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Discovery of *Aspergillus* mycoviruses and development of mycovirus-based vector for gene silencing in the human pathogenic fungus *Aspergillus fumigatus*

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Abstract

A collection of *Aspergillus* environmental isolates was screened for the presence of mycoviruses. Out of 178 isolates, 5.1% contained double-stranded RNA (dsRNA) elements ranging in size from approximately 0.7 to 6.0 kbp. The dsRNA elements were identified as belonging to four different categories comprising non-segmented dsRNA, bipartite dsRNA, quadripartite dsRNA and uncharacterized mycoviruses. *Aspergillus fumigatus* tetramycovirus-1 (AfuTmV-1) was exploited for development as a tool for silencing genes in the human pathogenic fungus *A. fumigatus*. Using a sequence of dsRNA2 belonging to the AfuTmV-1, a recombinant virus vector was engineered to generate the silencing vector AfuTmV-1-dsRNA2::ALB1 which carried a target *ALB1/PKSP* gene responsible for spore pigmentation. The engineered virus vector induced loss of conidial pigmentation in the fungal host at different levels and with low frequency. In addition, *in vitro* translation of mycovirus-based silencing vectors demonstrated that the engineered mycoviral vector was functionally transcribed and translated.

Keywords: *Aspergillus fumigatus*, Mycovirus, RNA silencing, Virus vector

1. Introduction

Mycoviruses are viruses which selectively infect fungi. Their genomes are commonly double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) encapsidated in a variety of particle morphologies, but mainly in isometric forms. Some unencapsidated mycoviruses have also been described [1]. Mycoviruses are widely distributed across a host range that includes the fungal phyla Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota, and plant pathogenic oomycetes. The diversity of known mycoviruses has grown rapidly over the last few years and this trend is expected to continue. To date, several mycoviruses have been discovered in *Aspergillus* spp. [2,3]. However, information concerning *Aspergillus* mycoviruses in Thailand has not yet been documented. Thus, the purpose of this study is to investigate the presence and prevalence of mycoviruses in *Aspergillus* spp. isolated from environmental samples collected in Thailand.

Aspergillus fumigatus is an opportunistic fungus and is the most encountered fungal pathogen of humans. The increasing incidence of fungal infections and the developing resistance of fungi to common antifungal drugs are currently major concerns worldwide. Investigation of the factors involved in fungal pathogenicity usually relies on the identification and targeting of genes associated with the process. Since most of the genes in the *A. fumigatus* genome are essential for its survival, knock-out mutants of the right genes could put an end to its pathogenicity. Therefore, a method such as RNA silencing, which down-regulates rather than deletes gene function, would have wider applications.

The use of hairpin RNA-expressing vectors is a commonly used versatile method of inducing RNA silencing in fungi. The approach presents problems ranging from the difficulty of plasmid preparation to the instability of hairpin constructs in the targeted fungal cells [4,5]. Therefore, an alternative tool for the fast and transient suppression of gene expression should be considered. In this regard, proposed approaches, such as the use of virus sequences to construct silencing vectors, have recently been developed and applied to several filamentous fungi. The demonstrated levels of silencing varied depending on the virus and target gene of interest [6,7].

The RNA silencing mechanism in several virus-infected fungi has been as a natural defense response against virus infection [8-10]. This concept has been adopted and, following the principle of virus replication, viral RNA derivatives have subsequently been developed that correspond to virus sequences that trigger the process of RNA silencing and reduce viral accumulation in the fungal hosts. Similarly, a fragment homologous to the host endogenous target gene can be engineered and inserted unto the virus genome. The technique leads to down-regulation of the targeted fungal transcripts. Thus, current knowledge of RNA silencing as an antiviral defense response, coupled with new insights into mycoviruses, have provided opportunities to develop tools which could be useful in the analysis of gene function and key elements involved in *A. fumigatus* virulence.

