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Overinduction of the *CYP51A* Gene After Exposure to Azole Antifungals Provides a First Clue to the Resistance Mechanism in the *Fusarium solani* Species Complex

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The genus *Fusarium* is largely represented in fungal infections, not only mostly in plants but also in humans. Fungi belonging to the *Fusarium solani* species complex (*FSSC*) are those that are most frequently isolated in invasive fusariosis and present elevated minimum inhibitory concentrations for the main antifungal drugs used in medicine. This study is the first to investigate the resistance mechanism in *FSSC* by monitoring *CYP51A* expression in the presence of azole antifungals. After exposure to voriconazole, an overinduction of *CYP51A* was observed irrespective of the concentration of antifungal used and the generation studied. The same observation is made for other azole antifungals, posaconazole and tebuconazole, but not for amphotericin B. This observation could contribute to explaining why some antifungal molecules used in agriculture or medical practices may have low susceptibility for some fungi.

Keywords: *Fusarium solani* species complex (*FSSC*), antifungal resistance, voriconazole, *CYP51*, over-induction

Introduction

OPPORTUNISTIC INVASIVE FUSARIOSES are severe fungal infections, refractory to treatment, and present a high mortality rate.¹ Recent guidelines have recommended voriconazole as the first-line therapy against these mycoses, in association with surgical debridement.²

However, clinical strains of the *Fusarium solani* species complex (*FSSC*) usually show high *in vitro* minimum inhibitory concentrations (MICs) for antifungal drugs and are also characterized by frequent *in vivo* resistance.³ Voriconazole has a mean MIC of 4.6 µg/mL and an epidemiological cutoff value (ECV) of 32 µg/mL.^{4,5} Unfortunately, clinical response to therapy cannot be predicted using ECVs that differ from breakpoints.⁵ A better understanding of the molecular mechanisms of resistance is therefore needed.

Azole molecules, such as voriconazole, target the 14 alpha-demethylase enzyme, block it, and inhibit the synthesis of cellular ergosterol. This protein is encoded by the *ERG11* and *CYP51* genes in yeasts and fungi, respectively. Resistance to azoles can be mediated by different mechanisms. Among them,

the best known is a mutation of the target gene, leading to a change in protein conformation and consequently to a decrease in the affinity between azoles and the target enzyme, as described for *Saccharomyces cerevisiae* with crystallography.⁶ This phenomenon has been identified for the *CYP51A* gene in *Aspergillus fumigatus* strains that are resistant to azole antifungals.⁷ Other mechanisms may also be involved, however, such as target gene upregulation.⁸ In *Candida albicans*, mutations in the transcription factor *UPC2* induce overexpression of *ERG11* and confer azole resistance.⁹

In the genus *Fusarium*, three paralogous *CYP51* genes have been described and designated as *CYP51A*, *CYP51B*, and *CYP51C*.¹⁰ The function of these genes has been investigated in *Fusarium graminearum* and *CYP51A* was shown to be responsible for the intrinsic variation in azole sensitivity.¹¹ On the contrary, *CYP51B* and *CYP51C* have not been described to play an essential role in antifungal activity.¹¹

To date, the mechanism involved in *FSSC* strains' low sensitivity to azoles is still unclear. The aim of this study was to explore *CYP51A* expression in *FSSC* cultures incubated with different azole antifungals to constitute a

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TABLE 1 CHARACTERISTICS OF THE *FUSARIUM SOLANI* SPECIES COMPLEX STRAINS USED IN THIS STUDY

Collection number	MIC voriconazole ($\mu\text{g/mL}$)		Genotype	Origin	
	CLSI	E-test		Location	Geography
CBS 115660	2	0.5	21 d	Potatoes	Egypt
CBS 124630	2	1	3+4 bbbb	Skin	France
CBS 124899	4	2	2 d	Nail	Gabon
CBS 124896	4	4	11 i	Blood	Belgium
CBS 124895	8	8	5 o	Skin	France
CBS 118931	4	32	9 a	Tomatoes	United Kingdom
CBS 124889	0.5	12	2 d	Nail	France
CBS 224.34	1	2	1 b	Toe nail	Cuba
CBS 102824	0.5	0.75	25 d	Plated litter fragment	Colombia
CBS 117608	1	0.38	6 f	Arm lesion dermis	Turkey

MIC, minimum inhibitory concentration.

first approach toward exploration of the mechanism of acquired resistance.

Materials and Methods

Isolates

Ten FSSC isolates were used for this study, among which different strains were selected depending on different experiments. These strains, previously studied, present different genotypes and MICs determined by both the CLSI and E-test methods.⁴ For all strains, the corresponding CBS-KNAW Fungal Biodiversity Centre collection numbers and characteristics are listed in Table 1. Strains were stored at -20°C in water/glycerol (1/1).

