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ORIGINAL ARTICLE

A novel double-stranded RNA mycovirus from *Fusarium graminearum*: nucleic acid sequence and genomic structure

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Abstract Ten *Fusarium graminearum* isolates from China were screened for dsRNA mycoviruses. Five dsRNAs (2.4 to 3.5 kbp) were purified from isolate China 9, cloned, and sequenced. BLAST analysis showed that the proteins encoded by dsRNA1 possess motifs that are conserved in RNA-dependent RNA polymerases, dsRNA2 resembles the hypothetical protein encoded by dsRNA3 of Magnaporthe oryzae chrysovirus 1, dsRNA4 shares no significant similarity to any published protein, and dsRNA5 has a C2H2 zinc finger domain. Tandem mass spectrophotometry, surface protein labeling of virus-like particles, SDS-PAGE, and protein BLAST results supports the notion that three of the virus segments code for structural proteins, of which dsRNA3 possibly codes for the capsid protein. Relative quantitative RT-PCR studies of the 5 dsRNAs suggested that the segments are encapsidated separately in unequal amounts. Genomic structure and phylogenic studies support the possibility that this virus may be a candidate for the type species of a novel genus in the family *Chrysoviridae.*

Introduction

Mycoviruses (fungal viruses) have been described in many fungal species, including phytopathogenic fungi [26]. Mycoviruses with dsRNA genomes are classified into four major families based on the number and sequence of their genomic segments. These families are *Totiviridae* (non-segmented: 4.6-7 kbp), *Partitiviridae* (2 segments: 1.4-2.3 kbp), *Chrysoviridae* (4 segments: 2.4-3.6 kbp), and *Reoviridae* (10-12 segments: 0.7-5 kbp). The genomes of mycoviruses belonging to theses families are usually encapsidated 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 [15, 26]. Mycoviruses with positive single-strand RNA genomes (9-17 kbp) and no true virions belong to the families *Narnaviridae*, *Endornaviridae,* and *Hypoviridae* [15]. Some of these viruses were classified originally as dsRNA mycoviruses, probably because they exist mostly as the dsRNA replicative form in their hosts [15, 25, 26]. With the exception of a few cases, most of the reported mycoviruses have been associated with cryptic or latent infections of their hosts [15]. Mycoviruses have limited routes of transmission. These include intercellular routes such as hyphal anastomosis and heterokaryosis or transmission *via* sexual and asexual spores [9, 15, 37, 38].

To date, only a few mycoviruses have been isolated from the phytopathogenic fungus *Fusarium graminearum* [8, 33, 39]. These viruses include Fusarium graminearum viruses 1, 2, 3 and 4 (FgV1, FgV2, FgV3, FgV4). Whereas the complete nucleotide sequences for FgV1, FgV3 and FgV4 have been reported, only a partial sequence with an RNA-dependent RNA polymerase (RdRP) motif has been published for FgV2. The dsRNA virus reported by Theisen et al. [33] has not been fully characterized at the molecular level. While these viruses have not yet been assigned to a specific genus, genome organization and phylogenetic analysis have shown that FgV1 is a ss (+) RNA mycoviruses, while FgV4 is a dsRNA virus and probably a member of the family *Partitiviridae*. The deduced amino acid sequence of the RdRP of FgV3 showed close relatedness to members of the families *Chrysoviridae* and

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Totiviridae [39]. In this study, we present the complete nucleotide sequence of a novel five-segmented dsRNA mycovirus from *F. graminearum*. Molecular characterization and phylogenetic studies suggest that this virus may be a candidate for the type species of a new genus in the family *Chrysoviridae*.

Materials and methods

Fungal isolates and culture conditions

Ten *F. graminearum* strains isolated from cereals in China (China 1-10) were grown on complete medium CM [21] for one week at 25°C in the dark. Agar blocks containing fungal mycelia were transferred to 100 ml of CM broth and incubated with shaking at 110 rpm for 4 to 7 days at 25°C. The mycelium was collected by filtration through two filter papers (Whatman No. 1), washed with distilled water, dried by blotting with paper towels and frozen at −70°C.

Phylogenetic identification of the China 9 fungal isolate

DNA from the *F. graminearum* China 9 isolate was extracted using the CTAB method [11] and subjected to PCR amplification using specific primers (CH1: 5′-GATA GCGAACAAGTAGAGTGA-3′ and CH2: 5′-GTCCGTGT TTCAAGACGGGC-3′) for part of the 28S rDNA [24]. The amplified products were purified using a Nucleo-Spin® Extract II Kit (MACHEREY-NAGEL, Dueren, Germany) and cloned into the pGEM®-T vector (Promega) according to the manufacturer instructions. Positive clones were sequenced by Eurofins MWG GmbH (Germany) using an ABI 3730XL sequencer.

Extraction, purification, and sequence determination of dsRNA

Double-stranded RNA was extracted and purified from 3-5 g of fungal mycelium using the cellulose chromatography method followed by DNase I and S1 nuclease digestion [10]. The sequences of the purified dsRNA segments were determined using the full-length amplification of cDNA (FLAC) method as described previously [10]. Briefly, 250 ng of PC3-T7 loop primer (5′-p-GGATCCCG GGAATTCGGTAATACGACTCACTATAT TTTTATAG TGAGTCGTATTA-OH-3′) was ligated to the 3′ ends of 200 ng of purified dsRNAs with 30 U of T4 RNA ligase at 37°C for 6 h in the presence of 10% (v/v) DMSO, 20% (w/v) $(PEG)_{6000}$, and 20 units of Ribolock RNase inhibitor. Excess unligated primer was removed using a Nucleo-Spin® Extract II kit. The purified dsRNAs were denatured in the presence of 1 M betaine and 2.5% (v/v) DMSO at 98°C for 2 min and then snap-cooled in ice. The cDNA was synthesized with 400 U of RevertAid™ Premium Reverse Transcriptase (Fermentas, St. Leon, Germany) at 50°C for 1 h, followed by 15 min at 55°C. After hydrolysis of the RNA with 0.1 M NaOH, the cDNAs were annealed at 68°C for 2 h. The dscDNAs were amplified in a TProfessional Thermo Cycler (Biometra, Goettingen, Germany) with 2.5 U of Go Taq DNA polymerase (Promega, Mannheim, Germany) and 1.25 µM of PC2 primer (5′-CC GAATTCCCGGGATCC-3′). The reactions were heated at 72°C for 2 min, and 95°C for 2 min, and then subjected to 35 cycles of 95°C for 25 s, with an increment of 1 s per cycle, 65°C for 30 s, and 68°C for 5 min, and a final extension step for 10 min at 72°C. Full-length PCR products were cloned into the pGEM-T vector (Promega), and their sequences were determined as described above. At least three independent clones per dsRNA were used for sequence determination at all nucleotide positions.

