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# Deployment of RNA Silencing Method to Mitigate Expression of a Cell Wall Protein (AFLMP1) in *Aspergillus flavus*

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## ABSTRACT

***Aspergillus flavus* is a major fungal phytopathogen and an opportunistic pathogen to humans and livestock. The fungus produces immunosuppressive and carcinogenic aflatoxins that act as a burden in food and feed industries. An earlier phylogenetic analysis indicated that the cell wall protein, *A. Flavus* Mannoprotein 1 (AFLMP1), is mainly present in Aspergillus section Flavi such as *A. parasiticus* and *A. flavus*. This makes AFLMP1 a great candidate for siRNA-based control of aflatoxigenic fungi in farms and processing units, and fungal therapy in hospitals. Here and for the first time, mode of action of a chemically synthesized RNA interference (siRNA) was investigated for the control of AFLMP1 synthesis. The efficacy of direct uptake of different concentration of siRNA on spore germination of *A. flavus* was monitored via Opera High Content Screening confocal microscope. siRNA caused growth inhibition at lower concentrations (0.65 nM) and germination failure (more than 90%) at higher concentrations (5 nM), most likely by interfering in mannoprotein biosynthesis. It is assumed that siRNA technology can be implemented as a promising suppressive agent in inactivation of target genes. It can be considered as an intervention in food/feed industries to control the development and reproduction of fungi to keep the fungal population below hazard critical points.**

**Keywords***:* Aflatoxin, *Aspergillus flavus*, Inhibition assay, Opera system.

## INTRODUCTION

Aspergillus genus holds more than 339 filamentous species that are mainly saprophyte (Schubert *et al*., 2018). Section Flavi of Aspergillus with numerous species have had a significant impact on human. Amongst which, *A. parasiticus* and *A. flavus* are infamous for producing aflatoxins found in cereals, nuts, crop roots and other agricultural products (Arias, 2015). *A. flavus* is the second most common cause of aspergillosis, and in its invasive form, it may lead to death, if no actions are taken (Van der Fels-Klerx *et al*., 2019; Kjærbølling *et al*., 2020; Vuong and Waymack, 2020; Warnatzsch *et al*., 2020). During COVID-19 pandemic, many of sever patients suffered from pulmonary aspergillosis, therefore, an initial screening for Aspergillus infections was suggested (Wasylyshyn *et al*., 2021). It seems global warming has led to greater inclination of Aspergillosis in recent years (Coleman *et al*., 2019). The aflatoxin production capabilities of *A. flavus* make the fungus even more harmful compared to other fungi

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that only infect and damage crops (Ismaiel and Papenbrock, 2015; Smith *et al*., 2017; Andrews-Trevino *et al*., 2020; Kyei *et al*., 2020). It is estimated that 25% of the food crops (~100 million tons) worldwide are contaminated with aflatoxins (Eskola *et al.*, 2020).

So far, Good Agricultural Practice (GAP *via* means of post-harvest management and integrated pest management) and Good Manufacturing Practice (GMP) have failed to fully control the impact of *A. flavus* that results in aflatoxins being present in food chain. Furthermore, the development of fungal resistance strains against all important fungicides that are being used in agriculture and clinics are considered as imminent threat (EI-Baky and Amara, 2021). Other new approaches including siRNA-mediated biocontrol methodologies (Nakayashiki *et al*., 2005; and genome editing tools (reviewed by Sauer *et al*., 2016) have started to shed lights on fungal crop resistance to lower the onset of fungal spread and their mycotoxin productions. Along with the development of resistant cultivars, use of specific antibodies (Schubert *et al*., 2018, 2019; Ansari *et al*., 2021) have shown to be vital in early detection of contamination, therefore, change in the storage conditions are considered to be necessary in order to avoid further fungal development. The early detection allows meeting Hazard Analysis and Critical Control Points (HACCP) standards, minimizing the devastating effects of the fungus. In future, it is expected that other biological (Masanga *et al*., 2015; Schubert *et al*., 2015), physical (Hossieini *et al*., 2016), and chemical (Bai, 2004; Werner *et al*., 2020) means would be implemented in order to collectively minimize the population of mycotoxin-producing fungi in food chain.