Here, the silencing vector system constructed for *A. fumigatus* was based on sequence of the mycovirus Aspergillus tetramycovirus-1 (AfuTmV-1). The selection of this virus was based on the following rationale. Previously, a virus-infected isolate and virus-free isolates of *A. fumigatus* showed indistinguishable colony morphologies, which indicated that the virus does not change the morphology of the fungus on any lines [1]. The chosen target of the present work was the fungal endogenous, *ALB1/PKSP*. The gene encodes polyketide synthase, which is responsible for the biosynthesis of the melanin necessary for conidial pigmentation. Thus, the aims of the present study were to screen for the presence of mycoviruses in *Aspergillus* spp. and to develop the virus as a silencing vector to study functions of the fungal genes.

2. Materials and methods

2.1 Fungal maintenance and DNA extraction

Aspergillus spp. were isolated from various environmental samples and maintained in 20% (v/v) glycerol solution in the storage tubes and kept at -80℃. A 20 µL of conidia suspension was inoculated on an *Aspergillus* Complete Media (ACM) agar plate and incubated for 4 days at 37℃. A 10 mL suspension of 5x108 conidia/mL was transferred to 500 mL ACM broth and incubated at 37℃ for 7 days on a rotary shaker at 150 rpm.

Aspergillus genomic DNA was prepared using the DNeasy® Plant Mini Kit (QIAGEN, UK) with mini protocol provided by the manufacturer.

2.2 Extraction of viral dsRNAs

Extraction of mycovirus dsRNAs was performed using LiCl fractionation as described by Hull and Covey [11]. Briefly, a 40 mL of extraction buffer (50 mL/L TrisHCl buffered phenol (Sigma), 60 g/L 4-aminosalicylic acid (Sigma) was added into a 25 g of ground mycelia. The mixture was shaken for 60 min, then centrifuged at 10,000 rpm for 40 min at 4℃. The supernatant was transferred and a 15 mL of Tris-saturated phenol was added and centrifuged. Then, a 15 mL of chloroform was added and the mixture was centrifuged. The supernatant was transferred then an equal volume of 4 M LiCl solution was added. The mixture was precipitated overnight at 4℃. and then centrifuged at 4℃ (10,000 rpm, 50 min). The precipitant was resuspended in 1 mL diethylpyrocarbonate (DEPC)-H_2O, then an equal volume of 8 M LiCl solution was added. The mixture was precipitated overnight at 4℃ and centrifuged. Prior to the next nucleic acid manipulation, dsRNA samples were purified by phenol-Sevag extraction [1]. DNA and ssRNA contamination were removed by sequential DNaseI and SI nuclease treatment. The presence of the dsDNA was checked by electrophoresis in a 1% (w/v) agarose gel in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer.

2.3 Construction of silencing vectors based on AfuTmV-1 mycovirus

A full-length complementary DNA (cDNA) clone of dsRNA2 was generated by RNA linker-mediated rapid amplification of cDNA ends [12] amplification using internal primers based on a *Bam*HI sequence in dsRNA2 (BamF and BamR primers; 5'-CTCAACAAGGATCCCATGCTCG-3' and 5'-CGAGCATGGGATCCTTGTT GAG-3' respectively) in combination with Lig-For and Lig-Rev primers [1], yielding two Polymerase chain reaction (PCR) fragments which were 1,195 and 1,130 bp in size and designated as amplicons A and B, respectively. Amplicons A and B were re-amplified using internal BamF and BamR primers in combination with respectively 5- and 3'- primers (RNA2FS;5'CCCAAGCTTT CTAGATAATACGACTCACTATAGGGTGAAT AAAAGATTTGTTCTG-3' and RNA2RS; 5' CCCGAATT CGGTACCTAATACGACTCACTATAGGGCC CCCAGGCCCGGCGGGGGG-3') to incorporate T7 RNA promoter sequences and restriction enzyme sites (*Hind*III, *Xbal*I, *Eco*RI and *Kpn*I) on both ends for subsequent *in vitro* transcription.