Purification of virus-like particles

Virus-like particles (VLPs) were purified from fungal mycelium as described previously [2] with some modification. Briefly, 6 g of frozen mycelium was 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 M 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 8,000 *x g* for 10 min (Rotor SS34 Sorvall). The chloroform:n-butanol step was repeated 2-3 times until the red pigment of *F. graminearum* was removed from the supernatant. Polyethylene glycol (PEG_{6000}, Merck, Darmstadt, Germany) and NaCl were added to the supernatant to final concentrations of 8% (w/v) and 1% (w/v), respectively. After 1 h of incubation at 4°C, the precipitate was collected by centrifugation (10,000 *x g*) at 4°C for 15 min, and the pellet was resuspended in 10 ml of 0.05 M sodium phosphate buffer (pH 7.0) at 4°C for 2-4 h. The suspension was layered onto a 20% (w/v) sucrose cushion and ultracentrifuged at 105,000 *x g* in a 50.2Ti rotor (Beckman, USA) for 2 h at 4°C. The pellet was resuspended in 1 ml of 0.05 M sodium phosphate buffer (pH 7.0) and further 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. Virus-like particles were stained with 2% (w/v) uranyl acetate and examined by transmission electron microscopy (LEO 906E, Zeiss, Germany).

Protein sequence analysis

Proteins of the purified VLPs were separated by 12% SDS-PAGE [20] fractionation for 2 h at 50 mA and stained with

0.1% (w/v) Coomassie brilliant blue R-250 (CBB, Bio-Rad, USA). To be able to identify the sequence of the viral structural proteins, the stacking and separation gels were prepared using fresh reagents and polymerized for 18-24 h. Viral proteins were separated as described above and electroblotted to a Roti®-PVDF membrane (Roth, Karlsruhe, Germany) for 1 h at 100 V. The membrane was stained for 10 s with Coomassie blue and then rinsed in ddH_2O. The protein bands were cut out, either directly from the gel or from the membrane, with a sterile razor blade and stored at 4°C until processing. The partial identification of the protein sequences was carried out at the Institut für Klinische Chemie of the Universitäts-Klinikum Hamburg-Eppendorf (UKE, Germany) by Q-TOF mass spectrophotometry using reverse-phase HPLC.

Viral surface protein labeling and immunoblotting

About 10–15 µg of purified VLPs was mixed with 5 mM freshly-prepared EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, Bonn, Germany) and incubated for 25 min at room temperature. The reaction was stopped by the addition of 1.5 µl of 1 M Tris-HCl (pH 7.5). One volume of 2x Laemmli buffer was added, and the mixture was heated for 5 minutes at 95°C and then quenched on ice. The denatured proteins were separated by 12.5% SDS-PAGE and blotted as described above. The membrane was blocked with 5% skimmed milk in 1x PBST and then incubated with ExtrAvidin® alkaline phosphatase conjugate suspension (Sigma-Aldrich) in 1% (w/v) skimmed milk for 1 h. Biotin-labeled proteins were detected using a CDP-Star kit or with Fast Red reagent (Sigma-Aldrich) and photographed.

Relative quantification by real-time RT-PCR

Double-stranded RNA was phenol-extracted from 50 µg of purified VLPs treated with DNAse I and S1 nuclease for 30 min at 37°C. In a second treatment, the dsRNA was purified from the mycelium by 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, incubated at 98°C for 2 min, and then quenched on ice for 5 min. cDNA was synthesized with 200 U of RevertAid™ Premium Reverse Transcriptase at 50°C for 45 min followed by 55°C for 10 min. The transcription reaction contained 50 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs and 20 units of RiboLock™ RNase Inhibitor (Fermentas). Serial dilutions of the synthesized cDNA (10^{-1}, 10^{-2}, and 10^{-3}) were made. Two µl of cDNA from each dilution was amplified in a reaction mixture containing 1x SYBR Green I Master (Roche) and 0.4 µM each primer (a forward and a reverse primer for each segment, Table 1) in a final volume of 20 µl. 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 s, 60°C for 15 s, and 72°C for 20 s, 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 measurements per °C. The products were cooled to 40°C for 10 sec. The denaturation and extension steps were carried out with a ramp rate of 4.4°C/s while the annealing and cooling steps were at 2.2 and 1.5°C/s, respectively.

Table 1 Primer sequences (5′–3′) used in the quantitative PCR study

Segment	Forward	Reverse
dsRNA1	gagtattaccagcaacaacca	ccagtgccttattgtaaccc
dsRNA3	atgaactgatacgaaacggtg	aggtgcatacacaaagttgag
dsRNA4	gcagctacaccagttaacag	aaagtgccgattctatacatgg
dsRNA2	gcctctcattctataacgcc	acatcaatcgaatgtcctcag
dsRNA5	agtagttatgacgatgatgcac	tcagttatcgggtaggtgtc

Data analysis

Sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI), Swissprot, and EMBL databases with the BLAST program. Multiple alignments of nucleic and amino acid sequences were carried out using 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 [31]. 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 3). 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). Zinc fingers were detected according to Persikov et al. [27] 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.

Results

Identification of the fungus isolate

The fungus isolate China 9 showed morphological properties similar to those described for *F. graminearum*,

Table2 Size and function of the genomic dsRNA segments and the deduced proteins of FgV-ch9

Segment (accession no.)	Size (bp)	Size of deduced protein (Da)	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 CP
dsRNA4 (HQ228216)	2746–3786[a]	91 303 (835 aa)	Unknown
dsRNA5 (HQ228217)	2423	79 872 (712 aa)	Contains a C2H2 zinc finger domain

[a] Due to the presence of 3′ terminal repeats, the size of dsRNA4 is variable

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 of the isolate was performed based on sequence analysis of the ribosomal RNA genes (rDNA), which are commonly used in identification and taxonomic studies at the species level [24]. Multiple alignment and phylogenetic results showed that China 9 isolate is closely related to members of the *F. graminearum* species complex (data not shown).