Host Induced Gene Silencing (HIGS) through RNAi is a molecular approach to post-transcriptionally silence the key genes involved in fungal development (Riechen 2007; Hernandez *et al*. 2009; Nowara *et al*., 2010; Tinoco *et al*., 2010; Yin *et al*., 2011; Zhang *et al*., 2011; Koch *et al*., 2013; Panwar *et al*., 2013). For instance, use of aflR-specific RNAi, a Transcription Factor (TF) involved in aflatoxin biosynthesis, reduced the expression of the TF, and thereby the aflatoxin, by 14-fold (Masanga *et al*., 2015). In contrast, transgenic lines harboring RNAi specific to Pathogenesis-Related maize seed gene (*ZmPRms*) were developed and the plants showed ~2.5-3.5 times more susceptibility to *A. falvus* with greater accumulation of aflatoxins (~4.5-7.5 fold) (Majumdar *et al*., 2017). Although somewhat contradictory, RNAi technology has the potential to shed light on the biology of aflatoxin-producing genes and the responsive host resistant genes towards developing the resistant lines.

RNA interference was used to investigate functional analysis of genes in the aflatoxin biosynthetic pathway (McDonald *et al*. 2005; Abdel-Hadi *et al*. 2011) and the developmental biology of *A. flavus* (Amaike and Keller, 2009). Earlier works have been illustrative of transgenic plants with siRNA to be able to suppress Aspergillus and Fusarium in production of toxins, when the relevant fungal genes were targeted (McDonald *et al*., 2005; Abdel-Hadi *et al*., 2011). siRNA offers the promise of controlling pests and pathogens in a sequence-specific manner without adversely affecting non-target species (Zhang and Hong., 2019; Wytinck, 2020).

siRNA has been introduced to fungi (Baldwin *et al*., 2018) *via* Polyethylene Glycol (PEG)-mediated transformation (Kadotani *et al*., 2003), microinjections (Mascia *et al*., 2014), electroporation (Rehman *et al*., 2016), and spray (Wang *et al*., 2017). siRNA uptake from the environment has been reported in *A. nidulans* (Khatri and Rajam., 2007), *Botrytis cinerea* (Wang *et al*., 2016), and *A. flavus* (Nami *et al*., 2017) that is called 'environmental RNAi' (Whangbo *et al*., 2008). The detailed mechanism of the uptake has been described in nematodes (Rosso *et al.*, 2005; Banerjee *et al*., 2017). RNAi can also be delivered to fungus via a





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method known as Spray-Induced Gene Silencing (SIGS), and sometimes, with the intervention of nanoparticles, a powerful, fast, non-GMO, and environmentally friendly approach, known as 'RNA fungicides' (Wang *et al*., 2017; Niu *et al*., 2021), through which fungal and oomycete activity/cell division are suppressed to control the disease or inhibit mycotoxin production (Wang and Jin, 2017; Song *et al*., 2018).

The extent and longevity of the reduction in abundance of fungal genes by means of siRNA (Khatri and Rajam, 2007; Jöchl *et al*., 2009) depends on its uptake efficiency that is mostly species- and size-dependent, half-life in host, and the possibility of signal amplification by the host (Wytinck, 2020). However, the exact mechanism by which exogenous RNAs enter the fungal cells is not fully understood. When using Host-Induced Gene Silencing (HIGS), the identification of suitable targets is sometimes the greatest challenge (Ebenezer, 2020). RNA-based disease therapeutics have been effective in both agriculture and therapeutic development for humans (Lieberman *et al*., 2018). The first sRNA drug, ONPATTRO (Akinc *et al*., 2019), and two of COVID-19 vaccines, developed by Pfizer and Moderna, were small RNA molecules targeting mRNAs (Forni *et al*., 2021). Along with clinical applications, RNA based technologies potentially can reduce the use of pesticides as an added-value strategy in integrated pest management (Mezzetti *et al*., 2020; Taning *et al*., 2021).

