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AGRICULTURE AND ENVIRONMENTAL BIOTECHNOLOGY

TWO NOVEL MYCOVIRUSES RELATED TO GEMINIVIRUS ISOLATED FROM THE SOIL-BORNE FUNGI *MACROPHOMINA PHASEOLINA* (TASSI) GOID. AND *MUCOR RACEMOSUS* BULL.

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ABSTRACT

Viral coat protein gene was detected in thirty isolates of different soil-borne fungi, using cotton leaf curl coat protein specific primers (geminiviruses). Out of them, seven fungal isolates showed positive results (a band with a molecular size of 680 bp). The products amplified from two isolates, Macrophomina phaseolina (Tassi) Goid. and Mucor racemosus Bull., were excised from the gel, purified and sequenced. The sequence analysis revealed that the amplicons were coat protein genes of geminivirus. The gene isolated from M. phaseolina was similar to that of wheat dwarf geminivirus with identity not exceeding 36 %; and the coat protein isolated from M. racemosus was 43 % identical with the maize streak geminivirus. These low identities suggest that the amplification products belong to new records of geminiviruses designated (MpDV) and (MrDV), respectively. The isolate from M. racemosus was selected to investigate the viral transmission into the phytopathogenic fungus Fusarium oxysporum Schlecht. Some phenotypic changes were observed in the infected mycelia of F. oxysporum, including growth rate reduction and increased pigmentation. The infected mycelia of both fungi were subjected to electron microscopy examination and the results revealed that different virus-like particles (VLPs) were observed. This finding may help in understanding and using the ssDNA mycoviruses in fungal biocontrol and expands our knowledge of virus ecology and evolution.

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Keywords: mycoviruses, *Fusarium oxysporum,* ssDNA, virus-like particles, viral transmission

Introduction

Fungi are the most economically significant type of pathogens that infect plants and are responsible for approximately two-thirds of the infectious plant diseases. These pathogens have the capability to diminish the economic values of all agricultural crops in the world (33). *Fusarium oxysporum* Schlecht. is an economically important fungus; it is an active saprophyte both in soil and organic matter (30). This fungus causes many plant diseases such as vascular wilt, yellows, corm rot, root rot, and damping-off (1). *Macrophomina phaseolina* (Tassi) Goid., is a necrotrophic soil-borne fungal pathogen that infects many important crops worldwide, e.g. soybean, corn and sorghum, and causes a disease known with the common name charcoal rot (18). *Mucor racemosus* Bull. is usually present as a saprophyte fungus and sometimes as a plant pathogen causing soft rot disease in succulent, fleshy fruits and vegetables, cut flowers, bulbs and corms (21).

Mycoviruses are viruses that infect fungi and have the potential to control fungal diseases of crops when associated with hypovirulence (38). Mycoviruses have characteristics that distinguish them from most other viruses (13). Different virus-like particles (VLPs) may be present in a single fungal strain (27). In yeast, mycoviruses can be transmitted vertically or horizontally via hyphal anastomosis or sexual mating (11). These viruses may cause a disease and kill the fungal cells. One of these viruses causes 'La France' disease of the white-button mushroom *Agaricus bisporus* (9). Some mycoviruses produce lethal toxins encoded by the viral dsRNA in some strains of *Saccharomyces cerevisiae* and *Ustilago maydis* (17, 29). These viruses may also stay dormant inside fungal cells and cause asymptomatic disease (26). Although many mycoviruses may have no significant impact on their hosts, some may cause hypovirulence in plant pathogenic fungi (6, 11, 26, 39). Such viruses, e.g. *Rosellinia necatrix* megabirnavirus 1 from the white root rot fungus *Rosellinia necatrix*, have shown significant potential for biological control of plant fungal diseases (5).

The typical genome of mycoviruses is made up of double-stranded (ds) or single-stranded (ss) RNA (26). The majority of the well known mycoviruses contain dsRNA particles with a diameter of 25 nm to 50 nm. On the other hand, a hypovirulence-associated circular ssDNA mycovirus from the plant pathogenic fungus *Sclerotinia sclerotiorum* was described by Yu et al. (38). Geminiviruses are plant viruses with twinned quasi-icosahedral particles and a circular ssDNA genome. This is the largest known family of ssDNA viruses (20). The presence of mycoviruses in fungal mycelia is commonly determined by electron microscopy (EM) as VLPs or by amplification of a specific nucleic acid locus directly from the fungal hayphae.

The aim of this study was to determine the viruses that infect or associate with some pathogenic fungi and to investigate the ability of these viruses to transfer to the fungus *F. oxysporum* through the vertical route.

