

Molecular characterization of a novel segmented dsRNA mycovirus and its association with hypovirulence of *Fusarium graminearum*.

Dissertation

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I confirm that the thesis of Mr. Omar Darissa was reviewed by me, a native English speaker, for English language accuracy. The thesis is well written, and therefore I would recommend that the dissertation be accepted in its current form. During my 42 years as a professor, I have read many theses and Mr. Darissa's thesis meets the standards for the University of Wisconsin-Madison.

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To my parents Mousa and Meriam Darissa
To my wife Laila Darissa
To my children Mousa, Mahmoud, and Mamoun

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ABBREVIATIONS

%	Percentage
°C	centigrade
μ	micro (10 ⁻⁶)
aa	amino acids
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	complementary Deoxyribonucleic Acid
CP	Coat protein
cv.	Cultivated variety; cultivar
DIG	Digoxygenin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Desoxynucleotide triphosphate (s)
DON	Deoxynivalenol
dsRNA	double-stranded RNA
DTT	Dithiotreitol
dUTP	Desoxyuracil triphosphate
EDTA	Ethylenediaminetetraacetic acid
<i>et al.</i>	et alii = and others
EtBr	Ethidium bromide
FHB	Fusarium Head Blight
Fig.	Figure
FLAC	Full Length Amplification of cDNA
h	hour
HCl	Hydrochloride
hph	Hygromycin B phosphotransferase
IPTG	Isopropylthio-β-D-galactoside
kb	kilo bases (= 1000 bp)
kDa	kilo Dalton (= 1000 Da)

LB	Luria-Bertani medium
M	Molar (mol/L)
mg	milligram
min	minutes
ml	milliliter
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
nt	nucleotide
OD	Optical Density
ORF	Open Reading Frame
PBS	phosphate-buffered saline buffer
PBS-T	PBS Tween
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
pH	Potentia Hydrogenii
qPCR	quantitative PCR
RdRP	RNA dependent RNA Polymerase
RNA	Ribonucleic acid
rPCR	random PCR
rpm	round per minute
RT-PCR	Reverse transcriptase PCR
SDS	Sodium Dodecylsulfate
sec	seconds
SNA	Synthetic Nutrient Agar
SPAT	Single Primer Amplification Technique
TE	Tris-EDTA
T _m	Annealing Temperature
Tris	Tris-(hydroxymethyl) aminomethane
U	unit
UV	Ultra violet

v/v	Volume per volume
vol	Volume
w/v	Weight per volume
WT	Wild type
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactoside

1. Introduction

The association of several viruses with hypovirulence of their fungal plant pathogenic hosts has motivated scientists to explore the world of mycoviruses or fungal viruses. As the majority of mycoviruses have dsRNA genomes, several methods for the handling and sequence determination of such genomes have been established. Molecular characterization and phylogenetic analysis of many of the so far reported mycoviruses have led to the initiation of new taxonomic virus families and genera to accommodate the diversity of accumulating mycovirus members.

The fungus *Fusarium graminearum* is the major causal agent of a worldwide disease of cereals called fusarium head blight. *F. graminearum* infections can lead to severe losses in the yield and quality of important crops like wheat and barley. The reported association of several isolates of *F. graminearum* with mycovirus infection might help in developing an effective and safe control method of the disease.

In this introduction, a brief description of each of the mycovirus families, including mycovirus members that infect *F. graminearum* and those that are associated with hypovirulence of their hosts is presented. Moreover, a short background about the fungus *F. graminearum* and its disease life cycle in wheat is included. Finally, a description of the molecular approaches for the sequence determination of dsRNA templates is illustrated.

1.1. Mycoviruses

Mycoviruses (fungal viruses) have been described in many fungal species including phytopathogenic fungi (Pearson *et al.*, 2009). Since the first report of a fungal virus which was in 1962 in diseased mushroom, *Agaricus bisporus* (Hollings), more than 200 mycoviruses classified into 10 families have been reported (Ghabrial and Suzuki, 2009). Although the majority of the mycoviruses are associated with dsRNA and to a lesser extent with ss (+) RNA genomes, few mycoviruses with ssDNA, or dsDNA genomes have been reported (Yu *et al.*, 2010). With the exception of few cases, most of the reported mycoviruses have been associated with cryptic or latent infections of their hosts (Buck, 1998). In figure 1, some properties of the major taxonomic families with mycovirus members are shown. Mycoviruses have limited routes of transmission. These include the intercellular routes such as hyphal anastomosis and heterokaryosis or via sexual and asexual spores (Xie *et al.*, 2006; Chu *et al.*, 2004; Buck, 1998). These transmission

limitations are also reflected on the natural host range of mycoviruses, which is restricted to fungal individuals who are vegetatively compatible.






	<i>Totiviridae</i>	<i>Partitiviridae</i>	<i>Chrysoviridae</i>	<i>Reoviridae</i>	<i>Hypoviridae</i>
Genome	monopartite	bipartite	tetrapartite	multisegmented	monopartite
Genome size	4.6 - 7 kbp	1.4 - 2.3 kbp	2.4 - 3.6 kbp	0.7 - 5 kbp	9 - 13 kb
Number of components					
Envelope	-	-	-	-	Unencapsidated membrane vesicle
Particle size	40-43 nm	30-40 nm	35-40 nm	~80 nm	
Associated hypovirulence	Yes	No	Yes	Yes	Yes
Host	fungi protozoa	fungi plants	fungi	fungi plants protozoa vertebrates	fungi

Fig. 1 Properties of the major viral families encompassing mycovirus members.

1.1.1. dsRNA mycoviruses

Mycoviruses with dsRNA genomes are classified into 4 major families based on the number and sequence of their genomic segments. These families are *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, and *Reoviridae*. In addition to the dsRNA mycoviruses, these families encompass members that infect organism other than fungi such as protozoa and plants. The genomes of mycoviruses belonging to these families are encapsidated usually in isometric particles with a diameter of 25-50 nm except for mycoreoviruses which have spherical double-shelled particles with a diameter of about 80 nm (Ghabrial and Suzuki, 2009; Pearson *et al.*, 2009). Interestingly, the proteins encoded by dsRNA mycoviruses, belonging to different genera, share little sequence similarities. Moreover, phylogenetic studies of the most conserved protein among dsRNA mycoviruses, their RNA-dependent-RNA-Polymerase (RdRp), indicate that these viruses are most likely polyphyletic (Ghabrial and Suzuki, 2009).

1.1.1.1. Family *Totiviridae*

Viruses belonging to this family have non-segmented dsRNA genomes (4.6 - 7 kb) and are encapsidated in isometric particles (single molecule per particle) of ~40 nm in diameter. *Totiviridae* genome encompasses two ORFs encoding the CP and RdRp, Virions have a buoyant density in CsCl of 1.40-1.43gcm⁻³. Members of *Totiviridae* that infect phytopathogenic fungi are classified into two genera; *Totivirus* reported in yeasts (e.i *Saccharomyces cerevisiae*) as well as the smut fungus *Ustilago maydis* and *Victorivirus* which infect filamentous fungi. Those members of *Totiviridae* that infect parasitic protozoa belong to either genus *Giardiavirus* or *Leishmaniavirus*.

The amino acid sequences of the RdRp of totivirus share 8 conserved motifs and an overall relatively significant sequence similarity. Diverse RdRp expression strategies have been reported for the different members of *Totiviridae*. For example, *Saccharomyces cerevisiae* viruses; ScV-L-A and ScV-L-BC express their RdRp as a fusion with the CP (*gag-pol*-like) due to a ribosomal frame-shifting. *Ustilago maydis* virus H1 (UmV-HI) on the other hand, produce such a fusion protein without a ribosomal frame-shifting. Proteins that expressed separately as a result of coupled translation have been reported for members of the *Victorivirus* such as *Helminthosporium victoriae* virus 190S (HvV190S).

Totiviruses of yeasts and smut fungi are usually associated with a satellite dsRNA (M-dsRNA) that encodes a toxin protein. The yeast and smut fungal isolates that host the M-dsRNA are immune against the encoded toxin. On the other hand, the isolates that are not associated with the M-dsRNA are sensitive to the produced toxin and are eliminated. This phenomenon is known as the yeast or smut killer system. Well-characterized mycoviral dsRNAs coding for the killer toxins include those associated with *Ustilago maydis* (Park *et al.*, 1996) and *Saccharomyces cerevisiae* (Bostian *et al.*, 1980). Interestingly, the multiplication of the helper totivirus and its satellite dsRNA are regulated by host genes. The overall result of such regulation is support of virus replication to a limit where the viral infection is not harmful to the host. Satellites and defective dsRNA of victorivirus, on the other hand, are not associated with the production of the killer toxin.

1.1.1.2. Family *Partitiviridae*

The viruses classified in this family have bipartite dsRNA genomes (1.4 - 2.3 kb) separately encapsidated in isometric particle of about 30-40 nm in diameter. The two dsRNA

segments are usually similar in size, one encoding the RdRP while the other encoding the CP. Association with a satellite dsRNA is common among members of the this family. Virions have a buoyant density in the range of 1.34-1.39 gcm⁻³.

Besides *Partitivirus*, this family contains two other genera *Alphacryptoviruses* and *Betacryptovirus*. However, only members of the *Partitivirus* have been reported to infect fungi (fungal partitiviruses). Members of the *Alpha*- and *Betacryptovirus* on the other hand, infect plants (plant partitiviruses). Fungal partitiviruses are transmitted mainly by asexual spores and hyphal anastomosis, while plant partitiviruses are transmitted only by pollen and ovule to the seed embryo.

In vitro transcription assays showed that the virions of all fungal and plant partitiviruses are associated with RdRp activity. The RdRp can function as a transcriptase and as a replicase. In the virions, the RdRp transcribe the parental positive strand RNA to produce ssRNA. The produced ssRNA displace the parental positive strand RNA and the later is released out of the virions and serve a template for protein synthesis by the host machinery or packaged in an assembling virion. The replicase activity of the RdRp produces a negative-strand RNA on the packaged positive-strand RNA to make a dsRNA genome.

The complete genomes of many fungal partitiviruses including *Atkinsonella hypoxylon virus*, *Fusarium solani virus 1*, and *Fusarium poae virus 1* have been sequenced. Phylogenetic analysis based on the amino acid sequences of the RdRPs showed that members of the family *Partitiviridae* are clustered into 4 groups. Most of the partitivirus members were grouped in either of two large clusters called subgroup 1 and 2. The average molecular weight of the CPs for the members of subgroup 1 and 2 was 47 KDa and 74 KDa, respectively. Interestingly, the third cluster encompasses fungal and plant partitiviruses together. The hosts of some of these viruses are phytopathogenic fungi. This evokes the probable horizontal transfer of partitivirus members between plants and fungi (Ghabrial *et al.*, 2008).

1.1.1.3. Family *Chrysoviridae*

Multipartite linear monocistronic dsRNA genomes (4 segments of 2.4 - 3.6 kb) separately encapsidated in non-enveloped isometric particles (35-40 nm in diameter) are characteristics for members of the family *Chrysoviridae*. The virions of chrysoviruses have buoyant densities in the range of 1.34-1.39 gcm⁻³. Besides the main components (virus particles, each encapsidating one

of the four genomic dsRNAs), the appearance of extra components, representing empty particles and replication intermediates with distinct densities, is frequent in purified chrysovirus.

Whereas the proteins encoded by dsRNA1 and 2 operate as the RdRp and CP, respectively, the role of those encoded by dsRNA3 and 4 is not obvious yet. Genomes of chrysovirus that infect fungi have relatively long 5' and 3' UTRs with regions of high sequence similarity. Moreover, a (CAA)_n sequence repeat upstream of the start codon exists probably in most of the characterized chrysovirus. Although the (CAA)_n repeat was reported in tobamovirus to act as a translational enhancer, the role of the (CAA)_n repeat in chrysovirus is not clear yet.

Although chrysovirus were classified previously as members of the family *Partitiviridae*, phylogenetic studies of the RdRp indicated that they are more related to toti- than to partitivirus. There is not much known about the replication of chrysovirus. However, *in vitro* transcription studies showed that the virus particles are associated with RdRp activity and that a full length mRNA is transcribed from each dsRNA in a conservative mechanism.

Many chrysovirus occur in mixed infections with other mycovirus or plant viruses and are associated with symptomatic infections of their hosts. Examples include Hv145SV and Hv190SV that infect the phytopathogenic fungus *Helminthosporium victoriae*, ACDACV and CCRSACV that co-infect cherry trees in combination with a partivirus and a suspected totivirus and the obscure chrysovirus AbV-1 and MBV (member of the family *Barnaviridae*) which are associated with the La France disease of cultivated mushroom (*Agaricus bisporus*). In most of the cases, the contribution of each virus in the disease development and the nature of the interactions between the co-infecting viruses are not clear yet.

1.1.1.4. Family *Reoviridae*

Members of the family *Reoviridae* that infect fungi are classified in the genus *Mycoreovirus*. *Mycoreovirus* have 11-12 dsRNA monocistronic genomic segments between 0.7 - 5 kbp in size encapsidated in double-shelled particles about 80 nm in diameter. Except for several RdRp motifs, the functions of the proteins encoded by *mycoreovirus* are not well characterized. To date, only three *mycoreovirus* have been identified. Two of these have been reported in *C. parasitica* and one in the root-rot fungus, *Rosellinia necatrix*. The three *mycoreovirus* are associated with hypovirulence of their fungal hosts.

1.1.2. Positive-strand RNA mycoviruses

Mycoviruses with positive-strand RNA genomes (9-17 kb) and no true virions belong to the families *Hypoviridae*, *Endornaviridae*, and *Narnaviridae* (Ghabrial and Suzuki, 2009). Some of these viruses were classified originally as dsRNA mycoviruses probably because they exist mostly as dsRNA replicative form (RF) in their hosts (Ghabrial and Suzuki, 2009; Pearson *et al.*, 2009; Nuss, 2005).

Members of the family *Hypoviridae* have linear RNA genomes in the size range of 9-13 kb. Their RdRp is closely related to those of *Bymovirus* in the family *Potyviridae*. *Cryphonectria hypovirus 1* (CHV1) is one of the mycoviruses that has been intensively studied for its potential use as a biocontrol agent of the chestnut blight fungus *Cryphonectria parasitica*.

The genomes of Endornaviruses consists of a large RNA (14-17 kbp as estimated from the dsRNA RF encoding a single protein with RdRp and an RNA helicase motifs. To date, only two Endornaviruses infecting a fungal host have been reported. These viruses are *Phytophthora endornavirus 1* and *Helicobasidium mompa endornavirus 1*.

The family *Narnaviridae* encompasses two genera; *Narnavirus* and *Mitovirus*. Members of this family have RNA genomes coding merely for RdRp and the viruses present as RNA-RdRp nucleoprotein complexes. Mitovirus infections associated with hypovirulence in phytopathogenic fungi were reported for *Cryphonectria mitovirus 1* and *Ophiostoma mitoviruses 3a, 4, 5, and 6*.

1.1.3. DNA mycoviruses

Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) is the only reported mycovirus with a DNA genome. The virus has a circular ssDNA segment of 2166 nt encapsidated in isometric particles of 22 nm in diameter. The ssDNA genome encodes two proteins; a replication initiation protein (Rep) and the coat protein (CP). Interestingly, SsHADV-1 is not only associated with hypovirulence of its host but also is transmissible between vegetatively incompatible strains of *S. sclerotiorum*.

1.2. Mycovirus associated hypovirulence.

Reduced or debilitated fungal virulence (hypovirulence) due to mycovirus infections have been reported for several phytopathogenic fungal isolates. This phenomenon has attracted the consideration of the prospective utilization of hypovirulent strains of phytopathogenic fungi and their associated mycoviruses as biocontrol agents as well as models to understand the

mechanisms of fungal pathogenesis. For example, hypovirulent strains of the chestnut blight fungus *Cryphonectria parasitica* were used effectively to control the disease in Europe (Anagnostakis, 1982). When hypovirulent fungal strains of *C. parasitica* were applied onto chestnut, the resident virulent strains were converted to the hypovirulent phenotype (Nuss D. L., 1992). Indeed, the interactions between a fungal host and its mycovirus and their utilization as biocontrol agents are best studied and described for *C. parasitica* and its associated hypoviruses (Dawe, A. L. & Nuss, D. L. 2001).

Mycovirus-associated hypovirulence was also reported in *F. graminearum* (Chu *et al.*, 2002), *Botrytis cinerea* (Castro *et al.*, 2003), *Helminthosporium victoriae* (Ghabrial, S. A. 2001), *Chalara elegans* (Park *et al.*, 2006), *Sclerotinia sclerotiorum* (Zhang *et al.*, 2009; Boland G.J, 1992; Yu *et al.*, 2010), *Sclerotinia homoeocarpa* (Deng *et al.*, 2003; Zhou, T. & Boland, G. J., 1997), *Sclerotinia minor* (Melzer & Boland, 1996), *Ophiostoma novo-ulmi* (Hong *et al.*, 1999), the white root rot *Rosellinia necatrix* (Chiba *et al.*, 2009), *Leucostoma personii* (Hammar S. 1989), and *Diaporthe ambigua* (Preisig *et al.*, 2000).

In most of the above examples, several aspects have hindered our understanding of the biology of hypovirulence and restricted the implementation of mycoviruses or their dsRNAs in biological control or genetic engineering programs. First, the absence of extracellular routes of infection for mycoviruses is the main barrier for their implementation as biological control agents. Second, the unavailability of effective gene transfer systems to utilize the power of hypovirulence-inducing dsRNAs for the control of plant pathogenic fungi or to study their interactions with their hosts. Fortunately, the successful use of *Agrobacterium tumefaciens* to transform yeast and several filamentous fungi has been achieved recently. The availability of agrotransformation protocols for the various species of phytopathogenic fungi would help to understand the interactions between mycoviruses and their fungal host. Finally, the generation of dsRNA from a cDNA clone is technically challenging. When this technical limitation was overcome for the hypovirus of *C. parasitica* as an example, tantalizing findings have been uncovered. The viral and host genes involved in the hypovirulence of *C. parasitica* are now well characterized. Moreover, all the spores produced by a transfected strain of *C. parasitica* have the integrated cDNA as well as the equivalent dsRNA. Natural hypovirulent strains on the other hand, produce dsRNA-free ascospores and many dsRNA free conidia, which would limit the spread of hypovirulence effects.

1.3. Mycoviruses of *F. graminearum*

To date, only few mycoviruses have been isolated from the phytopathogenic fungus *F. graminearum* (Yu *et al.*, 2009; Chu *et al.*, 2002; Theisen *et al.*, 2001). These viruses are *Fusarium graminearum* virus 1, virus 2, virus 3 and virus 4 (FgV1, FgV2, FgV3, FgV4). Whereas the complete nucleotide sequence for FgV1, FgV3 and FgV4 were reported, only a partial sequence with an RdRP motif was published for FgV2. The dsRNA virus reported by Theisen *et al.*, (2001) has not been fully characterized yet. While these viruses are still unassigned to a specific genus, genome organization and phylogenetic analysis showed that FgV1 belongs most likely to the ss (+) RNA mycoviruses while FgV4 belongs to the family *Partitiviridae*. The deduced amino acid sequence of the RdRP of FgV3 showed close relatedness to members of the families *Chrysoviridae* and *Totiviridae* (Yu *et al.*, 2009).

1.4. Fusarium head blight

Fusarium head blight (scab, FHB) is an important fungal disease of small-grain cereals like wheat and barley. Several species of the genus *Fusarium* can cause the disease of which *F. graminearum* is the most common. Other species includes *F. poae*, *F. culmorum*, and *F. avenaceum*. (Osborne and Stein, 2007). The disease epidemics have caused great yield and quality losses worldwide. The reduction of the kernels weight and the contamination of the infected grains with mycotoxins make them unacceptable and not safe for the animal and human consume. In North America, losses due to FHB were estimated to be more than \$1 billion per year (McMullen *et al.*, 1997). Moreover, the germination rate of infected seeds is highly reduced which contribute further to the yield loss (Argyris *et al.*, 2003).

1.4.1. The fungus *Fusarium graminearum*

The major causal agent of head blight in the United States, Canada, and Europe is *F. graminearum* Schwabe [teleomorph: *Gibberella zae* (Schwein.) Petch] (Goswami and Kistler, 2004). In addition to the FHB, the fungus is the main causal agent of the ear rot disease of maize (Tamburic-Ilinic and Schaafsma, 2009). Although phylogenetic studies of a worldwide collection of *F. graminearum* isolates showed that they could be classified into 11 distinct species, the original name *F. graminearum* is still used to describe all of these isolates (Starkey *et al.*, 2007; Leslie and Bowden, 2008). The economic losses and health consequences associated

with FHB are mainly because of the capability of *F. graminearum* to produce mycotoxins such as deoxynivalenol (DON), nivalenol (NIV), zearalenone, fusarin C and aurofusarin (Trail 2009; Bennett and Klich, 2003; Gilbert and Tekauz, 1995). For example, when DON is ingested in ample amounts, it can inhibit the protein biosynthesis in human and mammals, which may lead to death.

1.4.2. The disease cycle of *F. graminearum* in wheat.

As shown in Fig. 2, the disease cycle of *F. graminearum* starts when airborne spores settle on the wheat flower. At temperatures around 25°C and highly humid conditions, the spores germinate and enter the plant through natural openings or degenerating anther tissues. In the early stages of the infection, the fungus spreads intercellularly through the xylem and pith and the plants show no symptoms. At a later stage, the fungus spreads intracellularly and colonizes the tissues. In this phase symptoms such as necrosis, water soaking of the chlorenchyma, and tissue bleaching start to appear. The fungus produces a mycotoxin called DON which acts as virulence factor for wheat and most likely also maize. It causes tissue necrosis and facilitates the fungal spread into the rachis. Due to the accumulation of the DON and the colonization of the fungus in the developing seeds, the emerging wheat grains on an infected plant are small and shrunken. *F. graminearum* produces asexual (conidia) and sexual spores (ascospores) that allow the spread of the fungus to other plant individuals as well as infection inocula for the next seasons.

The sexual life cycle of *F. graminearum* starts when hyphal cells with two distinct nuclei are formed. These binucleate cells develop into the fruiting body initials (perithecium initials) filled with tubular sacs (asci) containing the ascospores. The ascospores are released and initiate the infection of other flowering wheat plants. Conidia, on the other hand, are produced from the sporodochia, which are small masses of hyphae bearing specialized stalks called the conidiophores, where the asexual spores or conidia are formed. Conidia are produced on the surface of infected plants. They serve mainly in the short distance spread of the fungus.

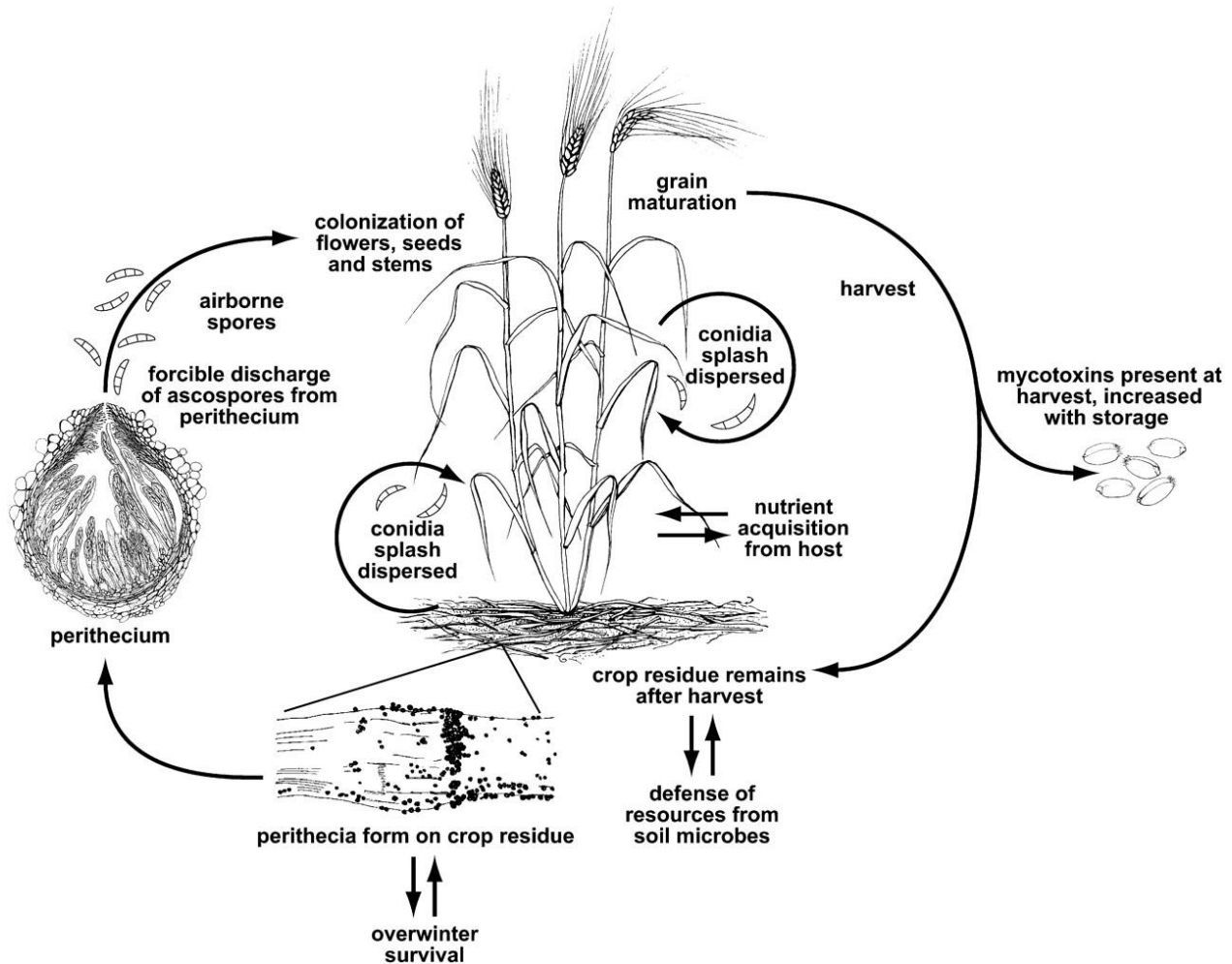


Fig. 2 The life cycle of *F. graminearum* (sexual phase, *G. zeae*), causal agent of Fusarium head blight on wheat.

Figure courtesy from Trail F. 2009.

1.5. Methods for the sequence determination of dsRNA templates.

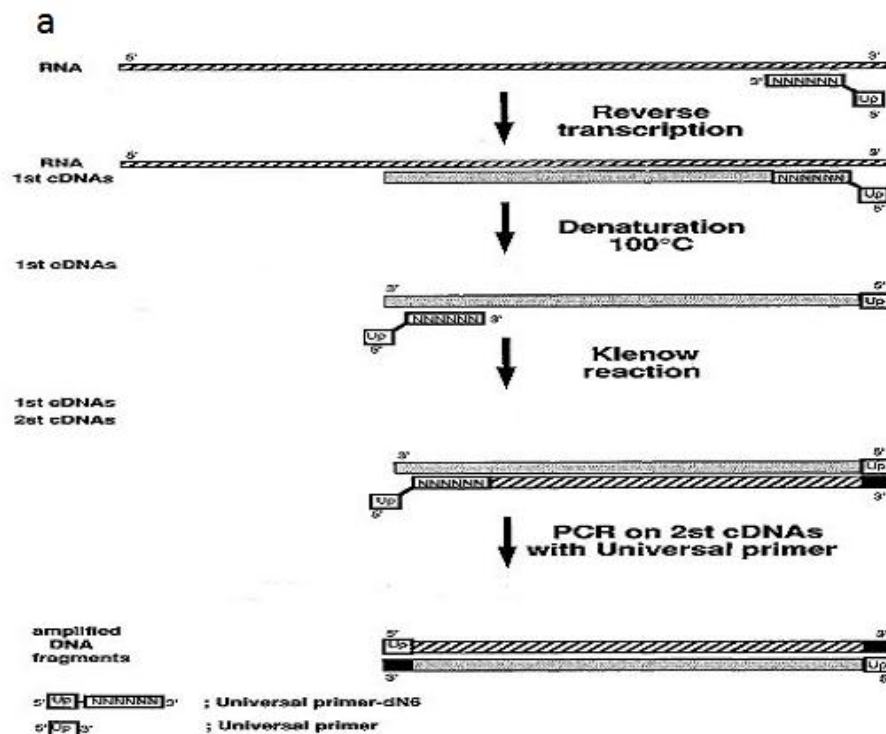
Several methods have been described for the sequence determination of dsRNA templates (Attoui *et al.*, (2000); Potgieter *et al.*, (2002) & (2009); Vreede *et al.*, (1998), Lambden *et al.*, (1992); Imai *et al.*, (1983); Coutts & Livieratos (2003)). These methods include the random PCR (rPCR), Single Primer Amplification Technique (SPAT), and Full Length Amplification of cDNAs (FLAC).

1.5.1. Random PCR (rPCR).

The random PCR (rPCR) involves the use of a primer with a random hexamer at its 3' end (Froussard 1992). This method was established for the random amplification of ssRNAs and was

applied thereafter for genomic dsRNA viral templates (Márquez *et al.*, 2007). An illustration of the basic steps of a rPCR on a dsRNA template is shown in Fig 3a, b. In brief, 1st strand cDNA is synthesized randomly using the hexamer sequences at the 3' of the primer. The 2nd strand cDNA is synthesized with the Klenow Fragment reaction as shown in Fig. 3a. Alternatively, this step might be omitted since the production of the 1st cDNA from a dsRNA template will result in several overlapping cDNAs that will anneal to form a dscDNA as shown in Fig. 3b. Finally, the dscDNA is PCR amplified using the primer employed in the cDNA synthesis but lacks the 3' hexamer sequence.

The sensitivity of rPCR was tested for ssRNA templates by Froussard (1992, 1998). He got intense amplification products starting from template amounts as little as 1 pg. So far, the sensitivity of this method for dsRNA templates was not tested. The need for cloning and sequencing of many PCR products, the filling of gaps, the use of other methods such as RACE (Rapid Amplification of cDNA Ends) for the terminal sequence determination and the requirement for highly pure template to avoid non-specific amplification make the rPCR methods time-consuming, laborious and costly.



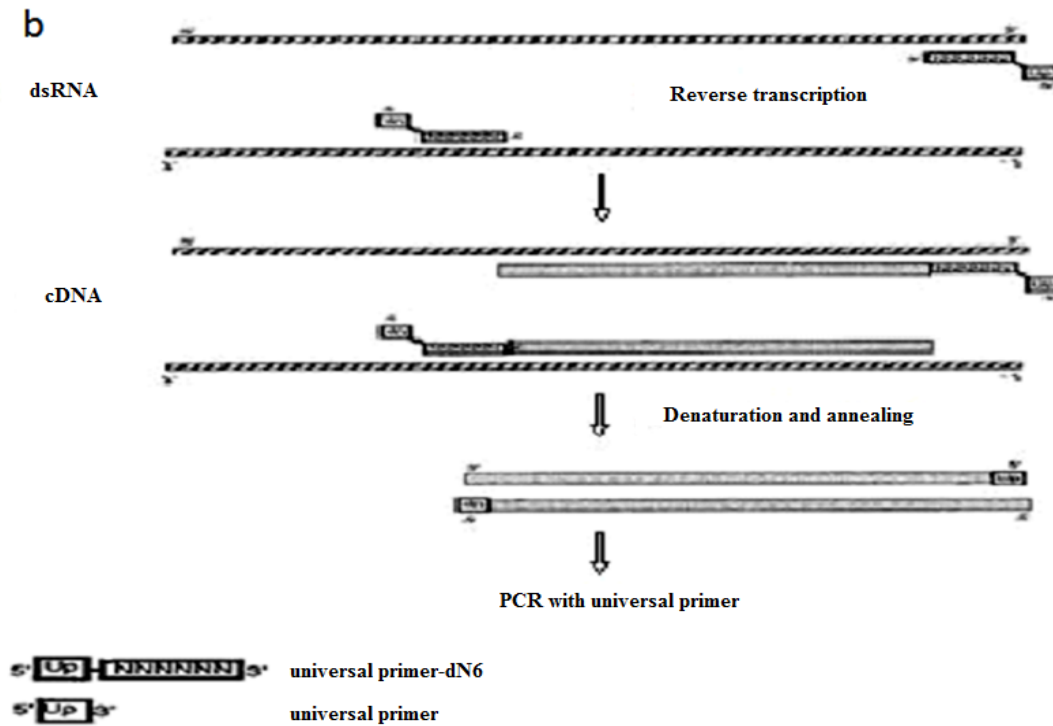


Fig. 3 A schematic illustration of the rPCR method for the sequence determination of dsRNA templates.

a: with, b: without the involvement of the Klenow Fragment reaction.

1.5.2. SPAT and FLAC methods.

A remarkable progress in dsRNA cloning was the establishment of sequence-independent single-primer amplification Technique (SPAT) by Lambden *et al.*, (1992). The method was employed either solely or with modifications to clone dsRNA of different sizes up to 2.5 kb (Bigot *et al.*, 1995; James *et al.*, 1999; Attoui *et al.*, 2000; Zhang & Rowhani, 2000; Chen *et al.*, 2002). The method was further modified to clone larger dsRNAs (>3 kb) (Vreede *et al.*, (1998); Potgieter *et al.*, (2002) and (2009); Mann *et al.*, (2007)). These modifications include enrichment of the longer dsRNA segments by means of sucrose gradients or purification from electrophoresis gels, labeling and size fractionation of cDNA, ligation of primers with extended lengths, the use of anchor primer, which prime themselves for full-length amplification of cDNAs (FLAC), the use of highly toxic chemicals like MMOH for the efficient denaturation of dsRNA. Although such modifications might be useful for the sequence determination of large dsRNA genomes, many of them might not be available in every laboratory e.g. ultracentrifugation, radioactive labeling of cDNA, MMOH's handling and disposal regulations.

As shown in Figures 4 and 5, the SPAT and FLAC methods involve the use of T4 RNA ligase to ligate a DNA oligonucleotide to both 3` ends of a dsRNA template. In the SPAT method the ligated primer is 3`-blocked to prevent concatemer while in the FLAC method the primer is designed to prime itself for cDNA synthesis, where it has two complementary halves separated by a spacer that has a loop structure. In the SPAT method, cDNA from each strand is synthesized using a primer complementary to the 3` part of the ligated primer. In both methods, excess RNA is removed by treatment with NaOH and the complementary cDNAs are annealed for few hours at 65-68°C. A primer complementary to the 5` part of the ligated oligonucleotide is used for PCR amplification of the template.