Here, controlling of *AFLMP1* mRNA was performed via application of different concentrations of chemically synthesized 27-mer RNA duplex RNAi to inhibit *A. falvus*. Phylogenetic analysis indicated that AFLMP1 that encodes a highly antigenic cell wall protein of *A. flavus* (Aflmp1p) contains 273 amino acid residues, is exclusively present in Aspergillus section Flavi (Woo *et al*., 2003). This cell-surface protein is homologous to proteins of *A. fumigatum* (Afmp1p) and *Penicillium marneffei* (Mp1p) present on their cell wall (Cao *et al*. 1998; Woo *et al*., 2003; Wang *et al*., 2012). Aflmp1 is the major cell wall galactomanoprotein of *A. flavus* that is located on the surface fungi hyphae revealed by indirect immunofluorescence (Figure 1). Aflmp1 is involved in cell wall assembly with roles in cell adhesion, the transportation of ions and nutrients, and cell-cell recognition (Ansari *et al*., 2021). It can be considered as a suitable target and excellent candidate for the RNAi-based control of aflatoxigenic fungi. Additionally, it may provide a ground for the biocontrol of the fungal population and reduced infection rate by intervention of new molecular methods. These can be envisaged as either via transgenesis of crops or through spraying over the formulations bearing RNAi within. Furthermore, RNAi can be used in fungal therapies within health-care units (Nölke *et al*., 2016).

## MATERIALS AND METHODS

### Fungal Strain and Spore Collection

*A. flavus* (DSMZ strain 818) was obtained from DSMZ (Braunschweig, Germany). It was cultured on potato dextrose agar (Merck, Darmstadt, Germany) medium at 37˚C in dark for 4-7 days. The spore suspension was prepared in 15 mL $dH_2O$ and filtered to separate mycelia over three layers of Miracloth™ (Merck, Darmstadt, Germany) after repeated washing by $dH_2O$. The spores were centrifuged at 5,000×g at 22˚C for 1 minute, and stored at 4˚C. The concentration of spore suspension was determined according to Caligiore-Gei and Valdez (2015) using a Neubauer chamber (Hagen, Germany).

### *AFLMP*1-Specific siRNA Synthesis

A machine-learning based algorithm developed by Ambion (https://www.thermofisher.com) was used to





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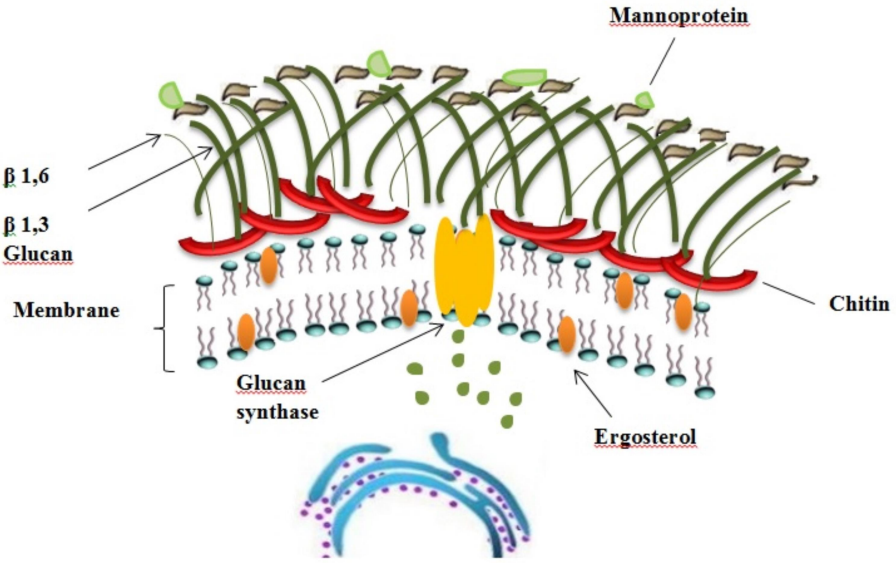


**Figure 1.** Schematic representation of fungal cell wall structure. Mannoprotein (Aflmp1p in this context) is located on hyphal surface, which contains a serine- and threonine- rich region for *O*-glycosylation.

develop a 27-mer *AFLMP*1-specific siRNA [Sense: 5′AGCAGGCTATCGATGACATTATCGCCA3′ and Antisense: 3′TGGCGATAATGTCATCGATAGCCTGCT5′]. The synthesized strands were HPLC purified via a desalting column (MP1) according to the manufacturer's instruction (https://www.thermofisher.com/de/en/home/life-science/rnai.html) and resuspended in sterile RNase-free dd$H_2O$ to have concentrations of 0.65, 1.25, 2.5 and 5 nM.

