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Quelling of Ochratoxin A Production by RNA Interference

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Abstract: Black aspergilli are important ochratoxigenic moulds known for its ubiquitous contamination of several pre and post harvest commodities including the ready to eat foods. On the consequent of this, we designed three siRNA sequences (pks_Ia, pks_Ib and pks_Ic) to target the mRNA sequence of the pks gene in order to determine the role of RNA interference in ameliorating ochratoxin A production. This study was carried out under varying degrees of concentration of siRNA while transfection was also done both in the presence and in the absence of lipofectamine. Results obtained portrayed significant disparity on the influence of varied concentrations of siRNA sequences on ochratoxin A inhibitions in *Aspergillus carbonarius* while no apparent statistical variation occurs in *Aspergillus niger* except for pks_Ia that had significant effectiveness at 25nM. The same cannot be said of transfection efficiency as concentration had no significant impact on it. Also, siRNA was significantly uptaken in the presence of lipofectamine than in its absence (t_{value} = 3.834, $p< 0.05$). On the inhibition of ochratoxin A, significant reductions were obtained with the three tested siRNA (80.9, 74.4 and 75.3% for pks_Ia, pks_Ib and pks_Ic respectively) while no apparent variation were found in the inhibitory activities of the three tested siRNA (Fvalue = 3.830, $p< 0.05$). The efficiency of the designed siRNA in inhibiting OTA production suggest that pks could be used as a target gene to develop means for ochratoxin A control using RNA silencing technology.

Key words: Quelling · Ochratoxin A · RNA Interference

INTRODUCTION

The production of ochratoxin A by certain species of *Aspergillus* and *Penicillium* [1-3] and its accumulated effect such as immunotoxicity, neurotoxicity, genotoxity and possibly carcinogenicity has been well documented [4]. However, studies suggest that such production is dependent on changes in water activity levels of food while the possibility of mycotoxigenic moulds thriving even in the presence of suboptimal conditions of antifungal compounds has been well reported [5]. It is thus imperative to consider approach which can potentially disrupt the biosynthetic pathway involved in ochratoxin A production in order to limit ochratoxin A contamination of feeds and foods.

Before now, RNA silencing in filamentous fungi has been carried out using plasmid constructs expressing a hairpin dsRNA structure controlled by an inducible or constitutive promoter [6-9]. But recently, the treatment of fungal protoplasts with synthetic siRNAs directed toward *Aspergillus flavus* and *Aspergillus parasiticus* aflD (Nor-1) has been shown to effectively silence gene expression [12]. In *Aspergillus nidulans*, siRNAs can also be up-taken during spore germination resulting in RNA silencing [11]. Although, such studies remain limited, their findings strongly suggest that the use of synthetic molecules would not only be an attractive means for studying RNA silencing in vivo but would also provide the platform for elucidating the possible role and targets of fungal small RNAs in growth, development and pathogenesis.

Not long ago, host induced gene silencing (HIGS) has been shown to be a promising strategy for controlling fungal diseases [13, 14]. In these studies, expression of dsRNA molecules in plants, specifically targeting fungal transcripts, resulted in RNA silencing of fungal targets and limited fungal infection. Tinoco *et al*. [15] also demonstrated how a plant expressing homologous dsRNA successfully down-regulate a *F. verticillioides* reporter gene during infection of the tobacco host. In another study, transgenic expression of dsRNAs in the

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host plant led to silencing of homologous genes in the invading and colonizing *Blumeria graminis* [13]. They further said that their results indicate that filamentous fungi may possess mechanisms for taking up dsRNAs or siRNAs from the environment and that expression of these siRNA molecules if producing the desired effect can be a mechanism used to reduce mycotoxin contamination. The sum process surrounding the delivery of siRNA into the fungal protoplast in order to ablate the specific messenger RNA (mRNA) species by inducing their degradation via cellular protein machinery collectively named the RNA-induced silencing complex (RISC; Ketting *et al*. [16]) is called RNA interference.