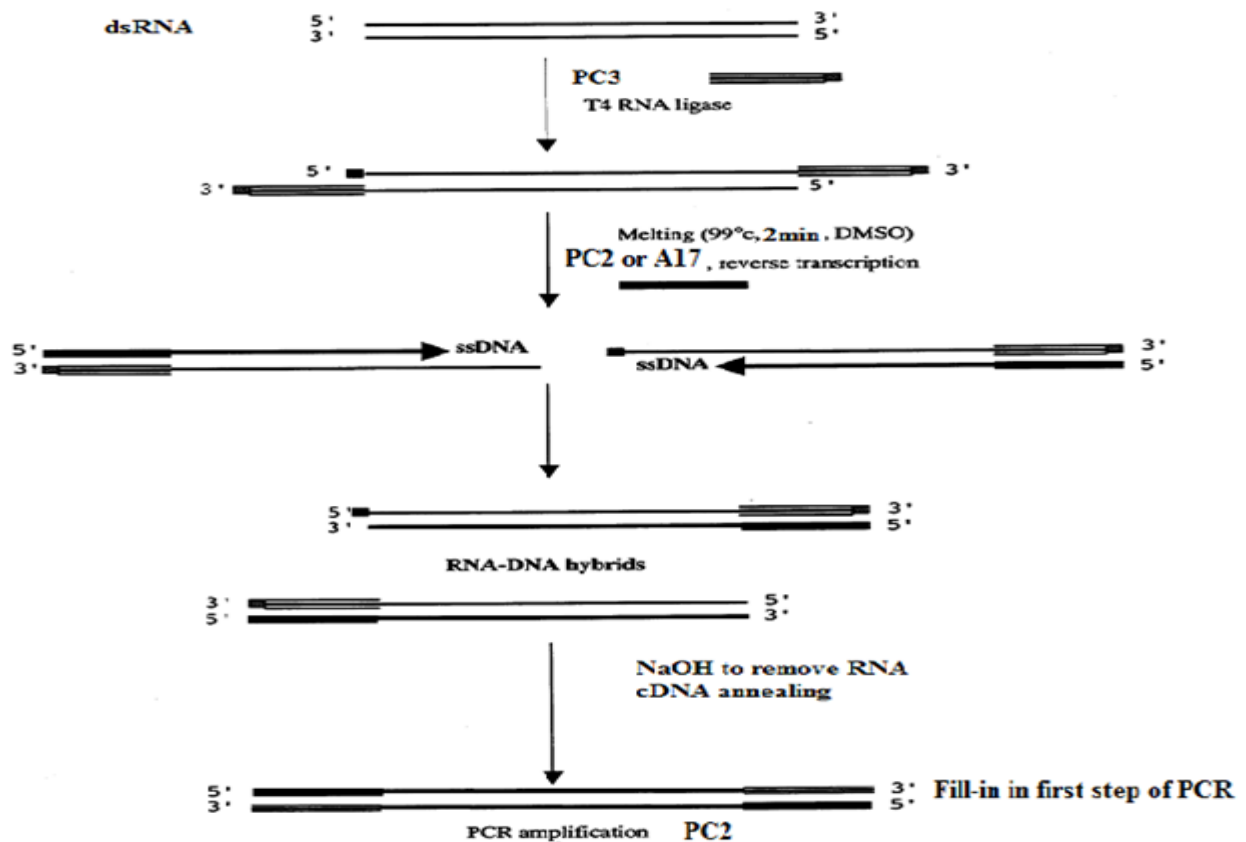


Fig. 4 A schematic illustration of the major steps of the SPAT method.

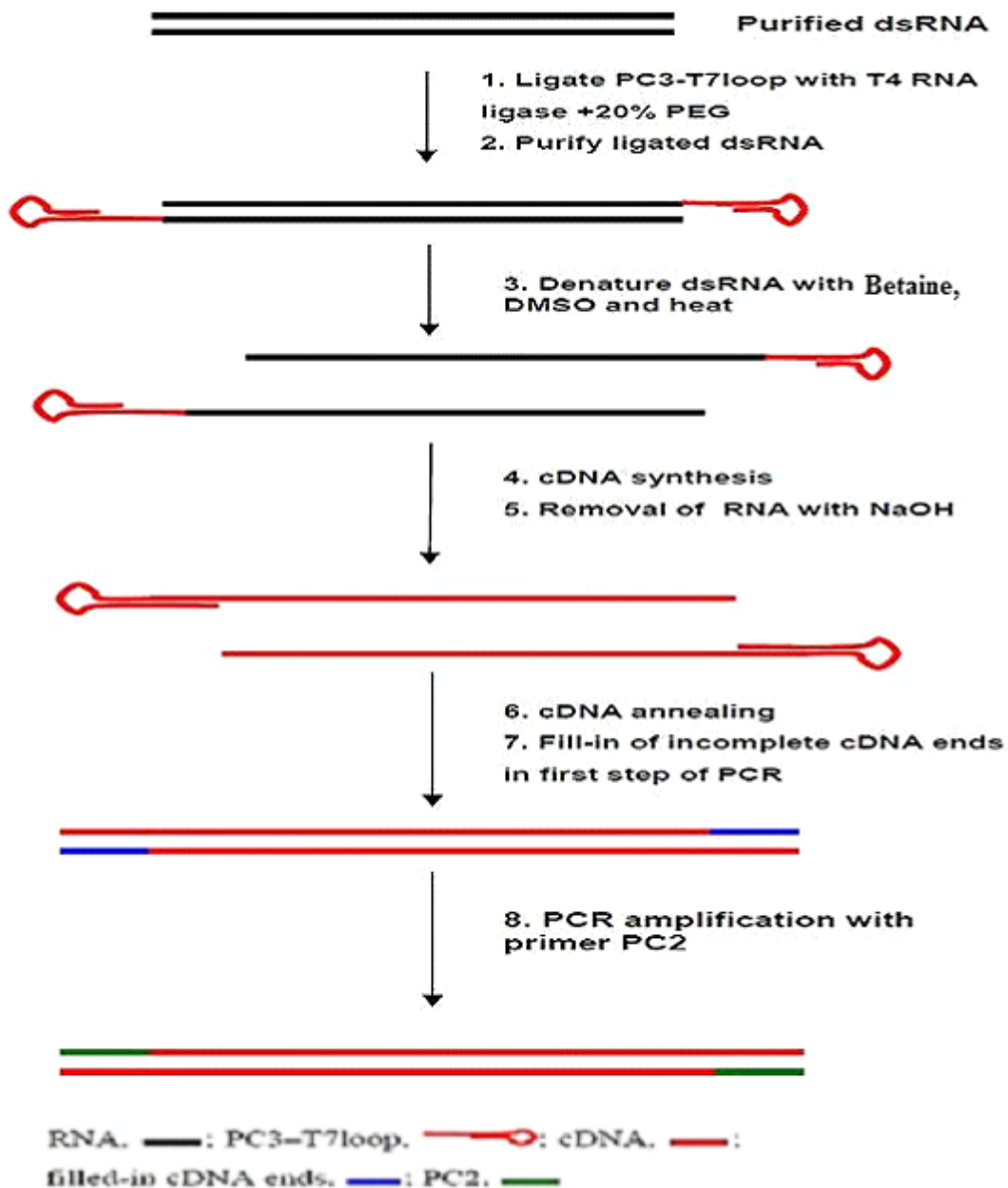


Fig. 5 An illustration of the major steps of the FLAC method.

1.5.3. Direct cloning of dsRNA into dsDNA vectors.

The cloning of viral dsRNAs directly into dsDNA vectors without any previous transcription and amplification steps was never reported so far except for one patent application by Skotnicki *et al.*, (1985). In the current study, the conditions, results, and evaluation of several attempts to clone genomic dsRNA into DNA vectors are presented.

1.6. Aims of this study

The first aim of this study was to characterize a novel mycovirus with segmented dsRNA genomes infecting a *F. graminearum* isolate from China. The characterizations involve the determination of the full-length nucleotide sequence of the virus. Furthermore, it includes molecular characterization and phylogenetic studies of the virus.

Second, the study aims at exploring possible association of the virus with hypovirulence of its fungal host. This involves a comparative phenotypic study of a virus-positive and a virus-free *F. graminearum*. These phenotypes include the growth rate of the fungus, conidiation, formation of perithecia, and infectivity on cereal plants such as wheat and maize.

2. Material and Methods

2.1 Material

2.1.1 Enzymes and chemicals

Restriction enzymes, T4 DNA and T4 RNA ligases, DNaseI, S1 nuclease, Klenow Fragment, RevertAid™ Premium Reverse Transcriptase, and Long PCR Enzyme Mix were obtained from Fermentas (St. Leon Roth, Germany). The Phusion® High-Fidelity DNA Polymerase, Platinum DNA polymerase, Go Taq DNA polymerase, and 5PRIME Taq polymerase were purchased from Finnzyme (Espoo, Finland), Invitrogen (Darmstadt, Germany), Promega (Mannheim, Germany), and 5PRIME (Hamburg, Germany) respectively. Chemicals used in the dsRNA purification, culture media and buffers were obtained from Sigma-Aldrich (Dorset, England), Duchefa (Haarlem, Netherland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), New England Biolab (Frankfurt am Main, Germany). Chemicals used in Southern, Northern, and Western blots were purchased from Roche (Mannheim, Germany).

2.1.2. Microbial strains and culture conditions.

Ten *Fusarium graminearum* strains were isolated from China By the group of Dr. Zhang Deyong, Institute of Plant Protection of the Hunan Academy of Agricultural Sciences in Changsha, China. The isolates were named China 1 to China 10, respectively and grown on complete medium CM for one week at 25°C in the dark. Agar blocks containing fungal mycelia were transferred to 100 ml of CM broth and incubated under shaking (110 rpm) for 4 to 7 days at 25°C. The mycovirus characterized in this study was purified from *F. graminearum* China 9 isolate and named *F. graminearum* mycovirus China 9 (FgV-ch9).

The wild type (WT) *F. graminearum* isolate PH-1 was obtained from T. Miedaner (Miedaner *et al.*, 2000). All of the fungal isolates were preserved on SNA plates as described by Nirenberg (1981) or stored in water at -70°C. For the cultivation of the WT *F. graminearum*, the fungus was grown on CM medium at 28°C under shaking (180 rpm) in the dark.

To induce conidiation, either a mycelial plug (0.5 cm²) or a drop of conidial suspension was place on SNA plates for 2 weeks at 18°C under near-UV light (TLD 36 W-08; Philips, Eindhoven, The Netherlands) and white light (TL 40 W-33 RS; Philips) with a 12h photoperiod.

All of the plasmid clones were propagated in *Escherichia coli* strains XL-1 blue (Stratagene) or NM522 (Pharmacia). Bacterial clones were stored in 50% glycerol at -70°C.

2.1.3. Media and buffers

CM medium (Leach *et al.*, 1982):

- Solution A (100x): 100 g/l Ca(NO₃)₂ x 4 H₂O.
- Solution B (100x): 20 g/l KH₂PO₄; 25 g/l MgSO₄ x 7H₂O; 10 g/l NaCl.
- Solution C (100x): 20% (w/v) Glucose.
- Suspension D (100x): 60 g/l H₃BO₃; 390 mg/l CuSO₄ x 5H₂O; 13 mg/l KI; 60 mg/l MnSO₄ x H₂O; 51 mg/l (NH₄)₆Mo₇O₂₄ x 4H₂O; 5.48 g/l ZnSO₄ x 7H₂O; 932 mg/l FeCl₃ x 6 H₂O.
- Solution E: 1 g Yeast extract; 0.5 g Casein, hydrolyzed by enzymatic cleavage; 0.5 g Casein, hydrolyzed by acid degradation.

Solution A, B and C are sterilized by filtration through 0.2 µm filters, while suspension D is sterilized by the addition of 2 ml chloroform. To prepare one liter of CM, solution E is dissolved in ddH₂O to a final volume of 929 ml and autoclaved. Solid CM is prepared by the addition of 16 g/l granulated agar. After autoclaving for 20 min at 121°C, 50 ml of solution C, 1 ml of solution D, and 10 ml of each solution A, and B are added. In cases where Hygromycin B is to be added for selection purposes, it was supplemented at a concentration of 250 µg/ml.

SNA synthetic nutrient poor medium (Nirenberg, 1981):

To prepare 1 l of SNA medium the following were dissolved in ddH₂O and autoclaved: 1 g KNO₃; 1 g KH₂PO₄; 0.5 g KCl; 0.5 g MgSO₄ x 7 H₂O; 0.2 g Sucrose; 0.2 g Glucose; 16 g granulated agar. Sterilize by autoclaving as described above.

Carrot Agar medium (Klittich and Leslie, 1988 with some modifications).

To prepare 1 l of Carrot agar medium, 400 g of fresh carrots were washed, peeled, cut into small pieces, and boiled in 400 ml of H₂O in a microwave for 10 min. The carrot was further blend in a blender and the juice was filtered through cheesecloth. Before autoclaving for 50 min at 121°C, 20 g of granulated agar were added and the medium volume was brought to 1 l with ddH₂O.

The media and buffers used for the preparation and transformation of *F. graminearum* protoplasts are (amounts per l):

YEPD: 3 g yeast extract, 10 g Bacto peptone, 20 g D-Glucose in ddH₂O.

STC: 20% Sucrose, 10 mM Tris-HCl pH 8.0, and 50 mM CaCl₂.

PEG: 40% PEG4000 and 60% STC.

TB3: 200 g Sucrose, 3 g yeast extract, 3 g casamino acids in ddH₂O.

1.5% agar-TB3: 1.5% granulated agar in TB3.

The media used in the *E. coli* transformation and Blue-White screening of the *E. coli* are prepared according to Sambrook *et al.* (2001) as follows:

TFB buffer:

(100 mM RbCl; 45 mM MnCl₂ · 4 H₂O; 10 mM CaCl₂ · 2H₂O; 3 mM Cl₃CoH₁₈N₆; 10 mM MES-KOH, pH 6.3)

DND:

12.5 M DMSO; 1 M DTT; 10 mM KAcO, pH 7.5

SOB-Medium (per liter):

20 g Tryptone; 5 g Yeast extract; 0.5 g NaCl; 0.2 g KCl. Adjust pH to 7.5.

SOC-Medium:

SOB medium; 20 mM Glucose; 20 mM MgCl₂.

LB-Medium (per liter):

10 g Tryptone; 5 g Yeast extract; 10 g NaCl.

LB-Agar (per liter):

add 15 g Micro-agar to the LB medium.

AIX-Agar (per liter):

Add to the LB-agar medium 150 mg Ampicillin; 47 mg IPTG; 40 mg X-Gal dissolved in 1 ml Dimethylformamid.

LB-amp: is LB-Agar supplemented with 150 mg/l Ampicillin.

All media were autoclave for 20 min at 121°C. Ampicillin, IPTG, and X-Gal were added to the media after autoclaving.

STE buffer (10x)

0.5M Tris-HCl, 1M NaCl and 10 mM EDTA, pH 7.0

2.1.4. Oligonucleotides (primers)

The primers used in this study were designed using the PerlPrimer v1.1.18 software and were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The sequences of primers, listed in 5'-3'-direction, are shown in the table below:

Table 1: List of the primers used in this study:

Name	Sequence and modifications
Primers used for the sequence determination of the mycovirus	
UP-dN ₆	CCTGAATTTCGGATCCTCCNNNNNN
UP	CCTGAATTTCGGATCCTCC
PC3	PO4-GGATCCCGGGAATTCGG(A) ₁₇ -NH ₂
PC3-T7 loop	p-GGATCCCGGGAATTCGGTAATACGACTCACTATATTTTTATAGT GAGTCGTATTA-OH
PC2	CCGAATTCCCGGGATCC
Primers used for the screening of <i>E.coli</i> transformants and sequencing of the clones	
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
T3	ATTAACCCTCACTAAAG
T7	AATACGACTCACTAT
Primers used for the identification of <i>F. graminearum</i>	
CH1	GATAGCGAACAAGTAGAGTGA
CH2	GTCCGTGTTTCAAGACGGGC
Primers used to screen fungal transformants	
HygF	GAATTCAGCGAGAGCCTGAC
HygR	GATGTTGGCGACCTCGTATT
gpdaF	TCCGAAGTAGGTAGAGCGAGT
Primers used in the real-time PCR	
rtRDRPF	GAGTATTACCAGCAACAACCA
rtRDRPR	CCAGTGCCTTATTGTAACCC
Rt3116F	ATGAACTGATACGAAACGGTG
Rt3116R	AGGTGCATACACAAAGTTGAG
rtFF	GCAGCTACACCAGTTAACAG
rtFR	AAAGTGCCGATTCTATACATGG
rt21F	GCCTCTCATTCTATAACGCC
rt21R	ACATCAATCGAATGTCCTCAG
rtzincF	AGTAGTTATGACGATGATGCAC

Name	Sequence and modifications
rtzincR	TCAGTTATCGGGTAGGTGTC
Primers used to construct the Dicer 2 knockout vector	
dic up F	GGGAGTTGCATGATGCAAGGAT
dic up R	agatgccgaccgaacaagagctgtccccGCAAGATAGGTTCGCAAGAATGGA
dic dow F	caatgetacatcaccacctcgtccccCGGGAGTTGGTAAAGATGGCA
dic dow R	TCAAGCGGGAAAACCACTCT
dic nes F	caagcttCAGCTTCGTAGCGTGAAT
dic nes R	caagcttAGACCTGAGAATGAGTATGC
Primers used to construct the overexpression vector	
startFBamHI	cgcggatccATAGGTGCGCGGGGAGAAA
capFBamHI	cgcggatccATGGCATCGAACGCATTGT
zincFBamHI	cgcggatccACATACCAGCAATTCGCCGAT
zincRBamHI	cgcggatccCATACTGCCTGGTGCCAAAACA
FRBamHI	cgcggatccGCATATGCCATTACGCGTTGA
21RBamHI	cgcggatccACAAGCATTTCCGAACCAA
capRBamHI	cgcggatccGTTGCTACTGGCGCCAATTT
Primers used in the duplex PCR, virus transmission, and the semi-Q-PCR	
Start F	ACATAGGTGCGCGGGGAGAAA
124 REV	CTGCAAACCGCTCTGATTCACT
3116RR	CAGTTAGCGGTGTGGTTGGC
4REV	ACAATGACGTTTCAAGCGCC
3100 REV	AAATTAGGCGTCCACTACAAGG
4mid3for	AGTCGGCATGGCAAACAGA
54for	GCAGCAGGAGGATAAGCAT
β -TubF	TGCTGTTCTGGTCGATCTTG
β -TubR	ATGAAGAAGTGAAGTCGGGG
3116F	TGGGTCGTGCGCAAGGAAA
21DR	CTGTACTGGCCATGGCATATTGT
21DI	TTGTCGTGCCTGGGTGCTTAT

Name	Sequence and modifications
3346up F	TGTTACGGCGAGCTAATGTACC
3346up R	CTGGAGGCGTAGCGTTTACT
zincF	GATTTGCTTCGAGCCACAACG
zincR	TGCTAGCTCCGGTGGTTCAAAT

2.2 Methods:

2.2.1 Isolation and purification of dsRNA

The fungus *F. graminearum* China 9 was grown on CM medium for 4-7 days under shaking (100 rpm) at 25°C in the dark. The mycelium was collected by filtration through two Whatman No. I filter papers, washed with distilled water, dried by blotting with paper towels and frozen at -70°C till use.

Double-stranded RNA (dsRNA) was isolated using the CF-11 fibrous cellulose chromatography (Sigma-Aldrich) as described by Preisig et al., (1998) with some modifications. Briefly, 5g of mycelium were put into a 50 ml reaction vessel with one stainless steel grinding ball (25 mm in diameter), frozen in liquid nitrogen and pulverized in a Mixer Mill MM 400 (Retsch, Haan, Germany) at a frequency of 30 Hz for 30 sec. The ground powder was then suspended in 10 ml of 2x STE buffer with 5mg/ml Bentonite and 1.5% (w/v) SDS at 60°C for 5 min. Ten ml of phenol:chloroform:isoamyl alcohol (5:1:1, pH 4.5) and 100 µl of β-mercaptoethanol were added and the mixture was shaken for 30 min at 37°C. After centrifugation at 7,818 \times g for 10 min at RT, the supernatant was mixed with 1g of CF11 and ethanol was added to a final concentration of 17% (v/v). The mixture was shaken for 10 min at 80 rpm and applied into a 15 ml syringe blocked with glass wool. The mixture was pressed into the column and the collected flow through was reapplied to the column. The column was washed with 50 ml of 2x STE containing 17% ethanol (v/v), the bound dsRNA was eluted with 10 ml of 1x STE buffer and finally precipitated with 1 vol of isopropanol for 1 h at -70°C. The pellet was collected by centrifugation, washed with 75% (v/v) ethanol, dried and resuspended in 60-100 µl of distilled water. Part of the extracted dsRNA was treated with DNaseI followed by S1 nuclease (Fermentas) for 30 min each as recommended by the supplier. Aliquots (10 µl) of digested and non-digested dsRNA were separated on 1% Agarose gel containing 0.5 µg/ml ethidium bromide for 1 h at 120 V in 1x TAE buffer and then visualized under ultraviolet light.

The dsRNAs were purified from the agarose gel using the NucleoSpin[®] Extract II (MACHEREY-NAGEL) and served as a template in the methods described below.

2.2.2 DNA extraction using the CTAB method

Grind fungal mycelia in liquid nitrogen with a pestle. Transfer 50 mg of ground powder to a 2 ml tube and add 900 μ l of CTAB buffer. Mix the contents of the tube and incubate at 65°C for 1 h. Centrifuge the tube at 10,000 \times g for 10 min at RT. Transfer the supernatant to a new tube, and add 900 μ l of chloroform and mix by inverting the tube 10 times. Centrifuge as above and transfer the supernatant to a new tube. Precipitate the nucleic acids with 1 vol isopropanol for 30 min at -20°C. Centrifuge the tube at 12,000 \times g for 20 min at 4°C. Wash the pellet with 70% ethanol and dissolve the N.A. in 50-100 μ l TE buffer containing 1-2 μ g RNase A for 1 h at 37°C.

CTAB Buffer: Prepare the CTAB buffer by mixing the following:

100 ml of 1 M Tris HCl pH 8.0

280 ml of 5 M NaCl

40 ml of 0.5 M EDTA

20 g of cetyltrimethyl ammonium bromide (CTAB)

Bring total volume to 1 L with ddH₂O and sterilize by autoclaving.

2.2.3 Phenol extraction method of total nucleic acids

About 100-400 mg mycelium was ground in liquid nitrogen using a mortar and pestle. To the ground mycelia, 0.5 ml of 1x STE buffer containing 1.5% SDS and 20 mg/ml Bentonite was added. The tubes were incubated at 60°C for 10 min. One vol of Phenol : chloroform : isoamyl alcohol (25:24:1) was added and the tubes were incubated at RT for 20 min with shaking. The tubes were centrifuged for 10 min at 7,818 \times g, and the supernatant was transferred to a new tube and re-extracted with Phenol: chloroform: isoamyl alcohol as described above. Nucleic acids were precipitated from the supernatant with 1 vol of isopropanol for 30 min at -70°C. The pellet was washed with 0.5 ml 70% ethanol, dried for 5 min at 50°C, and dissolved in 100-300 μ l ddH₂O.

2.2.3 Random PCR (rPCR)

Up to 100 ng of a mixture of the 5 dsRNA segments were mixed with 0.25, 0.5, 1 or 2 μ M of the up-dN₆ primer, incubated at 99°C for 2 min and quenched on ice for 5 min. Two hundred U

of RevertAid™ Reverse Transcriptase, 50 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, 1 mM dNTPs and 20 U of RiboLock™ RNase Inhibitor were added, and the mixture was incubated at 43°C for 1 h. At this stage, the cDNA was used either directly in the subsequent PCR or for the synthesis of second strand cDNA as follows. The cDNA was heated at 99°C for 2 min then quenched on ice for 5 min. Ten U of the Klenow Fragment, 50 mM Tris-HCl (pH 8.0 at 25°C), 5 mM MgCl₂, 1 mM DTT, 0.5 mM dNTPs and ddH₂O to a final volume of 50 µl were added. The reaction was incubated at 37°C for 30 min. The dscDNA was purified with the NucleoSpin® Extract II, eluted in 30 µl ddH₂O and stored at -20°C till use. To test the sensitivity of the rPCR, 10⁻⁸-10⁻¹ µg were reverse transcribed in the presence of 2 µM of the universal primer-dN₆ and directly amplified as described above without the Klenow Fragment reaction.

Amplification of the dscDNA took place in a reaction mixture containing; 1 µl of cDNA, 1x Taq Buffer advanced, 1.5 U of Taq DNA Polymerase (5 PRIME), 2 mM MgCl₂, 0.25 mM dNTPs and 1 µM of the UP primer. The thermal cycling was performed in a Biometra T1 thermo cycler as follows: one cycle at 94°C for 2 min, 65°C for 1 min and 72°C for 1 min, then 35 cycles of 94°C for 40 sec, 52°C for 30 sec and 72°C for 3 min followed by a final extension step at 72°C for 8 min.

2.2.4 Single Primer Amplification Technique (SPAT)

Primer PC3 described by Potgieter *et al.* (2002) was ligated to the 3' ends of the dsRNA as follows. About 250 ng of PC3 primer were ligated to 200 ng of a mixture of dsRNAs at a molar ration of >40:1. The ligation mixture included: 50 mM HEPES/NaOH, pH 8.0, 20 mM MgCl₂, 0.01% BSA, 1 mM ATP, 3 mM DTT, 10% (v/v) DMSO, 20% (w/v) (PEG)₆₀₀₀, 20 U of Ribolock Rnase inhibitor and 30 U of T4 RNA ligase in a final volume of 30 µl. The ligation components were incubated at 37°C for 6 h then at 18°C descending at a rate of 2°C per h down to 12°C. The dsRNA was purified with the NucleoSpin® Extract II kit, eluted in 40 µl ddH₂O, and concentrated in the SpeedVac vacuum concentrator (Savant Instruments Inc., USA) for 10-15 min.

In another treatment, the PEG₆₀₀₀, DMSO, BSA, and Ribolock RNase inhibitor were omitted from the ligation mixture and the reaction was incubated overnight at 16°C. The primer-ligated dsRNA was purified from excess primer with the NucleoSpin® Extract II and used in the subsequent RT-PCR.

The reverse transcription, removal of the RNA and annealing of the cDNAs were carried out basically as described for the FLAC method below with one exception: that is about 100 ng of the Oligo (dT)₁₈ were used to prime the PC3-dsRNA in the cDNA synthesis reaction.

2.2.5 Full length Amplification of cDNA (FLAC)

About 250 ng of PC3-T7 loop primer described by Potgieter *et al.*, (2009) were ligated to 200 ng of a mixture of dsRNAs as described for the SPAT method above. The purified primer-ligated dsRNA was denatured at 98°C for 2 min in the presence of 1M betaine and 2.5% (v/v) DMSO then quenched on ice for 5 min. The cDNA synthesis reaction contained: 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM dNTPs, 20 U of Ribolock RNase inhibitor and 400 U of RevertAid™ Premium Reverse Transcriptase. The reaction was incubated at 50°C for 1 h followed by 15 min at 55°C. RNA was digested with 0.1 M NaOH at 70°C for 20 min, followed by the addition of 0.1M Tris-HCl pH 7.5 and 0.1 M HCl to neutralize the reaction. The cDNA was then incubated at 68°C for one h followed by 1-2 h at 65°C.

The amplification mixture, calculated for a final volume of 25 µl, contained: 5 µl of cDNA, 1x of the provided DNA polymerase buffer, 320 µM of each dNTP, 2 mM MgCl₂ and 1.25 µM of PC2 primer and 2.5 U of one of the following DNA polymerases. Phusion® High-Fidelity DNA Polymerase with Phusion GC Buffer, Platinum DNA polymerase, Go Taq DNA polymerase with the colorless buffer, 5PRIME Taq polymerase with advanced buffer set, or Long PCR Enzyme Mix with the long PCR buffer. The mixtures were incubated in a Biometra T professional thermo cycler at 72°C for 2 min followed by 95°C for 2 min and then subjected to 35 cycles of 95°C for 25 sec with an increment of 1 sec per cycle, 65°C for 30 sec and 68°C or 72°C (as recommended by the manufacturer) for 5 min followed by a final step of 72°C for 10 min.

2.2.6 Direct ligation of dsRNA into pJET1.2 and pGEM®-T vectors:

About 200 ng of a mixture of the dsRNAs were ligated into the *E. coli* cloning vector pJET1.2 (Fermentas) or into pGEM®-T (Promega) at a molar ratio of about 4:1 (insert:vector). The ligation mixture contained 2.5 Weiss U of T4 DNA ligase and 25 U of T4 RNA ligase, 1 mM ATP, 5% (w/v) PEG6000 and 40 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT in a final volume of 15 µl. The reaction was incubated at 14°C for 24 h. In a second treatment, the reaction was

incubated at 14°C for 24 h then at 4°C for extra 24 h. Ligated plasmids were transformed into XL-1 blue *E. coli* competent cells by means of heat shock at 42°C for 1 min. The obtained clones were screened by means of either PCR or restriction digestion. Moreover, part of the positive clones was sequenced. The experiment was repeated 3 times.

2.2.7 Cloning and sequencing:

PCR products were purified from the agarose gel or directly from the PCR tube with the NucleoSpin[®] Extract II, cloned into pGEM[®]-T cloning vector or pJET1.2 and transformed into *E. coli* competent cells either by heat shock or by electroporation as described in the following sections. The sequences were determined using the Sanger sequencing with an ABI 3730XL sequencer (Eurofins MWG Operon) and assembled into contigs using the DNA Baser V2.90 RC.

2.2.7.1 Preparation of electrocompetent cells.

About 1-2 ml of *E. coli* XL-1 blue cells were cultured in 500 ml LB medium under shaking at 37°C until the optical density of the culture at wavelength of 600 measures 0.5- 0.6. The culture was incubated on ice for 20 min and then the bacterial cells were pelleted at 2,000 x g at 0-2°C for 15 min. The pellet was resuspended and washed two times with 250 ml and a third wash with 10 ml of ice-cooled ddH₂O. Each of the washing steps was performed at 3,000 x g for 15 min. The pellet was resuspended in 800 µl of 7% DMSO, divided into 50 µl aliquots, frozen in liquid nitrogen, and stored at -80°C

2.2.7.2 Preparation of chemical competent cells.

E. coli NM522 was cultured on LB-agar overnight at 37°C. Several colonies were transferred into 1 l Erlenmeyer flask with 30 ml of SOB supplemented with 20 mM MgCl₂ and cultured until the OD₅₅₀ reaches ~0.5. The culture was transferred into a sterile glass-tube and incubated on ice for 15 min. The tube was centrifuged at 1500 x g at 4°C for 10 min, and then the pellet was resuspended in 10 ml TFB buffer, and incubated on ice for 10 min. The suspension was centrifuged as described above, and then the pellet was resuspended in 4 ml TFB buffer, and incubated on ice for 10 min. DND solution (140 µl) was added to the suspension, mixed gently, and incubated on ice for 15 min. The last step was repeated once

more. The competent cells were transferred (200 µl aliquots) into Eppendorf tubes and used immediately for transformation.

2.2.7.3 Transformation of competent cells.

Chemical competent cells (50 µl) were mixed with the cloning vector in an Eppendorf tube and heat-shocked at 42°C for 1 min in a water bath. In case of electrocompetent cells, the cloning vector was purified from salts after the ligation reaction by ethanol precipitation. About 20-30 µl of the competent cells were mixed with the purified vector and electroporated at 1250 V for 4-6 msec.

After the heat- or electric-shock, the tubes were incubated on ice for 2 min, then 700 µl of SOC medium were added, and the cells were cultured for 1 h at 37°C. The bacteria were cultured overnight on AIX-LB agar (100 – 150 µl/plate) at 37°C. White colonies were screened by PCR, using vector-based primers flanking the cloning site. Colonies with positive PCR results were cultured in LB-amp overnight at 37°C and plasmids were purified as described in the following section.

2.2.7.4 MiniPreps and restriction digestion.

Plasmids were purified from transformed bacterial cultures using the E.Z.N.A. Plasmid Miniprep Kit I (PEQLAB Biotechnologie GMBH, Erlangen, Germany). Restriction digestions with the appropriate enzymes were performed according to the manufacturer instructions.

2.2.8 Molecular identification of China 9 isolate.

DNA from *F. graminearum* China 9 isolate was extracted with the CTAB method and subjected to PCR using primers CH1 and CH2 to amplify part of the 28S rRNA gene as described by Ninet *et al.*, (2005). The amplified products were purified with the NucleoSpin® Extract II and cloned into pGEM®-T vector according to the manufacturer instructions. Positive clones were sequenced by Eurofins MWG BmbH.

2.2.9 Purification of Virus-Like Particles

Virus-Like-Particles (VLPs) were purified from China 9 isolate as described by Aoki *et al.*, (2009) with some modification. Briefly, 6 grams of frozen mycelium were pulverized using a Mixer Mill MM 400 (Retsch, Haan, Germany) at a frequency of 25 Hz for 1 min and suspended in 60 ml of 0.1 sodium-phosphate buffer (pH 7.0). The suspension was shaken for 30

min at 4°C, mixed with 20% (v/v) of (1:1) chloroform:n-butanol and centrifuged at 8000xg for 10 min (Rotor SS34 Sorvall). The chloroform:n-butanol step was repeated 2-3 times until the supernatant was free of the red pigment from *F. graminearum*. To precipitate the virus, polyethelene glycol (PEG₆₀₀₀) and NaCl were added to the supernatant at a final concentration of 8% and 1%, respectively. After 1 h of incubation at 4°C, the precipitate was collected by centrifugation at 10,000 x g for 15 min at 4°C (rotor SS34 Sorvall) and the pellet was resuspended in 10 ml of 0.05 sodium-phosphate buffer (pH 7.0) at 4°C for 2-4 h. The suspension was layered onto 20% (w/v) sucrose cushion and ultra-centrifuged at 105,000 x g in a 50.2Ti rotor (Beckman) for 2 h at 4°C. The pellet was resuspended in 1 ml of 0.05 M sodium-phosphate buffer (pH 7.0) and purified by CsCl density equilibrium centrifugation (120,000 x g) at 5°C for 22 h in a SW 55Ti rotor (Beckman). The gradient was fractionated and VLPs were collected by pelleting at 120,000 x g for 2 h at 4°C in a 50.2Ti rotor (Beckman). The pellet was resuspended in 100 µl of 0.01 M sodium-phosphate buffer (pH 7.0)

2.2.9.1 Transmission Electron Microscope (TEM).

A copper grid was placed on a drop of purified virus particles on a hydrophobic surface (a piece of parafilm), incubated at RT for 5-10 min, then washed with 40 drops of water, and dried with a piece of filter paper. The virus particles were stained with 2% (w/v) of uranium acetate for 2 min, dried as above, and observed using a transmission electron microscope (LEO 906E, Zeiss, Germany).

2.2.10. Hyper immunization of rabbits.

Production of polyclonal antibodies for FgV-ch9 was carried out in the laboratory of Prof. F. Rabenstein, Julius Kühn-Institute, Quedlinburg, Germany. Every 2-3 days, a 100 µl suspension of purified VLPs in 0.9% NaCl was injected into a rabbit intravenously (5 injections). The amount of injected VLPs was 50 µg in each of the first two injections, 60 µg in each of the third and fourth injections, and 80 µg in the fifth injection. Blood samples (bleedings), 6- 9 ml each, were collected from the rabbit directly before the first injection (Null serum) as well as 20, 27, and 33 days after the first injection (sera 2- 4). Sera were obtained by spinning the collected blood samples after aggregation of erythrocytes at 8,000 x g at 4°C for 15 min to sediment the cellular debris. The supernatant (Serum) was stored aliquoted at -20°C until use.

2.2.11. Purification of the antibodies.

Virus-free *F. graminearum* PH-1 mycelium was ground in liquid Nitrogen using a mortar and pestle. About 50 mg of the ground mycelium was mixed with 750 µl of PBS buffer and 250 µl of serum in an Eppendorf tube. The tube was incubated at 37°C for 1 h under gentle shaking. Precipitated proteins (non-specific Antibodies bound to non-viral antigens) were pelleted by centrifugation at 10,000 \times g at RT for 15 min. The supernatant (supposed to contain the viral-specific antibodies) was collected and stored at -20°C until use. The purity and sensitivity of the antibodies were tested by means of Western blot analysis as described in section 2.2.17. For this purpose, dilutions of the antibodies (1:500 and 1:1000) were employed to bind to partially purified viral proteins. Anti-rabbit alkaline phosphatase conjugate (Sigma) was used as the second antibody.

2.2.12. Ultrastructural studies.

Fresh 3-days old mycelia cultures on CM-plates of *F. graminearum* China 9 originating from single conidia and exhibiting different growth rates (restricted, moderate, normal) were used for the ultrastructural studies. In addition, as a control the WT PH-1 isolate was included in the study.

2.2.12.1 Primary and secondary Fixations

Fixation of the mycelia were carried out by covering the plates with the fixation solution (2% Glutaraldehyde in 75 mM Cacodylate buffer pH 7,0) for 30 min at RT, then for 3 h on ice. This will preserve the tissues by means of protein crosslinking. Excess glutaraldehyde was removed by 3 times washing with 75 mM Cacodylate buffer. For the ease of handling aerial mycelia, 2% Agar in 75 mM Cacodylate buffer pH 7,0 was poured onto the plates. Small agar pieces containing mycelia tissues were cut out and used in the next steps. To preserve lipid saturated membranes, a second fixation, using 1% OsO₄ in 75 mM Cacodylate buffer pH 7.0 overnight at 4°C was performed. Excess osmium tetroxide was removed by 3 times washing with 75 mM Cacodylate buffer (1x 1 min, 1x 10 min, 1x 20 min).