PCR amplification was performed in a 50 µL reaction mixtures using Phusion® High-fidelity DNA polymerase (New England Biolabs (NEB); 5 units/µL). The reaction mixture and PCR conditions were set as follows; 5 µL cDNA, 28.5 µL DEPC-H_2O, 10 µL of 5x Phusion HF Buffer (NEB), 1µL of 10 mM Deoxynucleotide triphosphate (dNTPs) mix (Promega) and 2.5 µL of each forward and reverse primer (10 µM). PCR condition was performed as follows; 98℃ 30 sec, 35 cycles of denaturation at 98℃ 10 sec, annealing at 53-

72℃ 30 sec, extension at 72℃ 30 sec, and final extension at 72℃ 10 min. PCR amplicons A and B were respectively restricted with *Hind*III+*Bam*HI and *BamHI*+*Kpn*I and then ligated in a three-way ligation into pUC18 vector (Fermentas) doubly-restricted with *Hind*III and *Kpn*I, yielding pAfuTmV-1-RNA2 vector.

A truncated sequence of the full-length clone was constructed using two restriction sites for *Ngo*MIV (NEB) which deleted an internal fragment, leaving an internal sequence and the 5'- and 3'-untranslated regions. The resulting plasmid was designated as pAfuTmV-1-NgoMIV construct and was subsequently modified to express the transcripts of the engineered cDNA of dsRNA2 containing *ALB1/PKSP* gene fragment in both sense and anti-sense orientations. The targeting *ALB1/PKSP* fragment was amplified from *A. fumigatus* Af293 genomic DNA. The pAfuTmV-1-*Ngo*MIV plasmid and *ALB1/PKSP* PCR products were then digested with *Ngo*MIV, purified and ligated, resulting in a pAfuTmV-1-ALB1 construct.

2.4 Transfection and transformation assays

Protoplasts from *A. fumigatus* Af293 were prepared as described previously [1]. RNAs were synthesized from the pAfuTmV-1-*Ngo*MIV and pAfuTmV-1-ALB1 vectors using MAXI Script®T7 and MEGAscript RNAi Kit (Ambion). DNA and ssRNA were removed by treatment with DNase I and RNase (MEGAscript RNAi Kit, Ambion). Approximately 30 µg of *in vitro* synthesized dsRNAs was used to inoculate *A. fumigatus* Af293 protoplasts. Transfection was performed using the same protocol as described previously [1]. A solution of 200 µL and serial dilution (10^{-3}-10^{-6}) of the solution were plated gently onto the ACM selection plates. A 0.1 mg/mL ampicillin and IPTG/X-gal solution was added in the media when required. Single fungal colonies were randomly collected from the selection plates and transferred to fresh ACM plates, following incubation at 37℃ for 3 days.

2.5 In vitro translation of mycovirus-based silencing vectors

Double stranded RNAs were denatured at 95℃ for 5 min and immediately cooled on ice. Individual dsRNA segment was size-fractionated. The translation was performed in the TNT® Coupled Reticulocyte Lysate System (Promega, USA) by adding 1 µg of dsRNA to 25 µL of the translation mixture incorporating with [35S] methionine (PerkinElmer Cat.# NEG709A) and incubated at 30℃ for 2 h as described in Kanhayuwa et al. [1]. Aliquots of 5 µL of denatured translation mixture were loaded in a NuPAGE® Novex® 4-12% Bis-Tris protein gel (Invitrogen) and electrophoresed in 3-(N-morpholino) propanesulfonic acid sodium dodecyl sulphate (MOPS SDS) running buffer for 45 min at 190 V (Bolt® mini gel tank). Precision plus protein™ all blue standard (BIO-RAD) was used as marker proteins. The dried gel was exposed to Kodak® Biomax MR Film in the dark for 3 h to 10 days at room temperature.

