Control of Aflatoxin Production of *Aspergillus flavus* and *Aspergillus parasiticus* Using RNA Silencing Technology by Targeting *aflD* (nor-1) Gene

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Received: 18 April 2011; in revised form: 8 June 2011 / Accepted: 15 June 2011 / Published: 17 June 2011

**Abstract:** *Aspergillus flavus* and *Aspergillus parasiticus* are important pathogens of cotton, corn, peanuts and other oil-seed crops, producing toxins both in the field and during storage. We have designed three siRNA sequences (Nor-1a, Nor-1b, Nor-1c) to target the mRNA sequence of the *aflD* gene to examine the potential for using RNA silencing technology to control aflatoxin production. Thus, the effect of siRNAs targeting of two key genes in the aflatoxin biosynthetic pathway, *aflD* (structural) and *aflR* (regulatory gene) and on aflatoxin B1 (AFB1), and aflatoxin G1 (AFG1) production was examined. The study showed that Nor-1b gave a significant decrease in *aflD* mRNA, *aflR* mRNA abundance, and AFB1 production (98, 97 and 97% when compared to the controls) in *A. flavus* NRRL3357, respectively. Reduction in *aflD* and *aflR* mRNA abundance and AFB1 production increased with concentration of siRNA tested. There was a significant inhibition in *aflD* and AFB1 production by *A. flavus* EGP9 and AFG1 production by *A. parasiticus* NRRL 13005. However, there was no significant decrease in AFG1 production by *A. parasiticus* SSWT 2999. Changes in AFB1 production in relation to mRNA levels of *aflD* showed a good correlation (*R* = 0.88; *P* = 0.00001); changes in *aflR* mRNA level in relation to mRNA level of *aflD* also showed good correlation (*R* = 0.82; *P* = 0.0001). The correlations between changes in *aflR* and *aflD* gene expression suggests a strong relationship between these
structural and regulatory genes, and that aflD could be used as a target gene to develop efficient means for aflatoxin control using RNA silencing technology.

**Keywords:** siRNA; aflD (nor-1) gene; aflR gene; aflatoxin; real-time PCR

1. Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* that occur in nuts and cereal crops. These compounds have a high acute toxicity, as well as immunosuppressive, mutagenic, teratogenic, and carcinogenic activities and are classified as group 1 carcinogens by the International Agency for Research on Cancer [1].

Controlling aflatoxin production is of critical importance. Mainly traditional control methods including cultural practices such as pesticides and the development of resistant cultivars, pest resistance have been used. However, these have not always been successful in maize and in groundnuts. There is thus interest in exploring alternative means to control or reduce aflatoxin production.

RNA interference technology (RNAi) has received much attention in biology. The reason for this enthusiasm is that RNAi can rapidly ablate specific messenger RNA (mRNA) species by inducing their degradation via cellular protein machinery collectively named the RNA-induced silencing complex [2]. Short-interfering double-stranded RNA (siRNA) is synthesized and introduced through common transfection methods into cells, where they serve to guide the RNA degradation machinery to the select target gene. RNAi is an effective tool to investigate gene function, and may also be a useful tool to quench the expression of undesirable gene products.

RNA silencing in filamentous fungi has been carried out using plasmid constructs expressing a hairpin dsRNA structure controlled by an inducible or constitutive promoter [3–6]. Liu et al. [7] demonstrated silencing of the cryptococcal CAP59 and ADE2 genes by double-stranded RNA homologous to these genes in the basidiomycetous yeast *Cryptococcus neoformans*.

Application of siRNA-mediated RNAi has also been reported in cultured cells from fungi. Katri and Rajam [8] reported that ornithine decarboxylase (ODC) was specifically silenced by treating germinating spores with synthetic 23 nt siRNA in *Aspergillus nidulans*. Doubled-strand of RNA (dsRNA) was also delivered directly into protoplasts of *Phytophthora infestans*, which belongs to the fungus-like Oomycetes [9].