This whole process is initiated by both dsRNA or siRNA molecules, both are recognized by the dicer enzyme that will cleave dsRNA into siRNA molecules. Once the dsRNA has been cleaved into around 20 nucleotides siRNA molecules, these double stranded RNA molecules are unwound into single strand (ssRNA) with the RNA dependent RNA polymerase binding to the opposite end of the strand and this guide strand being incorporated into the RISC complex with an argonaute like protein [17]. The RISC complex endonuclease activity degrades the mRNA complementary to the guide ssRNA molecule. The RISC complex can also act at the genome level by heterochromatin formation and down regulate the gene pretranscriptionally [18].

In *A. niger* and *A. carbonarius,* the expression of an ochratoxin biosynthetic gene called pks gene is crucial and central to production of OTA. This study is therefore aimed at investigating the potential of siRNA for controlling phenotypic expression of ochratoxin A production in these organisms.

MATERIALS AND METHODS

Fungal Strains and Isolates Maintenance: The strains of *Aspergillus niger* and *Aspergillus carbonarius* used in this study were isolated and confirmed OTA producers in our previous study [19]. The cultures were maintained on Malt Extract Agar (MEA) (Biotech, United Kingdom).

Protoplast Generation: Protoplast was generated as described by Abdel-Hadi *et al*.[12] but with little modification. Briefly, spore suspension of *Aspergillus niger* and *Aspergillus carbonarius* were subcultured in 200 ml of YES broth in 500 ml conical flask. Cultures were incubated for 24 h in the dark at 25°C with shaking at 200 rpm. 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 H_2O, 2 ml of 0.2 M $NaPO_4$ [pH 5.8], 0.4 ml of 1.0 M $CaCl_2$, 1.4 g of NaCl, 200 mg of lysing enzyme [Sigma] and 50 mg of driselase [Sigma]. Mycelia were incubated at 30°C before shaking at (80 rpm) for 3 h. Protoplasts were separated from intact mycelia by passage through miracloth into sterile 50 ml tube and 20 ml of sterile STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl [pH7.5]) was added. Protoplasts were pelleted by low-speed centrifugation (1,000 rpm) for 5 mins. The supernatant was carefully removed and the protoplasts washed again in 20 ml of STC and pelleted by centrifugation as previously described. The protoplasts were then resuspended in 1.0ml of STC buffer and their concentration was adjusted using a spectrophotometer to 10^5 protoplasts ml^{-1}.

siRNA Design: Prior to siRNA design, the mRNA sequences of *A. niger* (Accession number XM_ 001394521.1) was obtained from NCBI database and used for designing the siRNA sequences. The designed sequences were subjected to i-score bioinformatics analysis [20]. This bioinformatics tool grade the sequences based on their biochemical attributes while the sequences with five higher scores were selected. To avoid off target effects, the selected siRNA sequences were challenged using the nucleotide alignment search tool (Blastn) against all reference sequences available and later against all filamentous fungi sequences [10]. Sequences with high homology to other genes were excluded. The top three siRNA sequences were then selected. These siRNA were named as pks_Ia, pks_Ib and pks_Ic designed to target *A.niger* (Accession number XM_ 001394521.1). The designed siRNA oligonucleotides were obtained from ambion custom silencer® select (Life technologies). Silencer® Negative Control (Life technologies) with no sequence homology and labelled with pks_I0 was used to assess transfection efficiency and influence on ochratoxin A production. All siRNAs were resuspended in RNase free water at a final concentration of 6.3, 12.5, 25 and 50nM. In a sterile 1.5 mL micro centrifuge tubes, 10 µL of each siRNA was mixed with 1 µL of Lipofectamine™ RNAi MAX (Invitrogen Life Technologies, UK) and allowed to stand for 15 min at 20 °C. 19 µL of protoplasts (1×10^3) were added and mixed gently. The tubes were incubated at 20°C for 24 h to allow transfection to proceed. The efficiency of the transfection protocol was evaluated mathematically relative to control using difference in their fluorescence levels. Fluorescence difference between 0nM siRNA control and the pks_I0 labelled negative

control with and without the presence of transfection reagent permitted the identification of protoplasts by their fluorescence levels. Protoplast regeneration was enhanced by adding 70 µL of YES broth with 1.2 M of sorbitol to the transfection mixture and incubation at 25°C for another 24 h of this suspension. The entire 100 µL of protoplast suspension were spread in wheat agar medium (wheat extract 1L, peptone 20g, yeast extract 10g, glycerine 10g, agar 20g, pH 6.8-7) and incubated at 25 °C in the dark. All experiments were carried out using three biological replicates.