3. Results

3.1 Screening of mycovirus in the Aspergillus environmental isolates

Screening for mycoviruses of the *Aspergillus* environmental isolates revealed that nine (5.1%) of 178 isolates contained dsRNA segments (Table 1). The profiles of isolated dsRNA mycoviruses found in the *Aspergillus* collection are shown in Figure 1. The genomic segment numbers, dsRNA profile and sizes of dsRNA segments found in each fungal isolate were varied. They were categorized into four tentative major groups. Four isolates of *Aspergillus* spp. (KW1-3, ASA23, ASA58 and ASA69) contained non-segmented dsRNA mycoviruses ranging from 1.1 to 6.0 kbp in size, indicating a characteristic of members of the family *Totiviridae* of the genus *Totivirus* (Figure 1; lane 3, 5 and 9). One isolate of *Aspergillus* spp. (AAA12) contained a dsRNA mycovirus with two dsRNAs ranging from 1.9 to 2.2 kbp in size, indicating a characteristic of members of the family *Partitiviridae* of the genus *Partitivirus* (Figure 1; lane 4). One isolate of *Aspergillus* spp. (S2-09) contained a dsRNA mycovirus with four dsRNAs, ranging from 1.3 to 3.0 kbp in size, indicating a characteristic of members of the family *Chrysoviridae* of the genus *Chrysovirus* (Figure 1; lane 2). Infection by mixed mycoviruses was observed in three isolates of *Aspergillus* spp. detected in lane 6, 8 and 10. These mycoviruses were placed into an uncharacterized category showing dsRNA segments of three and more than four elements.

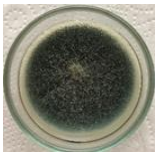
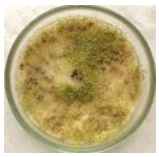
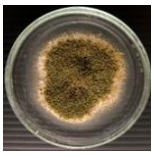
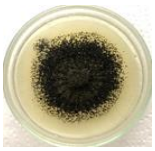
3.2 Construction of the silencing vector

The dsRNA2 segment of AfuTmV-1 was subjected to reverse transcription PCR (RT-PCR) to generate a full-length cDNA clone. The amplicon was designed by adding oligonucleotides to construct a clone containing a sequence of T7 RNA promoter and a sequence of restriction enzyme sites on both ends. The purified amplicon was then subjected to a three-way ligation into pUC18, making a pAfuTmV-1-RNA2 vector construct.

A truncated version of pAfuTmV-1-RNA2 was also generated by deleting of an internal sequence of the dsRNA2 ORF2 using *Ngo*MIV at two restriction sites to yield a pAfuTmV-1-*Ngo*MIV. The modified construct

contained the 5'- and 3'- untranslated regions (UTRs) of the AfuTmV-1, which are crucial sites for viral replication. Also, the N-terminal region of the open reading frame (ORF) was included. The pAfuTmV-1-*Ngo*MIV construct was subsequently used as a prototype vector to promote the insertion of either the fungal endogenous gene sequence or the non-viral fragment. Finally, the truncated ORF of the resulting plasmid pAfuTmV-1-*Ngo*MIV was inserted with an untranslatable sequence upstream of the *ALB1/PKSP* fungal gene, nominating pAfuTmV-1-ALB1 (Figure 2).

Table 1 *Aspergillus* environmental isolates containing dsRNA mycoviruses.

Isolate	Source	Date of isolation	Colony appearance	No. of segments	dsRNA size (kbp)
S2-09	Soil (Songkhla)	Aug-2017		4	1.3-3.0
KW1-3	Saline soil (Pattani)	Sep-2018		1	6.0
AAA12	Air (Songkhla)	Feb-2020		2	2.0-2.2
ASA23	Air (Phatthalung)	Feb-2020		1	1.0
ASA24	Air (Trang)	Feb-2020		3	2.5-3.5
ASA58	Air (Songkhla)	Feb-2020		1	0.9
ASA67	Air (Songkhla)	Feb-2020		5	0.7-6.0
ASA69	Air (Songkhla)	Feb-2020		1	1.4
ASA86	Air (Songkhla)	Feb-2020		5	1.0-2.8