Virus purification and dsRNA isolation

Isometric VLPs of 35-40 nm in diameter were purified from the mycelium of the *F. graminearum* China 9 isolate (Fig. 1) and thereafter named FgV-ch9. The buoyant density of the virions in cesium chloride was in the range of 1.42-1.45 g/cm^3. Cultures of *F. graminearum* China 9 originating from single conidia have been classified into three types according to the amount of viral dsRNA present per mg of mycelium; type I: China 9 with high virus titer, type II: China 9 with moderate virus titer, and type III: China 9 with low virus titer (unpublished data). Depending on the virus titer in the fungus and the electrophoresis conditions, the number of dsRNA segments that were visibly detectable by ethidium bromide staining varied from 2 to 5 (Fig. 2). However, northern blot analysis (data not shown) and nucleotide sequence analysis showed that five unique segments are associated with the purified VLPs. Several virus dsRNA preparations purified from infected mycelium by cellulose chromatography and treated with S1 and DNase I showed that the distribution of the segments in the virus population was not quantitatively equal and that the amounts of 2 or 3 of the dsRNAs were often below the limits of detection by ethidium bromide staining of gels after electrophoresis. Indeed, it was only possible to observe 4 or 5 distinct dsRNA segments from mycelium that contained a high titer of the virus (Fig. 2, lane 2) or from virions purified from such mycelium (Fig. 2, compare lanes 3 and 4). Furthermore, the resolution of the extracted dsRNA segments by 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. 2, compare lanes 1 and 2). The sizes of the five dsRNA segments and of the encoded proteins are summarized in Table 2.

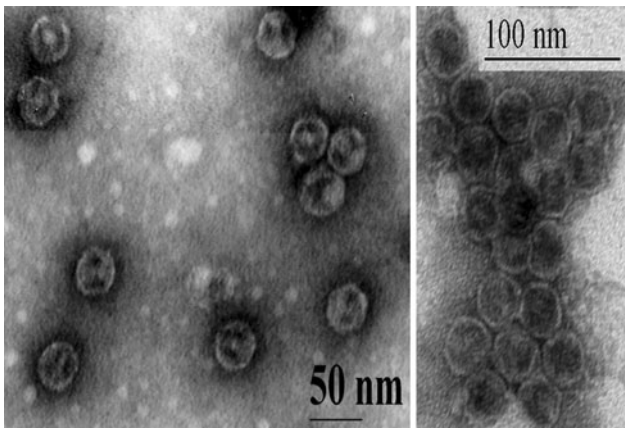

Fig. 1 Virus-like particles of FgV-ch9. The particles were purified from the China 9 isolate by CsCl gradient ultracentrifugation, negatively stained with 2% (w/v) uranyl acetate and observed under a transmission electron microscope

SDS-PAGE and peptide sequencing

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 gels (Fig. 3a 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. 4a and b, boxed). The irresolvable middle-double bands (Fig. 3a, lane 1) had peptide sequences identical to the protein deduced from dsRNA3 (Fig. 4c, boxed) and therefore were considered to be 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 the DNA clone sequences (compare Fig. 3 to Table 2).

Quantitative PCR

The various intensities of the 5 dsRNA segments isolated from purified virions or from the fungal mycelium as

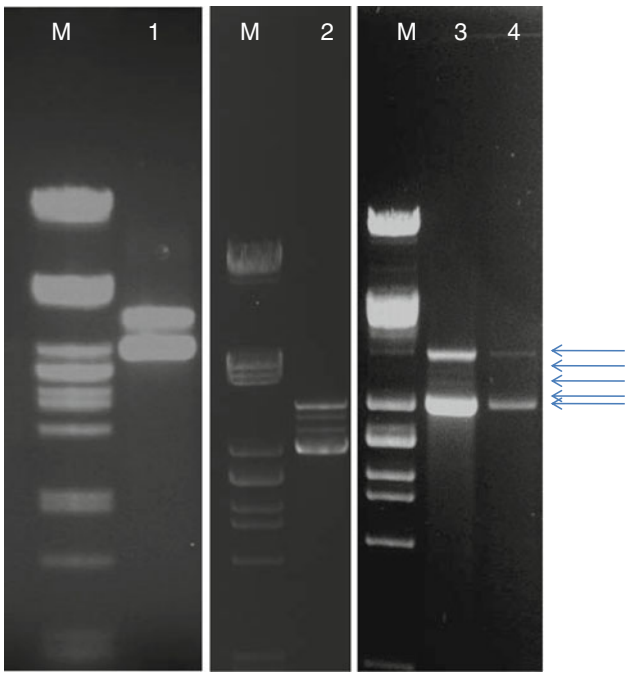

Fig. 2 Agarose gel electrophoresis of dsRNAs isolated from the fungus *F. graminearum* China 9 or from purified VLPs of FgV-ch9. The dsRNAs were purified either from the mycelium or from CsCl-purified VLPs by CF-11 chromatography and the standard phenol method, respectively. The dsRNAs were treated with DNase I and S1 nuclease and separated in a 1.2% agarose gel (arrows). **1**: dsRNAs purified from mycelium with a 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 a high titer of FgV-ch9. **4:** as in **3** but with a lower virus titer. **M**: λ DNA digested with *Pst*I