siRNA Effect on Ochratoxin A Production: OTA was extracted by adding one gram of sample into a bottle containing 25ml of 50% methanol. The content was shaken vigorously for 3 minutes on the horizontal shaker. A 5ml aliquot of the resulting solution was filtered through whatman no 1 filter paper. In all cases, OTA was quantified from the extracts by competitive direct ELISA in a microwell format using Neogen Veratox kit (Sigma, USA). The operating conditions were as follows: 100µl each of the extracted sample and ochratoxin A control were mixed, followed by the addition of 100µl conjugate enzyme solution. The resulting 300µl solution was mixed thoroughly using a multi channel pipettor. A 100µl aliquot was then transferred to an anti ochratoxin antibody (1:5000 Rabbit IgG in 0.5M potassium phosphate buffer pH 7.0) (Sigma, USA) coated microwell and incubated at 25°C for 10minutes. The content of the microwell was washed off and rinsed 3 times with sterile distilled water. Substrate solution (100µl) was then added to the microwell followed by incubation at 25°C for 10 minutes. The reaction was stopped by the addition of the stop solution (Tetraoxosulphate (vi) oxide). The microwell was wiped with a towel and the optical density (OD) was read on a ELISA reader (Bioline Technologies, India) at 650nm.

RESULTS

Table 1 depicts the effect of different concentrations of siRNA on the transfection efficiency. As shown in the above table, the optimum concentration enhancing the best transfection experiment was found to be 25nM for all the tested siRNA except for $pks_{1\text{-}a}$ that had the best transfection efficiency at 50nm on *Aspergillus niger* protoplast. Statistical analysis using Analysis of Variance suggest no significant difference in the variation observed among the tested concentrations. However, further analysis by Duncan test revealed concentration of

Table 1: Details of siRNA sequences used in this study

siRNA name		siRNA sequence
pks_Ia	Sense strand	CCUCAUAAAACCAGGUUAA
	Antisense strand	UUAACCUGGUUUUAUGAGG
pks_Ib	Sense strand	UAUUUGAAGUCUCUGGGUA
	Antisense strand	UACCCAGAGACUUCAAAUA
pks_Ic	Sense strand	AUGAGAGACACCGGGUAUU
	Antisense strand	AAUACCCGGUGUCUCUCAU

Table 2: Effect of different concentration of siRNA on Transfection efficiency

	Transfection efficiency (Mean±SEM)%	
Concentrations	*Aspergillus niger*	*Aspergillus carbonarius*
pks_Ia		
6.3	56.5±3.50	54.5±4.50
12.5	71.5±2.50	69.5±6.50
25	760±7.00	76.5±4.50^{NS}
50	76.5±8.50^{NS}	6.7.5±2.50
pks_Ib		
6.3	58.5.5±6.36	52.5±2.50
12.5	74.5±5.5	64.5±4.50
25	78.0±8.00^{NS}	81.0±12.^{NS}
50	75±4.00	69.5±6.50
pks_Ic		
6.3	58.5±4.50	52.5±2.50
12.5	74.5±5.5	64.5±4.50
25	78.0±8.00^{NS}	81.0±12.00^{NS}
50	75.0±4.00	69.5±6.50

Tabe 3: Effect of transfection reagent on transfection efficiency

	Transfection efficiency (%)	
Lipofectamine	*n*	*(Mean±SEM)*
Presence	24	71.54±1.85
Absence	24	62.08±1.63

n=number of replicates, tvalue = 3.834, P value<0.05

Table 4: Statistical evaluation of the effect of different concentrations of siRNA on OTA inhibition