In *A. flavus* and *A. parasiticus* the expression of the aflD (nor-1), a gene encoding an enzyme that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin [10,11] is a key structural gene in the biosynthetic pathway. Furthermore, aflR is a pathway regulatory gene coding for proteins shown to be involved in transcriptional activation of most of the structural genes [12]. Recent studies have shown that there may be a relationship between the ratio of aflR and aflS (the associated regulatory gene) genes which is influenced by environmental factors [13]. Recently, studies by Abdel-Hadi *et al.* [14,15] showed the potential use of aflD transcription as a good marker to discriminate between aflatoxigenic and non-aflatoxigenic strains contaminating peanuts while aflR failed to differentiate between these strains. They showed that the expression patterns of aflD were related to changing water activity in stored peanuts. In peanuts, aflR was found not to
change in the same consistent way with water availability in peanuts. Thus, the expression pattern of this structural gene was selected as a target gene for silencing.

The objective of this study was to determine the potential of siRNA for silencing the target gene (aflID) and phenotypic aflatoxin control in strains of A. flavus and A. parasiticus.

2. Materials and Methods

2.1. Fungal Strain and Growth Conditions

In this study, four aflatoxigenic strains (Aspergillus flavus NRRL3357, Aspergillus flavus EGP9, Aspergillus parasiticus NRRL 13005 and Aspergillus parasiticus SSWT 2999) have been used. The strains were sub-cultured on Malt Extract Agar (20 g malt extract, 2 g peptone, 15 g agar per liter) for 7 days at 25 °C in the dark.

2.2. Preparation of Protoplast

Protoplasts were prepared from actively growing mycelium; a spore suspension of the strains sub-cultured in 200 mL of Yeast Extract Sucrose (YES) broth (20 g yeast extract, 150 g sucrose per liter), then incubated for 24 h on a shaker at 200 rpm in the dark at 25 °C. The mycelium was harvested by filtration through Miracloth. One gram of mycelia was transferred into 20 mL of filter sterilized enzyme solution (per 20 mL: 17 mL of H2O, 2 mL of 0.2 M NaPO4 (pH 5.8), 0.4 mL of 1.0 M CaCl2, 1.4 g of NaCl, 0.2 mL of β-glucuronidase (105 U/mL; Sigma), 200 mg of lysing enzyme (Sigma), and 50 mg of driselase (Sigma). Mycelia were incubated at 30 °C with shaking (80 rpm) for 3 h. Protoplasts were separated from intact mycelia by passage through Miracloth into a sterile 50 mL tube, and 20 mL of sterile STC buffer (1.2 M sorbitol, 10 mM CaCl2, 10 mM Tris-HCl (pH 7.5)) was added. Protoplasts were pelleted by low-speed centrifugation (1000 rpm) at room temperature for 5 min. The supernatant was carefully removed, and the protoplasts were washed once more in 20 mL of STC and pelleted by centrifugation as described previously. The protoplast pellet was resuspended in 1.0 mL of STC buffer, and the protoplasts were counted on a haemocytometer and diluted to 1 × 10^5/mL [16].

siRNA design: Three siRNA sequences were designed by Ambion (Applied Biosystem) to target the mRNA sequence of the aflID gene of A. flavus (accession number EF565463) and purchased from the same company. These siRNA were named as Nor-Ia, Nor-Ib and Nor-Ic (Table 1). Annealing of RNA oligonucleotides and purification by HPLC were performed by the company. An siRNA (control-siRNA) with no sequence homology to any A flavus genome sequence database was also purchased from Ambion.

Table 1. Details of siRNA sequences used in this study.

| siRNA Name | siRNA Sequence |
|------------|----------------|
| Nor-Ia     | Sense strand: CAUGUAUGCUCGCCGUCCUAUU  
             Antisense strand: UAGGACGGGAGCAUAACAUGUU |
| Nor-Ib     | Sense strand: GCAACAGGCCAAGUUUGCUUU  
             Antisense strand: GACAAACUUGGCCUGUUGCUU |
| Nor-Ic     | Sense strand: CAGGCCAAGUUUGCUUGAUU  
             Antisense strand: UCAAGACAAACUUGGCCUGUU |
2.3. Delivery of siRNA to Protoplasts

All siRNAs were resuspended in water free of RNases at a final concentration of 25 nM and tested on A. flavus NRRL3357. In a sterile 1.5 mL micro centrifuge tubes, 10 μL of each siRNA was mixed with an equal volume of Lipofectin reagent (Invitrogen Life Technologies, UK) and allowed to stand for 15 min at 20 °C. 20 μL of protoplasts (1 × 10^5) were added and mixed gently. The tubes were incubated at 20 °C for 24 h to allow transfection to proceed [9]. Then the mixture was inoculated in 10 mL of YES medium with 1.2 M of sorbitol for 5 days at 25 °C in the dark. Different dilutions of Nor-Ib (5, 10, 15, 20, 25 nM) were tested on A. flavus NRRL3357. Twenty five nM of Nor-Ib was tested on Aspergillus flavus EGP9, Aspergillus parasiticus NRRL 13005 and Aspergillus parasiticus SSWT 2999. All experiments were carried out using three biological replicates.

