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# TRANSCRIPTIONAL FACTOR INFLUENCE ON OTA PRODUCTION AND THE QUELLING ATTRIBUTE OF SIRNA ON THE OTA PRODUCING STRAINS OF *ASPERGILLUS* SECTION *NIGRI*

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Abstract

This study determined the influence of some transcriptional factors on ochratoxin A production as well as investigates the quelling attributes of some designed siRNA on the OTA producing *Aspergillus* section *Nigri* using standard recommended techniques. Results obtained following comparison of the *pks* gene promoter sequences from 15 isolates depicts differences in length and homology with the *pks* gene ranging from 218bp in a strain of the *Aspergillus niger* to 700bp in *Aspergillus carbonarius*. The alignment of the *pks* gene promoter region revealed that six and two of the aligned genes have *Aba A* binding site corresponding to CATTCT and CATTCC respectively while *Brl A* binding site was absent in all the isolates. *Pac C* binding site corresponding to CCTGGC and GCCAAG was also found in two and three of the *pks* gene promoter region respectively. The three designed *siRNA* shows significant impact on OTA inhibitions with no significant statistical differences (80.9, 74.4 and 75.3% for *pks_Ia, pks_Ib* and *pks_Ic* respectively) (F= 3.830, p>0.05). It can be concluded that *Are A* and *Aba A* are potential enhancers for ochratoxin A biosynthesis and none of the investigated transcriptional factors is enough for the activation of ochratoxin A production. However, *pks* gene was seen as a good target gene for inactivation in order to develop efficient means for ochratoxin A control using RNA silencing technology.

Key words: Transcriptional factors, Ochratoxin A, siRNA, Quelling , *Aspergillus* section *Nigri*

# FACTEUR TRANSCRIPTIONNEL INFLUENCE SUR LA PRODUCTION D'OTA ET DE LA RÉPRESSION DE L'ATTRIBUT DE SIRNA SUR LES SOUCHES PRODUISANT DE L'OTA DE L'ASPERGILLUS LA SECTION NIGRI

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**Résumé**

**Cette étude visait à déterminer l'influence de certains facteurs de transcription sur l'ochratoxine A fait enquête sur la production ainsi que la répression de certains attributs conçu sur le siRNA Aspergillus la section Nigri produisant de l'OTA en utilisant des techniques recommandées. Résultats obtenus par la comparaison des séquences promotrices des gènes du pks à partir de 15 isolats illustre les différences dans la longueur et de l'homologie avec des gènes de pks allant de 218bp chez une souche d'Aspergillus niger de 700bp à Aspergillus carbonarius. L'alignement de la région promotrice du gène pks a révélé que six et deux de l'alignement des gènes ont un site de fixation de l'Aba à CATTCT CATTCC et correspondant respectivement alors que Brl un site de fixation était absent de tous les isolats. Cip C binding site CCTGGC GCCAAG correspondant à et a également trouvé dans deux et trois de la région du promoteur du gène pks respectivement. Les trois conçu siRNA montre un impact significatif sur les inhibitions OTA avec aucune différence statistique significative (80,9, 74,4 et 75,3 % pour la pks_Ia, Ib et pks pks__Ic respectivement) (F = 3,830, p >0,05). Il peut être conclu que sont un et de l'Aba A sont pour l'ochratoxine A enhancers potentiels biosynthèse et aucun des facteurs de transcription d'une enquête est suffisant pour l'activation de l'ochratoxine A la production. Cependant, le pks a été considérée comme une bonne cible pour l'inactivation de gènes afin de développer des moyens efficaces pour l'ochratoxine A contrôle en utilisant la technologie de neutralisation de l'ARN.**

**Mots clés ; facteurs transcriptionnels, ochratoxine A, siRNA, répression , Aspergillus la section Nigri**

## INTRODUCTION

Ochratoxins are a group of chemically related fungal contaminants produced by some strains of filamentous fungi. These toxins are classified into three major classes namely Ochratoxin A, B and C with Ochratoxin A being the most popular due to their recent classification as Group 2B human carcinogen following experiments on animals [1,2]. The presence of ochratoxin A in several foods and its accumulated effect such as immunotoxicity, neurotoxicity, genotoxity and possibly carcinogenicity has been well documented [3]. This toxin has also been suspected to be a risk factor for testicular cancer [4].