	Aspergillus carbonarius			*Aspergillus niger*		
siRNA (*nm*)	*(Mean±SEM)*	*Fvalue*	*Pvalue*	*(Mean±SEM)*	*Fvalue*	*Pvalue*
pks_Ia						
6.3	62.±2.5	51.6	0.001	68.5±1.5	50.7	0.001
12.5	94.1.±0.9	79.0.5±1.0				
25	90.5±1.5	88.0±1.0				
50	82.5±2.5	82.0±1.0				
pks_Ib						
6.3	62.±1.0	23.6	0.005	66.5±2.5	15.2	0.12
12.5	72.±1.0	80.5±1.5				
25	78.0.±2.0	85.0±2.0				
50	76.5±1.5	76.0±2.0				
pks_Ic						
6.3	75.±1.0	14.4	0.013	67.0±4.0	2.87	0.167
12.5	78.5±2.5	76.0±5.0				
25	84.5±1.5	81.0±2.0				
50	66.5±2.5	73.5±1.5				

Table 5: Relative measurement of the effect of siRNA On OTA Inhibition

SIRNA	% inhibition of siRNA on OTA	tvalue	pvalue
pks_Ia			
Aspergillus carbonarius	82.4±4.70	0.558	0.58
Aspergillus niger	79.4±2.71		
pks_Ib			
Aspergillus carbonarius	71.8±2.33	-1.432	0.174
Aspergillus niger		77.0±2.68	
pks_Ic			
Aspergillus carbonarius	76.13±2.57	0.506	0.62
Aspergillus niger		74.4±2.31	

Table 6: ANOVA for the Influence of siRNA on black Aspergilli

	% inhibition of siRNA on OTA		
SIRNA	*(Mean±SEM)*	*Fvalue*	*Pvalue*
pks_Ia	80.9±1.50	3.830	0.149
pks_Ib	74.4±2.60		
pks_Ic	75.3±0.86		

25nM as optimum for transfection. Lipofectamine effect on transfection efficiency was tested and evaluated statistically using student t- test (Table 2). This table disclosed that though, protoplast were easily transfected without lipofectamine but significant variation occurs between the protoplast transfected in the presence of lipofectamine and that done without lipofectamine (tvalue= 3.834 $p< 0.05$).

Table 4 disclosed that different concentrations of siRNA significantly influence inhibition rate of OTA in *Aspergillus carbonarius* while no statistical variation occurs on *Aspergillus niger* except for the activity of pks_Ia that was most effective at concentration of 25nm.

Table 5 and 6 reveals no significant difference in the sensitivity of the two aspergilli to the tested siRNA as shown by student t test and ANOVA respectively ($p > 0.05$).

DISCUSSION

The treatment of fungal protoplasts with synthetic siRNAs directed toward important gene has been shown to effectively silence gene expression [11, 12]. In this study, the siRNA designed to inhibit OTA production were successful while no significant variation was observed at concentration range of 6.3 to 50nM on the transfection efficiency. The opposite was the case with OTA inhibition as concentration significantly influence OTA inhibition in *Aspergillus carbonarius* while no apparent statistical variation occurs in *Aspergillus niger* except for pks_Ia that had significant effectiveness at 25nM. This observation corroborate that of Abdel-Hadi *et al*. [12] that reported 25nM concentration as the most effective for inhibiting biosynthetic gene AflD and the regulatory genes AflR/AflS in *Aspergillus flavus* and *Aspergillus parasiticus*. In another vein, Goncalo [5] reported concentration of 10nM as the optimum for silencing Otapks PV gene while 25nM was reported for Tri5. The disparity observed in the various study is an indication that optimum concentration for different siRNA varies according to the targeting gene. Our findings further assert that RNA interference could be the needed technique required to control ochratoxin A production as its effect was immediately noticed after just the fifth day of the experiment. This may not be unconnected to the fact that the fungal protoplast can maximally uptake siRNAs from the growth medium during growth [11]. The fact that efficient siRNA transfection was significantly higher in the presence of lipofectamine is not unexpected as this reagent has been reported to enhance uptake of siRNA in a medium [5, 12]. The lack of any effect on OTA production observed with the control (pks_I0) is an indication that inhibition observed with the designed siRNA in this study are not caused by transfection conditions and/or due to off target effects. Nevertheless, the inhibitory activities of the designed siRNAs on OTA were found to be relative with no significant statistical variation ($P> 0.05$). This finding suggest that such inhibitory activities may be due to sequence specific nature of our siRNA. Even though, this technique remain very promising, there is a need to further elucidate the exogenous expression of dsRNA by plant and the uptake of these molecules by their pathogens.

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