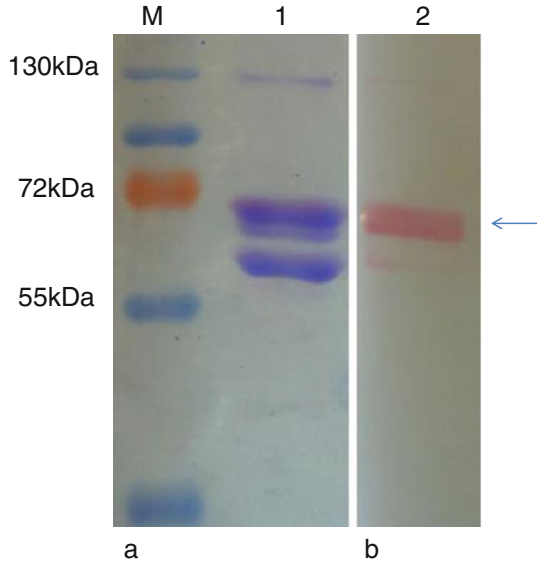

Fig. 3 SDS-PAGE and immunoblot analysis of FgV-ch9 structural proteins. CsCl-gradient-purified particles were denatured and subjected to SDS-PAGE (a, 1). The surface proteins of the purified VLPs were labeled with NHS-biotin, separated by SDS-PAGE, blotted, and detected with Fast Red reagent (b, 2). 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 CP. **M:** Prestained protein marker

shown in agarose gel electrophoresis (Fig. 2) might be correlated with unequal encapsidation and/or expression of the dsRNA segments. Relative quantitative real- time PCR of the five dsRNAs showed that they were encapsidated unequally. In relation to dsRNA4, dsRNAs1, 2, 3 and 5 isolated from purified virions were present at a ratio of 0.63 : 0.27 : 0.78 : 0.87. A similar trend was observed when dsRNAs were purified from the 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 provided by the real-time PCR calculations, we conclude that the dsRNAs are encapsidated separately.

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 purification preparations or dsRNAs purified from infected mycelium. The sequences were submitted to the GenBank database, and the accession numbers are shown in Table 2. Sequence analysis showed that each dsRNA segment is monocistronic with a single open reading frame (ORF).

dsRNA1

The complete nucleotide sequence of dsRNA1 revealed that it is 3581 bp in size and contains a single large ORF from nt positions 83 to 3496, coding for a protein of 1137 aa (Table 2). The calculated molecular weight of the encoded protein (127 kDa) is in agreement with the estimated size of the corresponding band in SDS-PAGE (Fig. 3a, lane 1; upper-faint band). Peptide sequences of the upper-faint band match perfectly with the deduced amino acid sequence of dsRNA1 (Fig. 4a). Sequence analysis of the deduced aa of dsRNA1 showed the presence of the eight conserved RdRP motifs that are characteristic for RNA viruses (Fig. 5) [5, 18]. BLAST searches of the deduced amino acid sequence of dsRNA1 showed that it has sequence similarities to the RdRPs (virus accession numbers are in Table 3) encoded by Aspergillus mycovirus 1816 (AsV1816) [37% identity, 53% similarity; e value 0], Magnaporthe oryzae chrysovirus1 (MoCV1) [33% identity, 50% similarity] and Agaricus bisporus virus 1 (AbV-1) [29% identity, 46% similarity]. Sequence similarities ($\leq$27% identity and $\leq$43% similarity, e^{-32} and below)

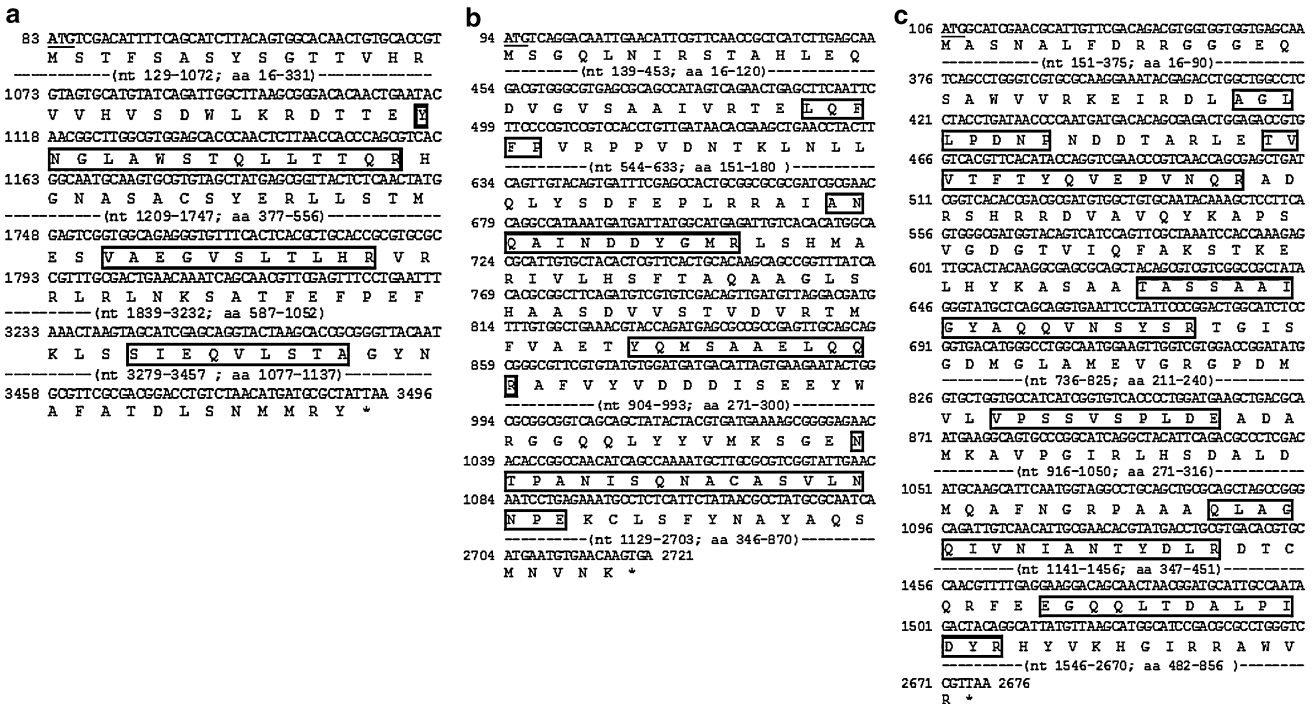

Fig. 4 Partial nucleotide sequences of FgV-ch9 dsRNA1 (4a), dsRNA2 (4b), and dsRNA3 (4c). The deduced amino acid sequences are written in the one-letter code below the nucleotide sequences. The translation start and termination codons are indicated by underlining and an asterisk, respectively. The tryptic-peptide-derived amino acid sequences, isolated by reverse-phase HPLC, are boxed

were also obtained for the RdRPs of viruses belonging to the family *Chrysoviridae*. These include CnV-1, Hv145SV, CCRS-CV, FuoxV1, PcV and others. Less significant similarities were obtained for RdRPs of viruses belonging to the family *Totiviridae* (BLAST hits of e^{-09} or below). 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 representation of the data is shown in Fig. 6a. Although AsV1816 is assigned as an unclassified member of the family *Totiviridae* and the classification of AbV-1 as a chrysovirus is not finalized [15], our results indicate that these viruses together with FgV-ch9 form a new cluster within the family *Chrysoviridae*.