2.4. Aflatoxin Extraction and HPLC Analysis

Five mL of filtrate was extracted with chloroform, and then the extract was evaporated. The residue was derivatized using TFA (Trifluoroacetic acid) as described by the AOAC [17]. Sample extracts were analyzed using an Agilent 1200 series HPLC (Agilent, Berkshire, UK) using a 470 fluorescence detector (FLD, G1321A, Agilent) (λexc 360 nm; λem 440 nm) and a C18 column (Phenomenex Luna ODS2 150 × 4.6 mm, 5 μm; Macclesfield, UK). The analysis was performed using a mobile phase of methanol: water: acetonitrile (30:60:10) at a flow rate of 1 mL/min and a run time of 25 min.

2.5. Isolation of RNA from the Samples and RT-PCR

Total RNA was extracted from mycelium using the RNeasy and Plant Mini Kit (Qiagen GmbH, Hilden, Germany). A 0.5–1.0 g sub-sample of the mycelia was ground in a mortar with a pestle in liquid nitrogen. Approximately 250 mg of the mycelial powder was then used for isolation of total RNA. RNA extraction from the ground mycelia was accomplished with the RNeasy and Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. Then RNA was treated with DNase I (RNase free DNase I, Amplification Grade, Sigma) to digest residual DNA in the samples.

TaqMan probes and primer design: Real Time RT-PCR was used to amplify the aflD gene (target gene) and aflR gene (regulatory gene). The two primers and an internal fluorescence labelled probe used in the reaction were nor-taq-1 5′-GTCCAAGCAACAGGCCAAGT-3′; nor-taq-2 5′-TCG TGCATGTTGTTGATGTTG-3′; nor-probe 6FAM TGTCTTGTGCTCAGGCCGCG- BHQ2 [18]; AflR-taq-1 5′-TCTCGTCTTATCGTTCTCAAGG-3′; AflR-taq-2 5′-ACTGTTTACAGCTGCTGCACT-3′; AflR-probe 6FAM AGCAGGCACCCAGTGCTACCTCAAC-BHQ2. To create a standard curve, a larger PCR fragment of the aflD (nor-1) gene was generated with the primer nor1 and nor2 [19] (Figure 1a). Different dilutions were prepared from a stock solution by a factor of 10 and the aliquots of the dilutions were used in standard reactions during each setup of the real-time PCR reaction. The concentration of this standard PCR product was determined in a spectrophotometer (WPA light wave Cambridge, UK) and the number of copies was calculated. The concentration of unknown samples was calculated by the CFX96 system (Bio Rad, Hercules, CA) according to the generated standard curve. To create a standard curve for aflR, a larger PCR fragment of the aflR gene was used. To create the
standard curve, a larger PCR fragment of the aflR with the following primers AflR1, 5'-CGAGTTGTGCCAGTTCAAAA-3'; AflR2, 5'-AATCCTCGCCCACCATACTA-3' was used (Figure 1b).

**Real-time PCR conditions:** Amplification was performed using a total reaction volume of 25 µL in a MicroAmp optical 96-well reaction plate (Applied Biosystems). For each reaction 12.5 µL of TaqMan Universal Master Mix (Applied Biosystems), 2.5 µL cDNA, 3 µL of primer and probe mix (0.5 nM primer and 0.2 nM probe), and 7 µL of free RNases water. Real Time reactions were performed using the Bio Rad CFX96 platform (Bio Rad, Hercules, CA) with the following conditions: an initial step at 95 °C for 10 min, and all 40 cycles at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 30 s.

**Statistical analysis:** All experiments were carried out with 3–4 replicates and repeated twice with similar results. Statistical tests were performed using Statistica version 8 (StatSoft, Inc, 1984–2007) for one-way ANOVA and LSD Fisher was determined at the 95% confidence limits.