2.2.12.2 Dehydration, infiltration, and embedding

The water content of the tissues was gradually decreased as follows. The tissues were incubated successively in 30, 50, 70, 90, and 100% acetone at 4°C for 10 min each, followed by

additional two incubations in 100% acetone for 10 min each, at RT. Infiltration of the embedding medium (Spurr) was performed by incubating the dehydrated tissues successively in:

1 part Spurr : 3 parts acetone for 1.5 h, 1 part Spurr : 1 part acetone for 3 h, 3 parts Spurr : 1 part acetone overnight, 100% Spurr for 4 h, then the Spurr was exchanged and the tissues were incubated in the fresh embedding medium overnight. The tissues were embedded in silicone molds and incubated at 70°C for 16 h to let the Spurr polymerize.

The Spurr medium: 5g Embedding Medium ERL 4221D (Serva 21041), 3g D.E.R. 736 (Serva 18247), 13g Nonenylsuccinic anhydrid (Serva 30812), 0.2g Dimethylaminoethanol (Serva 20130).

2.2.12.3 Sectioning and TEM.

Using an Ultramicrotome (Leica) with a diamond knife, ultrathin sections (~70 nm) were cut and mounted on a copper grid (150 mesh), filmed with Mowital. For contrasting, the grids were incubated on a drop of 2% Uranyl acetate for 10 min followed by 10 min on 2% lead citrate. The sections were observed in a TEM (LEO 906E, 100kV) with a CCD Gatan-Camera and the software Digital Micrograph 3.3.4.

2.2.12.4 Immunohistology.

To localize the particles of FgV-ch9 in its fungal host, fresh mycelia tissues were fixed, dehydrated, and sectioned as described above with little modifications. Briefly, the primary fixation was performed with 2% PFA (paraformaldehyde) in 50 mM MSB buffer pH 6.8 (100 mM PIPES, Sigma P-6757; 10 mM EGTA, Sigma E-4378; 5 mM MgSO₄; pH 6.8 with KOH). The secondary fixation was achieved with 0.5% OsO₄ for 1 h on ice. The washings after each fixation were done with 50 mM MSB buffer. Ethanol was used instead of acetone for the dehydration and infiltration. Embedding was carried out with LR-White medium and polymerized in gelatin capsules for 2 h at RT then for 36 h at 50°C in O₂ free atmosphere. The ultrathin sections (70-80 nm) were mounted on Mowital-coated nickel grids.

The grids were incubated on a drop of 50 mM MSB for 30 min and then were blocked with 3% BSA + 0.2% BSA-C in MSB. The grids were incubated on a drop of the purified antibodies (section 2.2.11) at a dilution of 1:800 for 1 h at RT and then washed 3 times with 1% BSA + 0,07% BSA-C in MSB buffer. The grids were incubated on a drop of the gold-conjugated antibody (1:40) for 1 h at RT, washed with 50 mM MSB, and then incubated on 1%

Glutaraldehyde in H₂O for 10 min. The grids were washed 3 times, 5 min each with H₂O and contrasted with 2% Uranyl acetate for 10 s and 0.2% lead citrate for 15 s.

2.2.13 Northern Blot analysis.

Purified dsRNAs were separated in 1% agarose gel containing 2% formaldehyde in TAE buffer (40 mM Tris-acetate, and 1 mM EDTA, pH 8.0) for 1 h at 120 V. The gel was soaked in 0.1 M NaOH for 30 min before neutralization with 1.5 M Tris-HCl pH 7.5 containing 0.5 M NaCl for 20 min at room temperature. The RNAs were capillary transferred onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) in 10x SSC buffer overnight. The RNAs were fixed on the membrane by UV cross-linking (Herolab) for 1 min then the membrane was blocked with DIG Easy Hyb (Roche) for 30 min and the RNAs were hybridized with DNA probes at 50°C overnight. Five DNA probes, P1-P5 specific for dsRNAs 1-5 respectively (P1: 2122-3021, P2: 1679-2850, P3: 36-1016, P4: 29-1226, P5: 884-2074) were digoxigenin (Dig)-11- dUTP-labeled by PCR amplification as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Chemiluminescent signals of RNA-probe hybrids were detected with a DIG detection kit and a CDP-Star kit (Roche) using LAS camera.

2.2.14 Southern Blot analysis.

Genomic DNA of the WT and transformed strains of *F. graminearum* were isolated using the CTAB method and digested overnight with the appropriate restriction enzymes. Digested DNA was then resolved in 1% agarose gel by electrophoresis for 1 h at 100 V. Denaturation, neutralization, capillary transfer, hybridization, and detection were carried out as described in section 2.2.13 with some modifications. Briefly, the 2% formaldehyde was omitted from the agarose gel and the denaturation with 0.1 M NaOH and the neutralization with 1.5 M Tris-HCl were performed for 10 min each. The capillary transfer was performed in 20x SSC buffer. Finally, the blocking of the membrane with DIG Easy Hyb was extended to 2 h. Signals were detected with the Fast Red Reagent (Sigma).

2.2.15 Protein sequence analysis.

Proteins of purified virus particles were separated in a 12% SDS-PAGE for 2 h at 50 mA and stained with 0.1% (w/v) Coomassie Blue R-250 (CBB, Bio-Rad). To identify the sequence of the viral structural proteins, the stacking and separation gels were prepared using fresh

reagents and were polymerized for 18 - 24 h. Viral proteins were separated as described above and electro-blotted to a Roti®-PVDF membrane (ROTH, Germany) for 1 h at 35 mA. The membrane was stained for 10 sec with Coomassie Blue and then rinsed in ddH₂O. The protein bands were cut out with a sterile razor blade and incubated at 4°C till processing. The partial identification of the protein sequences was carried out at the Institut für Klinische Chemie at the Universitäts-Klinikum Hamburg-Eppendorf (UKE, Germany) with the Q-TOF mass spectrophotometry using the reverse-phase HPLC.

2.2.16 Labeling of virus surface proteins.

About 10 – 15 µg of purified virus particles were mixed with 5 mM freshly-prepared EZ-Link Sulfo-NHS-Biotin (Thermo scientific) and incubated for 25 min at RT. The reaction was stopped by the addition of 1.5 µl of 1M Tris-HCl pH 7.5. Labeled proteins were denatured, separated in SDS-PAGE, blotted, and detected as described in the following section.

2.2.17 Western blot

Proteins were denatured by adding one vol of 2x Laemmli buffer (Laemmli, 1970), heating for 5 min at 95°C, and then quenching on ice. Denatured proteins were separated in 12.5% SDS-PAGE for 1.5 - 2 h at 30 mA, blotted to a PVDF membrane for 1 h at 100 V, which was then blocked with 5% of skimmed milk in 1x PBS buffer. Depending on the nature of the protein under study (Biotin-labeled, bound to Rabbit-IgGs), the membranes were incubated with either ExtrAvidin®- or antirabbit-alkaline phosphatase conjugate diluted as recommended (Sigma) in 1% (w/v) skimmed milk for 1 h at 37°C. Labeled proteins were finally detected with the CDP-Star (Roche) and LAS camera. or with the Fast Red Reagent and photographed

2.2.18 Relative quantification PCR.

Double-stranded RNA was phenol-extracted from 50 µg of purified virus particles treated with DNase I for 30 min at 37°C. In a second treatment, the dsRNA was purified from the infected mycelium by means of cellulose chromatography as described above. For each reverse transcription reaction, 200 ng of the extracted dsRNAs was mixed with 0.5 µM of a segment-specific primer (Table 1) in the presence of 10% (v/v) DMSO and incubated at 98°C for 2 min then quenched on ice for 5 min. The cDNA was synthesized with 200 U of RevertAid™ Premium Reverse Transcriptase (Fermentas) in a mixture containing 50 mM Tris-HCl (pH 8.3 at

25°C), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, 0.5 mM dNTPs and 20 U of RiboLock™ RNase Inhibitor (Fermentas) at 50°C for 45 min followed by 55°C for 10 min. Serial dilutions of the synthesized cDNA (10⁻¹, 10⁻² and 10⁻³) were made. Two µl cDNA from each dilution were amplified in a reaction mixture containing; 1x of SYBR Green I Master (Roche) and 0.4 µM of each primer (forward and reverse primer per each segment, see Table 1) in a final volume of 20 µl. The thermal cycling was performed in a LightCycler® 480 Multiwell Plate 96 (Roche) as follows: one cycle at 95°C for 10 min, then 35 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 20 sec followed by one cycle of melting at 95°C for 5 sec, 60°C for 1 min and 97°C at a continuous acquisition mode of 5 per °C. The products were cooled to 40°C for 10 sec. The denaturation and extension steps were carried out at a ramp rate of 4.4°C/sec while the annealing and cooling steps at 2.2 and 1.5°C/sec respectively.

2.2.19 Virulence assay on wheat heads.

Wheat plants Nandu c.v. (Lochow-Petkus, Bergen-Wohlde, Germany) were grown under greenhouse conditions till the flowering stage. Infection of the plants took place in an infection chamber that provides a 12h photoperiod, 20°C, and 70% humidity. At the anthesis stage, each of two alternate central spikelets was inoculated with a 10-µl suspension containing 500-1000 conidia. Inoculated spikes were covered with plastic bags sprayed with water for the first 3 days. Spikes were collected 3 weeks post-infection. As positive and negative controls, spikes were infected with the wild-type strain and water, respectively. For each treatment, three independent positive transformants were used to inoculate 30 spikes.

2.2.20 Virulence assay on maize cobs.

Maize inbred line A188 (Green and Philips, 1975) was used for the virulence assay. The plants were grown and infected in a greenhouse at 26°C-30°C, 60-80% humidity, and natural daily photoperiod. In short-day periods, artificial light was supplemented for 12 h. Pollination of the maize silks was manually performed three days before inoculation. Using a syringe and cannula, 4 ml conidia suspension (4 x 10⁴ conidia/ml) was injected into the silk channel of the primary ears of each maize cob. Inoculated cobs were covered with plastic bags misted with water for the first 3 days. As positive and negative controls, several maize cobs were inoculated with the wild-type *F. graminearum* PH-1 strain and water, respectively. Disease severity

measurements were determined 5 weeks after inoculation as described by Reid and Hamilton (1995).

2.2.21 Growth assays

The growth rate of *F. graminearum* China 9 isolate harboring various amounts of FgV-ch9 as well as of the wild-type transformants was determined by placing a mycelial plug (0.5 cm²) on agar plates of CM and SNA media. The cultures were incubated at 25-28°C in the dark. The diameters of the growing mycelia colonies were measured with a ruler daily for 1 week.

2.2.21.1 Production of Perithecia.

Carrot agar plates were inoculated with 10 µl of *F. graminearum* conidia suspension (~100 conidia) and were incubated at 25°C in the dark up to one week until the plate is covered with aerial hyphae. The mycelia were vigorously pressed down with 1 ml of sterile 2.5% (v/v) Tween 60 solution using a sterile glass rod while spreading the solution all over the plate. Remaining mycelia clumps were removed and the plates were incubated at 25°C in the dark or under a mixture of near UV and white light with a 12 h photoperiod up to three weeks. Produced Perithecia were counted under a stereoscope.

2.2.21.2 Transmission of FgV-ch 9 through conidia

Mycelial plugs of *F. graminearum* China 9 isolate with undefined, high, moderate, or low virus titers were cultured on SNA plates for 2 weeks to induce conidiation. Conidia were collected from the SNA plate by pouring 50 µl ddH₂O across the plate, pipetting the water out, and streaking it on 1.6% water agar plates. Using a stereoscope, a single conidium was cut out from the water agar plate under sterile conditions and cultured on a CM plate layered with a sterile cellophane membrane. Twenty single conidia were isolated as described above. The CM plates were then incubated in the dark at RT for 4-5 days. Mycelia was harvested and ground in liquid nitrogen. Total nucleic acids were extracted with the standard phenol-chloroform method and serve as a template in RT-PCR to detect FgV-ch9. The RT-PCR conditions are described in the following subsections.

2.2.21.2.1 Reverse transcription:

Phenol-extracted total nucleic acids (1 - 2 µg) were denatured in the presence of 1 µM of either of the antisense primers 4REV, or 3100REV and 10% DMSO in a final vol. of 10 µl at

98°C for 2 min and then quenched on ice for 3 min. To the denatured N.A., 10 µl of the RT mix were added and the reaction was incubated at 42°C for 45 min, then at 70°C for 10 min. The RT mix contained; 250 mM Tris-HCl (pH 8.3 at 25°C), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT, RiboLock™ RNase Inhibitor, 200 µM dNTPs, and 40 U of M-MuLV Reverse Transcriptase (Fermentas).

2.2.21.2.2 PCR:

The PCR reaction contained; 10% of the RT reaction (2 µl), 1x PCR buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 0.4 µM of the primer 4REV, 0.4 µM of the primer 54for (alternatively, primers 3100REV and 4mid3for were used), and 1 U of FirePol Taq polymerase (Espoo, Finland) in a final volume of 25 µl.

Cycling conditions: an initial denaturation step at 94°C for 3 min, then 30 cycles at 94°C for 30 sec., 57°C for 30 sec, and 72°C for 1 min. A final extension step was performed at 72°C for 5 min.

2.2.22 Expression of FgV-ch9 in *F. graminearum* PH-1

The ORFs of dsRNA 2, 3, 4, and 5 of FgV-ch9 were amplified by PCR with specific primers (Table 1). The PCR products were digested with BamHI and ligated into PAN 7.1 over-expression vector (Fig. 6) at the BamHI restriction site. The vector was excised with the appropriate enzyme and transformed into protoplasts of *F. graminearum* PH-1. Positive transformants were determined by means of PCR and Southern blot analysis. The expression of FgV-ch9 genes in the transformants was determined by means of semi-quantitative PCR as described in the following section.

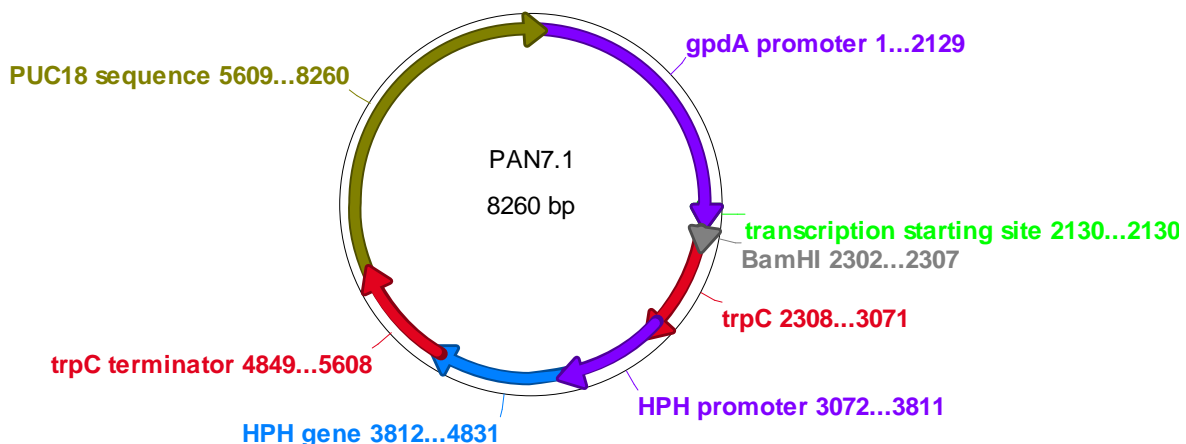


Fig. 6 PAN 7.1 over-expression vector.**2.2.22.1 Semi-quantitative PCR.**

Total nucleic acids were extracted from the positive transformants (section 2.2.22) with the phenol-chloroform method, treated with DNase I for 30 min at 37°C, and the RNA was purified with phenol-chloroform extraction. Reverse transcription was performed using Oligo (dT)18 primer and 200 U of RevertAid™ Premium Reverse Transcriptase as described by the manufacturer. The cDNA was amplified in the PCR (29 cycles, T_m 55°C) using β-tubulin specific primers (β-TubF and β-TubR, Table 1). PCR products were resolved in agarose gel electrophoresis, photographed, and analyzed using the Image software. A new set of PCRs were performed using a set of specific primers (dsRNA2; 21DI/21DR, dsRNA3; 3116F/3116RR, dsRNA4; 3346up F/3346up R, dsRNA5; zincF/zincR, Table1) for the correspondent transgenes. Based on the Image software calculations, the amount of cDNAs introduced into each PCR were supposed to be equal.

2.2.23 Dicer 2 gene disruption by double homologous recombination

Dicer 2 gene of *F. graminearum* was knocked out by the double homologous recombination method. A construct was synthesized so that a hygromycine cassette (selection marker) was flanked by two homologous fragments. The homologous fragments were produced by PCR (amplicon size 850-1000bp), gel purified and mixed with a double amount of SmaI-excised hygromycine fragment in a fusion PCR. A nested PCR was carried out using 100 ng of the fusion PCR product to amplify the desired product and to reduce the smear. The nested PCR product was ligated into pGEM-T vector, propagated in *E. coli*, and purified with a MiniPrep kit. The disruption construct was release from the plasmid by digestion with the appropriate enzymes and used for *F. graminearum* transformation.

2.2.24. Preparation of *F. graminearum* protoplasts.

About 1×10^6 conidia were inoculated in 100 ml YEPD medium and incubated overnight in the dark at 150 rpm at RT. Grown mycelia was harvested by filtration through a 200 μm Wilson-sieve, washed with 200 ml sterile water, and dried on a stack of sterile filter paper. Meanwhile the enzyme solution was prepared (2.5% (w/v) Driselase, 0.5% (w/v) Lysing enzymes, and 1.2 M KCl), stirred for 30 min, centrifuged at 3,000 \times g at RT, and the supernatant was filtrated through 0.2 μm sterile filters. About 1 g of the dried mycelia were mixed with 20 ml

of the enzyme solution and incubated for 90 min at 30°C with gentle shaking. The protoplasts were filtered twice through a 100 µm and 40 µm Wilson-sieves, respectively. The protoplasts were pelleted at 2000 \times g for 10 min at RT and washed with 10 ml STC medium. Protoplasts were finally resuspended in STC solution at a concentration of 1×10^7 protoplasts per ml.

2.2.24.1. Transformation of *F. graminearum* protoplasts with plasmid constructs.

In a 50 ml Falcon tube, 200 µl protoplasts (2×10^7) were gently mixed with 10 µg of linearized plasmid and incubated at RT for 20 min. To induce transformation, 1 ml of the PEG solution was added and the tube was incubated further at RT for 20 min. Cell wall regeneration was induced by adding 5 ml TB3 medium and incubating the tube overnight at RT at 100 rpm. Protoplasts were pelleted at 5000 rpm for 10 min at RT and resuspended in 300-500 µl of TB3 medium. Protoplasts were mixed with 50 ml of 1.5% agar-TB3 medium (cooled to 50°C) containing 250 µg/ml Hygromycin B and poured equally into 5 petridishes (96 mm). The plates were incubated overnight at 28°C in the dark and then overlaid with 10 ml/plate of 1.5% agar-TB3 medium containing 250 µg/ml of hygromycin B. The plates were incubated as above for few days and the emerging colonies were transferred onto CM plates supplemented with 250 µg/ml Hygromycin B.

2.2.24.2 Protoplast transfection with purified VLPs.

Protoplast transfection was carried out as described by Kanematsu *et al.*, (2004) and Sasaki *et al.*, (2006) with some modifications. CsCl-purified VLPs were filtrated through a 0.2 µm Millipore sterile filter and eluted in 300 µl of 0.01 Na-phosphate buffer, pH 7.0. The purified VLPs contained 60 ng/µl of dsRNA as was measured with a pico-drop spectrophotometer. In a 15 ml Falcon tube, 100 µl protoplasts (1×10^7) were gently mixed with 3-5 µg of VLPs and incubated on ice for 30 min. To induce transformation, 500 µl of sterile 60% (w/v) PEG₄₀₀₀ supplemented with 10 mM of MOPS pH 7 and 10 mM of CaCl₂ were added, the contents were mixed gently and incubated at 25°C for 25 min. Cell wall regeneration was induced by adding 2 ml TB3 medium and incubating the tube overnight at RT with no shaking. Protoplasts were pelleted at 4000 rpm for 10 min at RT and resuspended in 300 µl of TB3 medium. Protoplasts were cultured on CM plates (100 µl/plate) at 25°C in the dark for one week until the mycelia cover the whole plate. From each plate, 3-4 mycelial plugs ($\sim 0.5 \text{ cm}^2$) were cut out; each was

transferred to a cellophane-CM plate, and incubated for 4-5 days at 25°C in the dark. Part of the mycelia was used for Nucleic acid isolation and RT-PCR to check for positive transfectants.

2.2.25 Data analysis and accession numbers.

Sequence similarity searches were performed in the National Center for Biotechnology Information (NCBI), Swissprot, and EMBL databases using the BLAST program. The presented E values of the BLAST results indicate the reliability of the alignment results where it is defined as the probability due to chance that there is another alignment with a similarity greater than the scored result. Typically, a good E value from a BLAST search is e^{-5} or lower depending on the query size. Multiple alignments of nucleic and amino acid sequences were carried out using the CLUSTALX2 with the default parameters. The shading of multiple alignments was performed using the BOXSHADE Server (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic trees were constructed with the Molecular Evolutionary Genetic Analysis MEGA4 program (Tamura K. *et al.*, 2007). A bootstrap test was conducted with 100 replicates for the neighbor-joining (NJ) tree. Fungal and viral sequences, other than those isolated from *F. graminearum* China 9 isolate, were retrieved from the GenBank and EMBL databases, (Table 5). Primers were designed using the PerlPrimer v1.1.18 software. Relative quantification analysis was carried out using the LightCycler® 480 Software release 1.5.0 (Roche). The zinc fingers were detected according to Persikov *et al.*, (2009) using the online program (<http://compbio.cs.princeton.edu/zf/form.html>). FgV-ch9 genomic sequences were deposited in GenBank with accession numbers HQ228213-HQ228217 for dsRNA1 to dsRNA5, respectively. Statistical analysis of the results was performed using the Student's t-test of the Excel software. Values less than 0.05 ($P < 0.05$) were considered statistically significant.

3. Results

3.1. Optimization of the methods for the sequence-determination of dsRNA templates.

Several methods have been optimized in this study for achieving the full-length sequence of dsRNA templates. These methods include a set of PCR-based approaches such as the random PCR (rPCR), Single Primer Amplification Technique (SPAT), and the Full Length Amplification of cDNA (FLAC). Moreover, the direct cloning of dsRNA templates into a DNA vectors without any transcription or amplification steps was introduced and evaluated.

3.1.1. Random PCR (rPCR).

The rPCR products created different banding patterns after agarose gel electrophoresis depending on the conditions employed. In the presence of $2\mu\text{M}$ of the primer-dN6, several distinct bands were amplified from 100 pg of dsRNA (Fig. 7, lane 4). When more dsRNA served as a template, the size range and the intensity of the bands increased (Fig. 7, lanes 1-3). A single or no amplification products were obtained when 10 pg or less were applied into the rPCR (Fig. 7, lanes 5-8).

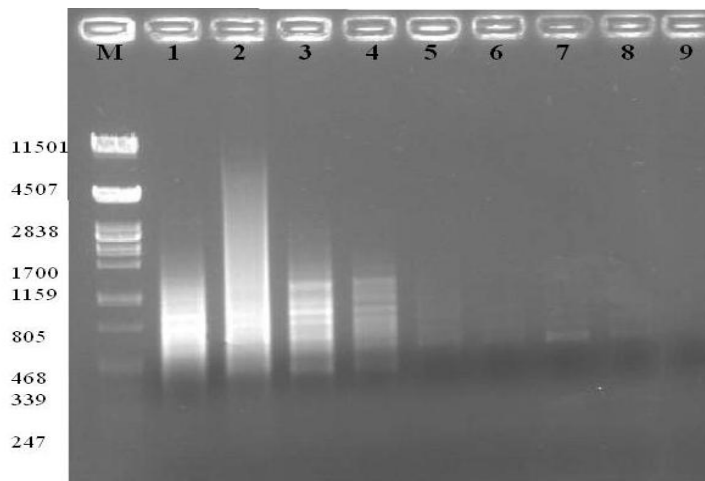


Fig. 7 Agarose gel electrophoresis showing the sensitivity of the rPCR for the amplification of dsRNA templates.

The dsRNAs were reverse transcribed with $2\mu\text{M}$ of the primer-dN6 and the cDNA was introduced directly into the PCR without a Klenow Fragment reaction. Different amount of the dsRNAs were employed: 1: 100 ng, 2: 10 ng, 3: 1 ng, 4: 100 pg, 5: 10 pg, 6: 1 pg, 7: 0.1 pg, 8: 0.01 pg, 9: no dsRNA negative control, M: λ -PstI marker.

The use of the Klenow Fragment for the second strand cDNA synthesis produced a banding profile with a size range of 0.4 - 2.2 kb compared to 0.4-3 kb when the cDNA was

applied directly into the PCR (Figs. 8A and 8B). In the absence of the Klenow Fragment reaction, increasing the concentration of the primer-dN₆ from 0.25 to 2 μ M substantially increased the intensity of the bands but not their size range (0.4 – 3 kb, Fig. 8B). On the other hand, both the size range and the intensity of the bands were reduced with decreasing amounts of the primer, when the Klenow Fragment reaction was included (Fig. 8A). Sequences of the rPCR products reveal an unequal representation of the 5 viral segments.

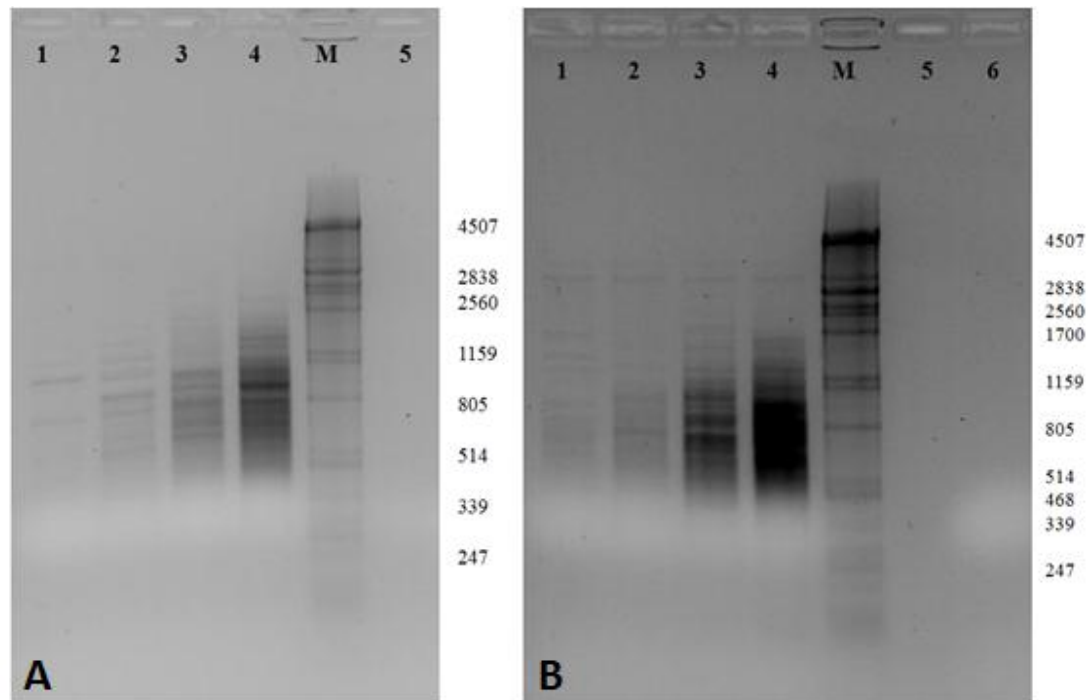


Fig. 8 Agarose gel electrophoresis of rPCR products amplified under different conditions.

A mixture of 5 dsRNAs (100 ng) was reverse transcribed into cDNA with different amounts of the primer-dN₆. The products were separated on a 1% agarose gel in 1x TEA buffer for 40 min at 120 V and visualized by ethidiumbromide staining. **A:** Second strand cDNA was synthesized using the Klenow Fragment. The concentration of the primer-dN₆ was **1:** 0.25 μ M, **2:** 0.5 μ M, **3:** 1 μ M, **4:** 2 μ M, **M:** λ -PstI marker, **5:** no dsRNA control. **B:** The cDNA was applied directly to the PCR without a Klenow Fragment reaction. The concentration of the primer-dN₆ was **1:** 0.25 μ M, **2:** 0.5 μ M, **3:** 1 μ M, **4:** 2 μ M, **M:** λ -PstI marker, **5:** empty, **6:** no dsRNA negative control.

3.1.2. Single Primer Amplification Technique (SPAT)

Null to 5 of the dsRNA segments were amplified in full when the ligation of the PC3 primer was performed at 37°C in the presence of 10% (v/v) DMSO and 20% (w/v) (PEG)₆₀₀₀ (Fig. 9, lanes 2, 3, 5, 7 and 9). Furthermore, when the primer-ligated dsRNAs were denatured

with DMSO alone, full-length cDNAs were not obtained as was reflected in the PCR results (data not shown). The type of the DNA polymerase employed in the amplification played a key role in the number of full-length products obtained (Fig 9, lanes 2, 3, 5, 7 and 9). For each DNA polymerase, the number of amplified segments is presented in Table 2. When the primer ligation reaction was carried out at 16°C in the absence of DMSO and (PEG)₆₀₀₀, several truncated PCR products with sizes less than 1 kb were obtained (data not shown).

Table 2. The efficiency of different DNA polymerases in the SPAT and the FLAC methods.

DNA polymerase	Producer	SPAT ¹	FLAC ¹
Platinum DNA polymerase	Invitrogen	0	0
Taq Polymerase	5 PRIME	2-3	4-5
GoTaq	Promega	4-5	1-2
Phusion [®] High-Fidelity DNA Polymerase	Finnzyme	2-4	5
Long PCR Enzyme mix	Fermentas	0	1-2

¹The values represent the numbers of full-length PCR products obtained.

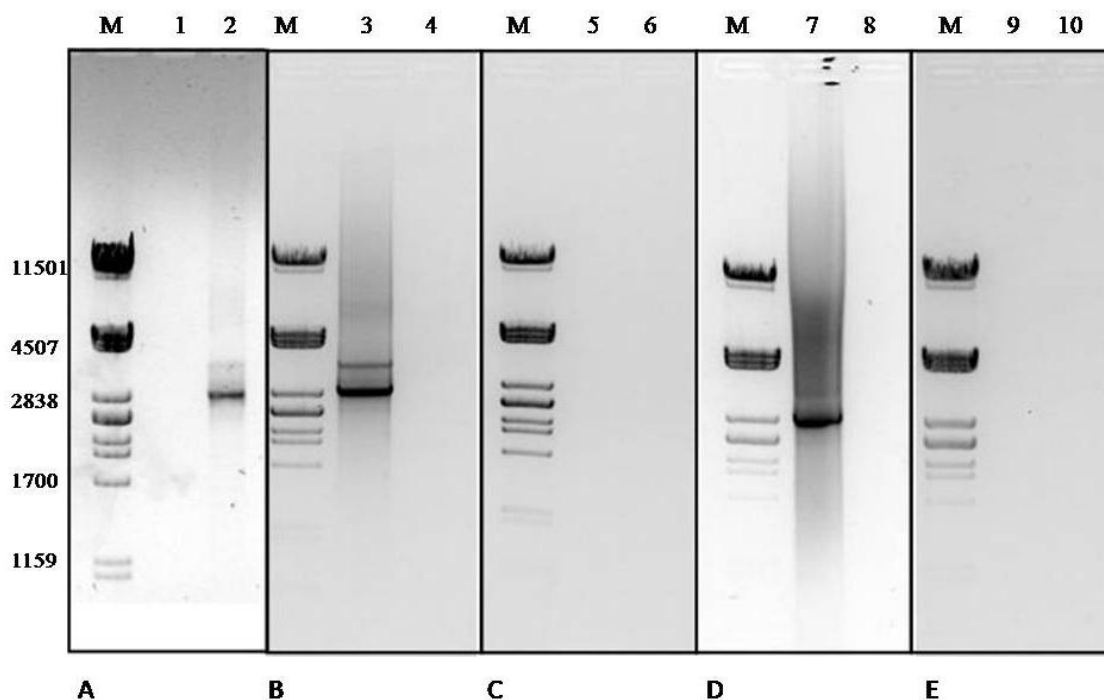


Fig. 9 Electrophoresis pattern of RT-PCR products obtained by the Single Primer Amplification Technique.

The ligation of the PC3 primer was performed at 37°C in the presence of 10% (v/v) DMSO and 20% (w/v) (PEG)₆₀₀₀. Different DNA polymerases were used in the PCR: **A:** Finnzyme, **B:** GoTaq, **C:** Platinum, **D:** 5 PRIME, **E:** Long PCR Enzyme Mix. Lanes **2, 3, 5, 7** and **9:** amplified products. Lanes **1, 4, 6, 8** and **10:** negative control. **M:** λ -PstI marker. Each polymerase was tested 3 times.

3.1.3. Full Length Amplification of cDNA (FLAC)

When the PC3-T7 loop primer was ligated to the mixture of the dsRNAs at 37°C in the presence of 10% (v/v) DMSO and 20% (w/v) PEG₆₀₀₀ as described above, full-length amplicons representing all or part of the segments were obtained depending on the DNA polymerase employed (Fig. 10). The use of the Phusion[®] High-Fidelity DNA Polymerase and the supplied Phusion GC Buffer resulted in the amplification of the 5 dsRNAs in one RT-PCR reaction (Fig. 10, lane 2). Similar results were obtained for the DNA polymerase supplied by 5 PRIME (Fig. 10, lane 7). Restriction digestion of the cloned full-length PCR products resulted in 5 different profiles representing all of the segments (data not shown). Clones with different digestion profiles were sequenced and analyzed. The 5 segments have conserved 5' and 3' terminal sequences, a known property of most segmented viral genomes.

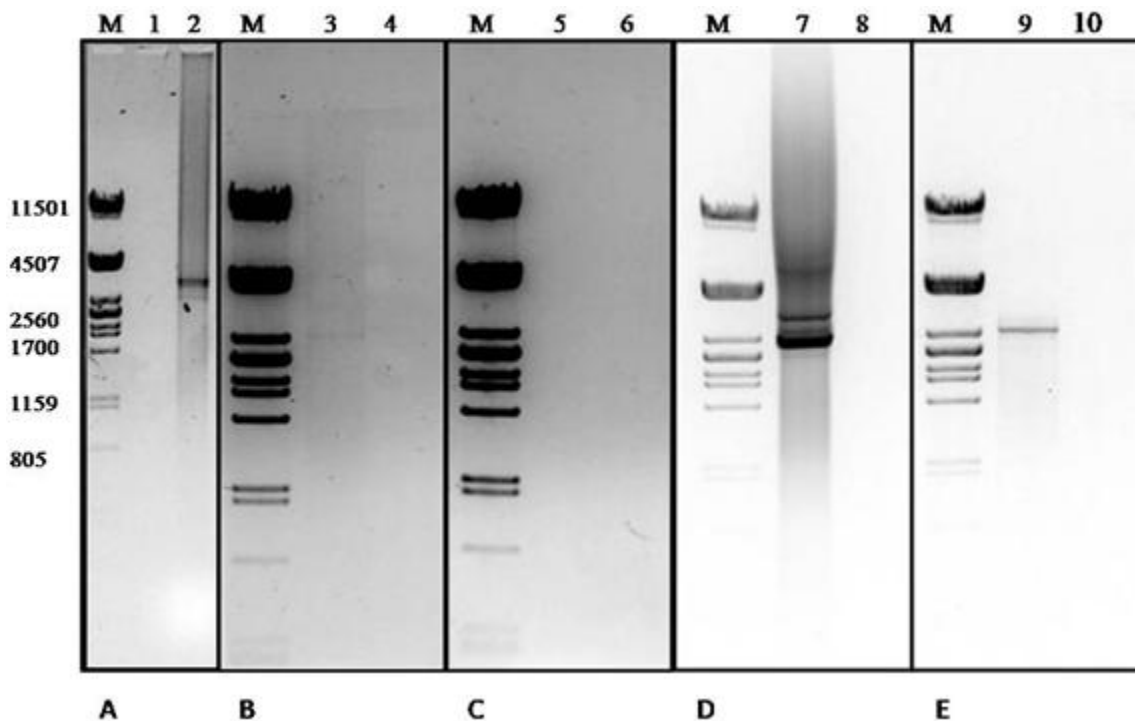


Fig. 10 Agarose gel electrophoresis of RT-PCR products obtained by the FLAC method.