dsRNAs 2

Sequence analysis of the DNA clones of the virus dsRNA2 showed that it is 2850 bp in length and contains a single ORF encoding a protein with a deduced molecular weight of 94 kDa. The estimated molecular mass of the protein encoded by dsRNA2 from SDS-PAGE is ~62 kDa (Fig.3, lane 1, lower band). BLAST searches of the deduced aa sequences of dsRNA2 resulted in 29% identity and 50% similarity (E value of $1e^{-49}$) with the hypothetical protein encoded by dsRNA3 of MoCV1.

dsRNA 3

Sequence analysis of full-length DNA clones of dsRNA3 showed that it consists of 2830 bp, coding for one large protein of 856 aa and a calculated molecular mass of 93 kDa. The estimated size of the protein in SDS-PAGE is ~70 kDa (Fig. 3a, lane 1; middle-double band). Surface protein labeling of purified virions with NHS-biotin showed a labeling preference for the middle-double band (Fig. 3b, Lane 2; arrow). The peptide sequences of this band match perfectly with the deduced aa sequence encoded by dsRNA3 (Fig. 4b). These results indicate that dsRNA3 most likely codes for the capsid protein (CP). Further support of this finding comes from BLAST searches, which showed sequence similarity of the deduced aa sequence of dsRNA3 to the L3 protein CP of AbV-1 (22% identity and 39% similarity at a BLAST hit of $5e^{-13}$). One other significant hit to the hypothetical protein encoded by segment 4 of MoCV1 was observed (23% identity, 39% similarity, and an E value of $1e^{-13}$). Phylogenic analysis of the CP encoded by dsRNA3 showed that although FgV-ch9, AbV-1, and MoCV1 cluster as a distinct clade, they are more closely related to members of the family *Chrysoviridae* than to those of the other families (Fig. 6b). Unfortunately, the sequence of the AsV1816 CP gene is not available.

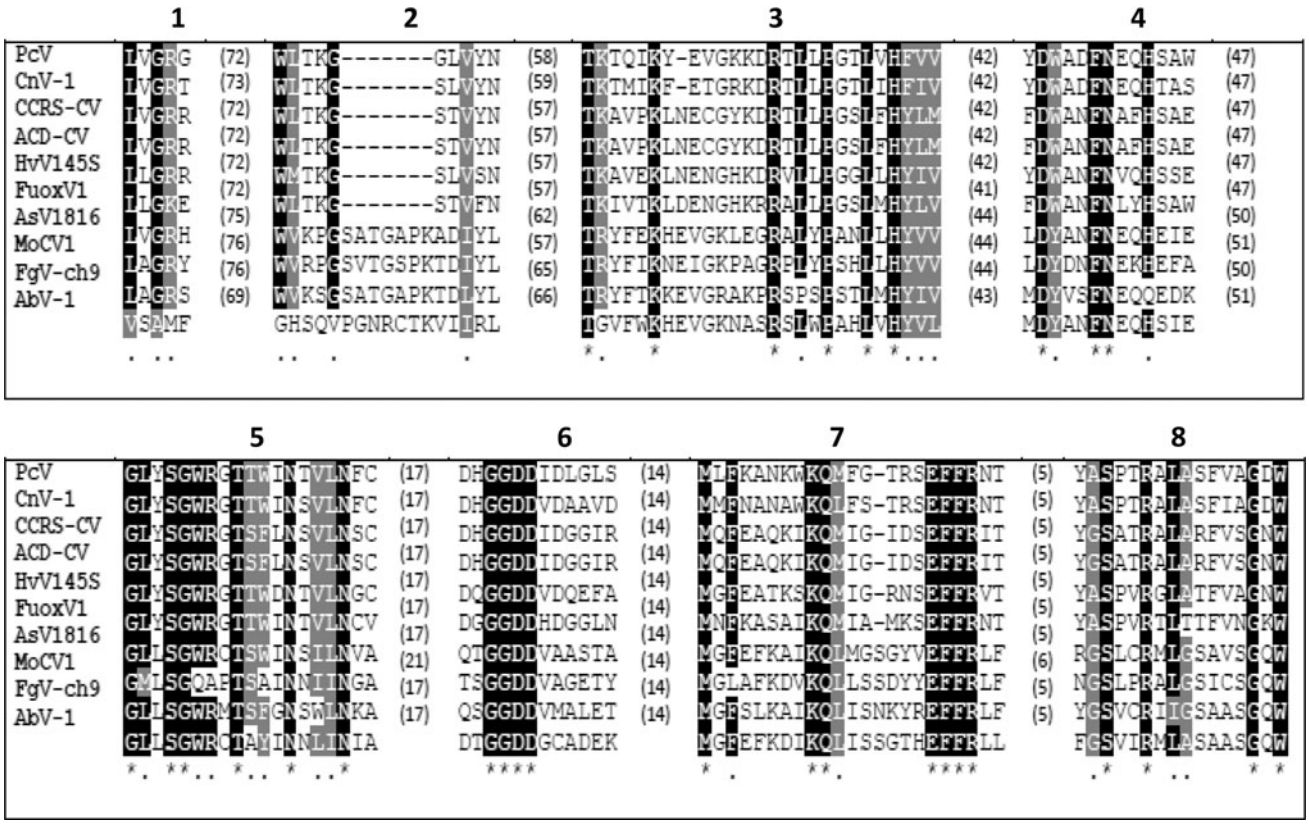

Fig. 5 Comparison of the conserved RdRPs motifs of several dsRNA mycoviruses, including FgV-ch9. Numbers 1-8 stand for the eight conserved motifs typical of RdRPs of RNA viruses. Multiple sequence alignments were carried out with the CLUSTALX (2.0) program. Dark shading and asterisks show identical residues 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 3

dsRNA 4

This segment has a minimum length of 2746 bp and includes a single ORF coding for a protein with a calculated molecular weight of 91 kDa. Interestingly, the last 146-208 nt 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). The protein encoded by dsRNA4 showed no significant similarities to any protein in the GenBank and EMBL databases.