Materials and Methods

Sample collection

Thirty fungal strains were isolated from different soil samples in Egypt (**Table 1**). The isolated fungi were grown on potato dextrose agar plates (PDA) (Difco, USA) supplemented with an antibacterial agent (L-chloramphenicol 5 mg/L and streptomycin sulphate 5 mg/L) and incubated at 28 °C for 4 to 6 days. Purification of the resulting isolates was done using the hyphal tip or single-spore technique to obtain pure cultures. The detected isolates were then transferred to PDA slants and kept at 4 °C for further studies. The pure cultures of the isolated fungi were identified based on their cultural properties, morphological and microscopic characteristics as described by Booth (4) and Domsch et al. (8).

TABLE 1

Presence or absence of Mycovirus in the examined fungi, based on specific PCR amplification of the geminivirus coat protein gene

No.	Fungal isolate	Mycovirus
1	*Acremonium* sp.	-
2	*Alternaria alternate*	-
3	*Asperigllus flavipes*	-
4	*A. flavus*	+
5	*A. fumigates*	-
6	*A. ochraceus*	-
7	*A. niger*	-
8	*A. nidulans*	-
9	*Botryodiplodia sp.*	+
10	*Chaetomium* sp.	-
11	*Cunninghamella* sp.	-
12	*Emericella* sp.	-
13	*Paecilomyces sp.*	+
14	*Fusarium graminearum*	+
15	*F. oxysporum*	-
16	*F. solani*	-
17	*Helmenthosporium* sp.	-
18	*Mucor racemosus*	+
19	*Macrophomina phaseolina*	+
20	*Neurospora crassa*	-
21	*Penicillium funiculosum*	-
22	*P. glabrum*	+
23	*P. italicum*	-
24	*Rhizoctonia solani*	-
25	*Rhizopus* sp.	-
26	*Sclerotium bataticola*	-
27	*Trichoderma harzianum*	-
28	*T. hamatum*	-
29	*T. viride*	-
30	*T. reesei*	-

(-) no viral infection; (+) presence of viral infection

DNA extraction

The viral DNA was extracted according to van Diepeningen et al. (36). The resultant nucleic acid was separated in an 0.8 % agarose gel, stained with ethidium bromide and visualized with UV transillumination. For obtaining pure mycovirus DNA, nucleic acid solutions were treated with RNase under low and high salt concentrations (23, 37).

Polymerase chain reaction (PCR) for detection of viral DNA

The cotton leaf curl coat protein (CP) specific primers were used for mycovirus detection in the extracted fungal DNA: forward primer, SAf, 5'-GTCGCAGGATTATTCACCG-3'; reverse primer, SAr, 5'-GTATGTAGATCAATATTCAGAAA-3' (14). Ten nanograms of supposedly previously extracted viral nucleic acid was added to a total volume of 25 µL PCR reaction mixture. The PCR mixture contained 10x PCR reaction buffer 10 pmol of each primer, 0.2 mmol/L dNTPs, 5 mmol/L $MgCl_2$, 1 unit Taq DNA polymerase (Promega, USA). PCR was carried out in a thermocycler (Uno II, Biometra, Germany), the PCR program was applied as follows: initial denaturation at 95 °C for 5 min; 34 cycles of 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 72 °C for 1 min; a final extension step at 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis, using 2 % (w/v) agarose in 0.5x TBE buffer. The size of each band was estimated using a DNA molecular weight marker. Finally, the gel was photographed with a gel documentation system (Alpha-chem Imager, USA).

Band isolation, purification and cloning

Expected PCR-amplified fragments were excised from the agarose gel and purified using the Qiagen Gel Extraction kit (Qiagen, Germany). Purified DNA fragments were then cloned in pCR4-TOPO vector with the TOPO TA cloning kit (Invitrogen, USA) in the competent *E. coli* strain TOPO 10. Plasmid DNA was isolated using the QIA Spin miniprep kit (Qiagen, Germany). Plasmid DNA was sequenced in both directions, using the BigDye Sequencing Kit and ABI 377 DNA sequencer (ABI, USA).

DNA sequencing and phylogenetic construction

DNA sequencing was performed by Macrogene Company (Korea). The sequence was submitted to NCBI GenBank database (http://www.ncbi.nlm.nih.gov). The sequenced CP gene was aligned to compare with other sequences available in the GenBank database and the phylogenetic tree was generated using MEGA4.1 for our gene in comparison to the other sequences published in GenBank.