### Spore Germination and siRNA Transfer

The effect of synthetic siRNA was examined via direct uptake by *A. flavus* to analyze spore growth inhibition. Serial dilutions of siRNA (20 µL) were added to 80 µL of spore suspension (200 spores $mL^{-1}$ RPMI medium, Merck, Germany). The treatment of putative fungal spores with 0.65-5.0 nM AFLMP1 duplex siRNA in 2% (w/v) RSA pre-blocked black 96-well Greiner-µClear plate was performed under sterile condition. The mycelial growth of untreated controls [$H_2O$ and an unrelated Negative Control siRNA (NC-siRNA), RPMI, and 1×PBS (2.7 mM KCl, 137 mM NaCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$.)] and siRNA treated samples were measured after the incubation of the plates at 37°C in three different time points (12, 18 and 36 hours). The experiments were repeated thrice with three replicates in each experiment.

### Monitoring siRNA Effect on Fungal Growth

Calcofluor White (20 µL of 1:20 in $H_2O$; Sigma) was used for fungal cell wall staining. In each fungal spore containing well of microtiter plate, Calcofluor White was added and gently mixed for 10 minutes at 22°C. Spore cell walls were imaged by Opera® system (Emission 440 nm/Absorption 355 nm) and processed by ImageJ software (NIH, Bethesda, USA). The surface of each cavity (~ 90%) was evaluated by the Opera® system; the image data were converted into statistically





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relevant information (Figure 4) in order to support the effect to the siRNA inhibition. To avoid image artifices and to calculate the actual surface coverage of the generated fluorescence images, ImageJ was used. Based on the shape of the spores and the mycelium, the image artifacts of all generated images were excluded by applying an algorithm (Particle size: 20-∞; Roundness: 0.0-0.5 nm) from the calculation of the relevant surface cover based on the pixel ($PX^2$).

## RESULTS AND DISCUSSION

Spore germination and growth retardation was monitored by Opera® system for all concentrations for a period of 36 hour. The visual results recorded by Opera High Content Screening microscope confirmed the inhibition of *A. flavus* spore germination and mycelial elongation retardation after 12 and 18 hours of incubation with different concentrations (0.65, 1.25, 2.5, 5.0 nM) of synthetic short interfering RNA (siRNA). siRNA showed prolonged spore germination (> 90%), when the spores inspected at 12 hours post-treatment. At 36 hours siRNA treatment, spores germinated only at 0.65 nM and Calcofluor White staining showed branched hyphae (Figure 2). However, when ungerminated spores were treated with different concentrations (0.65, 1.25, 2.5, 5.0 nM) of 1×PBS, dd $H_2O$, RPMI and unrelated siRNA (NC) after 12, 18 and 36 hours, we could not see any inhibitory effect in spore germination and germ tube growth (Figure 3).

After observing the whole stained mycelia with calcofluor under confocal microscope, relative surface coverage (%) was calculated by Image J. The graphical records after 12 and 18 hours (Figure 4) postulated