The ligation of the PC3-T7 loop primer was performed at 37°C in the presence of 10% (v/v) DMSO and 20% (w/v) (PEG)₆₀₀₀. Different DNA polymerases were used in the PCR: **A:** Finzyme, **B:** Platinum, **C:** GoTaq, **D:** 5 PRIME, **E:** Long PCR Enzyme Mix. Lanes **2, 3, 5, 7 and 9:** amplified products. Lanes **1, 4, 6, 8 and 10:** negative control. **M:** λ -PstI marker.

3.1.4. Direct cloning of dsRNA into DNA vector

The ligation of 200 ng of dsRNA mixture into 50 ng of the pJET1.2 vector produced as few as 5-9 transformants of which 2-4 had an insert longer than 150 bp regardless of the ligation conditions employed. PCR screening of the transformants showed that the longest insert length obtained under the conditions employed is 1 kb (Fig. 11).

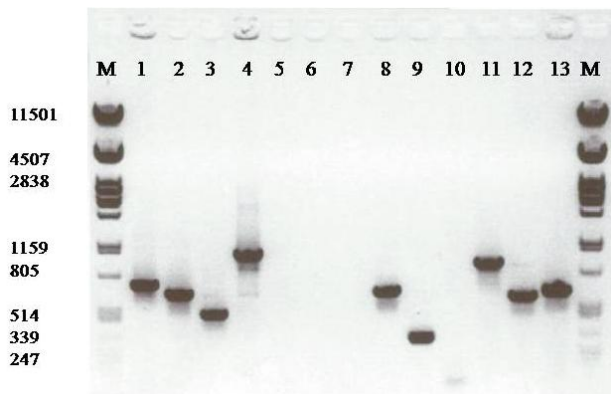


Fig. 11 Agarose gel electrophoresis of the PCR products obtained after direct cloning of dsRNA into pJET1.2 vector.

Bacterial colonies were transformed with pJET1.2 vector after ligation with dsRNA. The inserts were amplified with primers flanking the cloning site. **M:** λ -*Pst*I marker, **1-13:** each

When the dsRNA was ligated into pGEM[®]-T vector for 24 h about 7 transformants were obtained 3 of which contain an insert of 150 – 2100 bp in length. Restriction digestions of the clones with enzymes flanking the insert (*Nde*I and *Sac*II) produced variable profiles. While in some clones a complete release of the insert was observed (Fig. 12, lanes 2, 5), in other clones the insert was not released indicating that one of the restriction sites might be missing (Fig. 12, lane 3).

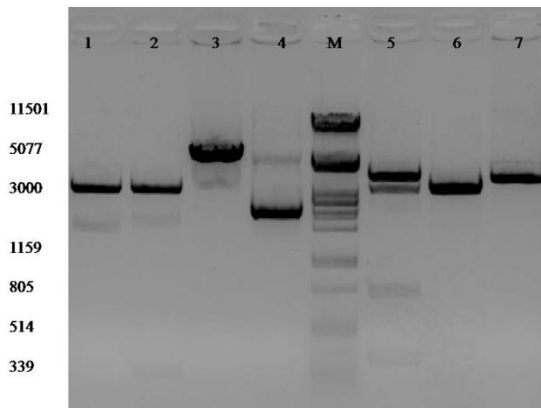


Fig. 12 Restriction digestion profiles of pGEM[®]-T vectors cloned with dsRNA segments.

The ligation reaction was carried out for 24 h. **M:** λ -*Pst*I marker, **1-7:** clones digested with *Sac*II and *Nde*I.

Although increasing the ligation time to 48 h resulted in improved transformation efficiency (Table 3), the size range of the cloned fragments remains similar to that obtained after 24 h of ligation. The identities of part of the clones were further checked by sequencing (data not shown). The calculated transformation efficiencies for the direct cloning experiment are summarized in Table 3.

Table 3. The transformation efficiency of dsRNA-ligated pJET1.2 and pGEM[®]-T vectors.

Cloning vector	Transformation efficiency ^a & insert size	
	Ligation time 24 h	Ligation time 48 h
pJET1.2	1.22e ¹ (up to 1 kp)	3.60e ¹ (up to 1 kp)
pGEM [®] -T	2.80e ¹ (up to 2.1 kp)	1.60e ² (up to 2.2 kp)

^a Calculated according to <http://www.sciencegateway.org/tools/transform.htm>. The results represent the average of 3 independent experiments.

3.2. Identification of the fungus isolate

The fungus isolate China 9 showed morphological properties similar to those described for *F. graminearum* including the size, shape, and septation of conidia, as well as the colony color and morphology in agar (Data not shown). To support these results, molecular identification was carried out based on sequence analysis of the ribosomal RNA genes (rDNA) which are commonly used in identification and taxonomic studies at the species level (Ninet. *et al.*, 2005). Multiple alignment and phylogenetic results (Fig. 13) revealed China 9 isolate to be closely related to the *F. graminearum* species complex.

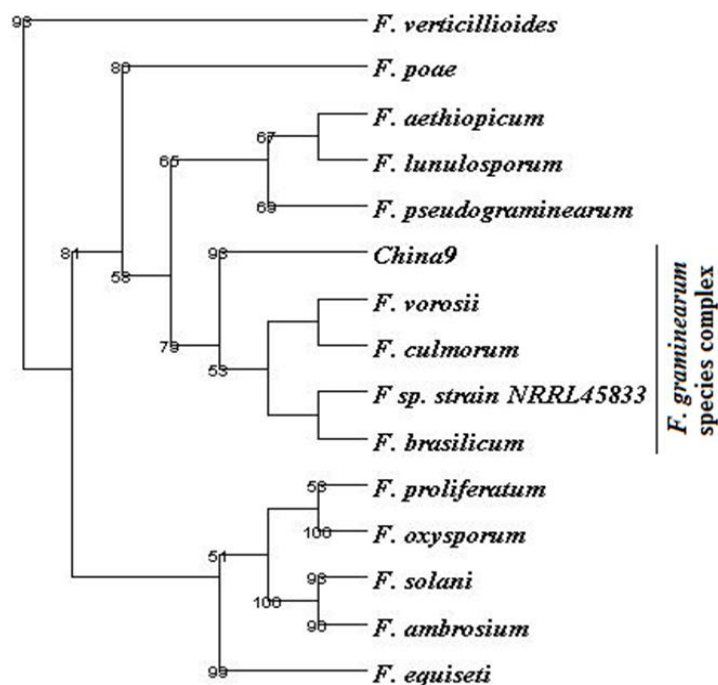


Fig. 13 Phylogenetic identification of China9 fungal isolate based on the 28S rDNA gene.

The fungal sequences were obtained from the NCBI gene bank database. Multiple-sequence alignment was performed using CLUSTALX (2.0). The tree was constructed based on the NJ method with 1000 bootstrap replicates. Bootstrap values of 50% or more are shown, *F. verticillioides* served as an out-group.

3.3. Molecular characterization of FgV-ch9

In this part of the study, I present the results on the molecular characterization of a novel mycovirus from *F. graminearum* China 9 isolate (FgV-ch9). This includes determination of the number and nature of the virus genomic segments, purification of the virus particles, the complete nucleotide sequence of the virus, the encapsidation pattern of the virus segments, production of polyclonal antibodies for the virus, sequence analysis of the virus genome and phylogenetic investigations.

3.3.1. Virus purification and dsRNA isolation

Isometric VLPs of 35-40 nm in diameter were purified from the mycelium of *F. graminearum* China 9 isolate (Fig. 14) and thereafter named tentatively FgV-ch9. The buoyant density of the virions in cesium chloride is in the range 1.42-1.45 g/cm³.

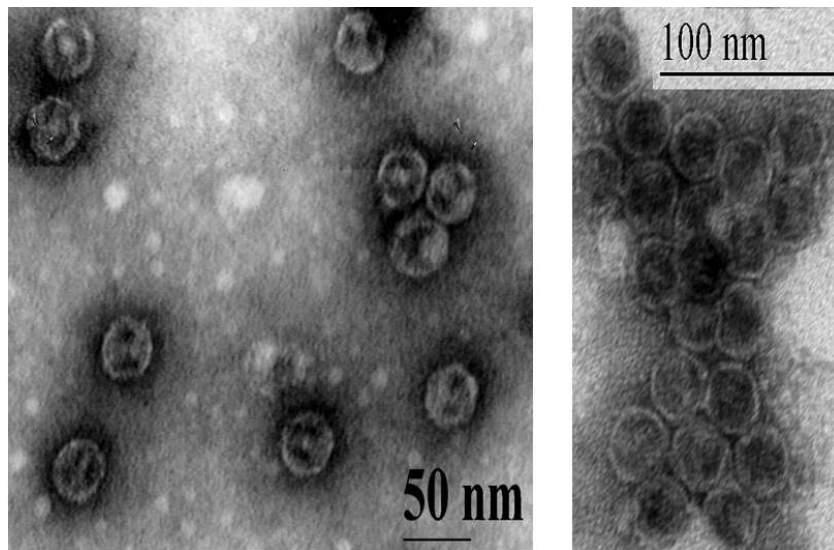


Fig. 14 Virus-like particles of FgV-ch9.

The particles were purified from China 9 isolate by CsCl-gradient ultracentrifugation, negatively stained with 2% (w/v) uranyl acetate and observed under transmission electron microscope.

Depending on the virus titer in the fungus and the electrophoresis conditions, the number of the visibly detectable dsRNA segments ranged from 2 to 5 (Fig. 15). However, Northern Blot analysis (Fig. 16) and nucleotide sequence analysis showed that always five unique segments are associated with the purified VLPs.

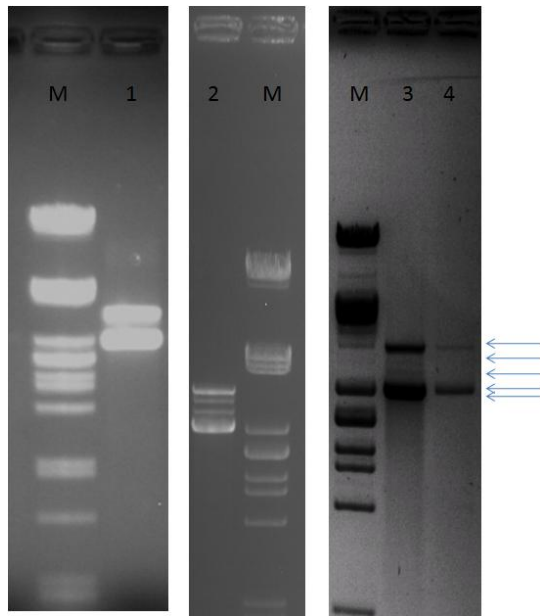


Fig. 15 Agarose gel electrophoresis of dsRNAs isolated from the fungus *F. graminearum* China 9 or from purified VLPs of FgV-ch9.

The dsRNAs were purified either from the mycelium or from CsCl-purified VLPs by CF-11 chromatography and standard phenol method, respectively. The dsRNAs were treated with DNase I and S1 nuclease and separated in 1.2% agarose gel (arrows). **1:** dsRNAs purified from mycelium with high titer of FgV-ch9 and electrophoresed for 30-40 min. **2:** as in **1** but electrophoresed for 90 min. **3:** dsRNAs extracted from viral particles purified from mycelium with high titer of FgV-ch9. **4:** as in **3** but with lower virus titer. **M:** λ DNA

Several purifications of the viral dsRNA from the mycelium by means of cellulose chromatography followed by S1 nuclease and DNase I digestions showed that the distribution of the segments in the virus population is not equal giving that 2-3 of the dsRNAs present below the detection threshold by agarose electrophoresis. Indeed, it was always possible to observe 4-5 distinct dsRNA segments only from mycelium containing a high titer of the virus (Fig. 15 lane 2) or from virions purified from such a mycelium (Fig. 15, compare lanes 3 & 4). Fungal cultures with various titers of FgV-ch9 were obtained by single conidiation. Furthermore, the resolution of the extracted dsRNA segments in 1.2% agarose gel electrophoresis into 4-5 distinct bands was only possible by extending the separation time to 90 min or more at 110 V (Fig. 15 compare lanes 1 & 2). The sizes of the five dsRNA segments and of the encoded proteins are summarized in Table 4.

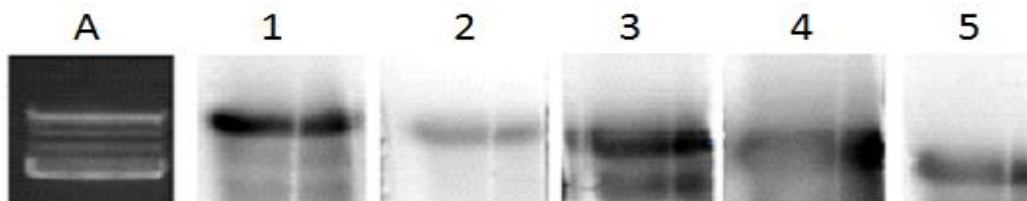


Fig. 16 Northern Blot analysis of FgV-ch9 dsRNA segments.

Purified dsRNAs were resolved in 1% agarose gel, blotted onto Hybond-N+ nylon membrane, and hybridized with Dig-labeled probes prepared by PCR amplification of cloned cDNAs. Lanes **1-5** correspond to dsRNAs1-5, respectively. Lane **A:** Purified dsRNAs separated on a 1.2% agarose gel and stained with ethidium bromide.

3.3.2. SDS-PAGE and peptide sequencing

Proteins from purified VLPs were resolved into three distinct protein bands (upper-faint ~130 kDa, middle-double ~ 70 kDa, and lower ~ 60 kDa) after electrophoresis in denaturing polyacrylamide gel (Fig. 17a lane 1). Tryptic peptide sequences from the upper-faint and the lower bands were identical to the deduced amino acid sequences of **dsRNA1** and **dsRNA2**, respectively (Fig. 19a and b, boxed). The irresolvable middle-double bands (Fig. 17a, lane 1) have peptide sequences identical to the protein deduced from **dsRNA3** (Fig 19c, boxed) and therefore were considered as a single band. With the exception of the band representing **dsRNA1**, the sizes of the other two bands were slightly different from the corresponding sizes calculated from the amino acid sequence deduced from DNA clones (compare Fig 17 to Table 4).

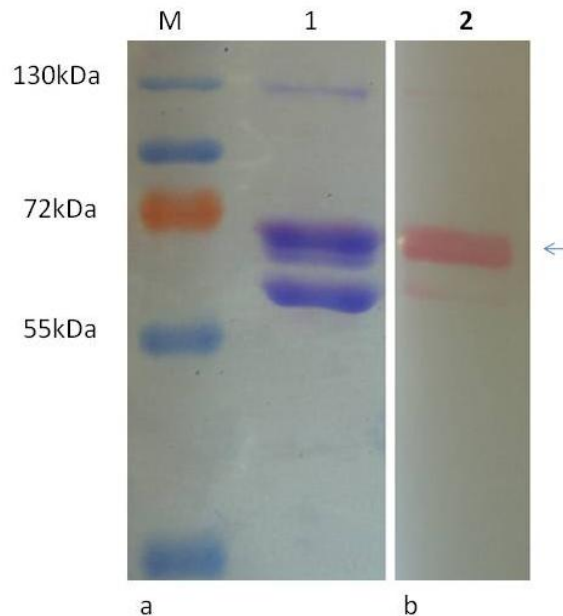


Fig. 17 SDS-PAGE and Western Blot analysis of FgV-ch9 structural proteins.

CsCl gradient-purified particles were denatured and subjected to SDS-PAGE (a, 1). The surface proteins of the purified VLPs were labeled with NHS-Biotin, separated in SDS-PAGE, blotted, and detected with the Fast Red reagent (b, 2). The preferential labeling of the protein encoded by **dsRNA3** (arrow), which appears as a double band probably due to protein degradation or modification, indicated that this protein might be the outer capsid protein. **M**: Prestained protein marker.

3.3.3. Specificity of the produced polyclonal antibodies for FgV-ch9

Immunoblot experiment using serum of the FgV-ch9-immunized rabbit (4th bleeding) against SDS-PAGE resolved FgV-ch9 structural proteins resulted in the detection of 2 of the viral proteins (RdRP, upper band and CP, lower double band) in the presence of a light background (Fig. 18, lane 1). In the control where Null serum was used, no signals were detected (Fig. 18, lane 2).

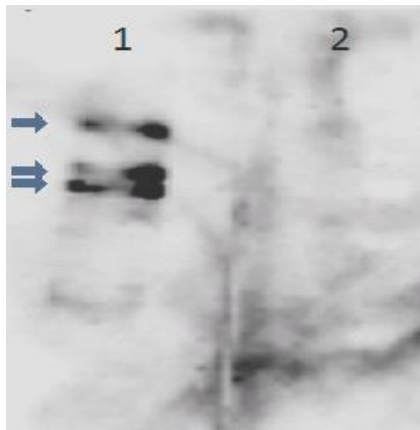


Fig. 18 Specificity of the FgV-ch9 polyclonal antibodies.

Null serum and serum of the 4th bleeding were diluted 1000x and applied in a immunoblot for the detection of FgV-ch9 structural proteins purified by means of CsCl gradient ultracentrifugation. The serum was purified by precipitating non-specific Abs with healthy *Fusarium* extract. **1:** serum of the 4th bleeding, arrows indicate FgV-ch9 specific signals, **2:** Null serum.

3.3.4. Quantitative PCR

The various intensities of the 5 dsRNA-segments isolated from purified virions or from the fungal mycelium as shown in agarose gel electrophoresis (Fig. 15) might be correlated to unequal encapsidation and/or expression of the dsRNA segments. Relative quantitative PCR of the five dsRNAs showed that they are encapsidated unequally. In relation to **dsRNA4** (was set as a reference because it has the lowest cross point value), dsRNAs1, 2, 3 and 5 isolated from purified virions, were present at ratios of 0.63 : 0.27 : 0.78 : 0.87, respectively. A similar trend was observed when the dsRNAs were purified from fungal host mycelia. Since the size of the purified isometric particles does not allow the encapsidation of the 5 dsRNA segments in one particle at the ratios determined by the real-time PCR calculations, I conclude that the dsRNAs are encapsidated separately.

3.3.5. Nucleotide sequencing

The complete nucleotide sequences of the 5 dsRNA-segments were determined from at least three full-length clones for each segment, originating from different virus purifications or dsRNAs purified from infected mycelium. The sequences were submitted to the Genbank and the accession numbers are shown in Table 4. Sequence analysis showed that each dsRNA segment is monocistronic with a single open reading frame (ORF).

Table 4: Size and function of the genomic dsRNA segments and the deduced proteins of FgV-ch9.

Segment (accession no.)	Size (bp)	Size of deduced protein (kDa)	Function
dsRNA1 (HQ228213)	3581	127 640 (1137 aa)	RDRP
dsRNA2 (HQ228214)	2850	94 872 (875 aa)	Unknown
dsRNA3 (HQ228215)	2830	93 606 (856 aa)	Putative capsid protein
dsRNA4 (HQ228216)	2746-3786*	91 303 (835 aa)	Unknown
dsRNA5 (HQ228217)	2423	79 872 (712 aa)	Contains a C2H2 zinc finger domain

*Due to the presence of 3`terminal repeats, the size of **dsRNA4** is variable.

3.3.5.1. dsRNA1

The complete nucleotide sequence of **dsRNA1** revealed that it is 3581 bp and contains a single large ORF from nt positions 83 to 3496 coding for 1137 aa (Table 4). The calculated molecular weight of the encoded protein (127 kDa) is in agreement with the estimated size of the corresponding band in the SDS-PAGE (Fig. 17a, lane1; upper-faint band). In addition, peptide sequences of this band match perfectly with the deduced amino acid sequence of the ORF on **dsRNA1** as shown in (Fig.19a).

Since sequence analysis of the deduced protein encoded on **dsRNA1** showed the presence of the 8 conserved RdRP motifs characteristic for RNA viruses (Bruenn, 1993) shown in (Fig. 20), I propose that RNA1 codes for the RdRP of the mycovirus.

This was further corroborated by BLAST searches of the deduced amino acid sequence of **dsRNA1** which revealed sequence similarities to several viral RdRPs (virus full names and accession numbers are in Table 5). For example: to AsV1816 with 37% identity, 53% similarity, and an E value of zero, MoCV1 with 33% identity, 50% similarity, and by AbV-1 with 29% identity, 46% similarity. Sequence similarities ($\leq 27\%$ identity and $\leq 43\%$ similarity, e^{-32} and lesser) were also obtained for viral RdRPs belonging to the family *Chrysoviridae*. These include CnV-1, Hv145SV, CCRS-CV, FuoxV1, PcV and others. Less significant similarities were obtained for RdRPs belonging to the family *Totiviridae* (BLAST hits of e^{-09} or lesser).

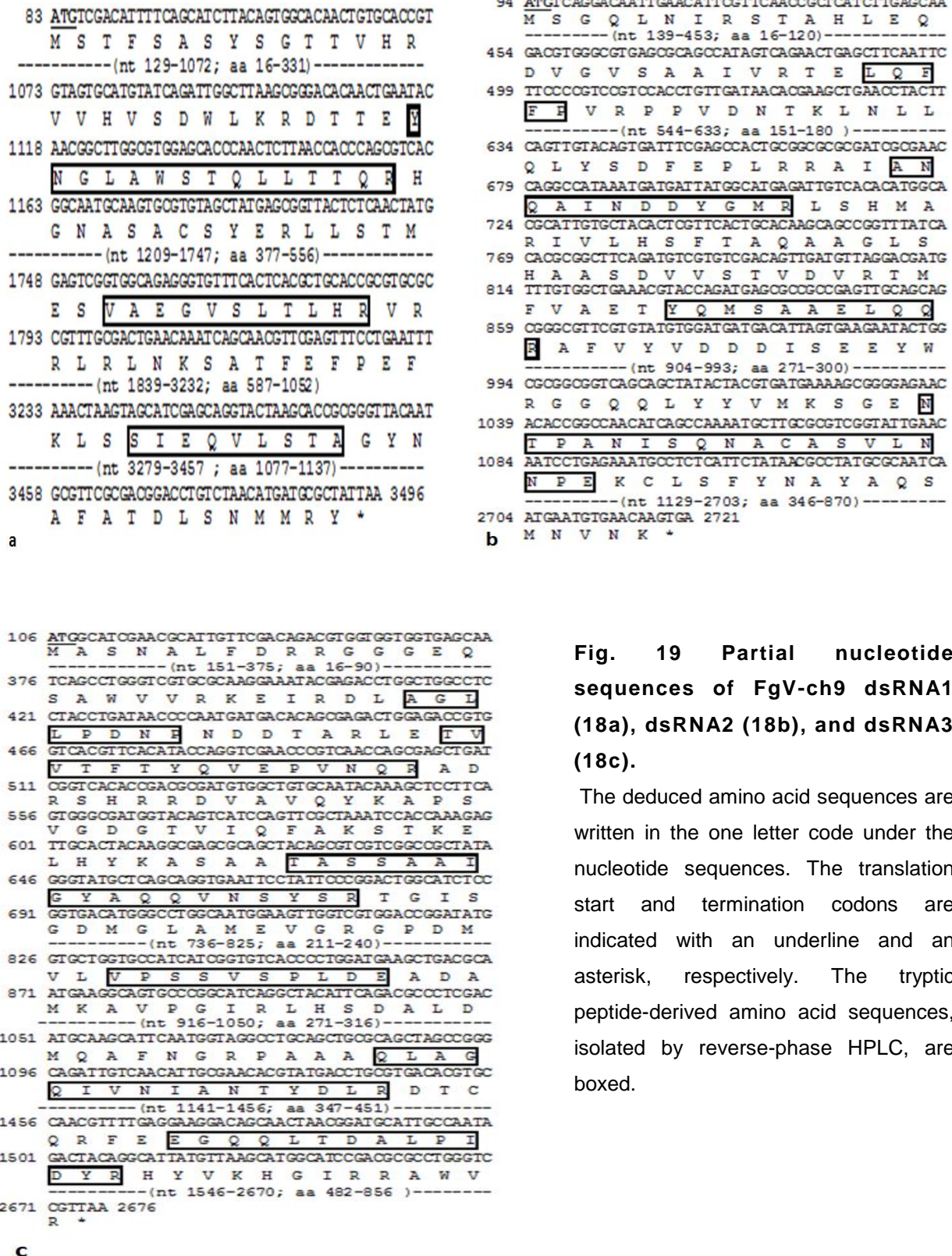


Fig. 19 Partial nucleotide sequences of FgV-ch9 dsRNA1 (18a), dsRNA2 (18b), and dsRNA3 (18c).

The deduced amino acid sequences are written in the one letter code under the nucleotide sequences. The translation start and termination codons are indicated with an underline and an asterisk, respectively. The tryptic peptide-derived amino acid sequences, isolated by reverse-phase HPLC, are boxed.

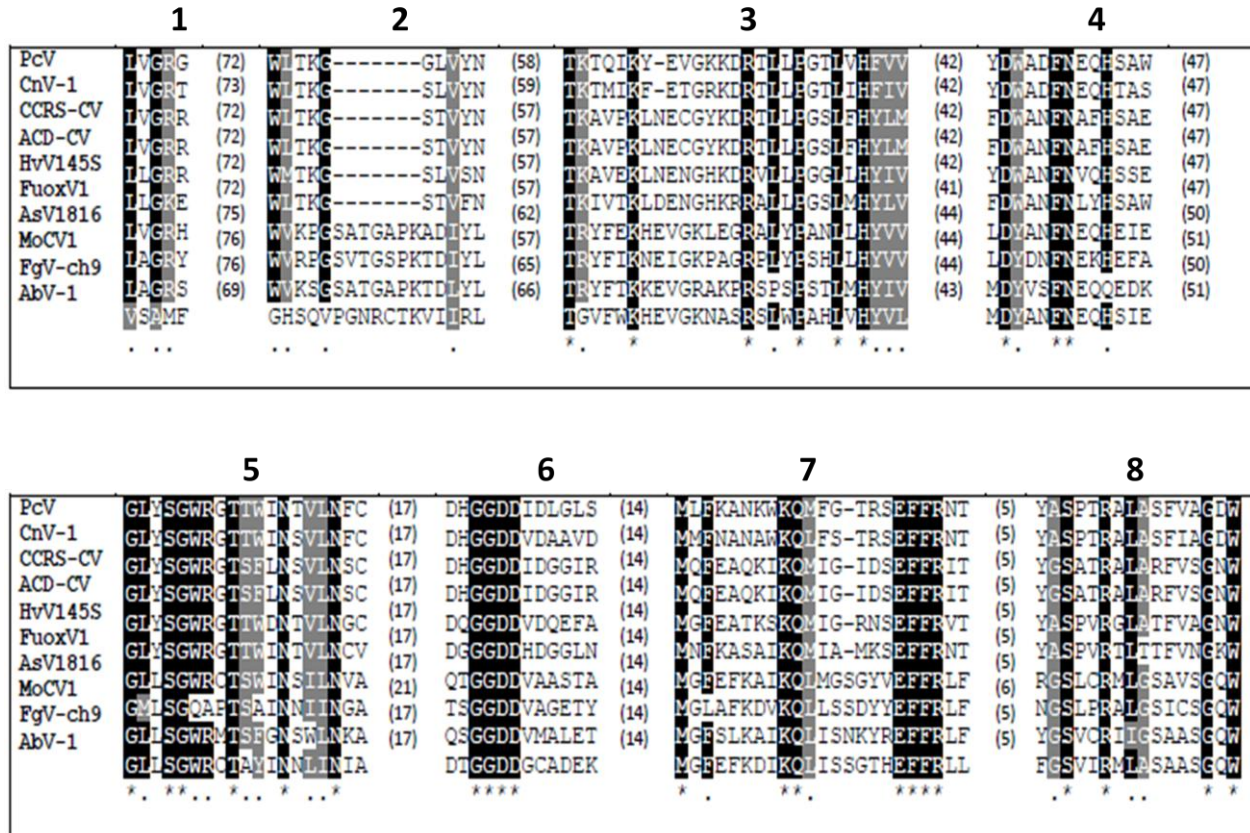


Fig. 20 Comparison of the conserved RdRPs motifs of several dsRNA mycoviruses including FgV-ch9.

Numbers 1-8 stand for the eight conserved motifs typical of RdRPs of RNA viruses. Multiple sequence alignments were carried out with the CLUSTALX (2.0). Dark shading and asterisks imply identical residues at the indicated position while light gray shading and single dots signify columns with similar residues. Numbers in parentheses relate to the number of amino acid residues between the motifs. The full names of the viruses are shown in Table 5.

Interestingly, an identity of 100% was found to the partial RdRP aa sequence (73 aa) available for *Fusarium graminearum* dsRNA mycovirus-2 (FgV2; No AAR17790). A phylogram presenting/summarizing the data is shown in Fig. 21a. Although AsV1816 is assigned as an unclassified member in the *Totiviridae* and the classification of AbV-1 as a chrysovirus is not finalized Ghabrial and Suzuki, 2009, the results in Fig. 21 indicate that these two viruses together with FgV-ch9 form a new cluster within the family *Chrysoviridae*.

Table 5: List of the viruses used in constructing the phylograms in Fig. 21a,b.

Virus	Acronym ^a	Accession no.		Family/ genus ^b
		RdRP	cp	
<i>Ustilago maydis virus HI</i>	UmV-HI	NP_620728.1	NP_620728.1	TT
<i>Saccharomyces cerevisiae virus L-A (LI)</i>	Sc V-L-A	NP_620495.1	NP_620494.1	TT
<i>Saccharomyces cerevisiae virus L-BC (La)</i>	ScV-L-BC	NP_042581.1	NP_042580.1	TT
<i>Gremmeniella abetina RNA virus L1</i>	GaRV-L1	AAK11656.1	NP_624332.2	TV
<i>Helminthosporium victoriae 190SV</i>	Hv190SV	NP_619670.2	NP_619669.2	TV
<i>Coniothyrium minitans mycovirus</i>	CmV	YP_392467.1	YP_392466.1	TV
<i>Epichloe festucae virus 1</i>	EfV1	CAK02788.1	CAK02787.1	TV
<i>Magnaporthe oryzae virus1</i>	MoV1	YP_122352.1	YP_122351.1	TV
<i>Magnaporthe oryzae virus 2</i>	MoV2	NP_047560.1	YP_001649205.1	TV
<i>Sphaeropsis sapinea RNA virus 1</i>	SsRV1	NP_047558.1	NP_047557.1	TV
<i>Sphaeropsis sapinea RNA virus 2</i>	SsRV2	YP_001649206.1	NP_047559.1	TV
<i>Botryotinia fuckeliana totivirus 1</i>	BfTV1	YP_001109580.1	YP_001109579.1	TV
<i>Atkinsonella hypoxylon virus</i>	AhV	NP_604475.1	NP_604476.1	P
<i>Aspergillus ochraceus virus 1</i>	AoV1	ABV30675.1	ABV30676.1	P
<i>Ceratocystis resinifera virus 1</i>	CrV1	YP_001936016.1	YP_001936015.1	P
<i>Discula destructiva virus 1</i>	DdV-1	NP_116716.1	NP_116742.1	P
<i>Discula destructiva virus 2</i>	DdV-2	NP_620301.1	NP_620302.1	P
<i>Fusarium poae virus 1</i>	FUPO-1	NP_624349.1	NP_624348.2	P
<i>Fusarium solani virus 1</i>	FsV-1	NP_624350.1	NP_624351.1	P
<i>Gremmeniella abietina RNA virus MS1</i>	GaRV-MS1	NP_659027.1	NP_659028.1	P
<i>Aspergillus mycovirus 1816</i>	AsV1816	ABX79996.1	NA ^c	uT
<i>Heterobasidion annosum partitivirus</i>	HaV	AAL79540.1	NA ^c	P
<i>Ophiostoma himal-ulmi partitivirus 1</i>	OPV1	CAJ31886.1	CAJ31887.1	P
<i>Penicillium stoloniferum virus S</i>	PsV-S	YP_052856.2	YP_052857.1	P
<i>Rhizoctonia solani virus 717</i>	RsV-717	NP_620659.1	NP_620660.1	P
<i>Penicillium chrysogenum virus</i>	PcV	YP_392482.1	YP_392483.1	C
<i>Helminthosporium victoriae 145S</i>	Hv145SV	YP_052858.1	YP_052859.1	C
<i>Agaricus bisporus virus 1</i>	AbV-1	CAA64144.1	BAA01612.1	?C
<i>Cryphonectria nitschkei chrysovirus 1</i>	CnV-1	ACT79255.1	ACT79251.1	C
Cherry chlorotic rusty spot associated chrysovirus	CCRS-CV	CAH03664.1	CAH03665.1	C
Amasya cherry disease associated chrysovirus	ACD-CV	YP_001531163.1	YP_001531162.1	C
<i>Fusarium oxysporum chrysovirus 1</i>	FuoxV1	ABQ53134.1	ABQ58816.1	C
<i>Magnaporthe oryzae chrysovirus 1</i>	MoCV1	YP_003858286.1	YP_003858288.1	uC

^a Provisional acronym^b TT: *Totiviridae*, genus *Totivirus*; TV: *Totiviridae*, genus *Victorivirus*; P: *Partitiviridae*; C: *Chrysoviridae*; ?: obscure; uT: unclassified.^c NA: not available.

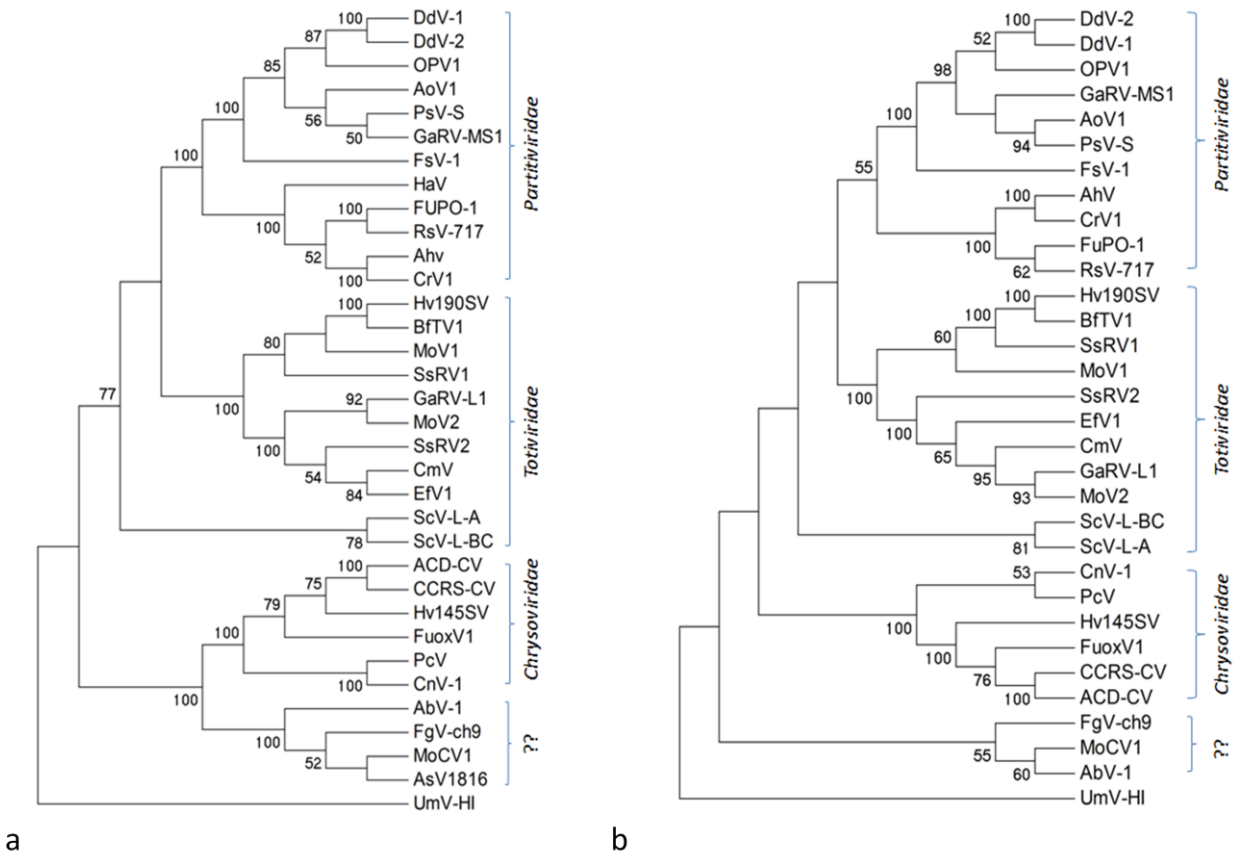


Fig. 21 Phylograms of the RdRP (a) and the CP (b) of FgV-ch9.