**Figure 1.** Standard curves from real-time PCR by plotting the threshold cycle (Ct) vs. log\(_{10}\) initial copy numbers of aflD gene (a) and aflR gene (b) amplified with the primer of labeled with FAM. Where E: The efficiency of PCR, R\(^2\) value: correlation coefficient.
3. Results

3.1. Treatment of Aspergillus flavus NRRL with siRNA

Quantification of the *aflD* based on the absolute quantification of copy number by using the calibration curve provided accurate, sensitive and highly reproducible data [20]. Figure 2 shows the changes in *aflD* and *aflR* mRNA expression and AFB$_1$ production by *A. flavus* NRRL3357 after treatment with control-siRNA or three siRNAs (Nor-Ia, Nor-Ib, Nor-Ic) specific to the *aflD* target gene. Treatment with the control-siRNA had no significant effect on AFB$_1$ production or *aflD/aflR* mRNA copy numbers. There was a significant decrease (95, 98, and 91% of the control level) in *aflD* mRNA abundance after treating with Nor-Ia, Nor-Ib and Nor-Ic siRNAs respectively, as assessed by real-time PCR. The lack of any effect using the control-siRNA and the knockdown seen with all three *aflD* siRNAs suggested that the results were not caused by transfection conditions or due to off-target effects.

**Figure 2.** Effect of siRNA for silencing *aflD* target gene on aflatoxin B$_1$ production, gene expression of *aflD* and *aflR* by using real-time PCR of *Aspergillus flavus* NRRL. Vertical bar indicates standard error, control (untreated with siRNA), and N. Control (treated with unrelated siRNA as a negative control).
Interestingly, a decrease (99% (Nor-la treatment), 97% (Nor-Ib treatment), and 72% (Nor-Ic treatment) of the control level in aflR mRNA abundance was also observed following knockdown of aflD. Subsequently, a decrease of AFL1 production as a result of a decrease of aflID and aflIR gene expression ((79% (Nor-la treated), 97% (Nor-Ib treated), and 76% (Nor-Ic treated)) of the control level was obtained. Statistical analysis of the effect of siRNA treatment on aflID gene expression, aflIR gene expression and AFL1 production were highly significant (Table 2a). There was a good correlation between siRNA effects on aflID and aflIR expression \((R = 0.82, P = 0.0001)\); aflID and AFL1 \((R = 0.88, P = 0.00001)\); and aflIR correlated significantly with AFL1 \((R = 0.66, P = 0.0074)\) (Table 3).

Table 2. (a) Analysis of Variance of the effect of siRNA silencing of the aflID target gene on AFL1 production, expression of aflID gene and aflIR gene, and (b) effect of different concentrations of siRNA (Nor-Ib) on log AFL1 production, log quantification of aflID gene and aflIR gene. Key: DF: Degrees of freedom, MS: mean square; P: Probability, F: F value.

|               | DF | MS       | F    | P      |
|---------------|----|----------|------|--------|
| (a) Factor    |    |          |      |        |
| aflID copy numbers | 4  | 8.58 x 10^8 | 42.47| 0.000003 |
| AFL1          | 4  | 1.24 x 10^8  | 18.74| 0.0001  |
| aflIR copy numbers | 4  | 8.16 x 10^9  | 8.63 | 0.0027  |
| (b) Factor    |    |          |      |        |
| log aflID     | 5  | 1.87     | 10.95| 0.0003  |
| log AFL1      | 5  | 0.41     | 199.13| 0.00000 |
| log aflIR     | 5  | 2.4659   | 6.05 | 0.005   |

Table 3. Statistical correlations between aflID gene, aflIR gene and AFL1 production of A. flavus NRRL3357 treated with siRNA (Nor-Ib). Key: R: correlation coefficient, P: Probability, F: F value.

| Correlations | R Value | F    | P     |
|--------------|---------|------|-------|
| aflID and aflIR | 0.82    | 28.41| 0.0001|
| aflID and AFL1 | 0.88    | 47.26| 0.00001|
| aflIR and AFL1 | 0.66    | 10.039| 0.0074|
| log aflID and siRNA conc. | 0.86 | 46.31| 0.00 |
| log AFL1 and siRNA conc. | 0.91 | 77.75| 0.00|
| log aflIR and siRNA conc. | 0.45 | 4.07 | 0.06|