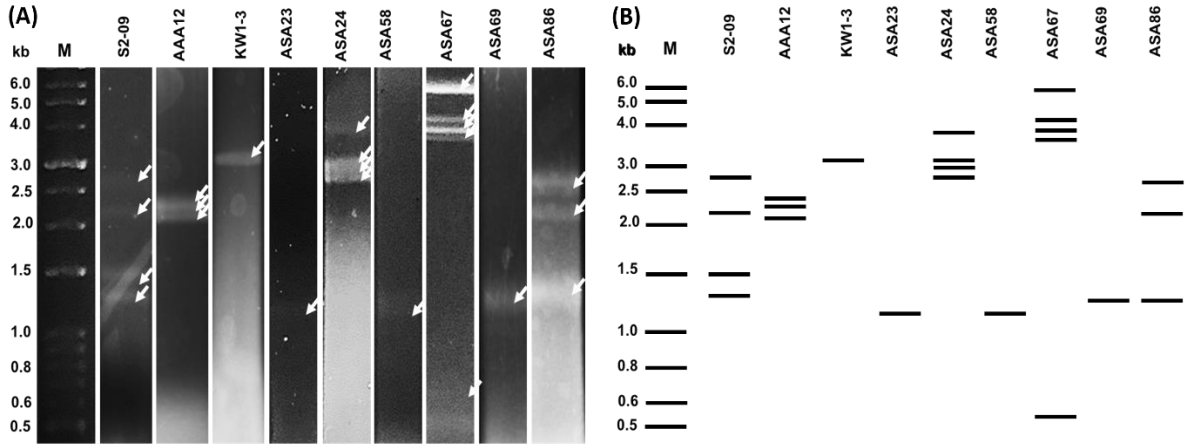


Figure 1 Double-stranded RNA profiling of dsRNA mycoviruses isolated from the *Aspergillus* environmental isolates analyzed on agarose gel electrophoresis. A 1 kb Plus DNA ladder (Lane M; 250bp-10kbp; Invitrogen) was used as a marker (A). The dsRNA profiles displayed in lines (B).

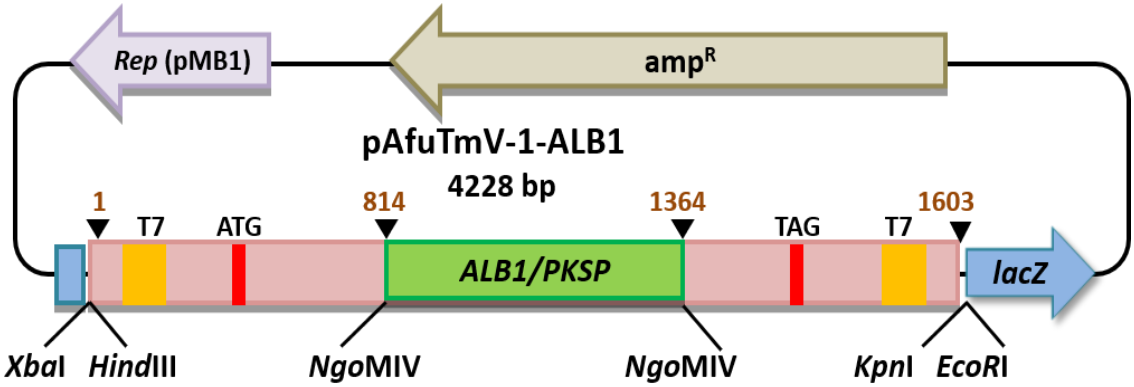


Figure 2 Schematic representation of the mycovirus-based silencing vector constructs of a modified pAfuTmV-1-ALB1 vector carrying untranslatable *ALB1/PKSP* sequence. Constructs are not drawn to scale.

3.3 In vitro translation of mycovirus-based silencing vectors

The mycovirus-derived silencing vectors (pAfuTmV-1-RNA2 and pAfuTmV-1-*Ngo*MIV) were examined for translational potency by *in vitro* translation in the TNT® coupled rabbit reticulocyte lysate system. The translation was performed in a time course study over a period of 90 min and the samples were investigated at 30, 60 and 90 min. A negative control sample without RNA was also included. Autoradiograms of *in vitro* translation products of the AfuTmV-1 silencing vectors showed two translation products: one of 76 kDa that corresponded to the dsRNA2 cDNA clone and another of 27.6 kDa that corresponded to the truncated dsRNA cDNA clone. These products confirmed the transcription and translation of functionally active plasmids (Figure 3).