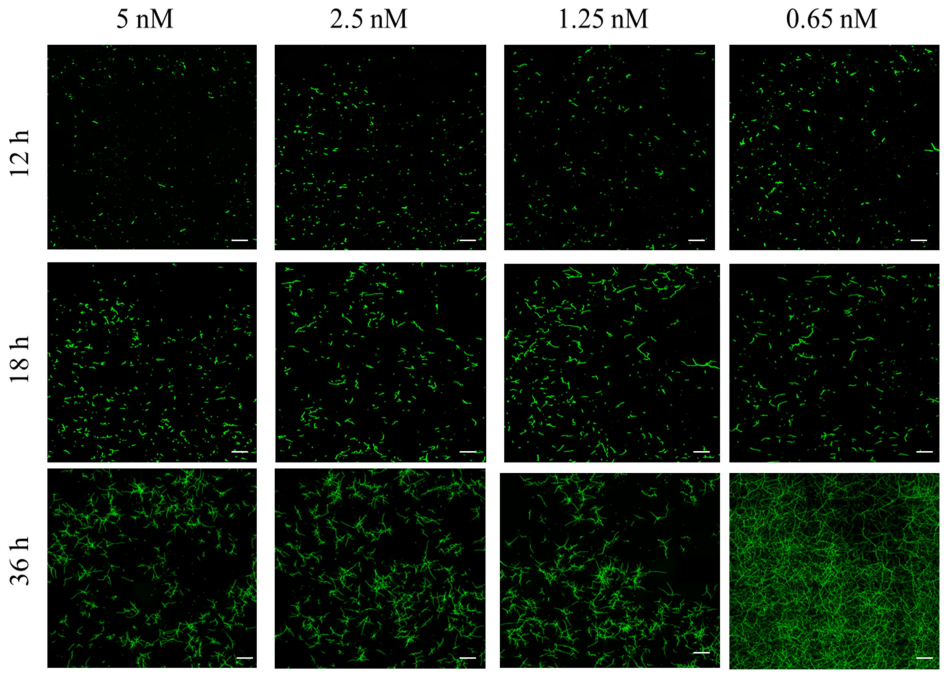


**Figure 2.** Effect of RNAi on the growth of *A. flavus*. Microtiter plates were inoculated with spores of *A. flavus* (200 spores/well) with RNAi (0.65 to 5.0 nM) for 12, 18 and 36 houra at 37°C incubated at dark. After adding Calcofluor White (1:20), stained hyphae were visualized by confocal microscope system in Opera® High Content Screening. Exemplified are fluorescence micrographs at a concentration of 0.65, 1.25, 2.5, and 5.0 nM RNAi *AFLMP*1. Scale= 100 microns.





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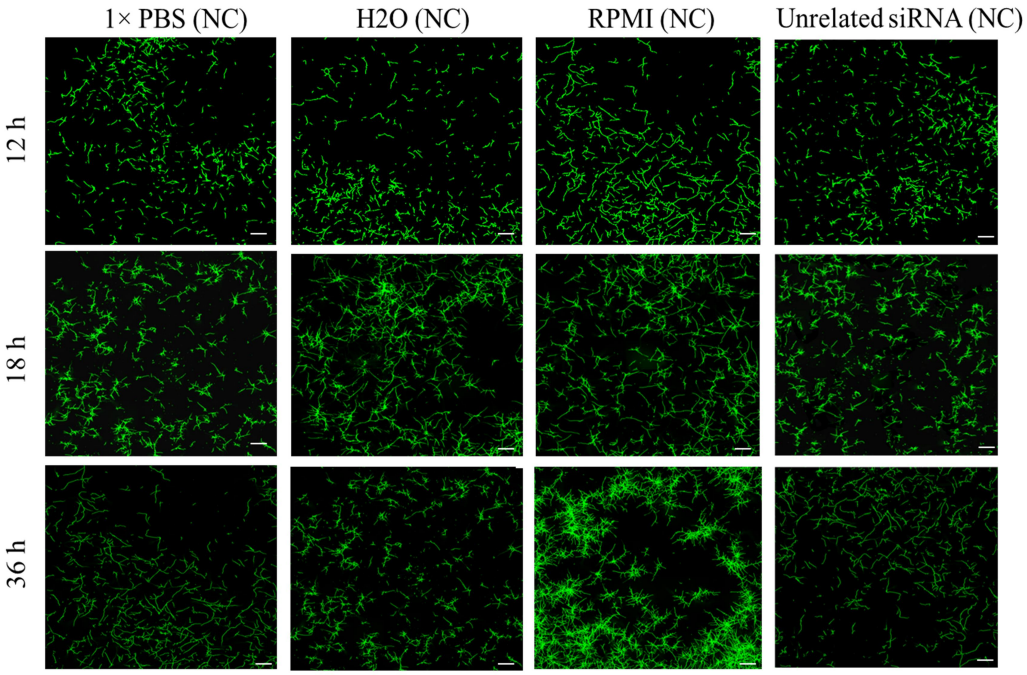