The organisms that produce ochratoxins are said to be ochratoxigenic and are said to be ubiquitous contaminants of pre and post harvest food commodities including the ready to eat foods [5,6]. The ability of this type of fungi to produce toxin is dependent on their strains, the composition of the food, the conditions of handling as well as storage conditions [7]. These organisms have also attracted attention through the damage it does to plants, animals and humans [1-2,8]. Their secondary metabolites (Ochratoxin A) is a developmental challenge to Africa and the world at large adversely affecting three major sectors namely; public health, trade and economy as well as food and nutrition security.

Some studies suggest that the mycotoxin biosynthesis genes are activated under different environmental conditions and so can easily be induced and not expressed constitutively [9,10]. Their induction can be determined some time before the detection of mycotoxin by analytical methods [11,12]. The signaling processes that switch on ochratoxin biosynthesis during ripening or in poorly stored crops are still not well understood. In order to state whether biosynthesis of OTA may be possible under certain transcriptional factor, *pks* promoter sequences of the isolated organisms were analyzed *in silico* and the effect of RNA interference on OTA silencing was investigated due to the recent evidences suggesting the possibility of some of these ochratoxigenic moulds growing in the presence of both suboptimal and even optimal concentration of both synthetic drugs and plant active ingredients.

## MATERIALS AND METHODS

**Sources of Processed *Manihot esculenta* Crantz (Cassava Flakes)**

Two hundred and fifty (250) samples each of processed *Manihot esculenta* Crantz were purchased between March 2013- December 2014 from the local markets in the four geopolitical zones of Ogun States, Nigeria viz; Yewa, Egba, Remo and Ijebu zones respectively during the dry and wet seasons to represent a total of one thousand samples according to a statistical sampling scheme recommended for microbiological testing of foods [13]. These samples were collected in pre sterilized aluminum pan. The samples in pre sterilized aluminum pan whose lids were opened before getting to the laboratory were rejected. Appearance of the garri samples, the sources of each collection site and the geopolitical zones were noted. autoclaved garri sample was used as control while the remaining one thousand (1000) processed *Manihot esculenta* samples were used as the test samples.

**Fungal Isolation and Identification**

**Microbiological identification**

One gram each of the processed *Manihot esculenta* Crantz sample was aseptically seeded in the middle of a sterile potato dextrose agar (PDA) plate in duplicate and incubated for one week at $25^0$C.After incubation, fungal isolates of public health significance were identified using rate of growth, colonial and microscopic morphology according to Larone [14].

**Molecular Identification of Fungi**

**DNA Isolation, Amplification and Sequencing**

Each specimen (fungal isolate) was stirred directly into 200 ml sterile saline and extracted using a QIAamp DNA mini kit (Qiagen) according to a protocol adapted for extraction of DNA from fungal cells, as described elsewhere [15]. In brief, each sample was pre-incubated at $99^0$C for 20 min and then processed as suggested by the manufacturer. After the addition of the cellular lysis buffer, the sample was incubated again at $99^0$C for 10 min. The extracted DNA was amplified by PCR using a pair of universal fungal primers (V9D: 59-TTAAGTCCCTGCCCTTTG TA- 39; LS266: 59-GCATTCCCAAACAACTCGACTC-39) encompassing highly conserved regions encoding fungal rRNA [16]. PCRs were performed in 0.2 ml reaction tubes in a final volume of 50 ml containing 2 to 10ng of DNA, 1.5 U Platinum Taq DNA polymerase (In vitrogen), 200 mM each of dATP, dGTP and dCTP, 400 mM dUTP (instead of dTTP), 20 mM Tris/HCl (pH 8.4), 50 mM $MgCl_2$, 0.4 mM each primer and 1 U uracil-N-glycosylase. The amplification reaction included a hold at $50^0$C for 5 min to allow uracil-N-glycosylase activity and an additional hold at $95^0$C for 5 min for Taq activation, followed by 35 cycles at $95^0$C for 30s, $62^0$C for 1 min and $72^0$C for 2 min, with a final extension step at $72^0$C for 5 min. The amplified product was visualized on agarose gels, purified and sequenced using a 310 auto

Genetic Analyzer (PerkinElmer, Applied Biosystems Div., Waltham, USA) with the same primers. For each sample, a pair of primers amplifying the human b-globin gene was included as an extraction/amplification internal control. DNA sequences were analysed using the BLAST database and assigned to the reference isolate sequences with the highest bit score

**Determination of level of ochratoxin A in processed *Manihot esculenta* Crantz (cassava flakes)**

Five grams each of processed *Manihot esculenta* was weighed into a weighing bottle. Then 25ml of 50% methanol was added. The content was shaken vigorously for 3 minutes on the horizontal shaker . A 5 ml aliquot of the resulting solution was filtered using the Neogen filter (45µm) syringe, into the Neogen collecting tube. The amounts of ochratoxin A (OTA) in the analyzed processed *Manihot esculenta* were determined using a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Screening method for the analysis was done using Neogen Veratox® testing kits with limits of detection of 1 µg/kg (ppb) for ochratoxin A. Free ochratoxin A in the samples and controls were allowed to compete with enzyme-labelled ochratoxin A (conjugates) for the antibody binding sites. After a wash step, substrate was added, which reacts with the bound conjugate to produce blue colour. More blue colour means less Ochratoxin A while more pink means more Ochratoxin A. The test is read in a microwell reader (Thermo lab system, Thermo, Finland) to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of ochratoxin A.