3.2. Effect of siRNA Concentrations on A. flavus NRRL3357

Figure 3 compares the effect of different concentrations of siRNA (Nor-Ib) on quantification of aflID and aflIR genes, and AFL1 production by A. flavus NRRL3357. Overall, the best reduction in aflID and aflIR mRNA abundance and AFL1 production was at 25 nM siRNA of the concentrations tested. Statistical analysis of the effect of different concentrations of siRNA treatment on aflID, aflIR gene expressions and AFL1 production was statistically significant (Table 2b). There was a good correlation in reduction as a result of siRNA treatment between log aflID and siRNA concentration \((R = 0.86,\)
Tables 4, 5 show the effect of treating three aflatoxigenic strains with the chosen concentration of the siRNA (25 nM, Nor-Ib). There was a significant effect on aflD (target gene), and a concomitant decrease in aflR mRNA abundance and AFB₁ production by A. flavus EGP9 treated with siRNA when compared to the control (99.7%, 83.4%, and 89%, respectively). Treating Aspergillus parasiticus NRRL 13005 with siRNA revealed a reduction in aflD (target gene) mRNA abundance and AFG₁ production which was statistically significant (89.4% and 77.2%, respectively). The data obtained with Aspergillus parasiticus SSWT 2999 after treatment with siRNA showed that there was only a significant effect in aflD mRNA abundance (92.3%).
Table 5. (a) Analysis of Variance of the effect of siRNA (Nor-Ib) for silencing aflD target gene on aflatoxin B1, aflatoxin G1 expression of aflD and aflR genes of three aflatoxigenic strains (a) A. flavus EPG9; (b) A. parasiticus NRRL13005; and (c) A. parasiticus SSWT2999. Key: DF: Degrees of freedom, MS: mean square, P: Probability, F: F value.

|                     | DF | MS     | F       | P     |
|---------------------|----|--------|---------|-------|
| (a) A. flavus EPG9 Factor |    |        |         |       |
| aflD copy numbers   | 1  | 5.7 x 10^10 | 11.71   | 0.026 * |
| AFB1                | 1  | 7.4 x 10^5  | 163.06  | 0.0002 * |
| (b) A. parasiticus NRRL13005 Factor |    |        |         |       |
| aflD copy numbers   | 1  | 6.3 x 10^5  | 17.07   | 0.01*  |
| AFG1                | 1  | 6.9 x 10^6  | 12.34   | 0.02*  |
| (c) A. parasiticus SSWT2999 Factor |    |        |         |       |
| aflD copy numbers   | 1  | 3.1 x 10^11 | 28.68   | 0.005* |
| AFG1                | 1  | 8.3 x 10^6  | 0.42    | 0.54   |
* Significant < 0.05 %.

4. Discussion

This is the first study to use RNA interference to silence one of the important structural genes in the aflatoxin biosynthesis pathway (aflD gene) in both A. flavus and A. parasiticus and to elucidate the function of this gene in aflatoxin production by direct delivery. Previously, it was reported that using direct delivery of dsRNAs or siRNAs could result in sequence specific suppression of this particular gene [21,22]. The application of direct delivery of synthetic siRNA, have been rarely attempted in fungi [23]. RNA interference was discovered after the injection of dsRNA into the nematode Caenorhabditis elegans lead to specific silencing of genes highly homologous in sequence to the delivered dsRNA [21]. Zamore et al. [22] reported that using the Drosophila in vitro system, dsRNA triggers the specific degradation of homologous RNAs only within the region of identity with dsRNA.

Our results showed that all three siRNAs designed to target aflD gene gave excellent levels of silencing. The transient gene silencing was observed at an early stage after 5 days of protoplast regeneration and hyphal growth, with no changes in fungal growth observed between siRNA treated and untreated samples. This suggests that protoplasts have the ability to take up siRNAs from the medium during growth. Recently, Khatri and Rajam [8] reported that germinated spores are capable of taking up siRNAs from the growth medium in the early stages of germ tube extension.