Viral transmission

Viral detection results showed that some fungi contained viral DNA and also nanoscale VLPs. The positively infected fungus *Mucor racemosus* was inoculated on PDA medium containing another virus-negative fungus (*F. oxysporum*). The inoculated plate was incubated at 25 °C for 7 days then, mycelia of *F. oxysporum* were collected and subjected to nucleic acid extraction and PCR amplification using the coat protein primers.

Electron microscopy (EM)

The collected mycelia of *Mucor racemosus* and *F. oxysporum* were examined by electron microscopy to detect viral particles. The samples were prepared according to Alves and Pozza (2) and kindly examined and photographed at the New Materials Institute, City of Scientific Research and Technology Applications (Borg El Arab, Alexandria), using JEOL 100 CX-II ASID-4D SEM.

Results and Discussion

Geminiviruses are a large family of plant viruses that infect a wide range of plants all over the world. In this study, the extracted nucleic acids from mycelia of the tested fungal isolates were separated in a 1 % agarose gel (**Fig. 1A**). The results revealed that the viral-DNA-free fungi had only one band (genomic DNA). On the other hand, the virus-infected fungi had either two bands or one band. These bands are circular dsDNA of the ss DNA geinivirus [which is called Replicative Form (RF-DNA)]. The RF-DNA was previously identified as concatameric dsDNA replicative forms produced during geminivirus infection (12). The molecular size of the viral RF-DNA was approximately either 3 kbp or 1 kbp. These results supported the report of Stenger et al. (32) that geminiviruses are ssDNA viruses and their genome size ranges from 2.5 kbp to 3.0 kbp. Our results are also in agreement with those of Yu et al. (38), who found two additional bands (2 kbp and 0.3 kbp) besides the fungal genomic DNA. They proved that the first band is the ssDNA of the mycovirus and the second band was defective viral DNA. Stanley et al. (31) described a hypovirulence-associated circular ssDNA mycovirus from the plant pathogenic fungus *Sclerotinia sclerotiorum*. They proved that this virus belongs to the geminiviruses and that viral particles and the extracted viral DNA were not able to infect the fungus *S. sclerotiorum* itself but succeeded in infecting the fungal protoplast.

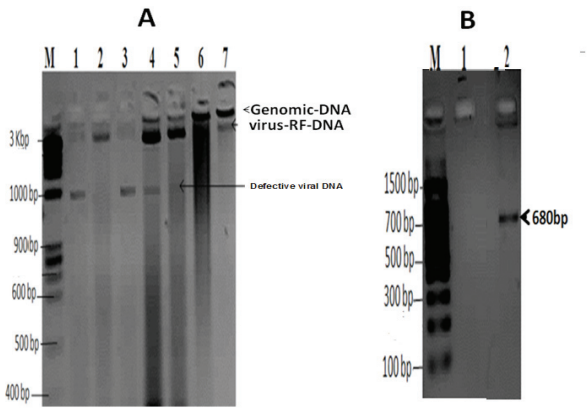

Fig. 1. Nucleic acids extracted from some of the examined fungi and geminiviral coat protein gene amplification using specific PCR. DNA band pattern of the extracted DNA from the tested fungi (**A**): Lane M: 3 Kb DNA marker; Lane 1: *M. racemosus*; Lane 2: *M. phaseolina*; Lane 3: *Paecilomyces sp*.; Lane 4: *F. graminearum*; Lane 5: *A. flavus*; Lane 6: *P. glabrum*; Lane 7: *Betryodipolida sp*. PCR amplification of the mycovirus coat protein gene (**B**): Lane M: 1.5 Kb DNA marker; Lane 1: negative control; Lane 2: *M. racemosus* (infected fungi showed the two extra band pattern).

The total extracted nucleic acids from the 30 examined fungi were amplified by PCR using the coat protein primers specific for geminiviruses. Out of these 30 isolates, seven different fungi showed positive results (**Table 1**). The positive isolates gave one band approximately 680 bp in size, but no bands were observed in the other isolates (**Fig. 1B**). The amplified products from two isolates, *M. phaseolina* and *M. racemosus*, were selected for DNA sequencing. A gene sequence of 269 bp was obtained for each isolate. The two gene sequences were aligned to compare with other sequences available in the GenBank database.