**Ochratoxigenic fungal isolates**

Twenty one of the forty isolated *Aspergillus* section *Nigri* are ochratoxin A producing isolates. The *Aspergillus* section *Nigri* used were *Aspergillus niger* and *Aspergillus carbonarius* . The PCR amplification of this isolates yielded a single fragment of an approximately between 600 and 700 bp. BLAST search using Genbank database showed that the isolate percentage similarity of sequence with GenBank range between 98-100% . All the isolates were found with differing levels of OTA

**Alignments and phylogenetic analyses**

DNA sequences were aligned using Clustal W in DNAMAN (Lynnon Biosoft, Vandreuil, Canada). Data sets included aligned DNA sequences from the *pks* region. Phylogenetic trees were obtained by parsimony analyses using heuristic search methods with stepwise sequence addition and the tree-bisection–reconnection (TBR) branch-swapping algorithm. Node support was assessed with 10000 bootstrap replicates. Gaps were completely deleted. The partition homogeneity test (PHT) in PAUP* was performed on parsimony informative sites only, with 10000 randomized data sets using heuristic search methods with stepwise sequence addition. A two-tailed Kishino–Hasegawa (KH) test using 10000 RELL bootstrap replicates in PAUP* was employed to further assess the likelihood of the different tree topologies.

**Protoplast generation from the ochratoxigenic Aspergillus section Nigri**

Protoplast was generated as described by Abdel-Hadi *et al.*[17] 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 [18]. This bioinformatic 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 [19]. 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 *pks* genes of *A.niger*

and *Aspergillus carbonarius*. 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 ($1 \times 10^3$) 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.

**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 |

**Effect of siRNA on ochratoxin A production**

OTA was extracted as described previously.

## RESULTS

Comparison of *pks* promoter region from 15 isolates of ochratoxin A producing *Aspergillus* section *Nigri* showed differences in length and homology. The *pks* gene ranged from 218bp in a strain of the *Aspergillus niger* to 700bp in *Aspergillus carbonarius*. The alignment of the *pks* region of fifteen isolates revealed the presence of putative binding sites for homolog's of some known fungal transcription factors namely *Are A*, *Aba A* and *Pac C* while all the studied isolates lack the binding site for *Brl A*. Consequently, eight of the fifteen studied isolates had at least one *HGATAR/YTATCD* site. Isolate 6 however contained two of these sites separated by 30bp (table 1). Sequences for *Aba A* binding site (*CATTCT*) were found ranging between 79-685bp while the *CATTCC* binding site was found in isolate 4 and 14. Isolates 14 had the CATTCC at 139bp. The other (isolate 4) however, harbored two of these sequence separated by 379bp. The sequence identical to binding site for the transcription factor involved in pH regulation (*Pac C*) was found in five of the isolates, three of the four isolates (isolate 1, 4 and 7) having Pac C binding site corresponding to GCCAAG with isolate 7 also having the binding site CCTGGC together with isolate 6 (table 2). 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* but not on *Aspergillus niger* except for *pks*_Ia that had significant effectiveness at 25nM. Nevertheless, the inhibitory activities of the designed siRNAs on OTA were found to be relative with no significant statistical variation (P>0.05) (Figure 1-3).

**TABLE: PRESENCE OF PUTATITIVE BINDING SITES FOR AREA IN THE PARTIALLY SEQUENCED *pks* GENE**

| Isolates | pks gene length | GATA1 | GATA2 | GATA3 | GATA4 | GATA5 |
|---|---|---|---|---|---|---|
| | | CTATCT | TTATCT | TTATCA | CTATCA | TTATCT |
| 1 | 700bp | - | - | - | - | 141 |
| 2 | 280bp | - | - | - | - | - |
| 3 | 647bp | 419 | - | - | - | - |
| 4 | 697bp | - | - | - | - | - |
| 5 | 683bp | 471 | - | - | - | - |
| 6 | 647bp | 455/425 | - | - | - | - |
| 7 | 649bp | - | - | - | 275 | - |
| 8 | 220bp | - | - | - | - | - |
| 9 | 622bp | - | - | - | - | - |
| 10 | 551bp | 433 | - | - | - | - |
| 11 | 650bp | 433 | - | - | - | - |
| 12 | 600bp | 433 | - | - | - | - |
| 13 | 625bp | 557 | - | - | - | - |
| 14 | 218bp | - | - | - | - | - |
| 15 | 526bp | - | - | - | - | - |

