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Point Mutations in the 14α-Sterol Demethylase Cyp51A *or* Cyp51C could contribute to azole resistance in *Aspergillus flavus*

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INTRODUCTION

Aspergillus spp. are opportunistic fungi that cause both allergic and invasive syndromes. This genus has become increasingly important because immunosuppressed patients are very susceptible to develop infection by these fungi. Currently, *Aspergillus flavus* is the second most common *Aspergillus* spp. causing invasive infections in humans. The first-line antifungal treatment against aspergillosis is broad-spectrum azole drugs. Recent reports of *A. flavus* isolates with *in vitro* azole resistance have been described [1] and their azole resistance mechanisms are being analyzed. As *A. flavus* is also an important crop associated pathogen [2], the use of azoles in agriculture raises the concern about *A. flavus* azole resistance related to the use of this class of antifungals in the fields.
Triazole drugs target the 14-α sterol demethylase (Cyp51), an enzyme that plays a key role in the ergosterol biosynthesis pathway [3]. One particular characteristic of *A. flavus* is the existence of three *cyp*51-related genes (*cyp*51A, *cyp*51B and *cyp*51C) encoding 14-α sterol demethylase-like enzymes [4], the target of azole drugs. To date, only one point mutation (Y319H) in *A. flavus* Cyp51C seems to be related to azole resistance although it needs further confirmation [5].
In this study we describe the analysis, characterization and phylogeny of *A. flavus cyp*51 genes, the presence of potentially relevant mutations and their potential role in *A. flavus* azole resistance. Isolates that showed reduced *in vitro* susceptibility to clinical azoles exhibited a different susceptibility profile to demethylation inhibitors (DMIs). The possible existence of a link between a medical azole resistance development route and resistance derived from the use of DMIs in agriculture is discussed.

MATERIAL AND METHODS

Twenty *A. flavus* clinical isolates were tested for antifungal susceptibility following EUCAST standard methodology. Clinical antifungal tested were amphotericin B, terbinafine, and caspofungin (ranges from 0.03 to 16 mg/L); itraconazole, voriconazole, posaconazole and isavuconazole (0.015 to 8 mg/L); anidulafungin (0.007 to 4 mg/L) and micafungin (0.004 to 2 mg/L). Besides clinical antifungals, two imidazole drugs, prochloraz and imazalil, and five triazole drugs, metconazole, tebuconazole, epoxiconazole, difenoconazole, bromuconazole and myclobutanil, that belong to the 14-α sterol demethylation inhibitors (DMIs) frequently used to protect crops, were also tested (ranges from 0.06 to 32 mg/L).
Minimum Effective Concentration (MECs) echinocandins and Minimal Inhibitory Concentrations (MICs) for the rest of clinical antifungals and DMIs were read after 24 and 48 hours of incubation.
The *cyp*51A, *cyp*51B and *cyp*51C genes, including their promoter region, were amplified and sequenced. The deduce protein sequence of *A. flavus* Cyp51A, Cyp51B and Cyp51C were compared with paralogs from different filamentous fungi including other *Aspergillus* species.

RESULTS

Antifungal susceptibility testing.

Three strains (CM8087, CM9326, and CM7668) were considered resistant to azole drugs with three different susceptibility profiles. There were no major differences in susceptibility to amphotericin B (AmB) except for one strain (CM8087) that could be considered AMB resistant (**Table 1**).

Strains	MIC ranges (mg/L)				
	AmB	ITC	VCZ	POS	ISV
ATCC 2004304	1	0.5	2	0.125	1
CM7668	0.5	> 8	0.25	1-2	0.5
CM8087	8->16	8 - 4	8 - 8	0.5 - 0.5	8
CM9165	1	0.25	0.5	0.125	0.25
CM9174	2	0.5-1	1-4	0.125-0.25	2-4
CM9189	1	0.5	1	0.25	1
CM9195	1	0.25	1	0.125	1
CM9228	1	0.5	0.5	0.25	2
CM9267	2	0.5	1	0.25	1
CM9298	1	0.5	1	0.125	1
CM9326	1 -2	8 - 4	8 - 8	2 - 1	>8
CM9329	2	0.5	1	0.25	1
CM9331	1	0.5	1	0.25	2
CM9684	0.5	1	>8	0.25	2
TP642	1	1	2	0.25	2
TP968	1	1-2	4	0.5-1	4
TP992	1	0.5	2	0.25	1
TP1004	1	1	4	0.5	4
TP1115	1	1	4	0.5	4
TP1179	1	1	4	0.5	4