**Figure 3.** Effect of negatives control on the growth of *A. flavus*. Microtiter plates were inoculated with spores of *A. flavus* (200 spores/well) with unrelated siRNA as NC for 16 h at 37 °C incubation in dark. After adding Calcofluor White (1:20), stained hyphae were visualized by confocal microscope system in Opera® High Content Screening. Exemplified are fluorescence micrographs at concentrations of 0.65, 1.25, 2.5, and 5.0 nM RNAi *AFLMP*1. NC-siRNA RNAi; NC $H_2O$: Sterile water; 1×PBS and RPMI. Scale= 100 microns.

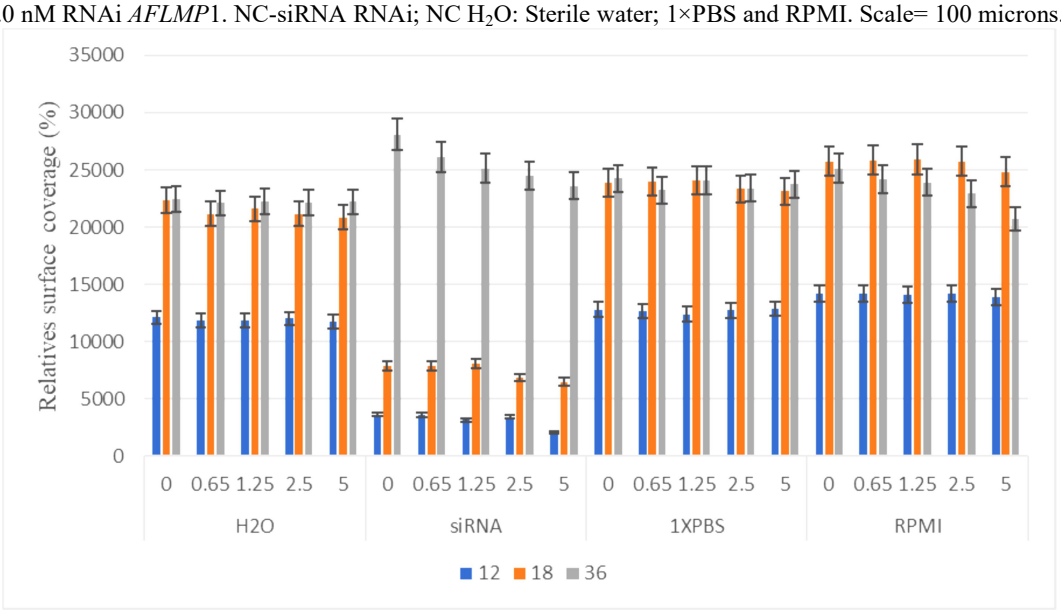


**Figure 4.** Quantitative representation of the inhibition of *A. flavus* by RNAi against *AFLMP*1. Based on the images recorded by the Opera® system, the evaluation of the surface coverage of the stained cell walls of *A. flavus* in various time points, including 12, 18 and 36 hours, was performed using ImageJ. The surface of the visualized hyphae was uniformly calculated according to the parameters particle size ($PX^2$) of 20-∞ and roundness of 0.0-0.5.





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suppression of fungal growth possible *via* silencing of AFLMP1 as compared to positive controls (1×PBS, dd $H_2O$ and RPMI) and a negatives control (unrelated siRNA). The images taken at 36 hours after the various concentrations of siRNA showed no inhibitory effect on the germination and elongation spores *of A. flavus* (Figure 4).