Multiple-sequence alignments of the deduced amino acid sequences of FgV-ch9 and selected chrysovirus, totivirus, and partitivirus were done using CLUSTALX (2.0) with default parameters. The trees were analyzed by the NJ method with 100 bootstrap replicates. The numbers near the branches refer to the percentage of bootstrap replicates. Only bootstrap percentages over 50 are shown. The trees were outgroup-rooted to the Totivirus UmV-HI. The full names of the viruses are shown in Table 5.

3.3.5.2. dsRNAs 2 and 4

Sequence analysis of the DNA clones of FgV-ch9 **dsRNA2** and **4** showed that they are 2850 and 2746 bp, respectively. Each segment has a single ORF with deduced proteins of molecular weights of 94 and 91 kDa for **dsRNA2** and **4**, respectively. From SDS-PAGE, the estimated molecular mass of the protein encoded by **dsRNA2** is ~62 kDa (Fig.17, lane 1, lower band). Interestingly, the last 146-208 nts of the 3'-end of **dsRNA4** were present in 6 out of 8 DNA clones as multiple tandem repeats. In the presence of these tandem repeats, various lengths of **dsRNA4** were measured for the different clones (2746-3786 bp). We have further confirmed the presence of such tandem repeats in **dsRNA4** by a PCR-based approach to exclude the

possibility of cloning and sequencing artifacts. Using a forward primer (F1, 5'-GCAGCTACACCAGTTAACAG-3') that anneals outside the repeat region and a reverse primer (R1, 5'-CGGATAAAACGTCAGTGGCAT-3') complementary to part of the repeat sequence, PCR amplification resulted in several amplicons with size differences corresponding to ~200 nucleotides (Fig.22, Lanes 1-3). In a control treatment, the F1 primer and a reverse primer (R2, 5'-AAAGTGCCGATTCTATACATGG-3'), designed to anneal outside the repeat region, were employed (Fig.22, lane 5).

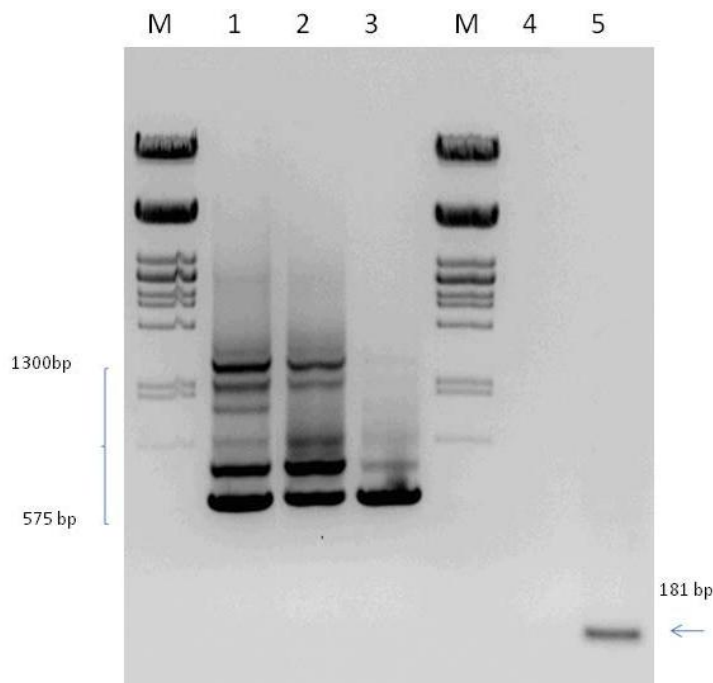


Fig. 22 Verification of the terminal repeats at the 3' terminus of dsRNA4 of FgV-ch9.

PCR products (brace) of dsRNAs templates extracted from purified VLPs (lane 1) or purified from independent mycelia cultures (lanes 2 and 3) and using a forward primer (F1) outside the repeated region and a reverse primer that anneal in the repeated region (R1). 4: PCR negative control (water), 5: PCR product (arrow) using F1 primer and a reverse primer (R2) that anneal outside the repeated region, M: λ DNA digested with *Pst*I.

Purified dsRNAs from independent mycelia cultures or VLPs were employed as templates in the described PCRs. BLAST searches of the deduced aa sequences of **dsRNA2** resulted in 29% identities and 50% similarity (E value of $1e^{-49}$) with the hypothetical protein encoded by **dsRNA3** of MoCV1. The protein encoded by **dsRNA4** showed no significant similarities to any protein in the GenBank and EMBL databases.

3.3.5.3. dsRNA3

Sequence analysis of full-length DNA clones of **dsRNA3** showed that it consists of 2830 bp coding for one large ORF of 856 aa and a calculated molecular mass of 93 kDa. The estimated size of the protein in SDS-PAGE is ~70 kDa (Fig. 17a, lane 1; middle-double band). Surface protein labeling of purified virions with NHS-Biotin showed a labeling preference for the middle-

double band (Fig. 17b, Lane 2; arrow). Peptide sequences of this band match perfectly with the deduced aa encoded on **dsRNA3** (Fig. 19b). BLAST searches showed significant sequence similarity of the deduced aa of **dsRNA3** to the L3 protein (CP) of AbV-1 (22% identity and 39% similarity at a BLAST match of $5e^{-13}$). One other significant match to the hypothetical protein encoded by segment 4 of MoCV1 was observed (23% identity, 39% similarity, and an E value of $1e^{-13}$). These results indicate that **dsRNA3** codes most likely for the capsid protein.

Phylogenetic analysis of the capsid protein encoded by **dsRNA3** showed that although FgV-ch9, AbV-1, and MoCV1 cluster as a distinct clade, they are more related to members of the *Chrysoviridae* than to those of the other families (Fig. 21b). Unfortunately, the capsid protein of AsV1816 is not identified yet.

3.3.5.4. dsRNA5

This segment has a length of 2424 bp with a single ORF (nt 97 to 2139) coding for a protein of 712 aa with calculated molecular mass of 79 kDa. BLAST searches of the deduced amino acid sequence of **dsRNA5** showed that while the N-terminus of the protein encoded by **dsRNA5** has no significant similarity to any published protein, the C-terminus of the protein (aa positions 326 to 711) shares high similarities to zinc finger proteins (BLAST matches of $2e^{-13}$). Using the online software developed by Persikov *et al.*, we have identified 12 fingers in this region. Multiple alignment of the 12 fingers showed that all of them share the typical characteristics of C2H2 zinc fingers (Fig. 23). Such fingers are composed usually of 2-3 β strands in their N-terminus and one α helix in the C-terminus. The specific binding affinity of zinc fingers to nucleic acids or proteins is conferred by some of the amino acid residues present in their α helix.

		C	C	-1123456H	H																					
ZF1	330	C	P	I	C	F	Q	R	S	R	R	S	L	A	A	H	R	S	S	H	351					
ZF2	356	C	G	Q	A	H	C	S	Y	T	T	D	V	P	A	D	F	A	F	H	M	D	E	E	H	379
ZF3	384	C	D	S	T	G	C	D	Y	T	T	R	S	S	Q	L	H	W	T	H	M	V	-	G	H	406
ZF4	409	C	-	-	T	V	C	G	L	L	S	A	N	L	A	A	A	D	H	I	R	-	E	H	429	
ZF5	435	C	-	-	S	L	C	N	E	P	F	E	D	A	F	S	L	N	E	H	N	Q	D	V	H	456
ZF6	460	C	-	-	T	Q	C	E	A	I	V	R	D	K	N	E	L	L	D	H	C	H	D	V	H	481
ZF7	486	C	R	E	V	G	C	A	F	R	T	F	E	P	P	E	L	A	A	H	H	D	-	W	H	508
ZF8	522	C	P	D	L	G	C	T	H	A	A	S	S	H	E	M	A	A	L	H	Y	N	-	T	H	544
ZF9	552	C	-	-	E	S	C	D	A	W	C	Q	Q	P	H	C	P	N	Q	H	S	R	-	M	H	572
ZF10	600	C	-	-	P	H	C	A	F	T	S	K	S	K	V	E	W	Q	D	H	I	N	I	N	H	621
ZF11	631	C	-	-	S	L	C	G	K	V	F	T	G	V	N	A	V	R	K	H	H	C	-	-	H	650
ZF12	690	C	-	-	Q	V	C	H	E	T	F	A	R	L	D	H	L	Q	R	H	V	R	R	R	H	711

Fig. 23 Multiple-sequence alignment of the 12 C2H2 zinc finger domain present at the C-terminus of the protein encoded by dsRNA5 of FgV-ch9.

C, H, and number above the aligned sequences refer to the residues, Cys and His, and residue number in the alpha helix, respectively. Bold indicates conserved amino acid residues. Multiple sequence alignment was done using CLUSTALX with some manual adjustments.

3.3.5.5. The 5` and 3`UTRs

The lengths of the 5`UTRs of the different FgV-ch9 dsRNA segments lay between 78 and 105 nt. Multiple alignment of the 5`UTRs of the five dsRNA segments showed regions of high sequence similarities (Fig. 24a). Stretches of identical sequences of 10 and 19 nt were found at positions (in regard to the consensus line) 1 to 10 and from 23 to 42, respectively. Sixty-one of the 5`-terminal 72 nts are identical in at least three of the five dsRNAs. Sequence similarities in the 5`UTR dramatically decreases in the region located directly upstream of the ATG initiator codon (specified in bold, Fig. 24a). The average GC content of the 5`UTRs of the five dsRNAs is 41% indicating a TA rich area. The CAA repeats, characteristics of the 5`UTR of chrysovirus (Jiang and Ghabrial, 2004) were observed in FgV-ch9, with a lesser frequency though. Whereas in PcV and CCRS-CV the CAA sequence is repeated 7-14 times per dsRNA, in FgV-ch9 they occur 2-6 times only (Fig 24a, in italics). In the 5`UTRs of tobamoviruses similar repeats were described to function as translational enhancer elements (Gallie and Walbot, 1992). The 3`UTRs of FgV-ch9 are relatively long, between 85 and 188 nt in length (Fig. 24b). In addition to the 6 nt

at 3'-terminal which are identical among the 5 dsRNAs, other regions with high similarity are distributed along the last 80 nt of the 3'UTR of the 5 dsRNAs (Fig. 24b).

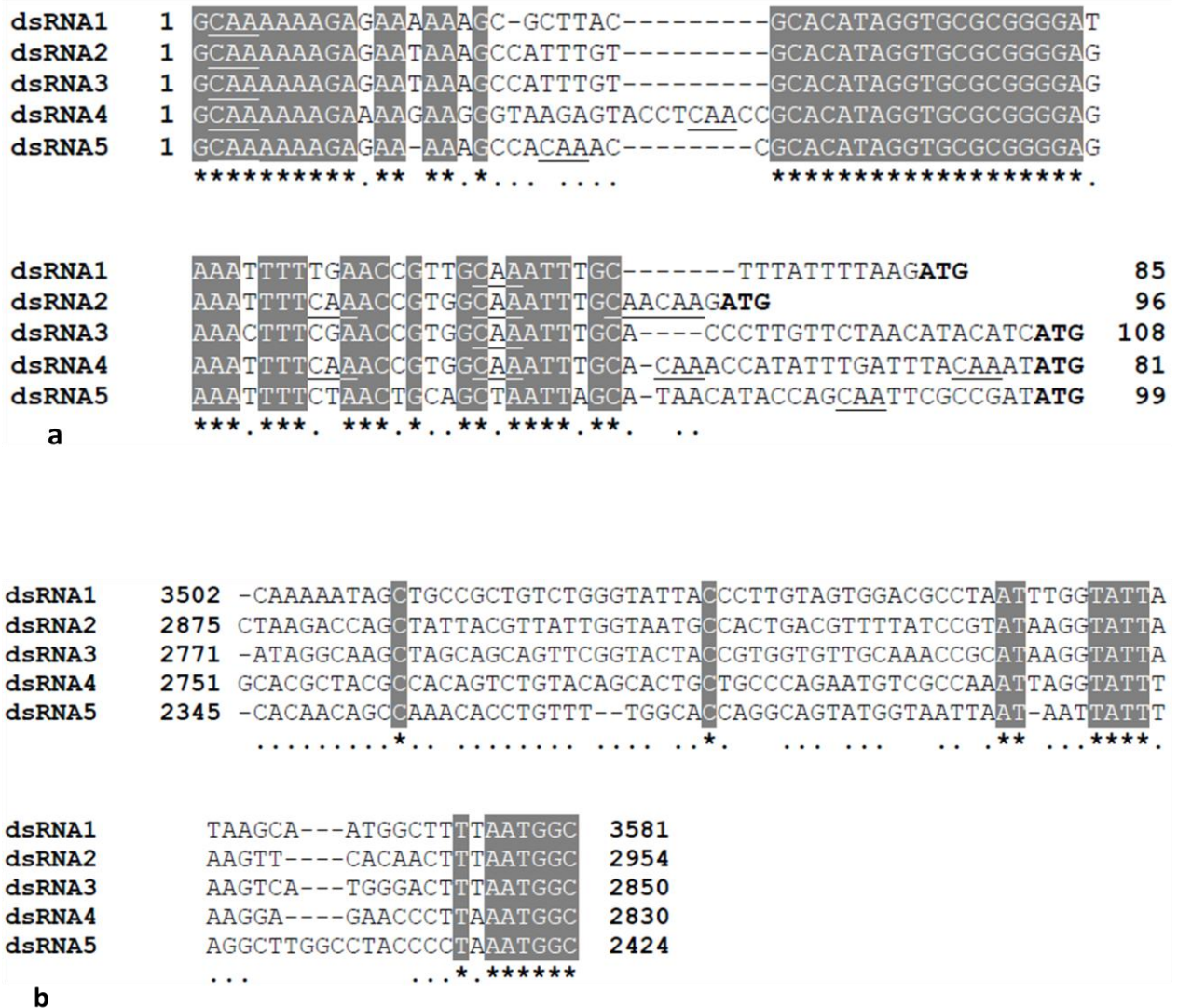


Fig. 24 Comparison of the 5' (a) and 3' (b) UTRs of the 5 dsRNA segments of FgV-ch9.

Multiple-sequence alignments were done with CLUSTAL X. Gray shading and asterisks specify columns with identical bases while dots indicate that three or four out of the five bases are identical in that column. The (CAA) repeats in (a) are underlined and the start codons are shown in bold.

A summary of the genomic organization of FgV-ch9 is illustrated in Fig. 25.

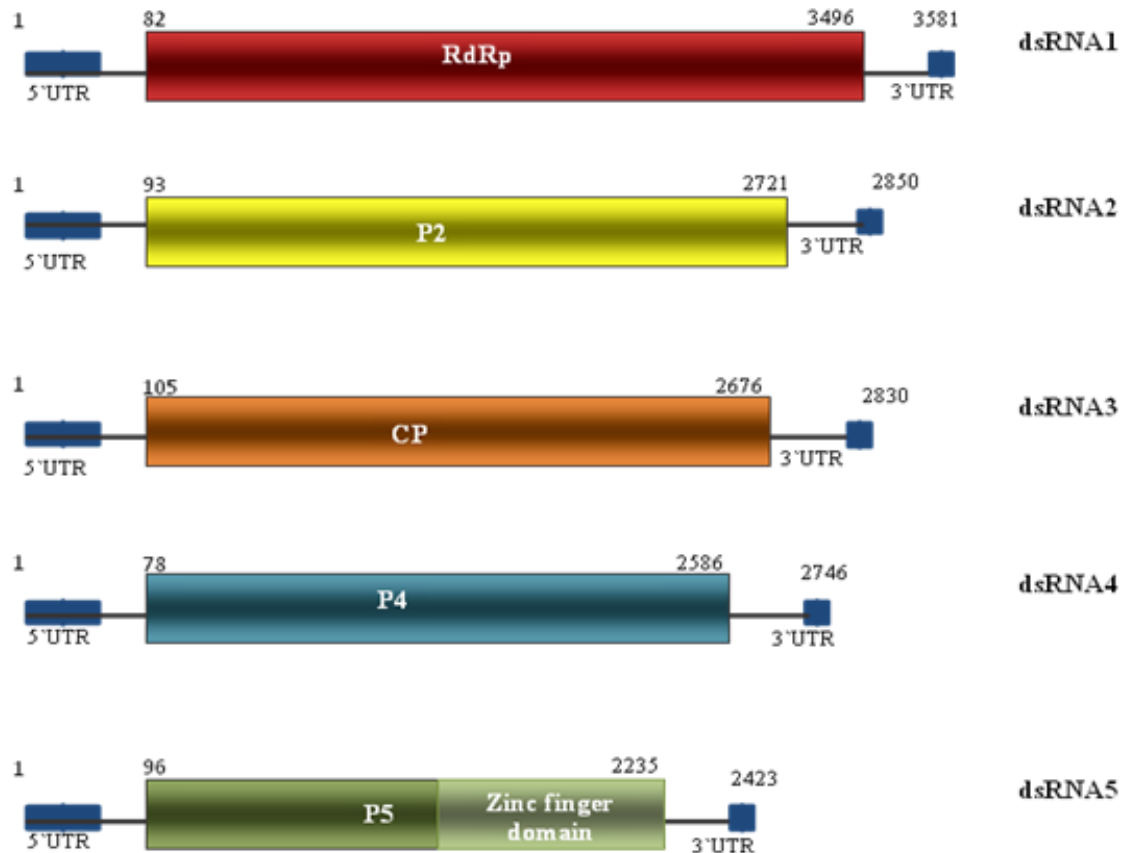


Fig. 25 A diagrammatic representation of the genomic structure of FgV-ch9.

Colored rectangles represent the ORF of each segment. P2 & P4 are proteins with unidentified function. RdRP: RNA dependent RNA Polymerase. CP: capsid protein. Conserved terminal motifs are indicated with blue boxes at the 5' and 3' UTRs. The numbers represent (from left to right) the first nucleotide, the first nucleotide of the ORF, the last nucleotide in the ORF, and the length of the segment, respectively.

3.6. Association of the virus with hypovirulence-traits of *F. graminearum* China 9.

This part of the results presents several hypovirulent features of *F. graminearum* China 9 in comparison to the WT isolate PH-1. These features are presumably due to the association of China 9 isolate with various titers of FgV-ch9. Evidences supporting this assumption are presented in the following sections of the results.

Cultures of *F. graminearum* China 9 originating from a conidium were found to be associated with different amounts of FgV-ch9 dsRNAs. These cultures were classified into three major categories (high, moderate, and low) depending on the amount of the viral dsRNA per gram of fresh mycelia. Mycelia of the high and moderate categories contained 50-150 and 8-30 μg viral

dsRNA/g fresh weight mycelia, respectively. The amount of viral dsRNA in mycelia with low virus titers was not visible in agarose gels but was still detectable by means of RT-PCR. In correlation to the amount of the viral dsRNA (virus titer), the three culture types of China 9 exhibited also differences in:

- cellular ultrastructure
- growth rate
- colony morphology
- conidiation capacity, and
- virulence on wheat and maize plants.

These differences are explained in details in the following sections.

3.6.1. Ultrastructural properties of China 9 isolate.

Ultrastructural examination of *F. graminearum* China 9 showed the presence of VLPs in membrane-bound vesicles, in vacuole-like structures, and in the abnormal looking cytoplasm (Figs. 26 A, 27 A-D, and 28 A and B). Severe cellular degeneration and regions with decreased electron density in the cytoplasm (Fig. 27, A), the presence of many large vacuole-like structures (Fig. 26, A), accumulation of membranous structures or vesicles (Fig.27, B), and disintegration of mitochondrial and nuclear membranes (Fig.27, B and C, respectively) are the major abnormal features observed for *F. graminearum* China 9 with high virus titer and severely restricted growth.

Although most of the examined cells of China 9 with moderate virus titer have a normal nucleus and abundance of ribosomes, many of them have partially disorganized cytoplasm (Fig. 26, B). On the other hand, the majority of the cells of China 9 that have low virus titer appeared normal with organized cytoplasm of usual electron density (Fig. 26, C). To compare the cell structure of China 9 isolate to another *F. graminearum* isolate, the cells of the WT PH-1 were also examined (Fig. 26, D). The use of polyclonal antibodies to allocate FgV-ch9 in the fungal cells showed that virus is scattered in the cytoplasm and the vacuole-like structures (Fig. 28, A and B).

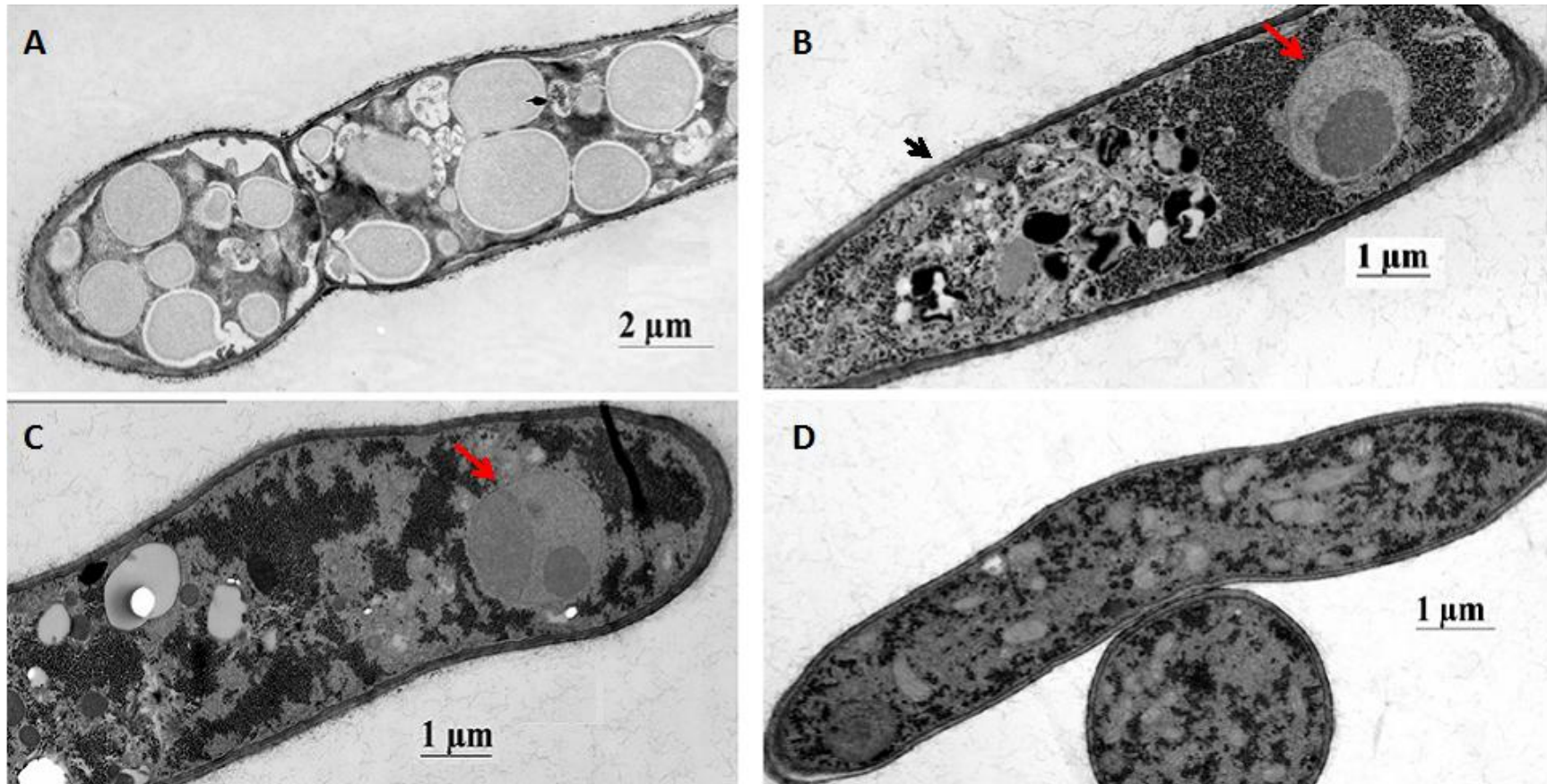


Fig. 26 Transmission electron micrographs of *F. graminearum* China 9 associated with different titers of FgV-ch9.

A: China 9 with high virus titers possesses abnormal cytoplasm with many large vacuoles that lack electron density.

B: China 9 with moderate virus titer showing normal nucleus (long arrow), abundance of ribosomes, but also a disorganized part of the cytoplasm (short arrow).

C: China 9 with low virus titer has normal cytoplasm and nucleus (arrow).

D: Wild type strain PH-1.

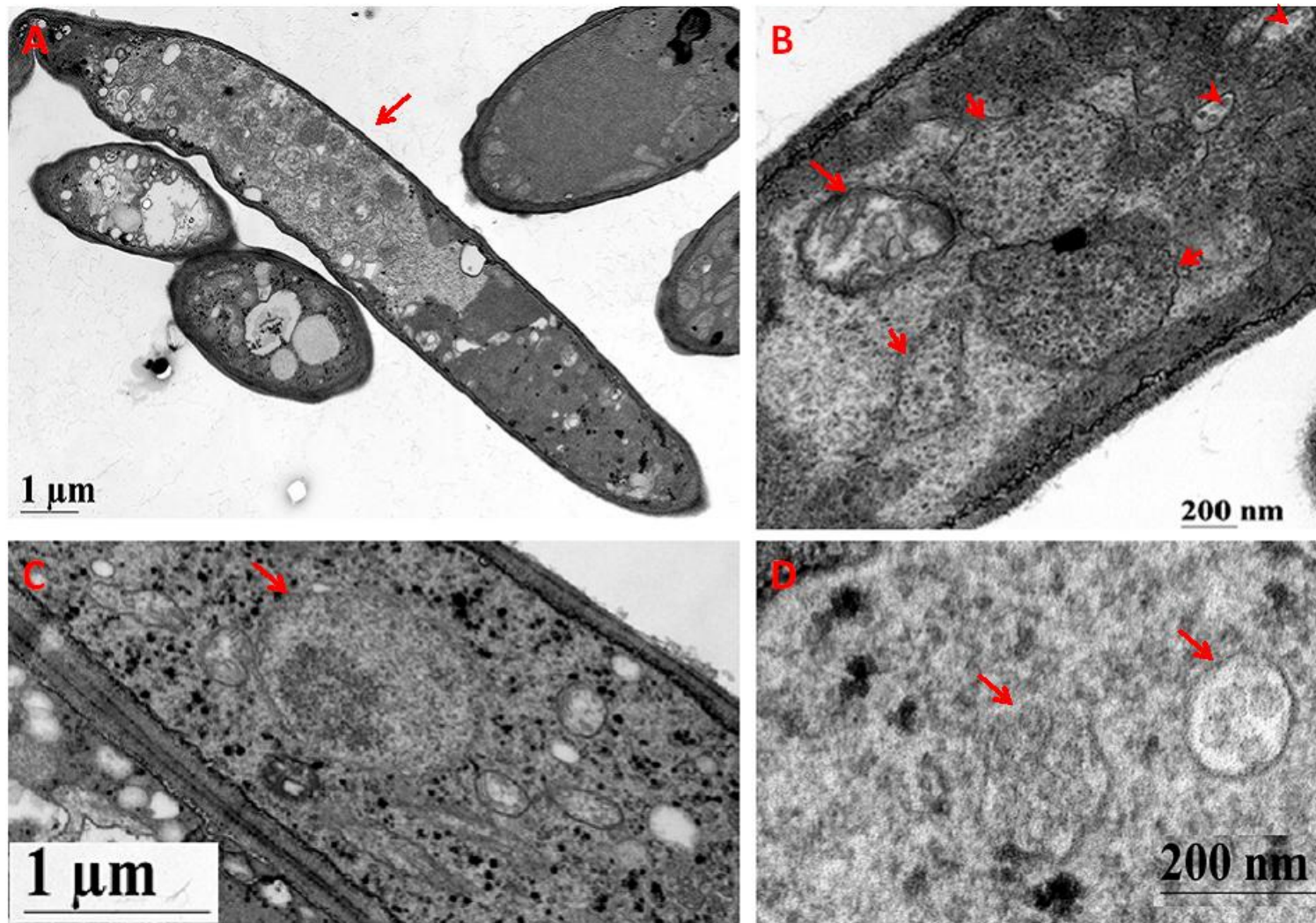


Fig. 27 Ultrastructural phenotypes of *F. graminearum* China 9 associated with high tiers of FgV-ch9.

A: Disorganized cytoplasm with decreased electron density and accumulation of membranous structures or vesicles (area indicated by the arrow). **B:** Membranous structures or vesicles (short arrow), some filled with VLPs (arrowheads), and abnormal mitochondrion with disintegrated membrane (long arrow). **C:** Disintegrated nuclear membrane (arrow). **D:** Vesicles filled with VLPs (arrows).

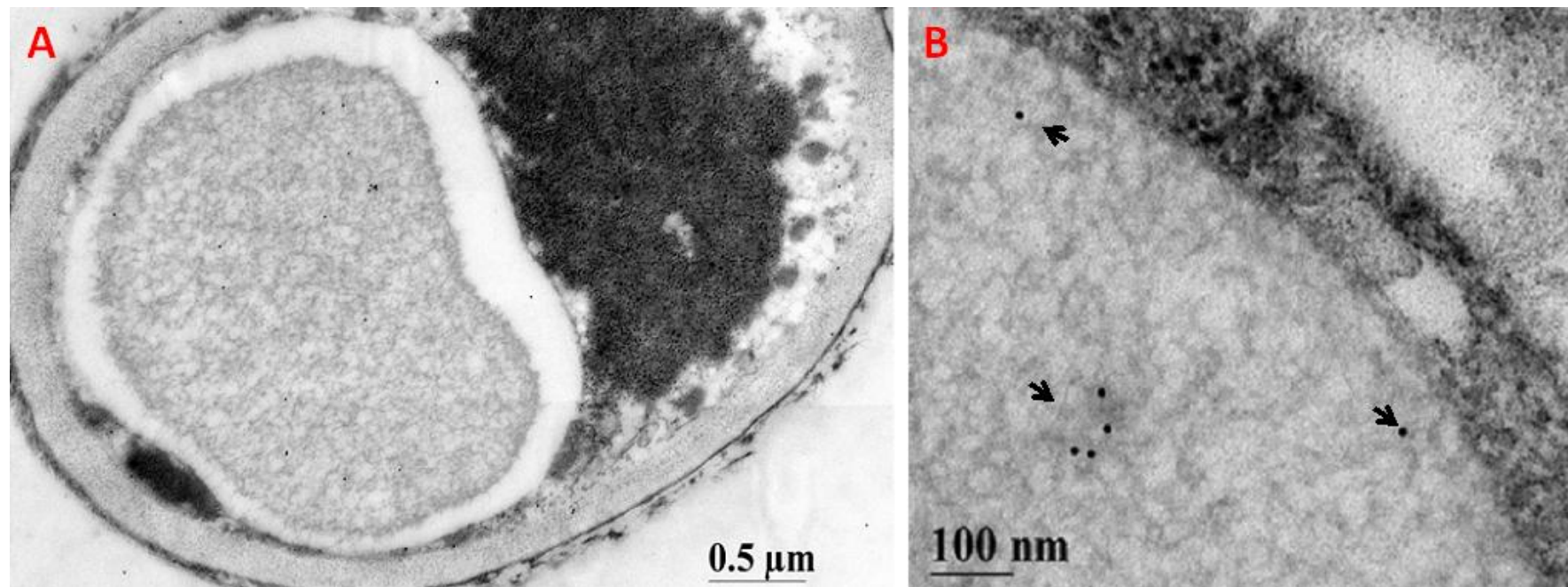


Fig. 28 Immuno-detection of VLPs in China 9 cells associated with high virus titer.

A: The VLPs of FgV-ch9 were mainly detected within the vacuole-like structures and scattered in the cytoplasm. Gold particles conjugated to the second antibody appear as black dots.

B: A high magnification micrograph of a section of the vacuole with gold particles (arrows).

3.6.2. Effect of virus titer on the growth rate of *F. graminearum* China 9.

The growth rate of *F. graminearum* China 9 on CM was significantly reduced in cultures associated with high and moderate virus titers (Figs. 29 and 30). The colony area of 6 days-old cultures associated with high or moderate virus titers were 23.6 and 60.6 cm², respectively. At low virus titer, the mycelia of 4 days-old cultures of *F. graminearum* China 9 cover the whole Petri-dishes (81 cm²).

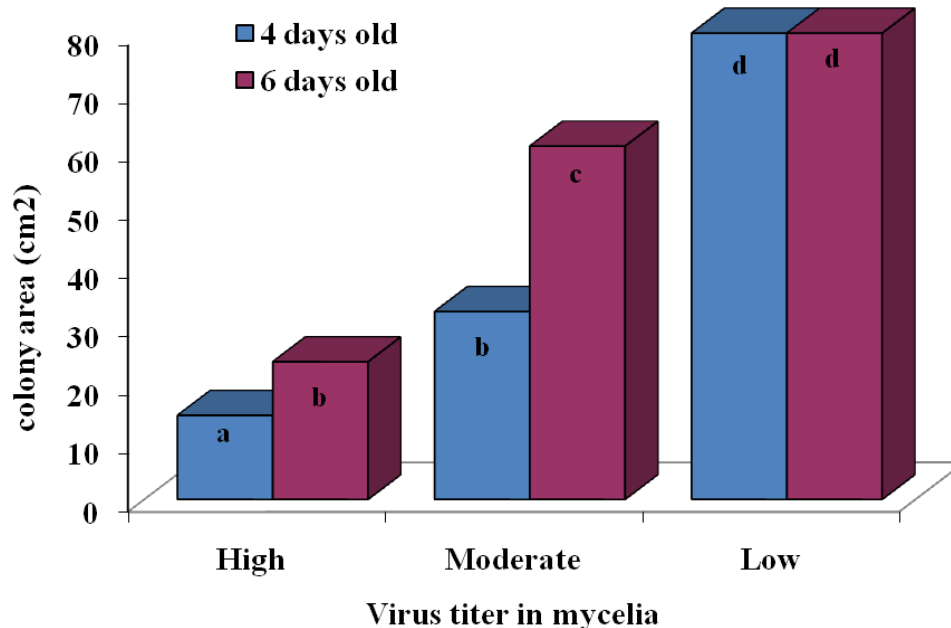


Fig. 29: Effect of FgV-ch9 titer on the growth rate of *F. graminearum* China 9 isolate.

The fungus was grown on solid CM medium at 25°C in the dark. The colony area was measured 4 and 6 days after inoculation. Values are the average area of 6 colonies from 2 independent experiments. Different letters indicate statistically significant differences.

3.6.3. Colony morphology of *F. graminearum* China 9 isolate

Besides the restricted growth rate in cultures associated with high virus titers, such cultures also exhibited abnormal growth phenotypes including dark pigmentation and altered (uneven) colony morphology with the frequent formation of ear-like structures at the colony margin (Fig. 30).