**TABLE 3: PUTATITIVE BINDING SITES FOR OTHER FUNGAL TRANSCRIPTIONAL FACTORS IN THE PARTIALLY SEQUENCED pks GENE**

| Isolates | *pks* gene length | Aba site A CATTCT | Aba site 2 CATTCC | Brl A site 1 AGAGGGG | Brl A site2 CAAGGGA | PacC site 1 CCTGGC | PacC site 2 GCCAAG |
|---|---|---|---|---|---|---|---|
| 1 | 700bp | - | - | - | - | - | 141 |
| 2 | 280bp | - | - | - | - | - | - |
| 3 | 647bp | - | - | - | - | - | - |
| 4 | 697bp | - | -306/685- | - | - | - | -17- |
| 5 | 683bp | -79- | - | - | - | - | - |
| 6 | 647bp | - | - | - | - | - | -594- |
| 7 | 649bp | -344- | - | - | -175- | - | -491- |
| 8 | 220bp | - | - | - | - | - | - |
| 9 | 622bp | 328 | - | - | - | - | - |
| 10 | 551bp | 109 | - | - | - | - | - |
| 11 | 650bp | 109 | - | - | - | - | - |
| 12 | 600bp | 109 | | - | - | | - |
| 13 | 625bp | - | - | - | - | - | - |
| 14 | 218bp | - | - | - | - | - | - |
| 15 | 526bp | - | - | - | - | - | - |