Table 1. Antifungal susceptibility profile of *A. flavus* isolates for: AmB, amphotericin B; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ISV, isavuconazole.

Susceptibility testing to 14-a sterol demethylase inhibitors (DMIs) fungicides.

Two strains (CM8087 and CM9326) had higher DMI values to all imidazole and triazole drugs and one strain (CM7668) could be considered as hyper-susceptible to all DMIs (**Table 2**).

Strains	MIC ranges (mg/L)							
	Imidazole		Triazole					
	Ima	Pro	Met	Tebu	Epoxi	Bro	Difeno	Myclo
ATCC 2004304	1-2	2-4	4-8	8	8-16	4-16	8->32	>32
CM7668	0.25	1	1	1	0.5-1	0.25-1	0.5-1	4
CM8087	16	>32	>32	>32	>32	>32	>32	>32
CM9165	2	1	8	8	8	8	16	>32
CM9174	4	2	16	16	32	8	16	>32
CM9189	2	1	8	8	8	8	16	>32
CM9195	2	1	8	4	8	8	16	>32
CM9228	2	1	8	4	8	8	16	32
CM9267	2	2	8	8	8	16	16	>32
CM9298	2	2	8	4	8	8	16	>32
CM9326	32	>32	>32	>32	>32	>32	>32	>32
CM9329	1	1	4	4	8	8	16	32
CM9331	2	1	8	8	16	8	32	>32
CM9684	8	2-4	16	16	16->32	32->32	16->16	>32
TP642	8	8	16	16	>32	32	>32	>32
TP968	8	32	16	32	>32	16	32	>32
TP992	2	2	8	4	16	8	>32	>32
TP1004	8	8	16	16	>32	>32	>32	>32
TP1115	8	8	16	16	>32	>32	>32	>32
TP1179	8	8	16	32	>32	>32	>32	>32

Table 2. Antifungal susceptibility profile of *A. flavus* isolates to imidazole and triazole demethylase inhibitors (DMIs). Ima, Imazalil; Pro, Prochloraz; Met, Metconazole; Tebu, Tebuconazole; Epoxi, Epoxiconazole; Bro, Bromuconazole; Difeno, Difenoconazole; Myclo, Myclobutanil.

*Aspergillus flavus cyp*51 genes expression.

RT-PCR amplification was used to show that all three genes are expressed during hyphal growth in submerged culture.
The sequencing of the amplified bands from DNA and cDNA confirmed the presence of introns in each of the genes analyzed (**Figure 1**).

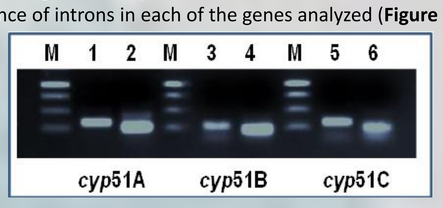

Figure 1. RT-PCR amplification of the three *A. flavus cyp*51 genes. A) *cyp*51A fragment from DNA (1) and RNA (2); B) *cyp*51B fragment from DNA (3) and RNA (4); C) *cyp*51C from DNA (5) and RNA (6). M; 1kb Ladder.

Sequence analysis: Protein similarity.

Comparison of the deduced Cyp51A, Cyp51B and Cyp51C proteins with other fungal Cyp51 resulted in a protein homology that ranged from 50 to 80%.

Phylogenetic analysis.