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 dsRNA 5 showed that the C-terminus of the protein (aa positions 326 to 711) shares high similarities to zinc finger proteins (BLAST hits of $2e^{-13}$). Using the online software developed by Persikov et al. [27] 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. 7). The N-terminus of the protein encoded by dsRNA5 has no significant similarity to any published protein.

5′and 3′ UTRs

The lengths of the 5′ UTRs of FgV-ch9 dsRNAs were between 78 and 105 nt long, and multiple alignment of these sequences showed regions of high sequence similarity (Fig. 8a). Stretches of identical sequences of 10 and 19 nt were found at positions (with regard to the consensus line) 1 to 10 and from 23 to 42, respectively. Sixty-one of the 5′-terminal 72 nt 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 AUG initiation codon (specified in bold, Fig. 8a). The average GC content of the 5′UTRs of the five dsRNAs is 41%, indicating an AT-rich area. The CAA repeats, characteristic of the 5′ UTRs of chrysoviruses [18] were observed in FgV-ch9, but at a lower frequency. Whereas in

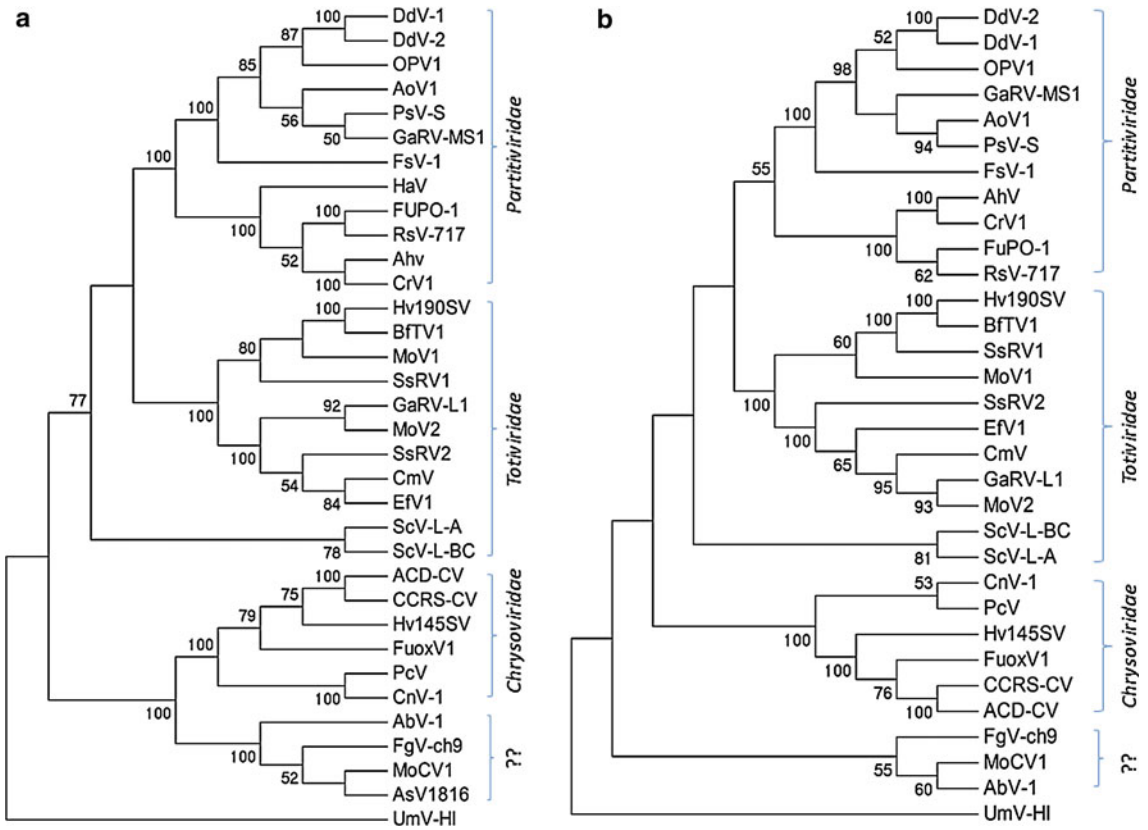

Fig. 6 Phylograms of the RdRP (9a) and the CP (9b) of FgV-ch9. Multiple-sequence alignments of the deduced amino acid sequences of FgV-ch9 and selected chrysoviruses, totiviruses, and partitiviruses were performed using CLUSTALX (2.0) with default parameters. The trees were analyzed by the NJ method 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 given in Table 3

PcV and CCRS-CV the CAA sequence is repeated 7-14 times per dsRNA, in FgV-ch9, they occur only 2-6 times (Fig. 8a, underlined). In the 5′ UTRs of tobamoviruses, similar repeats have been found to function as translational enhancer elements [14]. The 3′ UTRs of FgV-ch9 are relatively long, between 85 and 188 nt in length (Fig. 8b). In addition to the 6 nt at the 3′-terminus 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. 8b).

Multiple sequence alignment of most of the reported chrysoviruses showed that the first 15-30 nt of their 5' UTRs are rich in either adenosine (e.g. FgV-ch9, PCV1, Hv145SV) or adenosine and thymine (e.g. MoCV1, ACDCV1). On the other hand, the 3' UTRs of chrysoviruses are relatively conserved within the dsRNAs of the same species but not among different species.