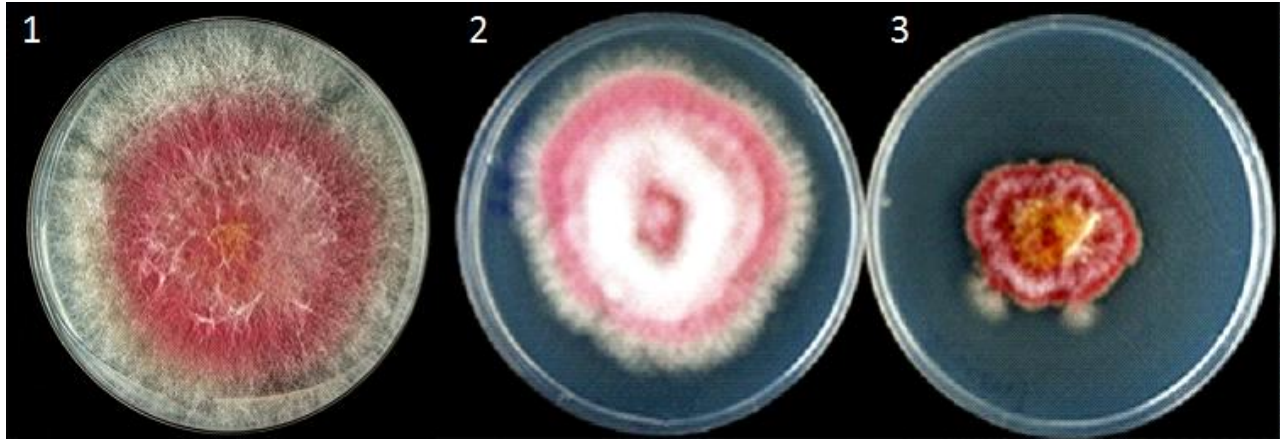


Fig. 30. Growth rate and colony morphology of *F. graminearum* China 9 cultures associated with different virus titers.

1: China + low virus titer, 2: China 9 + moderate virus titer, 3: China 9 + high virus titer. The cultures were photographed after 4 days of growth on CM medium at 25°C in the dark.

3.6.4. Effect of virus titer on the conidiation of *F. graminearum* China 9.

The capacity of *F. graminearum* China 9 to produce conidia was significantly reduced in cultures associated with high to moderate titers of viral dsRNAs. In correlation to the culture with low virus titers, the production of conidia per plate SNA medium in cultures with high and moderate virus titers was reduced to 16% and 60%, respectively (Fig.30). As a trend, the capacity for conidiation in all of the *F. graminearum* China 9 cultures is relatively lower than that of the *F. graminearum* PH-1 isolate (0.3-2.4 million in China 9 in comparison to 7-10 million conidia per plate in PH-1).

The reduced production of conidia was also associated with slower growth rate of the fungal mycelium on the SNA medium. For example, the area of one-week-old fungal colonies with high virus titer was less than half the area of those associated with moderate virus titers (Fig. 32).

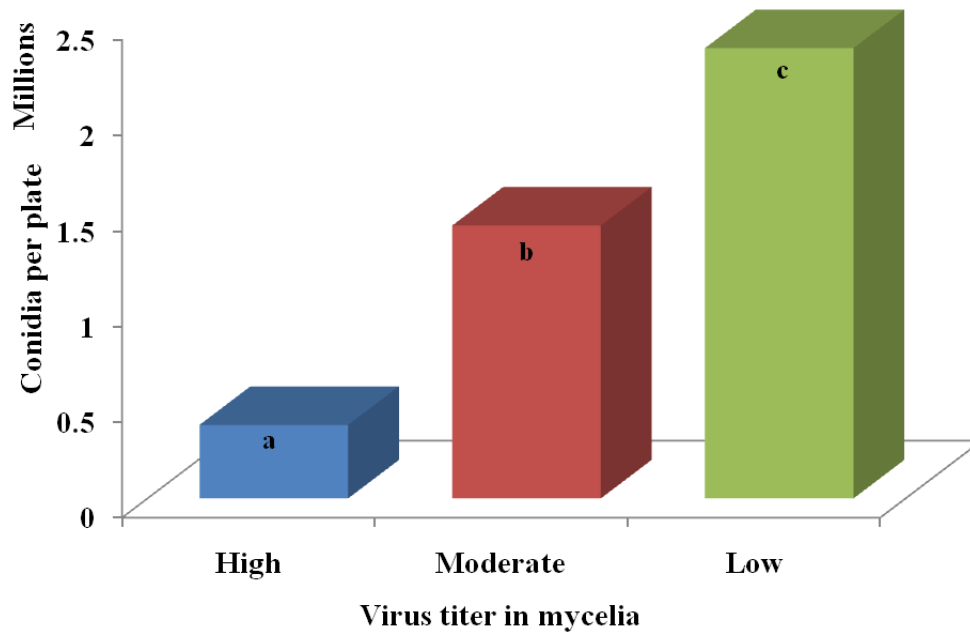


Fig. 31: Production of conidia in *F. graminearum* China 9 in correlation with the titer of FgV-ch9.

Conidiation was initiated in SNA medium at 18°C for 2 weeks. The results are the average of 5 independent experiments. Different letters indicates statistically significant differences.

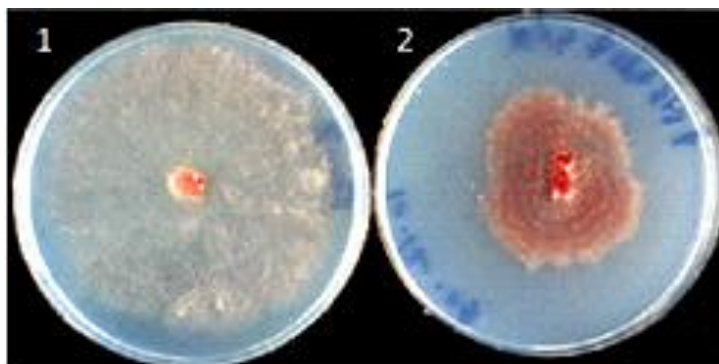


Fig. 32 Differences in the colony growth rate of mycelia associated with moderate and high virus titer on SNA medium.

1: China 9 + moderate virus titer, 2: China 9 + high virus titer. The cultures were photographed 7 days after inoculation.

3.6.4.1. Virus transmission through conidia

The transmission of FgV-ch9 through conidia was determined by means of RT-PCR using RdRP specific primers. Total nucleic acids from mycelia originating from single conidia cultures served as template in RT-PCR. Transmission of FgV-ch9 through conidia is dependent on the titer of the virus in the parent fungal inocula grown on SNA medium. When inocula with high

and moderate virus titers were used for conidiation, the virus transmitted into 100% of the emerging conidia (Fig. 33, A, B). The percent of virus-containing conidia produced from inocula of low virus titer was 20- 45 (Fig. 33, E, F). The use of inocula containing variable undefined virus titers for conidiation resulted in a virus transmission of 85-100% (Fig. 33, C & D).

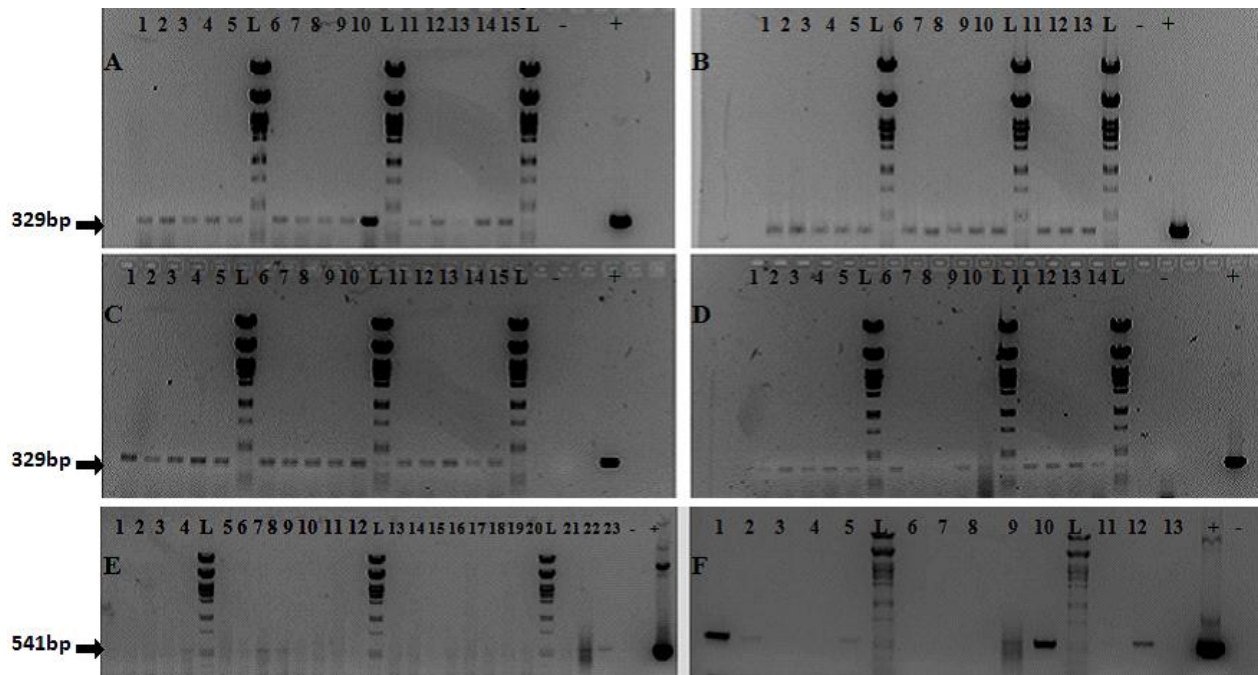


Fig. 33 RT-PCR products of the detection of FgV-ch9 in single conidia originating cultures of *F. graminearum* China 9 isolate.

The presence of the virus in conidia was tested by means of RT-PCR using primer specific to dsRNA1 (RdRP) of the virus. The conidia were produced on SNA using initial fungal inocula harboring titers of FgV-ch9 that are **A**: high, **B**: moderate, **C and D**: variable undefined, **E and F**: low. The expected sizes of the PCR products are indicated by arrows. L: λ DNA digested with *Pst*I, +: PCR positive control (a viral plasmid clone serve as a template in the PCR, -: PCR negative control (water).

3.6.5. Effect of virus titer on the pathogenicity of *F. graminearum* China 9 for wheat plants.

In addition to the reduced growth rate and conidiation of *F. graminearum* China 9 cultures associated with high and moderate titers of FgV-ch9, the virus is probably also associated with hypovirulence of its fungal host. Infection of wheat spikes with conidia originated from cultures associated with high and moderate virus titers resulted in typical Fusarium-blight symptoms that are restricted to the inoculated spikelet (10% of total kernels). However, the symptoms were visible in about 27% of kernels when conidia with low virus titers

were inoculated (Figs. 34 and 35). Inoculations with the WT PH-1 conidia and water were included as positive and negative controls, respectively.

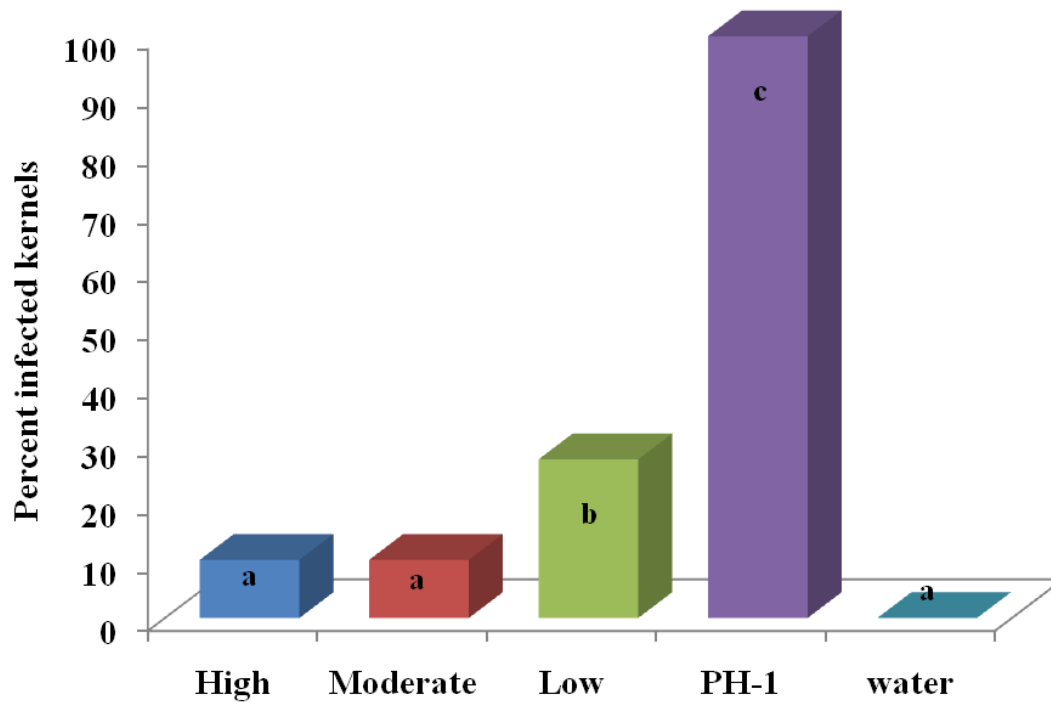


Fig. 34. Reduced pathogenicity on wheat plants of *F. graminearum* China 9 isolate is associated with the presence of FgV-ch9.

Results are the percent of infected kernels of 30 spikes per treatment except for PH-1 and water controls (8 and 6 spikes, respectively). The results were collected 3 weeks after inoculation. Different letters indicate statistically significant differences.



Fig. 35 Effect of the association of *F. graminearum* China 9 with different titers of FgV-ch9 on its pathogenicity on wheat.

Two central spikelets of wheat spikes were each inoculated with 500 conidia of the WT PH-1 (d) or China 9 harboring a: high virus titer, b: moderate virus titer, c: low virus titer. The infection was monitored for 3 weeks post inoculation. Arrowheads indicate inoculation sites.

3.6.6. Effect of virus titer on the pathogenicity of *F. graminearum* China 9 on maize plants.

Less than 6% of the maize kernels developed symptoms 5 weeks after of inoculation with *F. graminearum* China 9 cultures with high and moderate virus titers (Fig. 36). On the other hand, infection of maize cobs with conidia harboring low virus titers increase the percentage of infected kernels to 28% (Figs. 36 and 37). While cobs inoculated with water, (negative control) showed no disease symptoms, those infected with the WT PH-1 produced kernels with severe symptoms (Figs. 36 and 37).

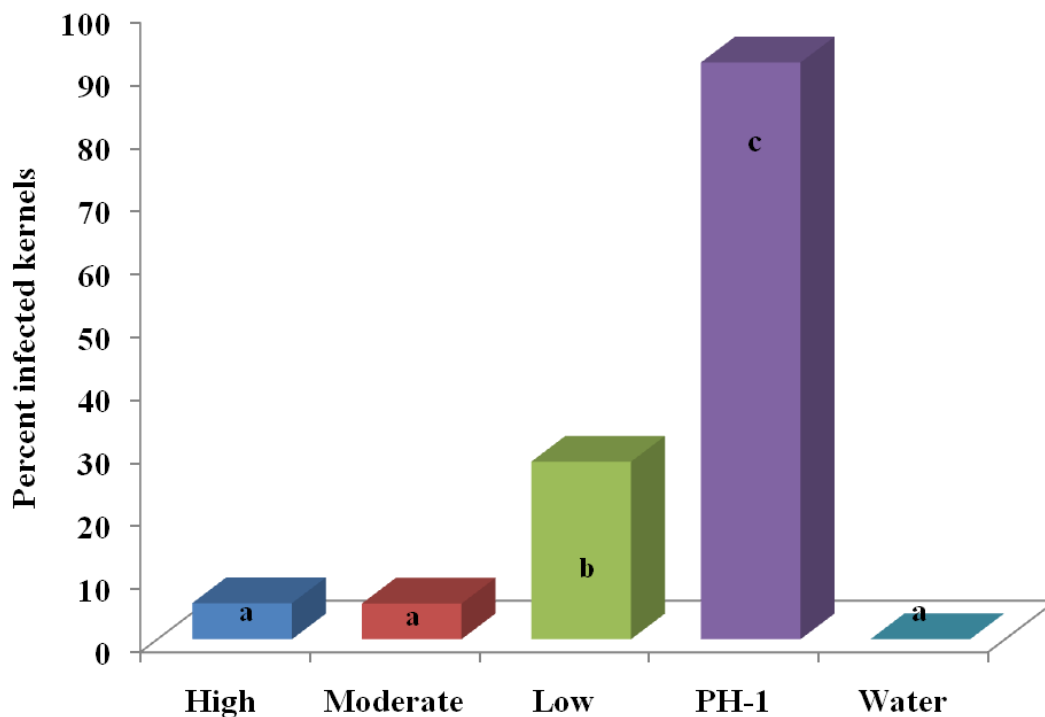


Fig. 36 Association of FgV-ch9 of hypovirulence of China 9 fungal isolate on maize.

Results are the percentage of infected kernels of 10 maize cobs per treatment. Each maize cob was inoculated with 4 ml of conidia suspension with a concentration of 40 conidia per μl or with purified water (control). Different letters indicate statistically significant differences.

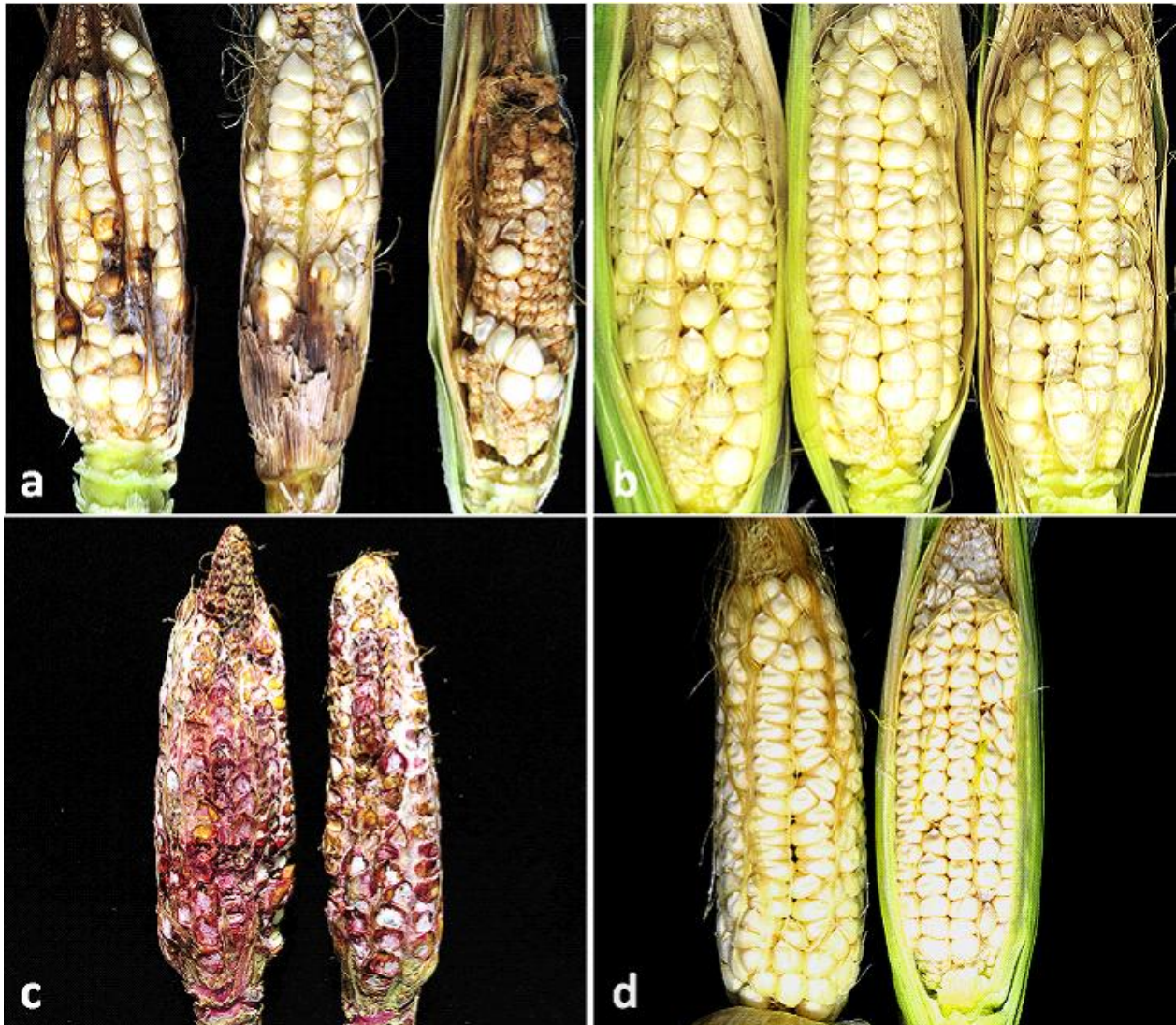


Fig. 37 Maize cobs inoculated with *F. graminearum* China 9 associated with different titers of FgV-ch9.

a: China 9 with low virus titer, **b:** China 9 with high and moderate virus titer, **c:** WT PH-1, **d:** water (negative control). The cobs were injected with the fungal conidia at the stage of early kernel formation. In each treatment, 10 cobs were injected. Cobs were Photographed 5 weeks post infection.

3.7. Transfection of *F. graminearum* PH-1 with particles of FgV-ch9.

In order to further proof that the hypovirulent features of China 9 isolate are due to its association with FgV-ch9, several attempts to cure the fungus completely from the virus were carried out. Unfortunately, all of these attempts failed. Alternatively, purified VLPs of FgV-ch9 were transfected successfully into the protoplasts of the WT isolate PH-1. Characterizations of the transfected cultures for possible hypovirulent traits are presented subsequent sections below.

Several mycelial plugs originating from transfected protoplasts were checked for the presence of FgV-ch9 by means of RT-PCR using two pairs of primers specific for 2 different viral genomic segments (Fig. 38). Two plugs with positive RT-PCR results were used for further studies. These plugs are named PH-1VLP1 and PH-1VLP11.

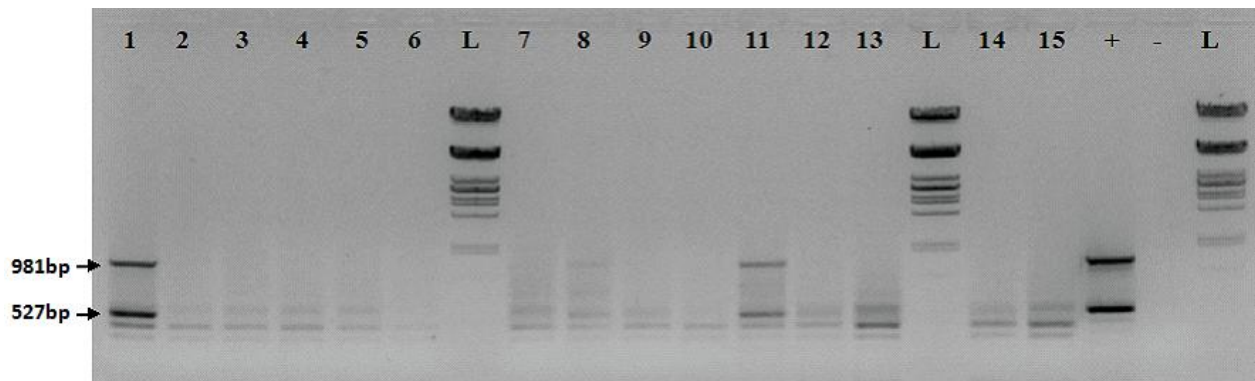


Fig. 38 Duplex RT-PCR of virus-transfected colonies of *F. graminearum* PH-1.

Reverse transcription was carried out using a primer (start F) that binds to a conserved region in the 5' UTR of all of the dsRNA segments. Duplex PCR was performed using the start F primer and other two primers 124REV and 3116RR that anneal to the RdRP and CP of FgV-ch9, respectively. Numbers **1-15** correspond to transfected colonies. **+** and **-** correspond to positive and negative PCR controls, respectively. **L:** λ DNA digested with *pst*I. Arrows indicated specific PCR bands.

3.7.1 Effect of FgV-ch9 on the conidiation of *F. graminearum* PH-1.

Conidiation of *F. graminearum* PH-1 was significantly reduced when the fungus was transfected with VLPs of FgV-ch9. The first generation of conidia produced from the virus-transfected colonies PH-1VLP1, and PH-1VLP11 were 1.8 and 0.85%, respectively, of the production in the control treatment (PH-1, Fig. 39). In the second generation of conidia, 13.3, and 10.5% of the WT production were produced by PH-1VLP1 and PH-1VLP11, respectively (Fig. 39). No further generations of conidia were produced.

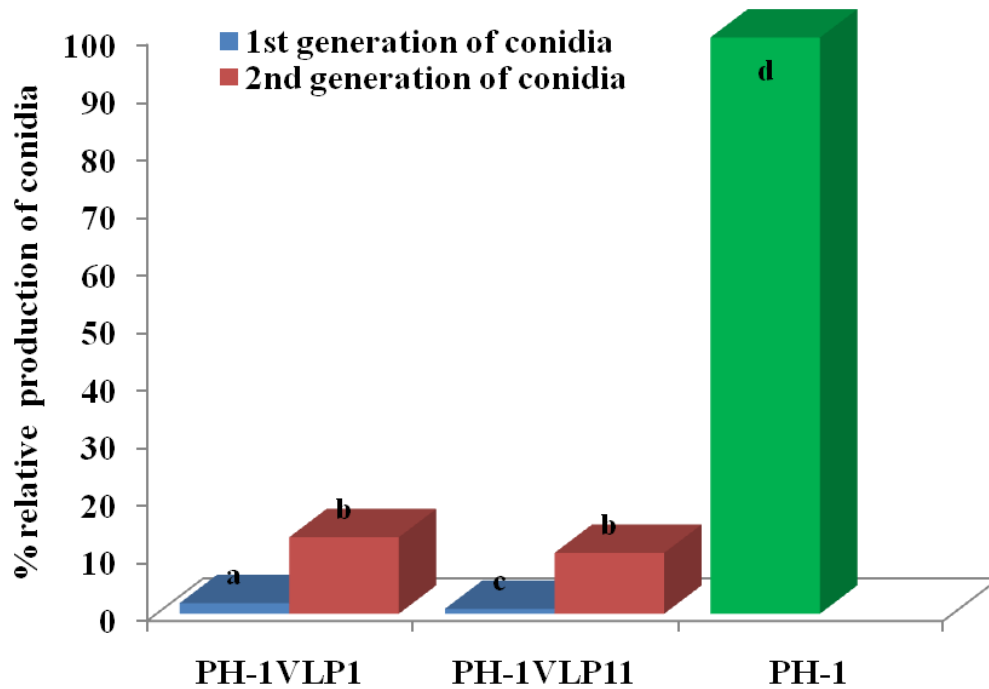


Fig. 39: Transfection of *F. graminearum* PH-1 with FgV-ch9 negatively affects its conidiation capacity.

The conidiation capacities of the strains are presented as percentage in relation to that of the WT PH-1. The results are shown for the first and the second generations of conidia produced from an FgV-ch9-transfected parent protoplast. Conidiation was initiated in SNA medium at 18°C for 2 weeks. The results are the average of 4 independent experiments. Different letters indicate statistically significant differences.

3.7.2 Effect of FgV-ch9 on perithecia development of *F. graminearum* PH-1.

Three *F. graminearum* PH-1 cultures originating from RT-PCR positive virus-transfected colonies were tested for their capacities to produce perithecia. For each culture, the number of perithecia is the average of perithecia counts of three plates. The capacity of the three virus-transfected cultures to produce perithecia was significantly reduced in comparison to the WT PH-1. The average number of produced perithecia in 9 plates representing 3 independent experiments was 45 in comparison to 220 in the WT PH-1 (Figs. 40 and 41). *F. graminearum* China 9 isolate did not produce any perithecia (Fig. 41, C).

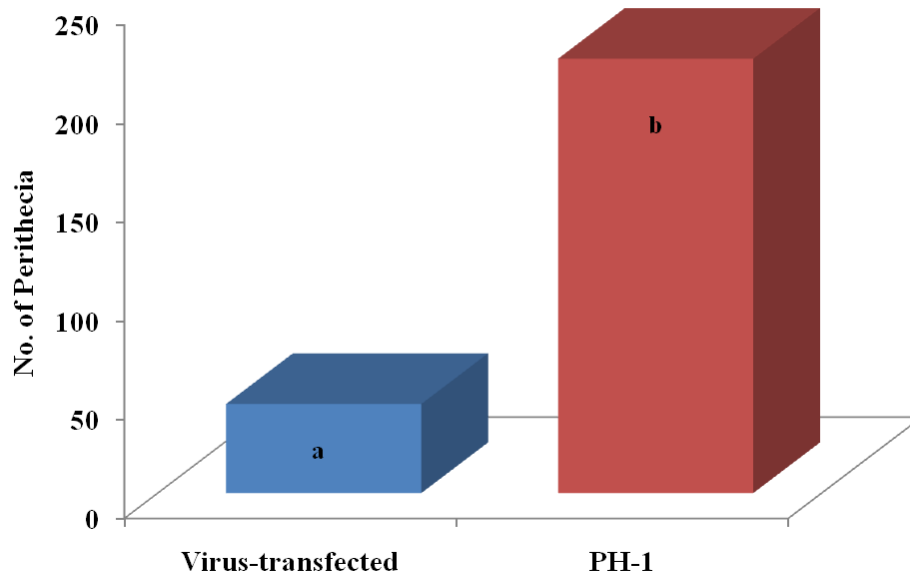


Fig. 40. Perithecia development of *F. graminearum* PH-1 before and after transfection with FgV-ch9.

Values are the average number of developed perithecia in 9 plates of carrot-agar medium representing 3 transfected-colonies. Produced Perithecia were counted under a stereoscope. Different letters indicated statistically significant differences.

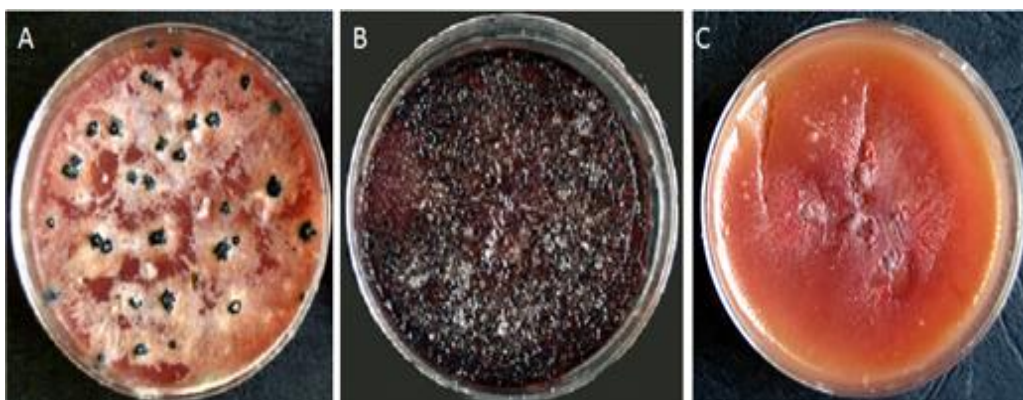


Fig. 41 Perithecia of *F. graminearum* PH-1 and China 9 isolates.

Perithecia were produced on carrot-agar medium and incubation at 25°C under a mixture of near UV and white light with a 12 h photoperiod up to three weeks. **A:** PH-1 isolate transfected with VLPs of FgV-ch9, **B:** WT PH-1, **C:** China 9 isolate.

3.7.3. Pathogenicity of virus-transfected *F. graminearum* PH-1 on wheat plants.

When wheat plants were inoculated with the first generation of conidia produced from PH-1VLP1 and PH-1VLP11, the percent of symptomatic kernels were reduced in comparison with untransfected WT PH-1. While the reduction in diseased kernels reaches 50% in case of PH-1VLP1, it was less than 20% for PH-1VLP11 (Figs. 42, 43, and 44). Symptoms developed due to infections with the second generation of conidia were comparable to those produced by the WT (Figs. 42, 43, and 44).

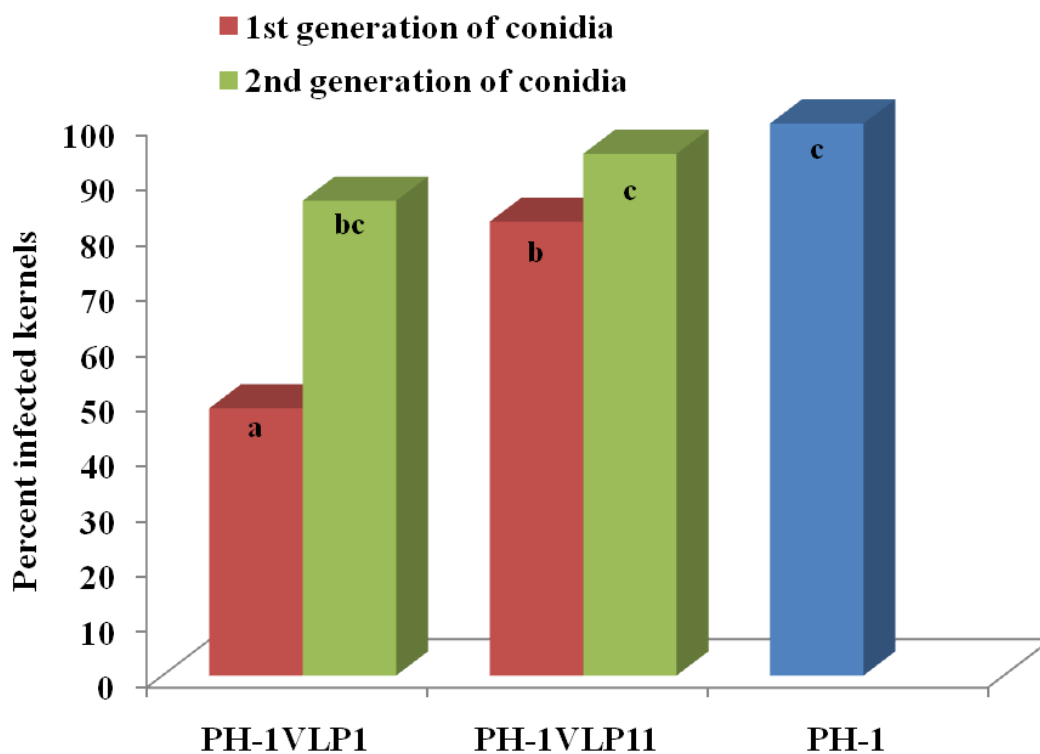


Fig. 42: Effect of transfection of *F. graminearum* PH-1 with VLPs of FgV-ch9 on its pathogenicity on wheat plants.

Results are the percent of infected kernels of 30-60 spikes per treatment except for the WT PH-1 (6 spikes). The pathogenicity was tested for two successive generations of conidia produced from two transfected cells (PH-1VLP1 and PH-1VLP11). The results were collected 3 weeks post infection. Different letters indicate statistically significant differences.