Well defined clusters could be detected for the Cyp51B-derived proteins and for the Cyp51A-derived proteins. However, the *A. flavus* Cyp51C sequences fall in a sub-cluster within the Cyp51A cluster. Interestingly, *Fusarium* spp. Cyp51C formed an independent cluster from Cyp51A or Cyp51B proteins (**Figure 2**).

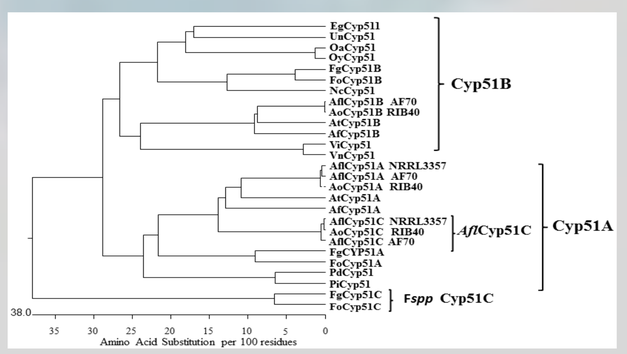

Figure 2. Phylogenetic tree obtained by CLUSTAL analysis. The length of each branch represents the distance between sequence pairs. Numbers at the bottom of the tree are represent the numbers of substitution events. The deduce proteins sequence of the *A. flavus* Cyp51A: Cyp51B: and Cyp51C, *A. oryzae* Cyp51A, Cyp51B and Cyp51C, *A. fumigatus* AfCyp51A and AfCyp51B; *A. terreus* AtCyp51A and AtCyp51B; *Fusarium graminearum* FgCyp51A, FgCyp51B and FgCyp51C; *F. oxysporum* FoCyp51A, FoCyp51B and FoCyp51C; *Penicillium italicum* PiCyp51 and *P. digitatum* PdCyp51; *Oculimacula yallundae* OyCyp51 and *O. acuformis* OaCyp51; *Neurospora crassa* NcCyp51; *Erysiphe graminis* EgCyp51; *Uncinula necátor* UnCyp51; *Venturia inaequalis* ViCyp51 and *V. nashicola* VnCyp51.

Sequence analysis of *cyp*51A, *cyp*51B and *cyp*51C.

Several synonymous and non-synonymous point mutations were found among both susceptible and non-susceptible strains. One azole resistant strain (CM7668) harbored a c641t base change in *cyp*51A resulting in a P214L point mutation that was only present in this strain **(Table 3)**. *cyp*51B only harbored four synonymous mutations and its protein showed a fully conserved amino acid sequence in all azole *A. flavus* strains **(Table 4**). One azole resistant strain (CM9326) showed an a1046g base change in *cyp*51C resulting in a H349R point mutation that was only present in this strain **(Table 5).**

Table 3. *Aspergillus flavus* Cyp51A sequence analysis.

Strains	Azl	Cyp51A																
		c132t	c165t	c342t	g390a	t546c	g595a	c641t	t723c	c738t	t907c	t927c	g966t	a985g	c1065t	t1164c	c1368t	g1371a
CM7668	R		c165t		g390a	t546c		c641t								t1164c		
CM8087	R		*c165t*		g390a	t546c								a985g		t1164c		
CM8098	S		*c165t*		g390a	t546c								a985g		t1164c		
CM9165	S		c165t			t546c			t723c							t1164c		
CM9174	S		c165t		g390a	t546c								a985g		t1164c		
CM9189	S		c165t		g390a	t546c										t1164c		
CM9195	S		c165t		g390a	t546c										t1164c		
CM9228	S					t546c					t907c		g966t			t1164c		
CM9267	S		c165t		g390a	t546c										t1164c		
CM9298	S	c132t	c165t	c342t	g390a	t546c						t927c				t1164c	c1368t	
CM9326	R		c165t		g390a	t546c										t1164c		
CM9329	S		c165t		g390a	t546c										t1164c		
CM9331	S		c165t		g390a	t546c										t1164c		
CM9684	S		c165t		g390a	t546c								a985g		t1164c		
TPH642	S		c165t		g390a	t546c										t1164c		
TPH968	S		c165t		g390a	t546c										t1164c		
TPH992	S		c165t		g390a	t546c										t1164c		
TPH1004	S		c165t		g390a	t546c										t1164c		
TPH1115	S		c165t		g390a	t546c										t1164c		
TPH1179	S		c165t		g390a	t546c										t1164c		
ATCC 2004304	S		c165t		g390a	t546c	g595a								c1065t	t1164c	c1368t	g1371a
A. oryzae RIB40	-		c165t		g390a	t546c	g595a								c1065t	t1164c	c1368t	g1371a
A. flavus AF70	-		c165t		g390a	t546c				c738t						t1164c	c1368t	
Protein		F44	P55	G114	K130	F182	**A199T**	**P214L**	Y241	N246	L303	P309	**K322N**	**T329A**	H355	P388	N456	L456