Discussion

Fungal viruses have stimulated interest because of the prospect of using potential hypovirulence-associated mycoviruses as biocontrol agents for plant pathogenic fungi [7, 22, 25]. Although most of the known mycoviruses are associated with latent infections of their hosts, several reports of reduced or eliminated fungal pathogenicity due to mycoviral infection have been described [6, 28, 40, 41]. In this study, ten Fusarium isolates from China were screened 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 identified phylogenetically as *F. graminearum* using the method described by Ninet et al. [24]. The novel virus was named F. graminearum mycovirus-China9 (FgV-ch9). The purified dsRNAs was resolved into 2-5 bands by 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 were resolved into 4-5 distinct bands. At low virus titer, however, dsRNA 2 and 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

Table 3 List of the viruses used in constructing the phylograms in Fig. 6a and b

Virus	Acronym[a]	Accession no.		Family/genus[b]
		RdRP	CP	
Ustilago maydis virus H1	UmV-HI	NP_620728.1	NP_620728.1	TT
Saccharomyces cerevisiae virus L-A (L1)	Sc V-L-A	NP_620495.1	NP_620494.1	TT
Saccharomyces cerevisiae virus L-BC (La)	ScV-L-BC	NP_042581.1	NP_042580.1	TT
Gremmeniella abetina RNA virus L1	GaRV-L1	AAK11656.1	NP_624332.2	TV
Helminthosporium victoriae 190SV	Hv190SV	NP_619670.2	NP_619669.2	TV
Coniothyrium minitans mycovirus	CmV	YP_392467.1	YP_392466.1	TV
Epichloe festucae virus 1	EfV1	CAK02788.1	CAK02787.1	TV
Magnaporthe oryzae virus1	MoV1	YP_122352.1	YP_122351.1	TV
Magnaporthe oryzae virus 2	MoV2	NP_047560.1	YP_001649205.1	TV
Sphaeropsis sapinea RNA virus 1	SsRV1	NP_047558.1	NP_047557.1	TV
Sphaeropsis sapinea RNA virus 2	SsRV2	YP_001649206.1	NP_047559.1	TV
Botryotinia fuckeliana totivirus 1	BfTV1	YP_001109580.1	YP_001109579.1	TV
Atkinsonella hypoxylon virus	AhV	NP_604475.1	NP_604476.1	P
Aspergillus ochraceus virus 1	AoV1	ABV30675.1	ABV30676.1	P
Ceratocystis resinifera virus 1	CrV1	YP_001936016.1	YP_001936015.1	P
Discula destructiva virus 1	DdV-1	NP_116716.1	NP_116742.1	P
Discula destructiva virus 2	DdV-2	NP_620301.1	NP_620302.1	P
Fusarium poae virus 1	FUPO-1	NP_624349.1	NP_624348.2	P
Fusarium solani virus 1	FsV-1	NP_624350.1	NP_624351.1	P
Gremmeniella abietina RNA virus MS1	GaRV-MS1	NP_659027.1	NP_659028.1	P
Aspergillus mycovirus 1816	AsV1816	ABX79996.1	NA[c]	uT
Heterobasidion annosum partitivirus	HaV	AAL79540.1	NA[c]	P
Ophiostoma himal-ulmi partitivirus 1	OPV1	CAJ31886.1	CAJ31887.1	P
Penicillium stoloniferum virus S	PsV-S	YP_052856.2	YP_052857.1	P
Rhizoctonia solani virus 717	RsV-717	NP_620659.1	NP_620660.1	P
Penicillium chrysogenum virus	PcV	YP_392482.1	YP_392483.1	C
Helminthosporium victoriae 145S	Hv145SV	YP_052858.1	YP_052859.1	C
Agaricus bisporus virus 1	AbV-1	CAA64144.1	BAA01612.1	?C
Cryphonectria nitschkei chrysovirus 1	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
Fusarium oxysporum chrysovirus 1	FuoxV1	ABQ53134.1	ABQ58816.1	C
Magnaporthe oryzae chrysovirus 1	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