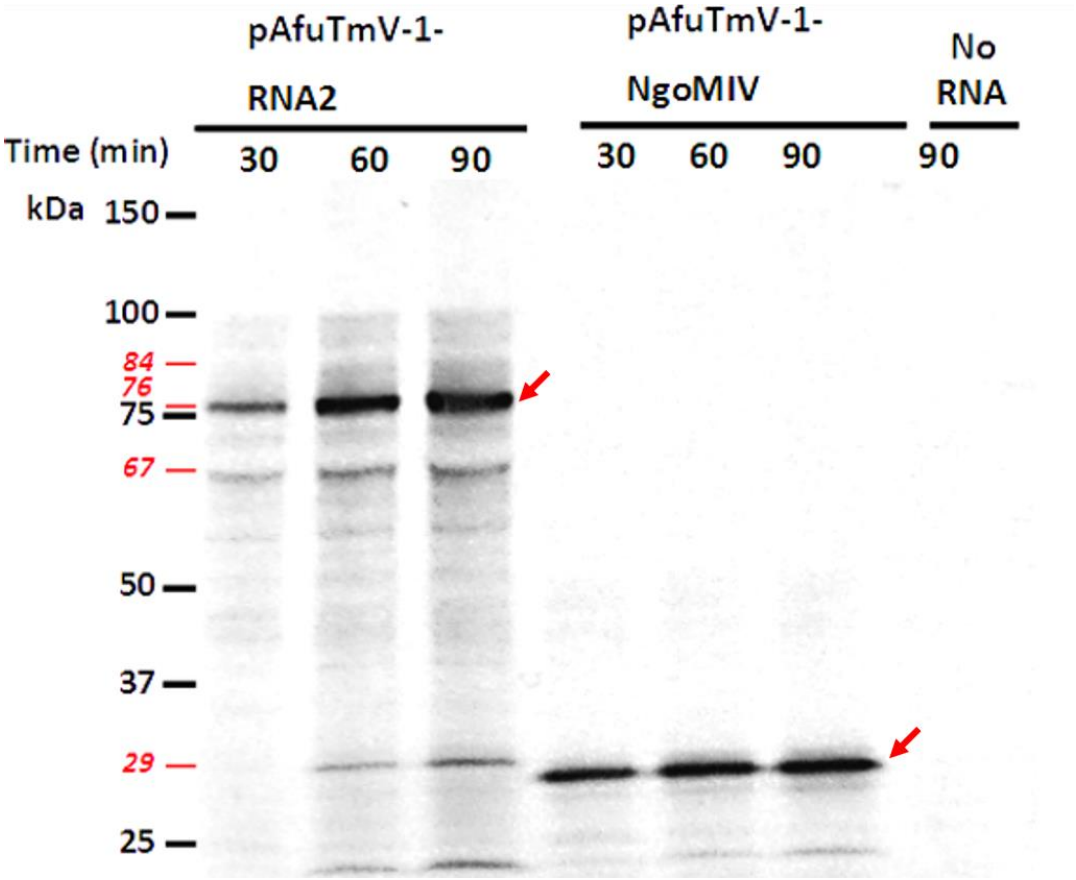


Figure 3 Autoradiograms of *in vitro* translation products of AfuTmV-1 derived silencing vectors. A time course over a period of 90 min was observe. Precision plus protein^{TM} all blue standard (BIO-RAD) were used as marker proteins. Films were exposed in the dark for 4 h. Arrows indicate major translation products from each sample.

3.4 Silencing efficiency of mycovirus-based silencing vectors

The silencing efficiency of the engineered mycovirus-based vectors was tested. The plasmids, pAfuTmV-1-*Ngo*MIV and pAfuTmV-1-ALB1, were *in vitro* transcribed at both ends to generate sense and anti-sense transcripts. The resulting dsRNA virus constructs of each plasmid were designated AfuTmV-1-dsRNA2ΔORF and AfuTmV-1-dsRNA2::ALB1, which were 1,056 and 1,562 bp long, respectively. Subsequently, each dsRNA virus construct was transfected into the protoplasts of AfuTmV-1-infected *A. fumigatus* Af293.