Table 4. *A. flavus* Cyp51B sequence analysis.

Strains	AZL	Cyp51B			
		c237t	c498t	t699c	a799g
CM7668	R				
CM8087	R				
CM9165	S	c237t	c498t		a799g
CM9174	S				
CM9189	S		c498t		
CM9195	S				
CM9228	S		c498t		
CM9267	S				
CM9298	S		c498t		
CM9326	R				
CM9329	S				
CM9331	S		c498t		
CM9684	S			t699c	
TPH642	S				
TPH968	S				
TPH992	S				
TPH1004	S				
TPH1115	S				
TPH1179	S				
ATCC 2004304	S				
A. oryzae RIB40	-				
A. flavus AF70	-		c498t		a799g
Protein		**C79**	**I166**	**N233**	**K267**

Table 5. *Aspergillus flavus* Cyp51C sequence analysis.

Strains	Azl	Cyp51C																	
		t161c	c174g	t718g	c748t	g760a	a761g	c826a	a853g	t876g	t894a	**g915a**	c978t	a1044g	**a1046g**	c1158t	g1196t	a1263g	a1267g	a1455g
CM7668	R	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
CM8087	R	t161c	G58G	t718g																
CM9165	S	t161c	c174g	t718g																
CM9174	S	t161c	G58G	t718g																
CM9189	S	t161c	G58G	t718g			a761g	c826a	a853g	t876g	t894a					c1158t		a1263g	a1267g	
CM9195	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
CM9228	S	t161c	G58G	t718g	c748t												g1196t			
CM9267	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
CM9298	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
CM9326	R	t161c	G58G	t718g											a1046g					
CM9329	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
CM9331	S	t161c	G58G	t718g		g760a		c826a	a853g	t876g	t894a					c1158t		a1263g	a1267g	
CM9684	S	t161c	G58G	t718g																
TPH642	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
TPH968	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
TPH992	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
TPH1004	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
TPH115	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
TPH1179	S	t161c	G58G	t718g			a761g						c978t	a1044g		c1158t		a1263g	a1267g	
ATCC 2004304	S	t161c	G58G	t718g		g760a			a853g	t876g	t894a					c1158t				a1455g
A. oryzae RIB40	-	t161c	G58G	t718g																
A. flavus AF70	-	t161c	G58G	t718g		g760a						g915a				c1158t		a1263g	a1267g	
Protein		**M54T**	G58G	**S240A**	**R250$_{ST}$**	**D254N**	**D254G**	**P276T**	**I285V**	A292A	S298S	**M305I**	L326L	L348L	H349R	S386S	**S399I**	**E421E**	**N423D**	V485V

CONCLUSIONS

1. Two different point mutations at *A. flavus* azole target Cyp51A (P214L) and Cyp51C (H349R) seem to be linked to the development of an azole resistance phenotype, although how these substitutions could affect the antifungal-enzyme interaction remains unclear.

2. The fact that reduced *in vitro* susceptibility to clinical azoles shows cross-resistance to DMIs may suggest the involvement of both in *A. flavus* azole resistance development.

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