be attributed to the small size differences between dsRNA 2, 3 and 4 (20-84 bp), the unequal band intensities, and the heterogeneous lengths associated with the 3′ UTR tandem repeats of dsRNA 4 (discussed below). Incomplete resolution in agarose gels have also been reported for PcV and AsV1816 [18, 36]. Our ability to detect the 5 dsRNAs segments in the various cultures originating from single conidia 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 that is closely related to those of dsRNA mycoviruses. The function of the proteins encoded by dsRNA2 and dsRNA4 are not known yet, since the one 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

```
                C    C    -1123456H    H
ZF1   330   C--PICFEPQRSRRSLAAHRSSSH  351
ZF2   356   CGQAHCSYTTDVPADFAFHMDEEH  379
ZF3   384   CDSTGCDYTTRSSQLHWTHMV-GH  406
ZF4   409   C--TVCGLLSANLAAAADHIR-EH  429
ZF5   435   C--SLCNEPFEDAFSLNEHNQDVH  456
ZF6   460   C--TQCEAIVRDKNELLDHCHDVH  481
ZF7   486   CREVGCAFRTFEPPELAAHHD-WH  508
ZF8   522   CPDLGCTHAASSHEMAALHYN-TH  544
ZF9   552   C--ESCDAWCQQPHCPNCHSR-MH  572
ZF10  600   C--PHCAFTSKSKVEWQDHININH  621
ZF11  631   C--SLCGKVFTGVNAVRKHHC--H  650
ZF12  690   C--QVCHETFARLDHLQRHVRRRH  711
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Fig. 7 Multiple sequence alignment of the 12 C2H2 zinc finger domain present at the C-terminus of the protein encoded by dsRNA5 of FgV-ch9. Such fingers are usually composed 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, H, and the number above the aligned sequences refer to the residues Cys and His and the residue number in the alpha helix, respectively. Boldface type indicates conserved amino acid residues. Multiple sequence alignment was done using CLUSTALX with some manual adjustments

identification of the protein encoded by dsRNA2 in SDS-PAGE might indicate that this protein is associated with virions. Interestingly, our analysis of eight independent DNA clones of dsRNA4 showed that the 3′-terminus of this segment contains tandem sequence repeats of 146-208 nt in six of the clones. Although the dominant pattern of the sequence repeats was $(146\ nt)_n$, in two of the analyzed clones, the repeated sequence was $(208\ nt)_n$ where $n = 2\text{-}5$. The significance of such a 3′-terminal repeat, which to our knowledge has not been reported for a dsRNA mycovirus, is unclear.

Evidence that dsRNA3 encodes the putative CP stems comes from two sources. First, 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. [19] to be the AbV-1 CP.

The molecular masses of the proteins encoded by dsRNA2 and dsRNA3 as estimated by SDS-PAGE (Fig.3, lane 2) are smaller than those calculated from the deduced aa sequences (Table 2). 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 (Fig. 4b and c). A similar observation was reported for protein p58, encoded by the tentative chrysovirus MoCV1 [35]. Furthermore, the appearance of the protein band encoded by dsRNA3 as a double band (Fig. 3, 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 has also been described for the totivirus Hv190SV CP [30].

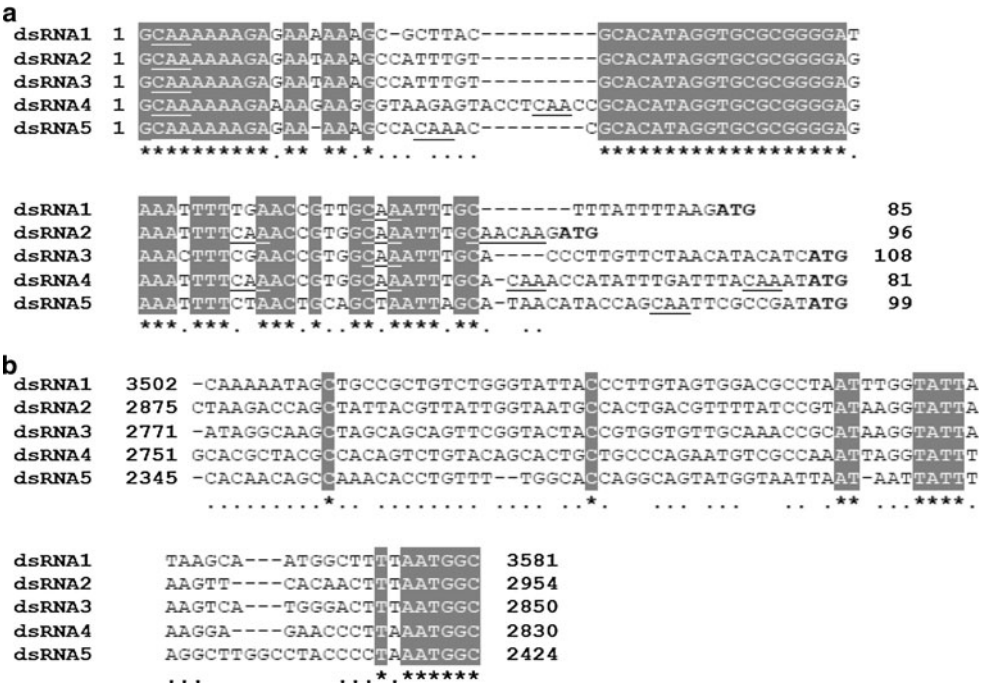

Fig. 8 Comparison of the 5′ (11a) and 3′ (11b) UTRs of the 5 dsRNA segments of FgV-ch9. Multiple sequence alignments were done with CLUSTAL X. Gray shading and asterisks specify columns with identical bases, while dots indicate that three or four out of the five bases are identical in that column. The (CAA) repeats are underlined and the start codons are shown in bold

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 a few dsDNA viruses such as Emiliania huxleyi virus 99B1 and Neodiprion abietis nucleopolyhedrovirus (NPV). Proteins with C2H2 zinc fingers are well characterized as regulatory proteins that bind to DNA, ssRNA, dsRNA or proteins [13, 17]. In the case of multiple-adjacent-C2H2 zinc fingers, binding to multiple different ligands has been reported [34]. In retroviruses such as human immunodeficiency virus 1 (HIV-1) and murine leukemia virus (MuLV), zinc fingers constitute part of the nucleocapsid protein and have been reported to have regulatory functions that are important for virus replication and infectivity [4, 32]. 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.

The relative quantitative RT-PCR results are in agreement with the unequal dsRNAs intensities in agarose gel electrophoresis as discussed above. These results indicate 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 (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 are a property that has been reported for ssRNA and dsRNA viruses with multipartite and multicomponent genomes [1, 3, 23]. These results lead 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 closely related to members of the family *Chrysoviridae* than to those of other dsRNA mycovirus families, it clusters distinctly (Fig. 6a and b). The new cluster encompasses, in addition to FgV-ch9, an unclassified totivirus (AsV1816), an obscure member of the chrysoviruses (AbV-1), and a Vietnamese isolate of MoCV1. Although AsV1816 is associated with at least four dsRNAs [37], 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 [16]. 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 white button mushroom virus and La France isometric virus, is the suspected causal agent of the La France disease of cultivated mushrooms. The disease is associated with isometric virus particles of AbV-1 and several (most common are L1-L5, M1 and M2) dsRNA segments [19, 29]. The deduced amino acid sequences of segments L1 and L3 encode the virus RdRP and the CP, respectively [19]. Recently, AbV-1 was listed as a tentative chrysovirus in the Eighth Report of the ICTV [12]. 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% identity and 40% similarity spanning 222 aa of the encoded proteins. The genomic sequences of the Vietnamese isolate of MoCV1 were deposited recently in the GenBank database and have not yet been subjected to final classification, although the authors related them to chrysoviruses. Although MoCV1 might be associated with up to eight dsRNAs, only the four most abundant and stable segments were sequenced [35]. In general, FgV-ch9, AbV-1, MoCV1, and AsV1816 have common features that discriminate them from related chrysoviruses. In addition to the phylogenic differences, these features include the number of genomic dsRNA segments (>4 in comparison to 4 for chrysoviruses) and point mutations in some of the RdRP conserved motifs (Fig. 5) as well as the mutations at other similar residues (data not shown).

The phylogenic analysis and genome organization of FgV-ch9 with 5 monocistronic dsRNAs, the presence of a zinc finger domain in dsRNA5 and 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 the family *Chrysoviridae* to accommodate the four viruses forming the new phylogenic cluster (FgV-ch9, AsV1816, AbV-1, and MoCV1).

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