10.1038/s41396-021-01005-w

Zhang, Y., Liang, X., Zhao, M., Qi, T., Guo, H., Zhao, J., Zhao, J., Zhan, G., Kang, Z., Zheng, L., 2022. A novel ambigrammatic mycovirus, PsV5, works hand in glove with wheat stripe rust fungus to facilitate infection. Plant Commun. 4(3):100505. https://doi.org/10.1016/j.xplc.2022.100505

Zhang, Y., Park, C., Bennett, C., Thornton, M., Kim, D., 2021. Rapid and accurate alignment of nucleotide conversion sequencing reads with HISAT-3N. Genome Res. 31(7):1290–5. https://doi.org/10.1101/gr.275193.120

Zhou, L., Li, X., Kotta-Loizou, I., Dong, K., Li, S., Ni, D., Hong, N., Wang, G., Xu, W., 2021. A mycovirus modulates the endophytic and pathogenic traits of a plant associated fungus. ISME J. 15(7):1893-1906. https://doi.org/10.1038/s41396-021-00892-3

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31(13):3406-15. https://doi.org/10.1093/nar/gkg595

Zwet, T.V., Keil, H.L., 1979. Fire blight a bacterial disease of rosaceous plants. USA. Department of agriculture. Agriculture handbook 510. https://lib.ugent.be/catalog/rug01:000124580

Appendix – Publications

Guo, J., Zhang, P., Wu, N., Liu, W., Liu, Y., Jin, H., Francis, F., Wang, X., 2024. Transfection of entomopathogenic *Metarhizium* species with a mycovirus confers hypervirulence against two lepidopteran pests. Proc Natl Acad Sci U S A 121(26):e2320572121. https://doi.org/10.1073/pnas.2320572121

Guo, J., Liu, W., Chen, C., Xu, Z., Francis, F., Wang, X., 2024. Complete genome sequence of a novel iflavirus from wheat sawfly (*Dolerus tritici*). Arch Virol. 170(1):21. doi: 10.1007/s00705-024-06206-0

Wang, X., Guo, J., Liu, Y., Zhang, P., Jin, H., 2024. A strain of M*etarhizium anisopliae* carrying the MfPV1 virus, named IPPMp818V, and its applications. Patent number: CN117586893A. (In Chinese)

Wang, X., Guo, J., Liu, Y., Zhang, P., Jin, H., 2024. A strain of *Metarhizium pingshaense* carrying the MfPV1 virus, named IPPMp818V, and its applications. Patent number: CN117586894A. (In Chinese)