The decrease in mRNA expression of aflD level caused a subsequent decrease in AFB1 production. Changes in AFB1 production in relation to mRNA level of AflD showed a good correlation (r = 0.88, P = 0.00001). This strongly suggest that aflD is absolutely essential for AFB1 biosynthesis and silencing of aflD gene expression by siRNA may result in accumulation of intermediate compounds and lead to blocking of AFB1 biosynthesis. In general, the aflatoxin gene cluster in A. parasiticus and A. flavus consists of 25 genes spanning approximately 70 kb [24,25]. Aflatoxin production could be disrupted if any step in the aflatoxin biosynthetic pathway is completely blocked by a specific
inhibitor. Using siRNA to target aflD (nor-1) gene expression that represents the early enzymatic steps in the aflatoxin biosynthetic pathway could be an appropriate target for inhibiting aflatoxin biosynthesis. Disruption or deletion of the aflD (nor-1) gene leads to the accumulation of norsolorinic acid and blocks the synthesis of all aflatoxins and their intermediates beyond norsolorinic acid [26]. Previously, it was reported that transformation of A. flavus and A. parasiticus with inverted repeat transgenes (IRT) containing sequence of aflatoxin-specific regulatory gene aflR suppressed aflatoxin production in both pathogenic fungi [27]. Also an aflR-specific IRT was successfully used to suppress the sterigmatocystin (ST) pathway in A. nidulans [28].

It was interesting to note that a decrease in aflR expression was observed following knockdown of aflD and changing in AflR mRNA levels in relation to mRNA level of aflD showed a good correlation ($R = 0.82$, $P = 0.0001$). One explanation for this reduction in aflR expression could be that there is a similarity in siRNA and the sequence of any of the global secondary metabolite regulatory machinery genes that regulate aflR like LaeA. Another explanation could be that accumulation of intermediate compounds resulting from aflD knockdown may have an indirect effect in suppression of aflR expression or any of the global secondary metabolite regulatory machinery genes. Butchko et al. [29] described a screen for detecting mutants defective in the sterigmatocystin (ST) gene cluster activity of A. nidulans by use of a genetic block early in the ST biosynthetic pathway that results in the accumulation of the first stable intermediate, norsolorinic acid. They found that three of the mutants were unable to express aflR, which encodes an ST zinc cluster (Zn(II)$_2$ Cys$_6$) transcription factor regulating ST biosynthetic gene expression. The biosynthetic and regulatory genes required for ST production in A. nidulans are homologous to those required for aflatoxin production in A. flavus and A. parasiticus [30,31].

The control siRNA did not lead to knockdown of aflD or aflR, suggesting that the results observed with aflD-specific siRNAs are not the result of a transfection artifact or an off-target effect. Our results support those obtained by Khatri and Ranjam [8]. They suggested that siRNA can cause specific silencing effects, in the polyamine biosynthetic pathway without any off-target effects. However, Jackson et al. [32] demonstrated that siRNAs may cross-react with targets of limited sequence similarity.

To confirm the effect of siRNA silencing, we treated three aflatoxigenic strains with siRNA. There was a significant decrease in aflD (targeting gene) of all three strains and an inhibition of AFB$_1$ production by A. flavus EGP9 and AFG$_1$ production by A. parasiticus NRRL 13005. However, there was no significant decrease in AFG$_1$ by A. parasiticus SSWT 2999. This suggests that perhaps uptake of siRNA by A. parasiticus SSWT 2999 protoplasts is not as efficient as in A. flavus and the other strain of A. parasiticus. Thus source of the strain may influence the effectiveness of the siRNA and the threshold concentrations required may vary. Another explanation may be that the biosynthesis of aflatoxin is slightly different in A. flavus and A. parasiticus. Wilkinson et al. [33] reported that the regulatory mechanism or mechanisms that control aflatoxin production in A. flavus and A. parasiticus are different in response to tryptophan (Trp), where, in the presence of Trp, three aflatoxin biosynthetic pathway genes (aflE (norA), aflD (nor-1), and aflO (omtB)) showed a decrease in expression and AFB$_1$ and AFB$_2$ production for A. flavus while, for A. parasiticus, an increase in expression profile and AFB$_1$ and AFG$_1$ production were observed.
5. Conclusions

The present study suggests that the aflD gene has a role in monitoring the biosynthetic direction of aflatoxin biosynthesis in A. flavus and A. parasiticus. This could thus be a good a target gene for inactivation, to develop efficient means of aflatoxin control by using RNA silencing technology. This can be applied, for example, by using mycoviruses as a candidate to mediate and propagate inactivation of the aflD gene.

Acknowledgments

Ahmed Abdel-Hadi is very grateful to Egyptian Higher Education Ministry and Al-Azhar University, Assuit branch, for financial support. We are very grateful to D. Bhatnagar and P. Cotty for the supply of strains.

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