Approximately 110 fungal regenerants were obtained from a single transfection reaction (calculated from each serial dilution of 200 µL sample/plate; 10^{-3}-10^{-5}, x3 replicates). Either an absence or reduction of ALB1/PKSP gene expression was found among the AfuTmV-1-dsRNA2::ALB1 transfectants (Figure 4A). Dark green colonies of the wild type *A. fumigatus* Af293 shown in Figure 4B and 4E. Fungal transfectants that produced completely white colonies, which indicated a possible completely silenced phenotype, were designated as AfM-W_N (N= 4) (Figure 4D and 4G; where AfM-W_1 is the example). Transfectants that produced light green colonies, possibly indicating and intermediate silenced phenotype, were designated as AfM-LG_N (N= 32) (Figure 4C and 4F; where AfM-LG1 is the example). The findings suggested that the fungal endogenous ALB1/PKSP gene was somehow partially or completely silenced by the dsRNA virus construct. However, following a second passage of the recovered regenerants on either solid or liquid ACM, all the silenced fungal transfectants returned to the normal phenotype of the wild type fungus, showing dark green colonies. This behavior could be explained by the transient silencing effect and instability of the phenotype. In addition, the identities of the fungal transfectants and the parental strain were confirmed by PCR amplification of the internal transcribed spacer (ITS) region.

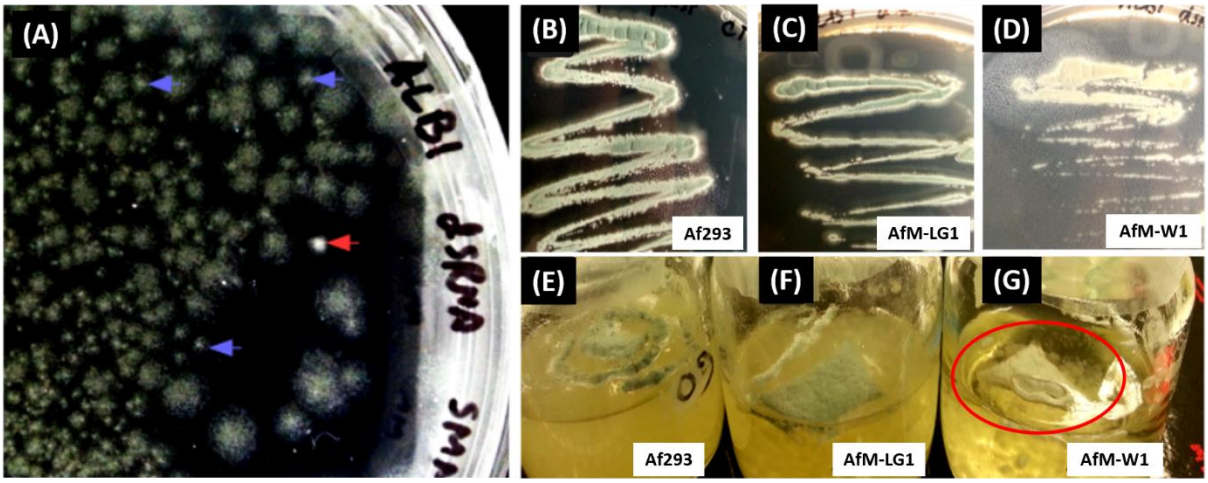


Figure 4 Phenotypic appearances of fungal transfectants obtained after ALB1/PKSP silencing. Regeneration plate (at 10^{-2} dilution) of the protoplast transfected with AfuTmV-1-dsRNA2::ALB showing white colonies (red arrow), light green colonies (blue arrow) and non-transfectant colonies (dark green) (A). Colony morphology of the wild-type *A. fumigatus* Af293 (green; B); intermediate phenotype (AfM-LG1; light green; C); and complete phenotype (AfM-W1; white; D) on ACM regeneration plates. Mycelial mass of the wild-type *A. fumigatus* Af293 (green; E); intermediate phenotype (AfM-LG1; light green; F); and complete phenotype (AfM-W1; white; G) in ACM liquid medium.