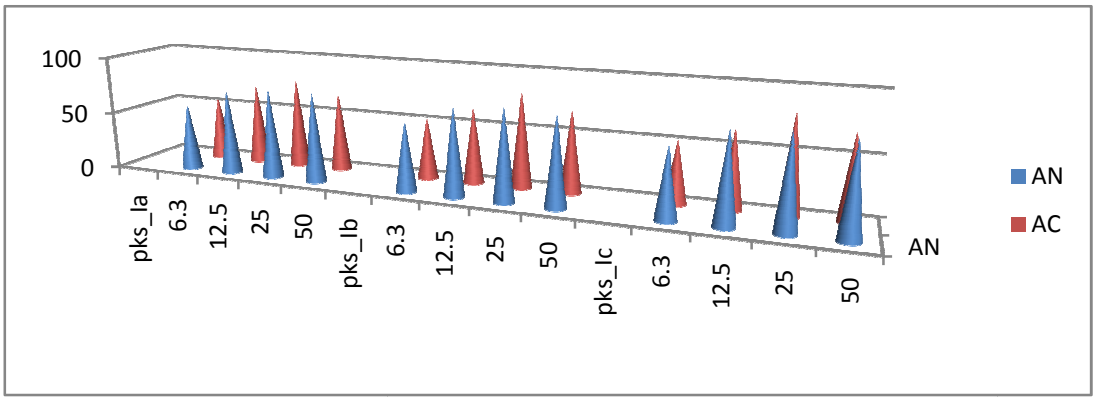


**FIGURE 1: INFLUENCE OF DIFFERENT CONCENTRATION OF siRNA ON TRANSFECTION EFFICIENCY**

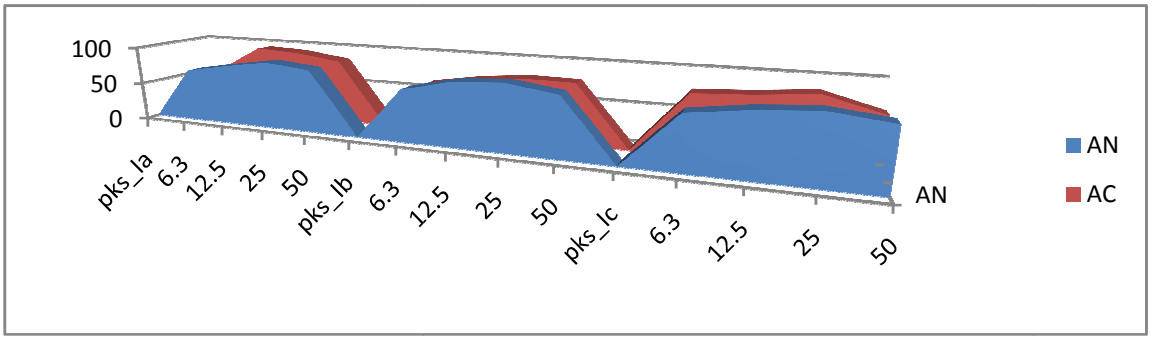


**FIGURE 2: INFLUENCE OF DIFFERENT CONCENTRATION OF siRNA ON OTA INHIBITION**

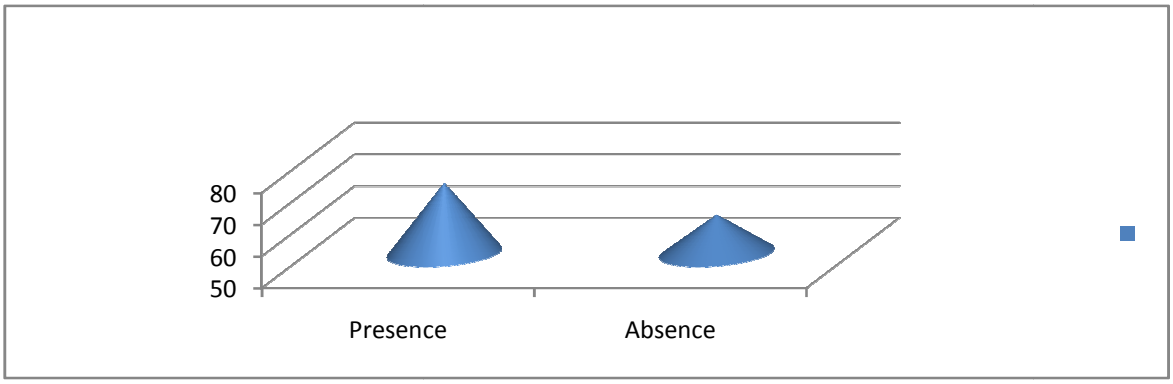


**FIGURE 3: INFLUENCE OF THE PRESENCE OF LIPOFECTAMINE ON TRANSFECTION EFFICIENCY**

## DISCUSSION AND CONCLUSION

The use of bioinformatic tools for sequence comparison in order to deduce important gene structure and functions have been well documented. In this study, *pks* gene promoter sequence as well as several fungal transcriptional factors compared revealed variation in homology and length. This observation is not unexpected as such observation has been reported for *Aspergillus* section *Flavi* [20](Ehrlich *et al.*,1999). However, our findings are a strong pointer that these genetic traits may not be necessary among the factors that triggers ochratoxin A production. Sequence differences in the *pks* region and promoter structure provide a basis for predicting the roles of environmental and developmental cues in

differential regulation of ochratoxin production among ochratoxin A producing fungi. The possibility of inhibition or stimulation of ochratoxin A production by different nitrogen sources was assessed with the presence or absence of *Are A*-binding sites of *pks* gene. Results obtained revealed a considerable 66.7% of the total isolates harboring these gene and associated high level of OTA. The variability of GATA sites in the *pks* region and differences among fungi in ochratoxin A production provides a way to test the role of nitrogen sources on transcriptional regulation of ochratoxin A biosynthesis genes. Transcription factors *Aba A* and *Brl A* may mediate expression of genes involved in development-specific processes in fungi [21], but have not yet been directly implicated in regulation of secondary metabolite biosynthesis. Putative *Brl A*-binding sites was absent in the *pks* region of all isolates. This observation may not be unconnected to the fact that this important gene is not involved in OTA biosynthesis. The presence or absence of *Pac C*-binding sites in the *pks* region could partly account for differential sensitivity of ochratoxin A production to pH in different species of *Aspergillus* [22]. Precedent for interference by *Pac C* in the expression of acid-expressed genes has been reported for the *gab A* gene in *A. nidulans* [23]. In this study, *Pac C* site was found in only five of the total of the fifteen isolates representing only 33.3%. This observation could be suggesting that pH may not be that important in the regulation of ochratoxin A gene. One of such method could be the use of RNA interference which is a post transcriptional silencing technique. In this study, the treatment of fungal protoplasts with synthetic siRNAs directed toward the *pks* gene has been shown to effectively silence ochratoxin A production [17,24]. 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* but not on *Aspergillus niger* except for *pks*_Ia that had significant effectiveness at 25nM . This observation corroborated that of Abdel-Hadi *et al.*[17] 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*. Moreover, Goncalo [25] 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 maximally uptake the designed siRNAs [17]. 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 [17,25]. 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 in future studies .

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