The results were indicative of germination inhibition and mycelial retardation as demonstrated by probable siRNA specificity and potency towards *AFLMP1*. Our data was verified when compared with NC-siRNA in different concentrations and buffers with no siRNA molecules. Negative controls did not show any inhibitory effect in all concentrations. The only comparable results to the negative controls were noted at 0.65 nM of siRNA. In addition to the careful design of siRNA (*i.e*., nucleotide content, sequence length and duplex thermodynamics) to alleviate off-targets, the mechanisms that facilitate RNA uptake and the accessibility of the target site on mRNA, the other consideration would be the impact of pest or pathogen RNAi machinery in the efficacy of siRNA technology (Hajeri and Singh, 2009; Gatta *et al*., 2018; Niu *et al*., 2021). As AFLMP1 previously demonstrated to be a good cell surface target to generate polyclonal (Woo *et al*., 2003) and monoclonal antibodies (Ansari *et al*., 2021), here, an alternative and attractive strategy based on a synthesized siRNA was put forward to target AFLMP1-encoding transcript. Upon successful interaction of the siRNA with AFLMP1 and biocontrol of spore germination and hyphal growth, many applications including RNA-based vaccine production and development of resistant transgenic plants with capability of suppressing fungal infiltration can be envisaged.

Our results were well in agreement with earlier studies (Nami *et al*. 2017; Schubert *et al*., 2018). Spore germination and growth retardation was monitored by Opera® system for all concentrations for a period of 36 hours. The results were indicative of germination inhibition and mycelial retardation, demonstrating siRNA specificity and potency towards *AFLMP1* as confirmed when compared with NC-siRNA in different concentrations and buffers with no siRNA molecules. The only comparable results to negative control were noted at 0.65 nM of siRNA.

## CONCLUSIONS

Our results confirmed AFLMP1-targeting siRNA has the potential of reducing growth of Aspergillus, and AFLMP1 has an essential role in life cycle of *A. flavus*; therefore, it is considered as a candidate strategy for generation of aflatoxin-free crops. Further studies and more detailed characterization are necessary to silence genes of interest in *A. flavus.*

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## خاموش سازی RNA به منظور کاهش بیان پروتئین دیواره سلولی (AFLMP1) در آسپرژیلوس فلاووس

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## چکیده

آسپرژیلوس فلاووس یک فیتوپاتوژن قارچی و فرصت طلب برای انسان و دام است. این قارچ تولید کننده آفلاتوکسین است که سرکوب کننده سیستم ایمنی است و سرطان زا می‌باشد که تولید سالم در صنایع غذایی و فرآوری خوراک دام را به خطر می‌اندازد. یک بررسی فیلوژنتیک پیشین روشن ساخت که پروتئین دیواره سلولی (مانوپروتئین های شماره یک) صرفا در آسپرجیلوس‌ها به‌خصوص در آسپرجیلوس فلاووس و پارازیتیکوس مشاهده می شود. بدین ترتیب، ار ان ای کد کننده مانوپروتئین شماره یک، می‌تواند هدف مناسبی برای خاموشی ژن و کنترل قارچ های آفلاتوکسیژنیک باشد تا بتوان این قارچ را در مزارع و واحدهای فرآوری تحت کنترل در آورد و همینطور در درمان بیماری¬های قارچی در بیمارستان‌ها مورد استفاده قرار داد. در اینجا و برای اولین مرتبه، نحوه عمل تداخل اس ای آر ان ای سنتز شده شیمیایی بر روی مانوپروتئین شماره یک مورد بررسی قرار گرفت. اثر جذب مستقیم غلظت‌های مختلف اس آی آ. ا. ای در جوانه‌زنی اسپورها از طریق میکروسکوپ کانفوکال غربالگری اپرا با دقت بسیار بالا و در شرایط کاملا استریل بررسی شد. اس آی آر ان ای باعث مهار رشد در غلظت‌های پایین‌تر (٦٥/. نانومولار) و جلوگیری از جوانه زنی (بیش از ٩٠%)، به احتمال زیاد با دخالت در بیوسنتز مانوپروتئین می شود. بدین ترتیب، فناوری خاموشی اس آی آر ان ای را می توان به عنوان عامل مهار کننده امید بخش در غیرفعال سازی ژن های هدف به کاربرد و همچنین آم را به عنوان نوآوری در کنترل رشد و تکثیر قارچ‌ها در صنایع غذایی/خوراکی در نظر گرفت تا جمعیت قارچ‌ها را در زیر نقطه بحرانی خطر نگاه داشت.





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