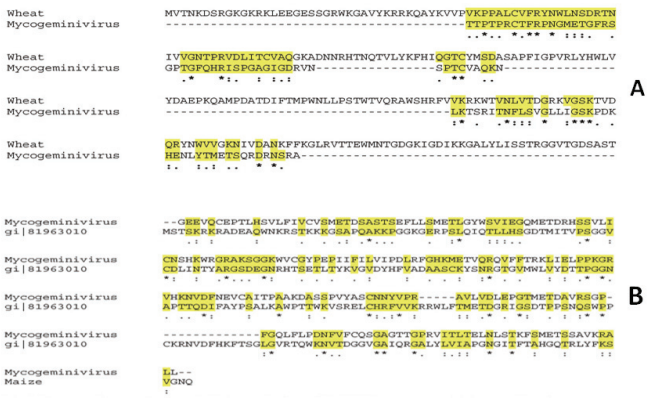

Fig. 2. Sequence alignment between the obtained DNA sequence of the coat protein genes amplified from the two examined fungi and those of other geminiviruses. Sequence alignment between the wheat dwarf virus (gb|ACP28912.1|) and mycogeminivirus MpDV (**A**); and between the Maize streak virus (gi|81963010) and mycogeminivirus MrDV (**B**). The alignment was performed using the ClustalW 1.4 program, based on the amino-acid sequences deduced from the obtained nucleotide sequence: similarity (*), degeneracy (:), substitution (.).

The sequence analysis revealed that the two sequences for the positive strands showed similarity with some geminiviruses with 63 % and 34 % identity, respectively. Moreover, the ClustalW alignment results confirmed the above results and showed some similarities between the Wheat Dwarf Virus, Maize Streak Virus and the other two Mycogeminiviruses (**Fig. 2**). The evolution of the coat protein genes was illustrated by construction of phylogenetic trees (**Fig. 3** and **Fig. 4**) with the other similar coat protein genes (29 coat protein genes in the case of *M. phaseolina* and eight ones in the case of *M. racemosus*). The results revealed that the obtained coat protein shares the origin of the other genes. The low identities obtained with the two genes suggest that they are new records of geminiviruses. These viruses were named *M. phaseolina* DNA virus (MpDV) and *M. racemosus* DNA virus (MrDV).

It has been demonstrated that mycoviruses can infect and propagate in all families of the kingdom Fungi (24). Until recently, it was generally accepted that mycoviruses have either dsRNA or ssRNA genomes and DNA viruses are unable to infect fungi (26). It was reported that DNA viruses can infect animals, plants, parasites, and prokaryotes, but not fungi (10). Indeed, Dawe and Kuhn (7) proved that the fungus *Rhiziodiomyces* sp. was infected with a dsDNA fungal virus but scientists considered the *Rhiziodiomyces* sp. a water mold

belonging to the kingdom Protista not to the kingdom of fungi. Recently, Yu et al. (38) described a hypovirulence-associated circular ssDNA mycovirus from the plant pathogenic fungus *Sclerotinia sclerotiorum* (SsHADV-1) and identified this virus as a geminivirus. Moreover, they suggested that the host range of SsHADV-1 could be expanded to include other important plant pathogens.

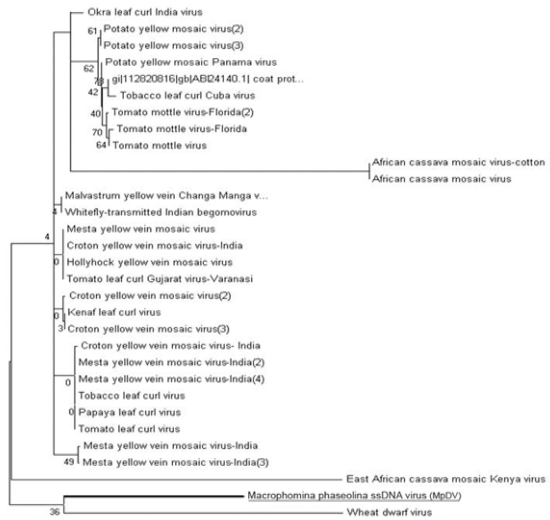

Fig. 3. Phylogenetic tree showing the evolutionary relationship between the coat protein gene of MpDV and the coat protein genes of the plant *Geminivirus* accessions in GenBank. The Neighbour-joining method (28) was used to construct the tree. The numbers on the branches represent bootstrap support for 1 000 replicates. Names refer to the accession number of the nucleotide sequences that encode the corresponding capsid protein genes.

Mycelia of the PCR-positive isolate of *M. racemosus* and the treated isolate of *F. oxysporum* were examined by EM to detect viral particles. The results presented in **Fig. 5** (**A** and **B**) revealed that different VLPs were observed. The VLPs were detected in mycelia of the fungal strains and the particle sizes ranged from 25 nm to 30 nm in diameter. The number of particles was high in all the examined fungi. The VLPs observed in *Mucor* appeared like stones with a different size. But when the virus was transferred into *Fusarium*, the VLPs were in the form of a nanoscale isometric shape (**Fig. 5C**), which is in agreement with the results obtained by Boland et al. (3).