4. Discussion

Several mycoviruses were detected during the screening of the *Aspergillus* environmental isolates. The screening revealed that 5.1% of 178 isolates contained dsRNA mycoviral elements. Aspergillus mycoviruses showed different genomic profiles in terms of genome segment numbers and sizes. The mycoviruses were categorized into four major groups comprising members of the family *Totiviridae* of the genus *Totivirus*, members of the family *Partitiviridae* of the genus *Partitivirus*, members of the family *Chrysoviridae* of the genus *Chrysovirus* and a group of uncharacterized mycoviruses.

The present study indicated that AfuTmV-1, an unencapsidated mycovirus with a small genome [1] can be developed for gene silencing in fungi. The virus naturally initiates RNA silencing and is targeted by the defense response of the fungal host. According to this finding, RNA silencing by this mycovirus would be activated in transfected fungal protoplasts when fungal RNAs or transgenes are integrated into the viral genome. In addition, the infection of *A. fumigatus* by AfuTmV-1 is cryptic. This is a useful feature of the virus since alterations to the phenotype caused by inserted genetic information in virus-based constructs would not be confused with symptoms elicited by the virus itself.

The AfuTmV-1-based silencing vector was constructed and tested. The *A. fumigatus* Af293 endogenous gene, *ALB1/PKSP*, which has been successfully silenced elsewhere [13,14], was selected for the study. Introduction of the engineered AfuTmV-1-dsRNA2::ALB1 caused loss of conidial pigmentation in the fungal host but to different levels. Complete silencing was demonstrated by the generation of white colonies, and where the silencing event was transient, the silenced phenotypes generated light green colonies. However, transfection occurred at a very low frequency with only 4 fully silenced isogenic lines and 32 partially silenced isogenic lines being generated. Moreover, after second subculturing, some lines of fully silenced (white) and partially silenced (light green) transfectants showed signs of recovering the wild-type phenotype by producing dark green colonies. These recoveries suggest that the silencing was transient and unstable. These also suggest that the virus vector lost its replication capacity or the insert was unstable in the construct.

The instability of the silenced phenotype might be explained by loss of vector replication competence due to deletion of the insert fragments. If the virus contributed to the silencing effect, it is unclear how a range of silencing events could occur when the virus-based silencing vector or dsRNA fragments were used. One explanation for this phenomenon could be differences in the replication rates of recombinant virus vectors which would lead to uneven distribution and variation in the silencing level and silencing stability of fungal cells. The ability of the host to mediate RNA silencing mechanisms must also be considered as a factor in the variation of silencing efficacy, which has a potential effect on the degradation of the mRNA target. However, the presence of partially silenced and fully silenced phenotypes among isogenic lines has been observed in other microorganisms, for instance *Cryptococcus neoformans* [15]; *Neurospora crassa* [16]; *Magnaporthe oryzae* [17]; *Aspergillus fumigatus* [13,15] and *Trypanosoma brucei* [18].

5. Conclusion

Here, the first silencing vector for *A. fumigatus* was developed based on mycovirus sequences. A recombinant virus vector was manipulated using the AfuTmV-1 mycovirus to generate the AfuTmV-1-dsRNA2::ALB1 silencing vector which carried a target ALB1/PKSP gene responsible for spore pigmentation. The engineered silencing vector caused loss of conidial pigmentation in the fungus at different levels in some lines and with low frequency. From the present findings, a mycovirus-derived vector seems to be an alternative candidate for use as a silencing tool to study gene function in certain fungi. However, many aspects of the use and performance of the construct should be further investigated before applying a mycovirus-based silencing vector system as a high-throughput tool for functional genomic studies of human pathogenic fungal species.

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