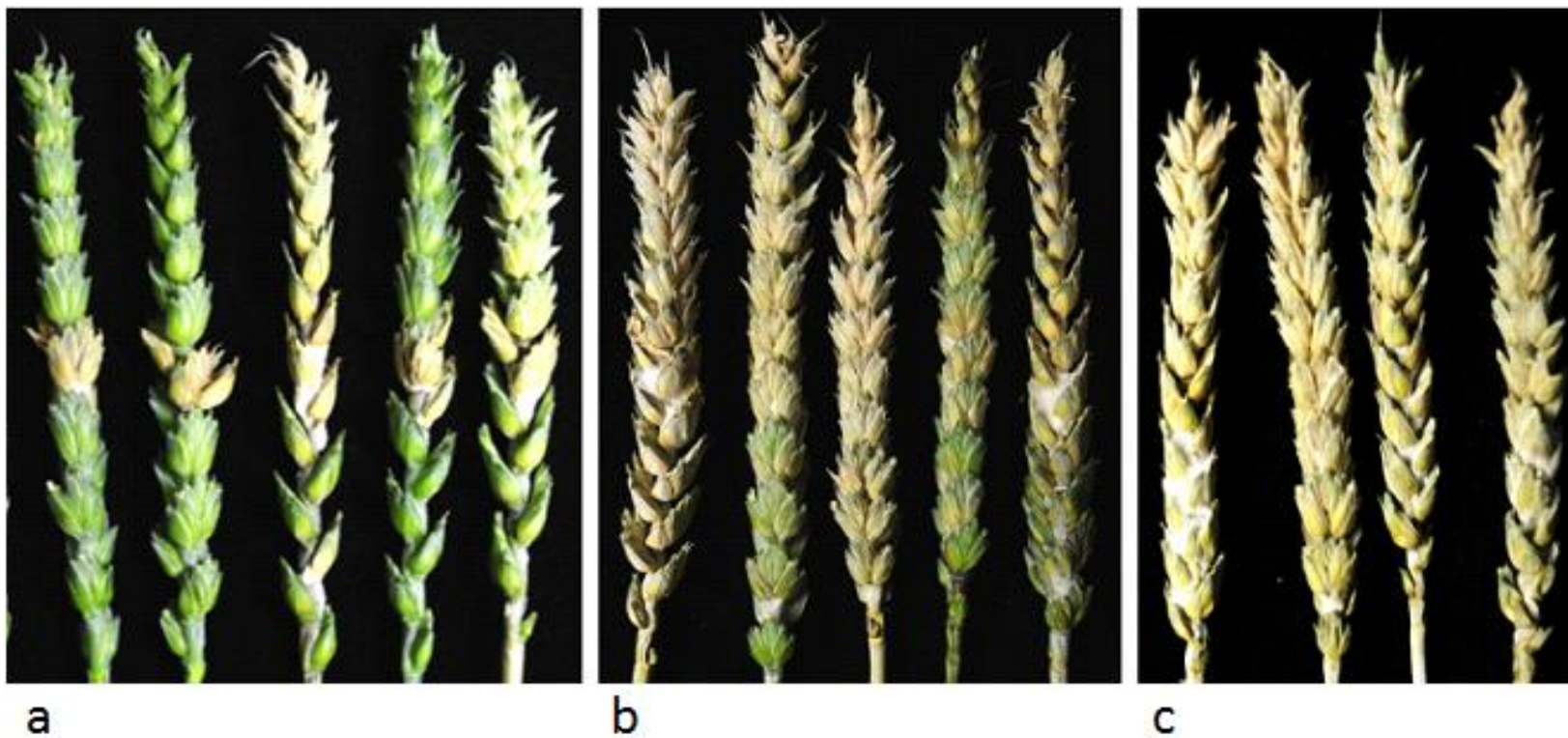


Fig. 43. Symptom development on wheat spikes after 3 weeks of inoculation with conidia of PH-1VLP1.

Each of two alternate central spikelets was inoculated with a 10- μ l suspension containing 500 conidia. **a:** First generation conidia of PH-1VLP1. **b:** Second generation conidia of PH-1VLP1. **c:** Conidia of WT PH-1. The infection was monitored for 3 weeks post inoculation.



Fig. 44. Symptom development on wheat spikes after 3 weeks of inoculation with conidia of PH-1VLP11.

Each of two alternate central spikelets was inoculated with a 10- μ l suspension containing 500 conidia. **a:** First generation conidia of PH-1VLP11, **b:** Second generation conidia of PH-1VLP11 and **c:** Conidia of the WT PH-1. The infection was monitored for 3 weeks post inoculation.

3.7.4. Pathogenicity of virus-transfected *F. graminearum* PH-1 on maize plants.

Infection of maize with PH-1VLP1 and PH-1VLP11 conidia resulted in 68 and 82% of diseased kernels, respectively (Fig. 45 and 45). In comparison to PH-1, these results are not significantly different.

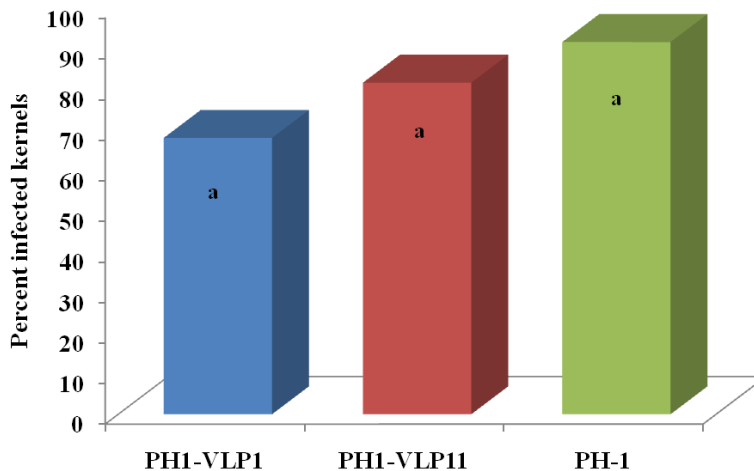


Fig. 45: Effect of transfection of *F. graminearum* PH-1 with VLPs of FgV-ch9 on its pathogenicity on maize plants.

Results are the percentage of infected kernels of 8-10 maize cobs per treatment. Each cob was inoculated with 4 ml of conidia suspension with a concentration of 40 conidia per μl . Different letters indicate statistically significant differences.

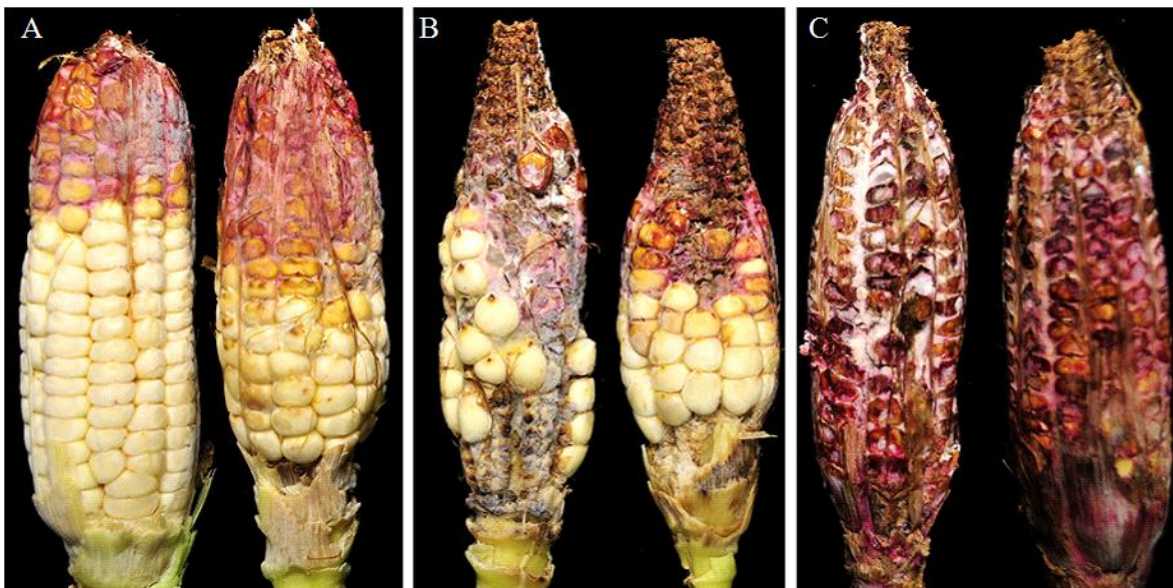


Fig. 46 Pathogenicity of *F. graminearum* PH-1 transfected with FgV-ch9 for maize.

Maize cobs were infected with conidia of PH-1 transfected with FgV-ch9. **A:** PH-1VLP1, **B:** PH-1VLP11, **C:** WT PH-1. The cobs were injected with the fungal conidia at the stage of early kernel formation. In each treatment, 8-10 cobs were injected. Cobs were Photographed 5 weeks post infection.

3.8. Co-infection of maize with *F. graminearum* China9 and PH-1

Simultaneous introduction of plant pathogenic fungi to the host could either decrease or increase the threshold of the disease. Following this concept, I have co-applied the virulent PH-1 and the hypovirulent China9 isolates into maize cobs to evaluate the outcome of such an interaction on the disease development.

The results showed that simultaneous inoculation of maize cobs with conidia of *F. graminearum* China 9 and PH-1 isolates significantly reduces the percentage of infected kernels in comparison to infections with PH-1 alone (Figs. 47 and 48). While infection due to China 9 or PH-1 resulted in 6 and 92% of diseased kernels, respectively, those resulting from a mixed infection of both isolates were 29% (Figs. 47 and 48).

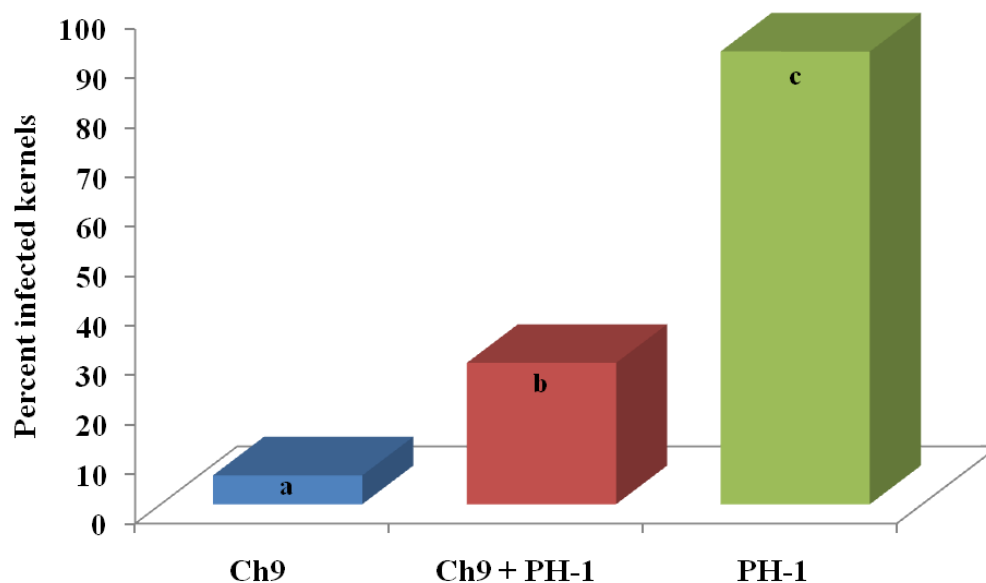


Fig. 47: Simultaneous inoculation of conidia of *F. graminearum* China 9 and PH-1 isolates into maize.

Results are the percent of infected kernels of 6-10 cobs per treatment. Each maize cob was inoculated with 4 ml of conidia suspension with a concentration of 40 conidia per μl . In case of simultaneous infection, 2 ml of conidia suspension from each isolate were mixed and injected into the maize cobs. Different letters indicate statistically significant differences.

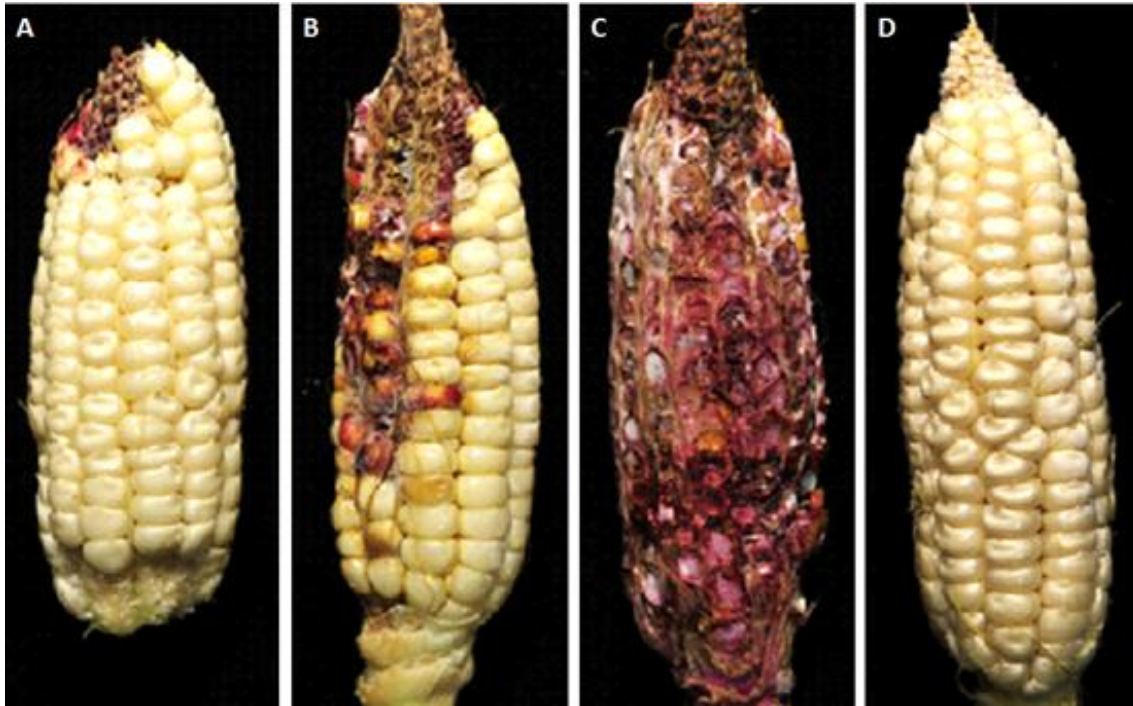


Fig. 48 Co-infection of maize with *F. graminearum* China 9 and PH-1 isolates.

Maize cobs were inoculated with conidia of **A:** China 9, **B:** China 9 and PH-1, **C:** PH-1 and **D:** water (negative control). In each treatment, 6-10 cobs were injected with the fungal conidia at the stage of early kernel formation. Maize cobs were photographed 5 weeks after inoculation with the conidia.

3.9. Consequences of the over expression of FgV-ch9 putative genes in *F. graminearum* PH-1.

In order to reveal the role of each of the genes encoded by the dsRNAs of FgV-ch9 in hypovirulence, the genes were cloned into a plasmid vector under a constitutive fungal promoter (*gpdA*) and transformed into protoplasts of the WT isolate PH-1. The effects of each constitutively expressed gene on the virulence of PH-1 isolate are presented.

The transformation of *F. graminearum* PH-1 protoplasts with Pan 7.1 vector cloned with the genes encoded by dsRNA2, 3, 4, or 5 resulted in several positive transformants checked by PCR. In each case, the expression vigor of 5 positive transformants was determined by means of semi quantitative PCR (Fig. 49). Colonies with the highest expression levels (Fig. 49, arrows) were double-checked for successful integration of the transgene by means of Southern blot analysis (Fig. 50). These colonies were used for conidiation as well as for pathogenicity assays on wheat and maize plants.

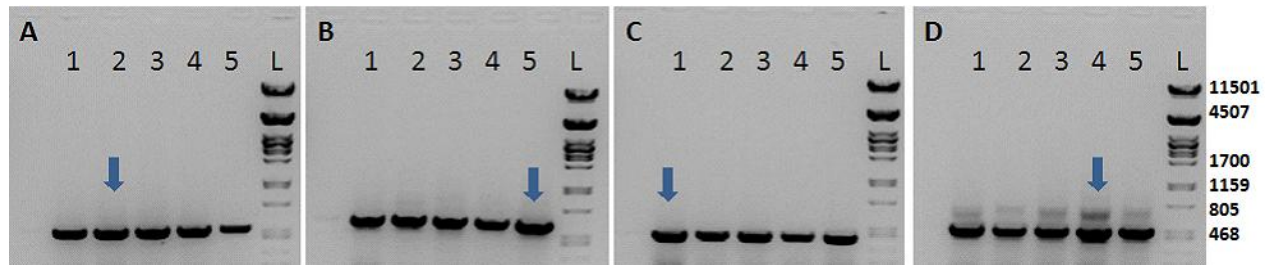


Fig. 49 Semi-quantitative PCR of *F. graminearum* PH-1 mutants expressing genes encoded by FgV-ch9.

The genes are encoded by **A:** dsRNA2, **B:** dsRNA3, **C:** dsRNA4, and **D:** dsRNA5 of FgV-ch9. For each gene, 5 mutants were tested (1-5). L: λ DNA digested with *pstI*. Mutants with the highest expression (arrows) were selected for further studies.

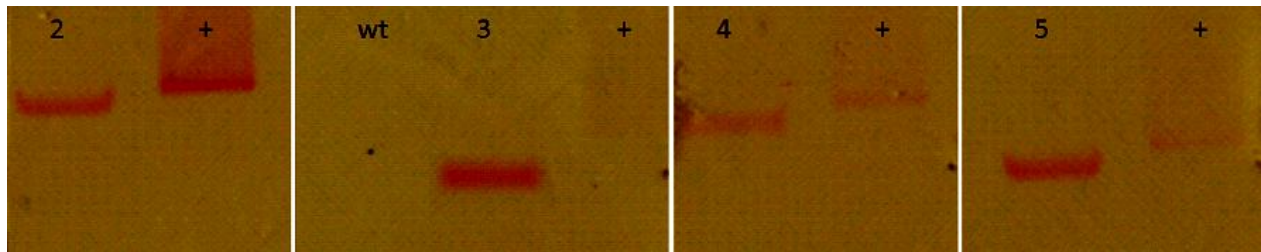


Fig. 50: Southern blot analysis of *F. graminearum* PH-1 mutants expressing genes encoded by FgV-ch9.

The DNA of the wild type and mutant strains were digested with the appropriate restriction enzyme and hybridized to the corresponding specific digoxigenin-labeled probe. Probes were prepared by PCR amplification. The mutants express genes encoded by **2:** dsRNA2, **3:** dsRNA3, **4:** dsRNA4, and **5:** dsRNA5 of FgV-ch9. **wt:** wild type PH-1, **+**: positive control (pAN7.1 construct digested with *Bam*HI). Signals were detected by the Fast Red Reagent.

3.9.1. The conidiation capacity of *F. graminearum* PH-1 expressing genes encoded by FgV-ch9.

In correlation to the WT PH-1, the percent conidia produced from PH-1 transformants expressing the gene encoded by **dsRNA2**, 3, 4, or 5 were 50, 53, 12, 23, respectively (Fig. 51). Statistically, these results for all overexpressed proteins are significantly different from those produced by the WT isolate. Moreover, the reduction in the capacity for conidiation was stricter in transformants with **dsRNA4** and 5 than those of **dsRNA2** and 3 (Fig. 51).

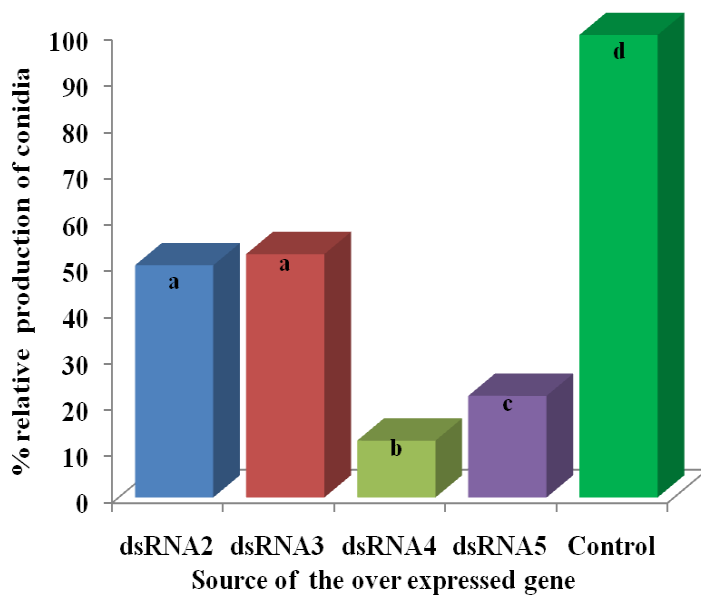


Fig. 51: Effect of FgV-ch9 genes on the conidiation of *F. graminearum* PH-1.

The results are presented as the percent conidia production of each treatment in relation to the control (PH-1). Conidiation was initiated in SNA medium at 18°C for 2 weeks. The results are the average of 3 independent experiments. Different letters indicate statistically significant differences.

3.9.1. Effect of the expression of genes encoded by FgV-ch9 on the pathogenicity of *F. graminearum* PH-1 on wheat.

The expression of genes encoded by dsRNAs 2, 3, 4, or 5 of FgV-ch9 in *F. graminearum* PH-1 significantly reduced the pathogenicity of the later on wheat plants. The pathogenicity was measured as the percentage of infected (diseased) kernels. While the percent of diseased kernels in the control treatment (PH-1) was 98%, those for PH-1 expressing the gene encoded by dsRNA2, 3, 4, or 5 were 58, 60, 46, 71, respectively (Figs. 52 and 53).

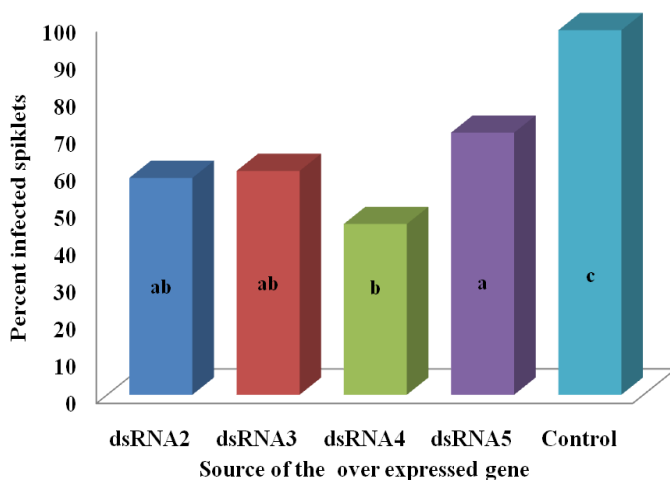


Fig. 52: Effect of the expression of FgV-ch9 genes on the pathogenicity of *F. graminearum* PH-1 for wheat.

Results are the percent of infected kernels of 25-50 spikes per treatment except for PH-1 control (14 spikes). The results were collected 3 weeks after inoculation. Different letters indicate statistically significant differences.

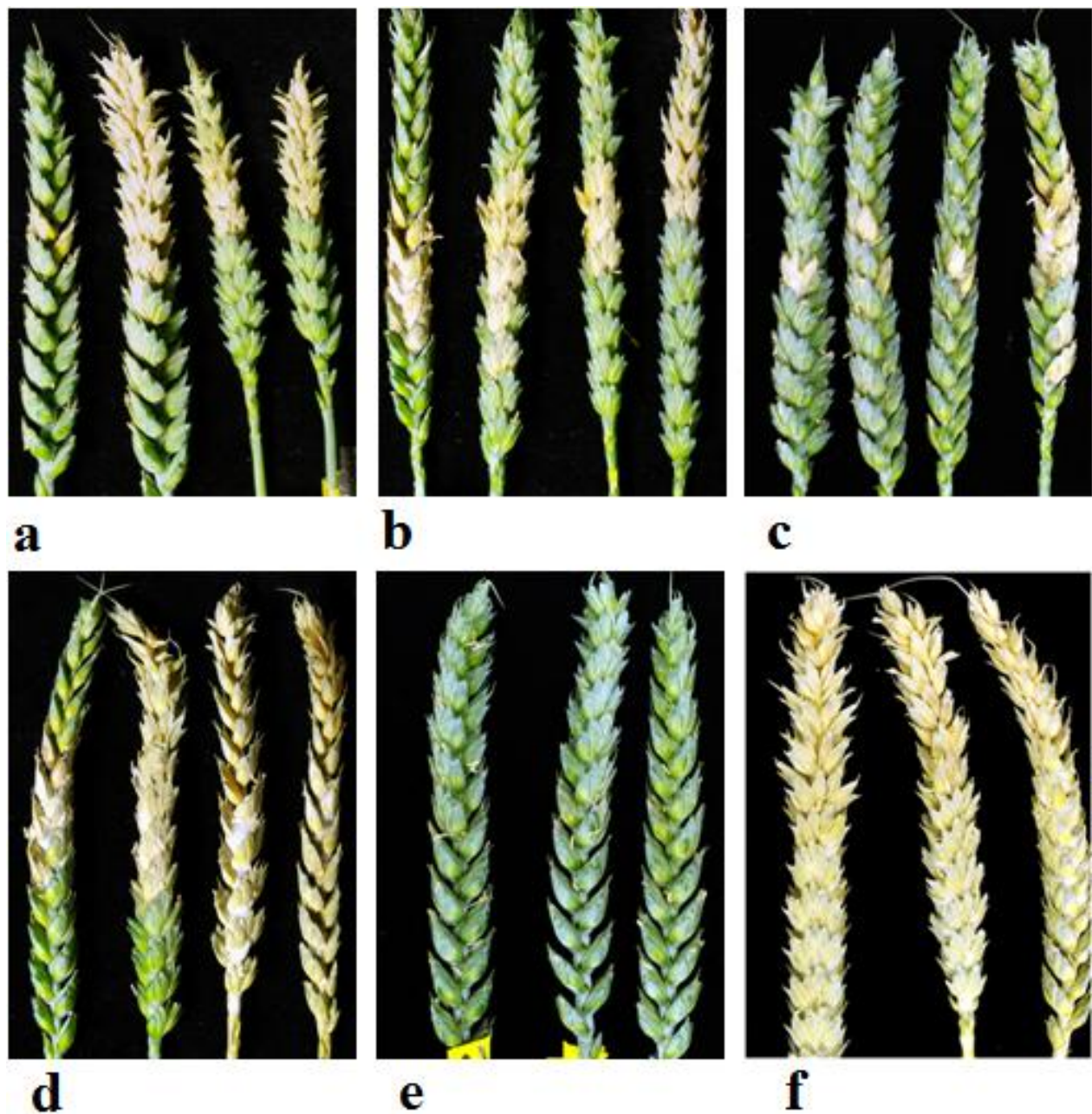


Fig. 53: The effect of the expression of FgV-ch9 genes on the virulence of *F. graminearum* PH-1 for wheat.

Two central spikelets of wheat spikes were each inoculated with 500 conidia expressing the gene encoded by **a: dsRNA2**, **b: dsRNA3**, **c: dsRNA4**, and **d: dsRNA5** of FgV-ch9. **e: water** (negative control), **f: WT PH-1**. The infection was monitored for 3 weeks post inoculation.

3.9.1. Effect of the expression of genes encoded by FgV-ch9 on the pathogenicity of *F. graminearum* PH-1 for maize.

The percentages of infected kernels in maize plants after 5-weeks of inoculation with *F. graminearum* PH-1 expressing the gene encoded by dsRNAs 2, 3, 4, or 5 of FgV-ch9 were 54, 56, 62, 79, respectively (Figs. 54 and 55). Except for the data collected for **dsRNA5**, the above results are significantly different from the percent of infected kernels in the WT treatment, PH-1, (92%).

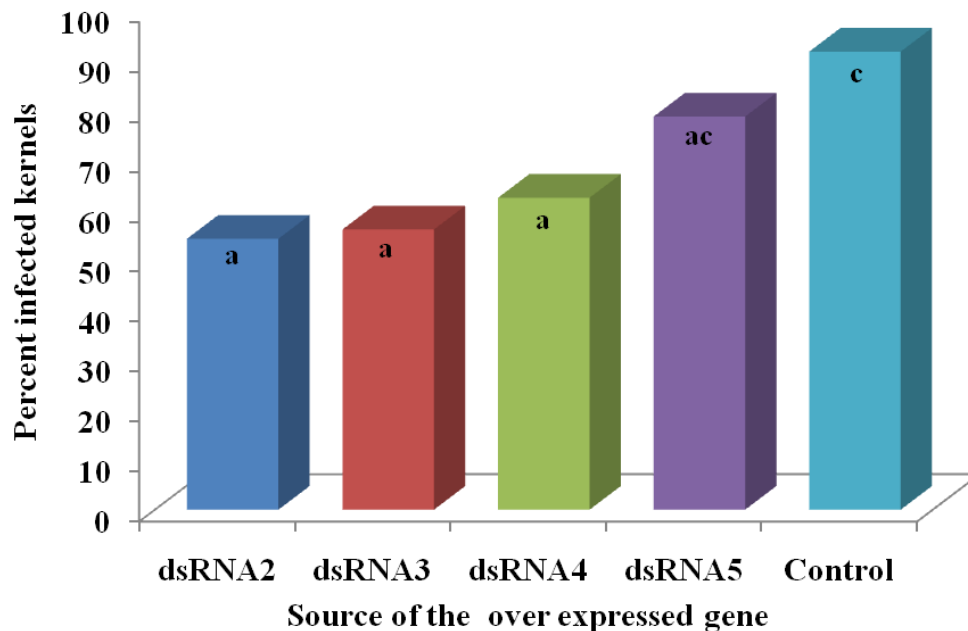


Fig. 54: Effect of the expression of FgV-ch9 genes on the pathogenicity of *F. graminearum* PH-1 for maize.

Results are the percentage of infected kernels of 10-20 cobs in each treatment except for the PH-1control (6 cobs). Each maize cob was inoculated with 4 ml of conidia suspension with a concentration of 40 conidia per μl . Different letters indicate statistically significant differences.



Fig. 55: Symptom development on maize cobs after 5 weeks of inoculation with conidia of *F. graminearum* PH-1 expressing genes of FgV-ch9.

The genes are encoded by **a**: dsRNA2, **b**: dsRNA3, **c**: dsRNA4, and **d**: dsRNA5 of FgV-ch9. **e**: water, **f**: WT PH-1. The cobs were injected with the fungal conidia at the stage of early kernel formation. In each treatment, 10-20 cobs were injected. Cobs were Photographed 5 weeks post infection.

In conclusion, all of the PH-1 mutants that express one of the genes of FgV-ch9 are hypovirulent in comparison to the WT PH-1 isolate. However, the threshold of the hypovirulence seems to be dependent on the expressed gene and the nature of the host plant (wheat or maize).

3.10. Effect of disruption of Dicer 2 gene on *F. graminearum* PH-1 and China 9 isolates.

To check if the levels of FgV-ch9 dsRNAs are regulated by the fungal host through quelling, I have knocked out the Dicer 2 gene of China 9 isolate. This experiment aimed also at evaluating the outcomes of eliminating the Dicer 2 gene on the vigor of the fungus. As a control experiment, the Dicer 2 gene of the WT PH-1 isolate was knocked out.

The results showed that the disruption of the Dicer 2 gene has no significant effect on the growth rate or on the pathogenicity of either *F. graminearum* PH-1 or China 9 isolates on wheat or maize plants. Furthermore, *F. graminearum* China 9 dicer 2-mutant cultures behave like WT cultures with low virus titers in term of the mycelium growth rate and virus titer in the mycelium.

4. Discussion

4.1. Optimization of the methods for the sequence determination of dsRNA templates.

The isolation of dsRNA from plant tissues has been established by Morris & Dodds (1979) as a tool to generally detect virus infections. Thereafter, the method, which requires two working days, has been frequently employed to purify dsRNA from plants and fungi as a source for the subsequent molecular biology techniques such as RT-PCR, cloning and sequence determination of viral genomes. I have optimized the method enabling better isolation of intact dsRNA in one day. First, the use of Bentonite in the extraction buffer as an RNase inhibitor helped to protect the dsRNA before and after the phenol treatment. Although dsRNA is more resistant to RNase A than ssRNA, it could be partially or fully degraded by this enzyme at low salt concentration. (Nosek *et al.*, 1993). Also, we observed that a combination of acidic phenol:chloroform:isoamyl alcohol at a ratio of 5:1:1 is optimal in term of the amount of dsRNA recovered in the aqueous phase (Sambrook *et al.*, 1989). Acidic phenol mixed with chloroform at the 5:1 ratio probably prevents the loss of RNA in the interphase due to insoluble protein:RNA complexes. Moreover, applying the phenol for 30 min at 37°C might substitute the incubation for 1 hour at room temperature. Finally, precipitation of the dsRNA in 1 volume of isopropanol for 1 hour at -80°C is equivalent in our hands to overnight precipitation with ethanol at -20°C. Alternatively, we have precipitated the dsRNA with isopropanol by 3 times freeze-thawing in liquid nitrogen.

The rPCR method has been established by Froussard (1992) as a tool to amplify whole RNA sequences. Several publications appeared later reporting the use of the method for the random amplification of viral dsRNA genomes (Marquez *et al.*, 2007 & Willenborg *et al.*, 2009). I have tested the sensitivity of this method for amplifying a mixture of dsRNA templates and optimized the conditions to produce distinct amplicons of considerable sizes. Such optimization reduces the time and costs for cloning and sequencing of many clones. In the range of 0.1 – 100 ng of dsRNA, the optimum concentration of the universal primer-dN₆ for achieving intense products suitable for cloning lies between 1-2 µM. The use of the Klenow Fragment for the second strand cDNA synthesis followed by the removal of excess universal primer-dN₆ has increased the number of smaller amplicons (Fig 2A). Using dsRNA as a template for reverse transcription provides dscDNA for the PCR amplification in the first step without any need for the Klenow Fragment reaction. In theory, the production of a second round of dscDNA would

result in shorter DNA templates for the subsequent PCR amplification. Although Froussard (1992) was able to amplify from 1 pg of ssRNA using the rPCR method, in our hands only amounts equal to 100 pg or more of the dsRNA templates resulted in considerable amplification products. However, we were still able to obtain few faint bands when 10 pg of dsRNA served as a template for the rPCR. Using a mixture of dsRNAs of 2.5 - 3.6 kb has resulted in a maximum rPCR product of about 3 kb under the conditions described in this study. Probably bigger sized PCR products could be achieved when longer templates are used. These results indicate that the rPCR is a powerful and sensitive method for the random amplification of dsRNA templates. The uneven representation of the 5 dsRNA segments among the rPCR products as revealed from their sequences, might be mainly due to their unequal abundance in the host.

The SPAT and FLAC methods constitute powerful means for the full-length amplification of long dsRNA templates when performed under optimum conditions. First, incubating the ligation reaction at 37°C in the presence of 20% PEG₆₀₀₀ and 10% DMSO highly improved the ligation of the primer to the 3' ends of the dsRNA. T4 RNA ligase is in principle an ssRNA ligase but was shown to ligate DNA oligos to dsRNA (Imai *et al.*, 1983) with a lesser efficiency though (Higgins *et al.* 1979). The ligation reaction can be inhibited by RNA secondary structures and can be enhanced with the addition of 10% DMSO and also by PEG which act by macromolecule crowding (Harrison and Zimmerman 1984). Probably, under the conditions described above, partial denaturation of the dsRNA termini occurs, providing single stranded 3' ends to the ligation enzyme. Second, denaturing the dsRNA with 1M betaine and 2.5% DMSO has resulted in full-length cDNAs. This treatment replaces the use of the highly toxic chemical MMOH, which is used frequently for such purposes (Potgieter *et al.*, 2009). The employment of DMSO alone at various concentrations for the denaturation of dsRNA was not sufficient for the production of full-length cDNAs. Whereas low DMSO concentrations (up to 15%) might not be enough to denature relatively long dsRNAs, the use of higher concentrations of DMSO might inhibit the reverse transcriptase (Jucà and Aoyama, 1995). Third, the ligations of linkers, which loop back to prime themselves, make the introduction of primers in the reverse transcription step superfluous. This eliminates the chances for initiation of cDNA synthesis from places other than the ends and reduces the production of truncated cDNAs. Fourth, the use of high annealing temperature in the PCR prevents non-specific amplification. Although the calculated melting temperature (T_m) for the primer PC2 is 54 - 56°C, going up to 67°C has not affected the specific annealing of the PC2 primer as revealed by the PCR results (Potgieter *et al.*, 2009). Finally, we have compared 5

commercial DNA polymerases, designed to amplify long DNA templates (4 – 20 kb), of which only two have produced the complete set of full length amplifiable products with different intensities, though. The other polymerases produced 0-3 full-length PCR products. We have no clue to interpret these results since the 5 polymerases were applied under optimal conditions using identical number of units, the same cDNA preparation, and the same thermocycler machine.