Fig. 4. Phylogenetic tree showing the evolutionary relationship between the coat protein gene of MrDV and the coat protein genes of the plant *Geminivirus* accessions in GenBank. The Neighbor-joining method (28) was used to construct the tree. The numbers on the branches represent bootstrap support for 1 000 replicates. Names refer to the accession number of the nucleotide sequences that encode the corresponding capsid protein genes.

Our results showed that the virus affected the phenotype and growth of *F. oxysporum*. Some phenotypic changes were observed in the treated mycelia of *F. oxysporum*, including growth rate reduction and increased pigmentation as compared to the untreated fungus, in PDA cultures. These findings are in agreement with those of Chu et al. (6), who reported morphological changes, including reduction in mycelial growth and increased pigmentation, in the virus-infected strains of *F. graminearum.* Fungi are commonly infected with viruses and these viruses are typically associated with no symptoms and mostly have valuable effects for controlling some of their hosts (15). Mycoviruses may play an important role in diminishing the growth of their fungal host (35). For example, some hypovirulence-associated fungal viruses have been used for controlling the plant fungal disease chestnut blight (25).

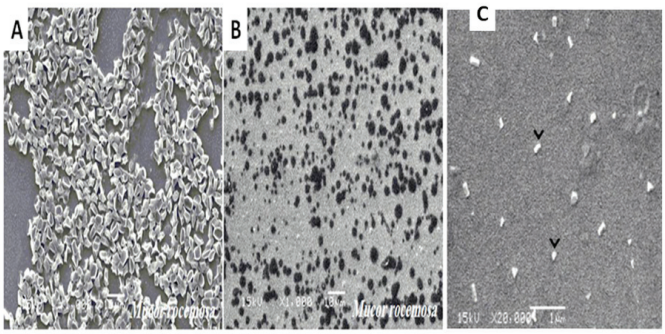

Fig. 5. EM micrograph of the virus-like particles in the *Mucor racemosus* isolate. Spore shape (virus-like particles) of the examined fungus (**A**). The virus-like particles on the same slide (**B**). EM micrograph of the virus-like particles in the *F. oxysporum* fungal isolate after viral transfer (**C**).

It has previously been reported that the vegetative incompatibility between two fungal strains mostly limits the mycoviruses transmission, and that the transmission mostly affects the fungal morphology and the hyphal cells (19, 34). Yu et al. (38) postulated that the extracted viral DNA isolated directly from the infected fungal mycelium was able to transfer to *S. sclerotiorum* protoplasts. Lee et al. (22) indicated that protoplast fusion can be used for introduction of FgV1-DK21 dsRNA into other *Fusarium* species and into *C. parasitica*, and that FgV1-DK21 can be used as a hypovirulence factor and, thus, as a biological control agent. Moreover, it has been demonstrated that transmission of hypoviruses between two vegetatively incompatible fungal strains could happen (16). Yu et al. (38) suggested that the DNA mycovirus described by them may have a different way to initiate infection of its host than that of known RNA mycoviruses. Moreover, in their subsequent study, Yu et al. (39) reported that virus particles isolated from an infected host can directly infect the hyphae of virus-free *S. sclerotiorum* when applied to hyphae grown on potato dextrose agar or sprayed on leaves of *Arabidopsis thaliana* and *Brassica napus*, regardless of vegetative compatibility affiliation. In this context, our finding may help in understanding and using the ssDNA mycoviruses in fungal biocontrol and expands our knowledge of virus ecology and evolution.

Conclusions

Pathogenic fungi cause many diseases in different plant crops all over the world and, at the same time, these fungi could be infected with different types of viruses. Mycoviruses could be used as biocontrol agents against most of the economically significant pathogenic fungi and, in this way, the usage of fungicides could be reduced. In this study we showed different families of fungi infected with mycoviruses belonging to the Geminivirideae family. When these viruses were transferred into the plant pathogen *Fusarium oxisporum*, the virulence of this pathogenic fungi was decreased. This step could justify the potential use of these viruses in the control of some plant pathogenic fungi, especially *Fusarium sp*. Future work will focus on purification of the mycovirus particles and their use in a biocontrol bioassay on different plant pathogenic fungi on plat and on the infected plant tissues as well.

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Conflicts of Interest

The authors declare no conflict of interest.

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