Our attempts to clone viral genomic dsRNA into DNA vectors without prior transcription and amplification steps showed that the principle is valid. Although under the conditions applied in this study we achieved relatively long sequences (up to 2.2 kp), we did not obtain full length clones. Surprisingly, the cloning of S1 nuclease-treated dsRNA into the pGEM®-T vector resulted in more transformants and bigger inserts than those obtained for ligation into the blunt end pJET1.2 vector. To my knowledge, there is no information in the literature explaining the mechanism by which such direct ligation and transformation take place. Skotnicki et al., (1985) claimed that it is possible to clone, in full or partially, dsRNAs from RF or replicative intermediate (RI) stages of several ssRNA viruses such as tobacco mosaic virus (TMV), alfalfa mosaic virus (AMV), Fiji disease virus (FDV), barley yellow dwarf virus (BYDV) and beet western yellows virus (BWYV).

However, the patent does not clearly state when it was possible to obtain full length clones, does not present data on the transformation efficiency, and to our knowledge was not published in any referred journal so far. I think that this method should be further explored to understand the molecular mechanisms behind it that might enable further optimizations and eventually lead to cloning of full-length dsRNA segments.

In conclusion, the optimization described in this study resulted in reproducible tools for the isolation, transcription and amplification of full length products from a mixture of dsRNA templates without prior need for separation or for the use of noxious chemical and expensive or time-consuming approaches. Furthermore, the implementation of the direct cloning of dsRNA into DNA vectors as a laboratory protocol requires more molecular investigations and evaluations. Such investigations may help to optimize the method for the cloning of full length RNA molecules, enhance their transformation efficacy and thereby might improve virome research.

4.2. Molecular characterization of FgV-ch9

We have isolated 10 *Fusarium* isolates from China and screened them for the presence of viral dsRNA. In one of the isolates, namely China 9, five dsRNA segments, associated with isometric VLPs of 35-40 nm in diameter were identified. The China 9 fungus isolate was phylogenetic-identified as *F. graminearum* using the method described by Ninet *et al.* (2005). The novel virus was named tentatively *F. graminearum* mycovirus-China9 (FgV-ch9). The purified dsRNAs were resolved into 2-5 bands following agarose gel electrophoresis depending on the virus titer in the fungal mycelium and the electrophoresis conditions. At high virus titer and relatively long electrophoresis times (~2 h), the dsRNAs could be resolved into 4-5 distinct bands. At low virus titer however, dsRNA 2, 3, and in some cases dsRNA1, were below the detection threshold of agarose gel electrophoresis. Additional reasons for the inconsistent resolution of the dsRNAs in agarose gel electrophoresis might be attributed to the small size differences between dsRNA 2, 3 and 4 (20-84bp), the unequal band intensities, and the heterogeneous lengths associated with the 3'UTR tandem repeats of dsRNA 4 (discussed below). Incomplete resolution in agarose gel was also reported for PcV and AsV1816 (Jiang and Ghabrial, 2004; van Diepeningen *et al.*, 2006). Our ability to detect the 5 dsRNAs segments in the various single-conidia originated cultures or in the purified virions by means of RT-PCR and northern hybridisation (data not shown) indicates that FgV-ch9 particles encapsidate 5 genomic segments.

The complete nucleotide sequence of the five dsRNA-segments was determined. Each of the segments was found to have a single unique ORF. BLAST searches with the deduced aa sequences showed that dsRNA1 encodes a putative RdRP, closely related to those of dsRNA mycoviruses. The function of the proteins encoded by dsRNA2 and dsRNA4 are not known yet since that encoded by dsRNA2 is only similar to the hypothetical protein encoded by MoCV1, while that of dsRNA4 has no significant similarity to any published protein. The identification of the protein encoded by dsRNA2 in SDS-PAGE might indicate that this protein is associated with virions. Interestingly, our analysis of 8 independent DNA clones of dsRNA4 showed that the 3'-terminus of this segment contains tandem sequence repeats of 146-208 nt in 6 of the clones. Although the dominant pattern of the sequence repeats was (146nt)_n, in two of the analyzed clones the repeated sequence was (208nt)_n where n = 2-5. The significance of such a 3' terminal repeat, which to my knowledge has not been reported for another dsRNA mycovirus, is unclear.

Evidence that dsRNA3 encodes the putative CP stems for two sources. First, *in vitro* surface protein labeling of CsCl gradient-purified virions followed by SDS-PAGE and immunoblot analysis showed preferential labeling of the protein band encoded by dsRNA3. Tryptic peptide sequences of this protein band were identical to those deduced from the DNA clones of dsRNA3. Second, BLAST searches showed relatively high sequence similarities between the protein encoded by dsRNA3 and the L3 protein of the AbV-1 associated with La France disease. The L3 protein was reported by Kuang *et al.* (2004) to be the AbV-1 CP.

The molecular masses of the proteins encoded by dsRNA2 and dsRNA3 as estimated in the SDS-PAGE (Fig.5, lane 2) are smaller than those calculated from the deduced aa sequences (Table 4). These differences might be due to protein processing or immature termination of translation since none of the tryptic-peptide sequences belonged to the C-terminus of the deduced aa sequences of dsRNA2 and dsRNA3 (Figs. 6b and 6c). A similar observation was reported for the protein p58 encoded by the tentative chrysovirus MoCV1 (Urayama *et al.*, 2010). Furthermore, the appearance of the protein band encoded by dsRNA3 as a double band (Fig.5, lane 2) might be due to posttranslational modifications. The double band, though encoded by the same gene, might represent closely related polypeptides associated with the capsid protein. Such a phenomenon was also described for the totivirus Hv190SV CP (Soldevila *et al.*, 2000).

Whereas, the N-terminus of the protein encoded by dsRNA5 shares no significant similarity with any known protein, we have identified 12 multiple-adjacent-C2H2 zinc fingers at its C-terminus. The detected fingers showed high similarity to zinc finger domains of higher eukaryotic organisms such as *Pan troglodytes* and *Homo sapiens*, lower eukaryotes such as the fungi *Neurospora crassa* and *Penicillium chrysogenum* and relatively moderate to low homology with fingers of few dsDNA viruses such as *Emiliania huxleyi* virus 99B1 and *Neodiprion abietis* nucleopolyhedrovirus (NPV). On the other hand, the zinc fingers of FgV-ch9 share no significant matches with any of the published proteins in the genus *Fusarium*. Proteins with C2H2 zinc fingers are well characterized as regulatory proteins that bind to DNA, ssRNA, dsRNA or proteins (Finerty and Bass, 1997; Iuchi 2001). In the case of multiple-adjacent-C2H2 zinc fingers, the binding to multiple different ligands was reported (Tsai and Reed, 1998). In retroviruses such as the human immunodeficiency virus 1 (HIV-1) and murine leukemia virus (MuLV), zinc fingers constitute part of the nucleocapsid protein and were reported to have regulatory functions important for the virus replication and infectivity (Boukhvalova *et al.*, 2010; Tanchou *et al.*, 1998). The significance of the detected C2H2 zinc finger motifs detected in FgV-

ch9 is still unexplored. To our knowledge, the presence of zinc finger domains has not been reported for a mycovirus before.

The relative quantitative RT-PCR results are in agreement with the unequal dsRNA intensities in agarose gel electrophoresis as discussed above. These results indicated that the dsRNAs, purified directly from the virions or from infected mycelium, must be present in unequal amounts. If all of the dsRNAs are encapsidated in one particle, which is probably not possible in this case because of packaging space constraints, they should be present in equimolar amounts. Moreover, the conserved terminal sequences present at the 5' and 3' UTRs of all five dsRNAs of FgV-ch9 is a property reported for ssRNA and dsRNA viruses with multipartite and multicomponent genomes (Ahlgquist, 1999; Attoui *et al.*, 1997; Mertens and Sangar, 1985). These results, together with one unique ORF on each segment, led to the conclusion, that the new virus indeed possesses five genomic dsRNA segments that are encapsidated separately and in unequal amounts.

Phylogenetic analysis of the RdRP and the putative CP of FgV-ch9 showed that although the virus is more related to *Chrysoviridae* than to other dsRNA mycovirus families, it clusters distinctly (Figs. 9a and 9b). The new cluster encompasses in addition to FgV-ch9, an unclassified totivirus (AsV1816), an obscure member of the chrysovirus (AbV-1), and a Vietnamese isolate of MoCV1. Although AsV1816 is associated with at least 4 dsRNAs (van Diepeningen *et al.*, 1998), only the sequence of the segment encoding the RdRP is known. The two smallest segments were inconsistently detected probably because they were obscured by the rRNA of the host fungus (Hammond *et al.*, 2008). Further characterization of this virus would be interesting since its RdRP shares the highest amino acid similarity with that of FgV-ch9 (90%, the sum of identical and similar aa residues). AbV-1, sometimes called the *white button mushroom virus* and *La France isometric virus*, is the suspected causal agent of the La France disease of cultivated mushroom (*Agaricus bisporus*). The disease is associated with isometric virus particles of AbV-1 and several (most common are L1-L5, M1 and M2) dsRNA segments (Kuang *et al.*, 2004; Romaine and Schlagnhauer, 1995). The deduced amino acid sequences of segments L1 and L3 encode the virus RdRP and the CP, respectively (Kuang *et al.*, 2004). Recently, AbV-1 was listed as a tentative chrysovirus in the 8th report of ICTV (Fauquet *et al.*, 2005). Besides the phylogenetic relatedness of the RdRP and putative CP encoded by FgV-ch9 and AbV-1, the

proteins encoded by dsRNA4 of FgV-ch9 and L5 dsRNA of AbV-1 share 23% identities and 40% similarities spanning 222 aa of the encoded proteins. The genomic sequences of the Vietnamese isolate of MoCV1 has been recently deposited in the Genbank and still not subjected to final classification although the authors related it to chrysovirus. Although MoCV1 might be associated with up to 8 dsRNAs, only the 4 most abundant and stable segments were sequenced (Urayama *et al.*, 2010). In general, FgV-ch9, AbV-1, MoCV1, and AsV1816 have common features that discriminate them from their relative members of the chrysovirus. In addition to the phylogenetic differences, these features include the number of genomic dsRNA segments (>4 in comparison to 4 for chrysovirus), and the point mutations in some of the RdRP conserved motifs (Fig. 5) as well as the mutations throughout other similar residues (Data not shown).

The phylogenetic analysis and genome organization of FgV-ch9 with 5 monocistronic dsRNAs, the presence of a zinc finger domain in dsRNA5 and a relatively long 3'UTR sequence repeats in dsRNA4 distinguish the virus from the mycoviruses reported so far. Based on the results described above, we propose the establishment of a new genus in *Chrysoviridae* to accommodate the 4 viruses forming the new phylogenetic cluster (FgV-ch9, AsV1816, AbV-1, and MoCV1).

4.3. Association of FgV-ch9 with hypovirulence of its host.

Fungal viruses have raised the interest of several scientific groups for the prospect potential use of hypovirulence-associated mycoviruses as biocontrol agents of plant pathogenic fungi (Nuss, 2005; McCabe *et al.*, 1999; Chiba *et al.*, 2009). Although most of the known mycoviruses are associated with latent infections with their hosts, several reports on a reduced or eliminated fungal pathogenicity due to mycoviral infection have been introduced recently (Zhang *et al.*, 2009; Castro *et al.*, 2003; Yu *et al.*, 2010; Preisig *et al.*, 2000; Mertens, 2004). As part of this study, the association of FgV-ch9 with hypovirulence-traits of its fungal host is explored and discussed.

The association of China 9 cultures originated from single conidia with different amounts (titers) of FgV-ch9 might be due to one of the following scenarios. First, the unequal and separate encapsidation of the virus dsRNAs might lead to their unequal representation in different single conidia. Second, different titers of the virus in the parent mycelium or conidial inocula, which

were used for the production of conidia, might result in different titers in the progeny ones. Finally, the virus replication, transmission, or accumulation might be regulated somehow by an unknown host mechanism. Possible mechanisms include silencing RNA (siRNA) or quelling. Due to possible genetic variations among fungal cultures stemming from single conidia, because of a point mutation as an example, such cultures may have differential accommodation capacity for the virus. To check this possibility, we have knocked out the Dicer 2 gene of China 9 and PH-1 isolates. The results showed that Dicer 2 gene has no significant role on the colony morphology, growth rate, conidiation capacity, and virulence of both isolates on wheat and maize plants. The titer of FgV-ch9 in Dicer 2 mutants of China 9 isolate was low (PCR detectable) which is probably because it originated from a single cell (protoplast). Although, Dicer 2 has most likely no effect on the titer of FgV-ch9, the possibility that the virus is regulated by a host siRNA is still valid. This is because of the presence of a second Dicer-like gene in the genome of *F. graminearum*. This gene is related to the Sms-3 endoribonuclease. Unfortunately, the Sms-3 gene was not subjected to knockout experiment in this study. Evidence that RNA silencing in fungi can function as virus defense were reported for *Aspergillus nidulans* (Hammond *et al.*, 2008) and *C. parasitica* (Segers *et al.*, 2007). They showed that *C. parasitica* with a mutation on its Dicer exhibit severe debilitation in comparison to the WT when it was infected with *Cryphonectria hypovirus 1* (CHV1, Segers *et al.*, 2007). On the other hand, some mycoviruses like *Aspergillus mycoviruses 1816* was reported as a suppressor of RNA silencing (Hammond *et al.*, 2008). Such a suppression effect was also reported for a protein encoded by CHV1 (Segers *et al.*, 2006).

The transmission of FgV-ch9 through conidia is dependent on its titer in the mycelium of the parent culture. The higher the virus titer in the host mycelium, the higher is its chances to be transmitted into every emerging conidium. Since the viral genome is unequally and separately encapsidated, the chance to obtain a conidium which lack one or more of the viral particles (segments) is possible. This is true for conidia originated from mycelium associated with low virus titers. However, the ability of mycoviruses for intracellular transmission would substitute for such lack of a certain particle in cultures originating from high number of conidia. The frequent observation of less than 5 dsRNA segments in agarose gels in some cultures of the China 9 isolate might be in part due to the lack or inadequate representation of certain virus particles. In the literature, the transmission of mycoviruses through conidia varied from 1-100%

depending on the mycovirus and its fungal host (Chu *et al.*, 2002; Demarini *et al.*, 1977; Ihrmark *et al.*, 2002; Hunst *et al.*, 1986; Lima *et al.*, 2010).

We have shown that the fungal isolate China 9 that contain high virus titer exhibits abnormal and disorganized cytoplasm. Such observation was reported for several mycoviruses including reports on the accumulation of vacuoles (Kwon *et al.*, 2007), disintegration of nuclei and mitochondrial membranes (Zhang *et al.*, 2009), cytoplasm of electron light density (Kwon *et al.*, 2007, Zhang *et al.*, 2009). Moreover, the aggregation of fungal virus particles in membranous structures or vesicles was observed in *Sclerotinia sclerotiorum* (Zhang *et al.*, 2009), *Penicillium chrysogenum* (Volkoff *et al.*, 1972; Yamashita *et al.*, 1973), *P. cyaneofulvum* (Border *et al.*, 1972), *P. funiculosum* (Border *et al.*, 1972), *P. brevicompactum* (Hooper *et al.*, 1972), *A. bisporus* (Albouy, 1972; Dieleman-van Zaayen, 1972a; Dieleman-van Zaayen, 1972b), *P. stoloniferum* (Adler and MacKenzie, 1972; Corbett, 1972; Hooper *et al.*, 1972), *Peziza ostracoderma* (Dieleman-van Zaayen, 1970), *Saccharomyces cerevisiae* (Border, 1972), *Thraustochytrium sp.* (Kazama and Schornstein, 1972; Kazama and Schornstein, 1973), and *A. foetidus* (Banks *et al.*, 1971). Similar pattern of virus particle aggregation was also observed for several animal viruses such as Herpes virus (Nii *et al.*, 1968; Shipkey *et al.*, 1967) and adenovirus (Yamamoto, 1969) as well as plant viruses (Weintraub and Ragetli, 1970).

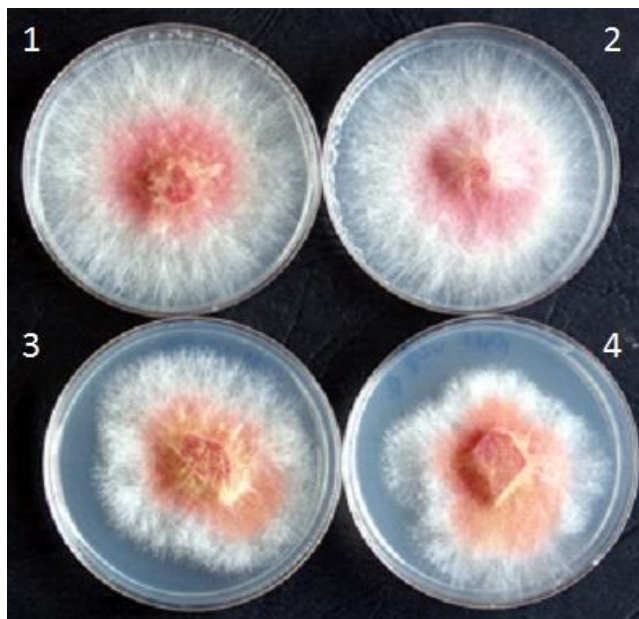


Fig. 56 Growth rate differences between PH-1 cells after transfection with FgV-ch9.

1 and 2: not transfected. **3 and 4:** successfully transfected. Transfected cells were grown on CM medium at 25°C in the dark. Mycelia were photographed after 4 days of culturing.

The reduced growth rate and altered colony morphology of fungal colonies due to the presence of viruses in their hyphae was reported for many fungal species including *F. graminearum* (Kwon *et al.*, 2007). In the case of FgV-ch9, such alterations seem to be correlated with the virus titer in the hyphal cells. Moreover, the reduction in the growth rate of *F. graminearum* cultures was observed for FgV-ch9 transfected colonies (Fig. 56, plates 3, 4), but not in virus free colonies (Fig. 56, plates 1, 2), of the WT isolate PH-1. Furthermore, these restrictions in the growth rate of FgV-ch9 transfected cells was observed in part of the colonies originating from the first (Fig. 57), but not the second generation of conidia produced from such virus-transfected cells. Such reduction in the growth rate of fungi associated with mycoviruses was reported for *F. graminearum* (Chu *et al.*, 2002) and many other fungal species (Jiang *et al.*, 1998; Xie *et al.*, 2006; Zhang *et al.*, 1993; van Diepeningen *et al.*, 2006).

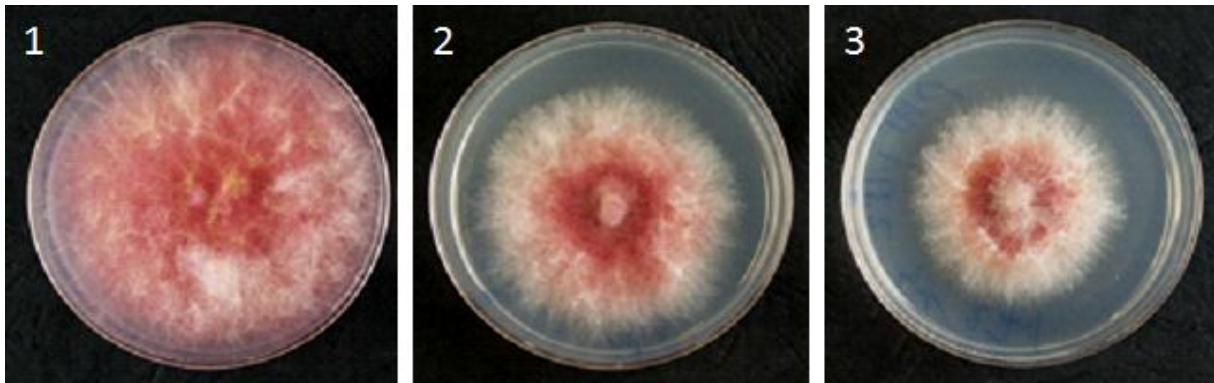


Fig. 57 Single conidia originating cultures of virus transfected PH-1 cells display growth rate differences.

The cultures originated from the 1st generation conidia. Conidia were grown in CM medium at 25°C in the dark. Mycelia were photographed after 4 days of culturing.

More important is the association of FgV-ch9 with significant reduction on the conidiation capacity of its fungal host. This was observed for China 9 isolate with high, moderate and low titers of FgV-ch9, where the fungal capacity to produce conidia was negatively correlated with the virus titer. This phenomenon was also observed in FgV-ch9 transfected cultures of the WT PH-1 isolate, where a severe reduction (~95%) was documented. In addition to that, transfection of PH-1 isolate with FgV-ch9 significantly reduced the former capacity to produce perithecia. Furthermore, the expression of the genes encoding by FgV-ch9 in the WT isolate also reduced the conidiation capacity of the later. Such association of the virus, or its genes, with reduced conidiation and perithecia formation might be of biological and economical

importance. Conidia and ascospores of *F. graminearum* play a key role on the spread of the fungus in the field, which would contribute to the disease catastrophe. In addition, perithecia can constitute an infectious dose in future culturing seasons of cereals because of their ability to survive and overwinter. Reduced conidiation capacity of some fungi due to their association with mycoviruses was reported for several fungal species (Castro *et al.*, 2003; Deng and Nuss, 2008; van Diepeningen *et al.*, 2006).

In addition to the restricted growth, reduced conidiation capacity, and the disorganized cytoplasm of China 9 associated with moderate to high virus titers, the association of FgV-ch9 with hypovirulence of its fungal host was observed in wheat and maize plants inoculated with the fungus conidia. *F. graminearum* China 9 ability to infect wheat and maize is 3-10 folds (depending on the virus titer) lower than that of the WT PH-1. Further studies were conducted to check if this difference in the virulence of the two isolates is due to the association of China 9 with FgV-ch9. First, the percentages of the infected wheat and maize kernels (50% and 73%, respectively) in the plants inoculated with the first generation of conidia produced from FgV-ch9 transfected PH-1 cells were significantly lower than those observed due to infections of the WT PH-1 (> 95%). However, the inoculation of the plants with the second generation of conidia produced from FgV-ch9 transfected PH-1 results in disease symptoms comparable to those of the WT isolate. Although, this might be an indication of an instable transfection, the results obtained for the first generation of conidia may indicate that FgV-ch9 is associated with hypovirulence of its host.

In a second experiment, conidia of both isolates (China 9 and PH-1) were mixed at a ratio of 1:1 and inoculated into the maize cobs. This resulted in 60% reduction in the number of diseased kernels. The mechanism by which such an effect took place is not fully understood. However, it might be due to the transfer of the virus to the WT isolate by anastomosis or due to competition between the fungal isolates. Decreased ability to colonize cereals, reduced production of mycotoxins, and reduced virulence because of competition between isolates of *F. graminearum* (Xu *et al.*, 2007) as well as between different *Fusarium* species was described by several researchers independently (Velluti *et al.*, 2000; Ramakrishna *et al.*, 1996; Miedaner *et al.*, 2004).

An extra proof of the association of FgV-ch9 with hypovirulence of its fungal host is supported by the results of expressing the individual genes encoded by the virus in cells of the WT isolate PH-1. In general, the expression of any of the genomic segments of FgV-ch9

(dsRNA2-5) resulted in reduced virulence of the fungal host (PH-1) either on wheat or on maize. However, the percentage of diseased wheat kernels due to infections with PH-1 mutant expressing dsRNA4 was less than those resulted from infections of the other mutants. This probably indicates an association of the gene encoded by dsRNA2 with hypovirulence of the fungal host on wheat. In maize however, the results reveal a probable association of the genes encoded by dsRNA2 and dsRNA3 with hypovirulence of the fungal host. This indicates that dsRNA3 might possess other functions in addition to the particle formation. Such a phenomenon, multiple functions of certain viral genomes, was frequently reported (Leupin *et al.*, 2005; Yu and Summers, 1994; Villordo *et al.*, 2010). The resolution of the CP of FgV-ch9 as a double band in SDS-PAGE, which might be due to a phosphorylation event, may have a role in the association of this protein with multiple functions. Phosphorylation of the CP of the hepatitis B virus was reported to result in multiple functions of the protein in the virus replication (Yu and Summers, 1994). They also found that phosphorylation of a certain amino acid of the CP is required for the initiation of infection.

As a conclusion, the information collected in this study on the novel virus FgV-ch9, including the complete nucleotide sequence, molecular characterization, and its association with hypovirulence of its fungal host, might constitute a solid basis for further studies that would employ the virus or its genes for the control of diseases of *Fusarium graminearum*.

5. Summary

Ten *Fusarium graminearum* (*F. graminearum*) isolates from China were screened for the presence of dsRNA mycoviruses. One of them, namely *F. graminearum* China 9 showed 5 dsRNA segments after agarose gel electrophoresis with sizes ranging from 2.4 to 3.5 kb. Isometric Virus-Like particles (VLPs) of about 40 nm were successfully purified by means of CsCl equilibrium-centrifugation and observed under the Transmission Electron Microscope.

As part of this study, several PCR-based methods for the sequence determination of dsRNA templates were optimized. These methods include random PCR (rPCR), Single Primer Amplification Technique (SPAT), and Full Length Amplification of cDNA (FLAC). Sequences obtained by the rPCR revealed partial information of 4 of the virus genomic segments. Moreover, the modified versions of the SPAT and FLAC methods allowed the amplification of full-length segments representing all or part of the viral dsRNAs in a single RT-PCR. In a less efficient approach, viral sequences up to 2.2 kb in length were obtained by cloning purified dsRNA segments directly into DNA vectors.

Using the above methods, the 5 dsRNA segments of *F. graminearum* mycovirus China 9 (FgV-ch9) were completely sequenced and a single ORF per segment was identified. BLAST results showed that dsRNA1 possess RdRP conserved motifs, dsRNA2 resembles the hypothetical protein encoded by dsRNA3 of *Magnaporthe oryzae* chrysovirus 1, dsRNA4 share no significant similarity to any published protein, and dsRNA5 has a C2H2 zinc finger domain. Tandem Mass Spectrophotometry, surface protein labeling of purified VLPs, SDS-PAGE, and protein BLAST results support that three of the virus segments code for structural proteins of which dsRNA3 possibly codes for the capsid protein. Relative quantitative PCR studies of the 5 dsRNAs isolated from purified VLPs suggested that the segments are encapsidated separately in unequal amounts. Genomic structure of the virus and the phylogenic study of its RdRP and capsid proteins support that FgV-ch9 would possibly be the candidate as a type species for a novel genus in the family *Chrysoviridae*.

Single conidia originating cultures of *F. graminearum* China 9 isolate were classified into three categories based on their association with various amounts of FgV-ch9 dsRNAs. Moderate and high virus titers of FgV-ch9 are associated with hypovirulence-related traits of China 9 isolate. These include disorganized cytoplasm with large vacuoles and membrane-bound structures, abnormal colony morphology with restricted growth rate, low conidiation capacity,

inability to form perithecia, and reduced virulence on wheat and maize plants. To check if these phenotypes are due to the direct association with FgV-ch9, purified Virus-like Particles (VLPs) were transformed into the WT fungal isolate PH-1. The results showed that FgV-ch9 transfected cultures of PH-1 exhibit reduced pathogenicity of 10% - 50% for wheat and 20-30% for maize, a significant reduction in their conidiation capacity (>90%), and 5 folds reduction in perithecia formation. Simultaneous application of the China 9 and PH-1 isolate on maize plants resulted in 3 folds reduction of the diseased kernels.

When the genes encoded by FgV-ch9 dsRNA2, 3, 4, or 5 were constitutively expressed in cells of PH-1 isolate, a reduction of 50-90% on the conidiation capacity of these cells was observed. Moreover, the results indicated that the genes encoded by dsRNA 2, 3, and 4 significantly reduced the pathogenicity of PH-1 for wheat (42, 40, 54%, respectively) as well as for maize (46, 44, 38%, respectively). The over-expression of the gene encoded by dsRNA5 resulted in a less profound effect in either plant. These results confer that FgV-ch9 is associated with hypovirulence of *F. graminearum* and might constitute a prospective material for the control of FHB and ear rot diseases.

6. Zusammenfassung

Zehn *Fusarium graminearum* (*F. graminearum*) Isolate aus China wurden auf die Anwesenheit von dsRNA und damit Mycoviren gescreent. Eines von ihnen, nämlich *F. graminearum* China 9 enthielt 5 dsRNA Segmente nach Agarosegelelektrophorese mit Größen von 2,4 bis 3,5 kb. Isometrische Viruspartikel von etwa 40 nm wurden erfolgreich durch CsCl-Gradienten Zentrifugation gereinigt und konnten unter dem Transmissionselektronenmikroskop beobachtet werden.

Als Teil dieser Studie, wurden PCR-basierte Methoden für die Bestimmung der Sequenzen der dsRNA Segmente optimiert. Diese Methoden umfassten random PCR (rPCR), Single Primer Amplification Technique (SPAT) und Full Length Amplifikation der cDNA (FLAC). Sequenzen, durch rPCR lieferten Teilinformationen von vier der viralen genomischen Segmente. Darüber hinaus erlaubten die modifizierten Versionen der SPAT und FLAC Methoden die Amplifikation voller Länge Segmente für die meisten der viralen dsRNA mit einer einzigen RT-PCR. In einem weniger effizienten Ansatz wurden virale Sequenzen bis zu 2,2 kb Länge durch Klonieren gereinigter dsRNA-Segmente direkt in DNA-Vektoren erhalten.

Mit diesen Methoden wurden die fünf dsRNA Segmente von *F. graminearum* Mycovirus China 9 (FGV-ch9) vollständig sequenziert. In jedem Segment wurde ein einziger ORF identifiziert. BLAST Ergebnisse zeigten, dass die dsRNA1 konservierte RdRP Motive beinhaltet. Das dsRNA2 Segment ähnelt dem hypothetischen Protein von dsRNA3 des *Magnaporthe oryzae* chrysovirus 1. Für die dsRNA4 konnten keine signifikanten Ähnlichkeiten zu bekannten Proteinen nachgewiesen werden. Für das dsRNA5 Segment konnte eine C2H2 Zinkfinger-Domäne im C-terminalen Bereich identifiziert werden. Tandem Massenspektrometrie, Oberflächenprotein-spezifische *in vitro*-Markierung mit Fluoreszenzfarbstoffen von gereinigten VLPs und SDS-PAGE, sowie Protein BLAST Ergebnisse belegen, dass drei Segmente des Virus Strukturproteine sind. Möglicherweise kodiert die dsRNA3 für das Kapsidprotein.

Ergebnisse von relativen quantitativen PCR Untersuchungen der Mengenverhältnisse der fünf dsRNAs aus gereinigten VLPs führten zur Hypothese, dass die Segmente einzeln in ungleichen Mengen verpackt werden. Die genomische Organisation des Virus und phylogenetische Untersuchung basierend auf der RdRP und dem Kapsidprotein unterstützen in hohem Maße die Zugehörigkeit von FGV-ch9 zur Familie der *Chrysoviridae*, und wo es möglicherweise die Typspezies einer neuen Gattung darstellt.

Kulturen aus vereinzelt Konidien von *F. graminearum* China 9 wurden in drei Kategorien auf der Basis ihrer Assoziation mit verschiedenen Mengen von FGV-ch9 dsRNAs gruppiert. Dabei waren moderate und hohe Virustiter von FGV-ch9 mit Hypovirulenz in China 9 verbunden. Dies umfasste desorganisierte Zytoplasmastrukturen mit großen Vakuolen und Membran-gebundenen Strukturen, abnorme Koloniemorphologie mit eingeschränktem Wachstum, reduzierte Konidienproduktion, Unfähigkeit Perithezien zu bilden, und verminderte Virulenz des Pilzes für Weizen und Maispflanzen.

Um zu überprüfen, ob diese Phänotypen auf direkter Interaktion mit FGV-ch9 beruhen, wurde ein virusfreies *F. graminearum* Isolat, PH-1, mit gereinigten Virus-ähnlichen Partikeln (VLP) transfiziert. Die Ergebnisse zeigten, dass FGV-ch9 transfizierte Kulturen des PH-1 eine um 10 – 50% reduzierte Pathogenität für Weizen und 20-30% für Mais, eine deutliche Verringerung ihrer Kapazität, Konidien zu produzieren (> 90%) und eine fünffache Reduktion der Perithezienbildung aufwiesen. Co-Inokulation von Maispflanzen mit China9 und PH-1 ergab eine dreifache Reduktion der Zahl erkrankter Körner.

Wenn die Genprodukte von FGV-ch9 dsRNA2, 3, 4 oder 5 konstitutiv in Zellen des PH-1 exprimiert wurden, war eine Reduktion der Konidienproduktion um 50-90% dieser Zellen zu beobachten. Darüber hinaus zeigten die Ergebnisse, dass die Gene, die von dsRNA2, 3 und 4 kodiert werden, die Pathogenität von PH-1 für Weizen um 42, 40, 54% und für Mais um 46, 44, 38% reduzierten. Die Überexpression des Gens von dsRNA5 führte zu einer weniger auffälligen Wirkung in beiden Pflanzen. Diese Ergebnisse unterstützen die Hypothese, dass FGV-ch9 mit Hypovirulenz von *F. graminearum* korreliert ist, und somit eine mögliche Methode für die Kontrolle der Krankheiten darstellt, die durch *Fusarium graminearum* an Getreide hervorgerufen werden.

7. References

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9. Curriculum Vitae

Personal information:

Name: Omar Mousa Mohamad Dar-Issa.

Date and place of birth: May 28, 1975, Bethlehem, Palestine

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Education:

Secondary school:

Completed in 1993 at Al-Khader Secondary School, Bethlehem by passing the Tawjihi Certificate.

Undergraduate:

BSc. 1997: Biological Sciences, Minor chemistry, Bethlehem University, Palestine.

Graduate:

MSc. Completed in 2000 at An-Najah National University, Nablus. Thesis title: Interaction between the biocontrol agents; entomopathogenic nematodes, *Serratia marcescens*, and *Beauveria bassiana* isolated from Palestine.

PhD. March 2010 at Hamburg University, Germany. Thesis title: Molecular characterization of a novel segmented dsRNA mycovirus and its association with hypovirulence of *Fusarium graminearum*.

Languages:

English and Arabic: conversation, reading, and writing.

Deutsch: B2T level.

Conferences and workshops:

1. Attended the VIIth International Plant Virus Epidemiology Symposium: Plant Virus Epidemiology: Current status and future prospects. Held in Aguadulce (Almeria), Spain, 11-16 April, 1999.
2. Attended a workshop on: Development of a Regional Viral Indexing and Certification Program for Plant Propagation materials in the Middle East (MERC), 1st workshop, Egypt, March, 2001.
3. Attended and participated in a workshop entitled: Science for Peace and Regional Scientific Cooperation in Molecular Biology, Microbiology and Biotechnology. Park Plaza Hotel, Jerusalem. 29 & 30 November - 1 December 1999.
4. Attended a workshop on Integrated Pest management in Palestine. Planning, Research, Implementation, Extension, and Implementation of IPM. A program for Palestinian-Israeli Cooperation. Shepherd's Hotel, Bethlehem, November 3-4, 1999.
5. Participated in a scientific meeting and workshop on molecular marker-assisted plant breeding. Cyprus, February 2002. (Funded by MERC-USAID)
6. Attended a scientific workshop on entomopathogenic nematodes. Eilat, Israel, November 2003.

7. Participated by giving lectures and by lab demonstration in a workshop on: Detection of grapevine viruses. Aghadeer, Morocco, December 2003. (Funded by MERC-USAID)
8. Participated by giving lectures reporting the last scientific achievements in the UNESCO BETCEN laboratory at Bethlehem University in two MERC projects related to plant viruses in the Middle East. 12-21 July 2004, Chania, Crete, Greece.
9. Participated by giving a lecture on Agrobacterium-mediated transformation in the meeting of the TYLCV project funded by MERC-USAID. February, 2005, Aqaba, Jordan.
10. Participated by giving lectures and by lab demonstration in a workshop on: Agrobacterium-mediated transformation of tomato for GROEL and GUS genes. Giza, Egypt July 2005. (Funded by MERC-USAID).
11. Attended the American Phytopathological Society (APS) meeting which was held in Austin, Texas, USA during the period 30 July – 3 August 2005.
12. Attended the fifth Joint Meeting of the DPG Working Group "Plant Viral Diseases" and the "Nederlandse Kring voor Plantevirologie". 8 - 9 April, 2009, Hamburg, Germany.
13. Participated in the 42nd annual meeting of the DPG working group of "Plant Viral Diseases". 11-12 March, 2010, Goettingen, Germany.

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