Cultivation

Fusarium strains were grown on yeast extract sucrose medium and incubated at 30°C with stirring at 180 rpm for half a week.¹² The medium was supplemented with various concentrations of antifungals: $\frac{1}{4}$ or $\frac{1}{2}$ of the previously determined MIC for each selected strain for each of the following molecules: voriconazole (Sigma-Aldrich, St Quentin Fallavier, France), posaconazole (Sigma-Aldrich), tebuconazole (Sigma-Aldrich), and amphotericin B (Sigma-Aldrich). A culture without any antifungal was used as a control. For this study, culture generation is defined as about 80 hours of growth.

RNA extraction

RNA was extracted from liquid cultures using the RNeasy[®] Plant Mini Kit (Qiagen, Hilden, Germany) after filtration through Miracloth paper (Merck Millipore, Fontenay sous Bois, France) and mycelium washings with sterile water.

The RNA concentration was determined using a Nanodrop 2000 system (Thermo Scientific, Wilmington).

Primer and probe design

To ensure the specificity of our quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments, the forward primer was designed over the junction, obtained after pre-mRNA splicing, between two different exons (Fig. 1). Sequences of primers and probes are shown in Table 2. Specificity of the *CYP51A* primers was verified against *CYP51B* and *C* genes by bioinformatics and sequencing.

Multiplex RT-qPCR

To quantify *CYP51A* expression, a multiplex RT-qPCR was developed using *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) mRNA as the reference. RT-qPCR multiplex assays were carried out in triplicate using the QuantiTect Multiplex RT-PCR NR Kit (Qiagen) in 25- μL reaction volumes containing 5 μL of RNA 10 $\mu\text{mol/L}$. Experiments were run in an iCycler MyIQ2 Thermal cycler (Biorad, France). To optimize multiplex PCR and reduce competition between the target and reference transcripts, various tests were applied according to suppliers' recommendations. Optimal conditions were obtained by mixing 12.5 μL of Master Mix, 2 μL of MgCl_2 (25 mmol/L), 4 μL of RNase-free water, 0.25 μL of reverse transcriptase mix, 0.25 μL of each *GAPDH* primer (20 $\mu\text{mol/L}$), 0.125 μL of *GAPDH* probe (20 $\mu\text{mol/L}$), 0.25 μL of each *CYP51A* primer (40 $\mu\text{mol/L}$), and 0.125 μL of *CYP51A* probe (40 $\mu\text{mol/L}$). This mix was subjected to an initial 20-minute reverse transcription step at 50°C , followed by an activation step at 95°C for 15 minutes, and then 45 PCR cycles at 94°C for 45 seconds and 60°C for 45 seconds with fluorescence acquisition.

FIG. 1. Location of primers (**bold and underlined**) and probe (*gray*) used to perform RT-qPCR experiments. Intron is shown in *italics*. RT-qPCR, quantitative reverse transcription polymerase chain reaction.

CTCTCATCTTCCACTGGCTTCCCTTCATCGGCAATGCTGTGTCGTAT
GGCCTAGATCCGTGTAACCTTCTTCATGAAGTG**TCCGAGAGAAG***gtaga*
gtaccaactctagcacactgcaattcttcgctgactgtttgggtccgta
*tcttcttttag***CACGGCG***ACGTCTTACCTTTGTCTCTTTGGCCGA*
AGATTGCTGCTGTCTCGGAGT*CGAAGGCAACGACT***TTGTCTCAAC**
AGCCGA*CTCCAAGACGCCAACGCCGAAGAGATCTACGGTCCCCTCAC*
GATCCCCGTCTTCGGTAGCGATGTCGTA

TABLE 2. SEQUENCES OF PRIMERS AND PROBES USED FOR MULTIPLEX RT-qPCR (*GAPDH*: REFERENCE mRNA; *CYP51A*: TARGET mRNA)

Name	Sequence
GAPDH F	5'-CTCAAGGGCGTTCTCGCTTA-3'
GAPDH R	5'-CACTCGTTGTCGTACCAGGA-3'
GAPDH S	5'-FAM-CACCAACTCCTCCATCTTC GATG-TAMRA-3'
CYP51A F	5'-TCGAGAGAAGCACGGC-3'
CYP51A R	5'-TCGGCTGTTGAGGACAA-3'
CYP51A S	5'-HEX-CGACTCCGAGACAGCAGACA ATCTTTG-BHQ-3'

RT-qPCR, quantitative reverse transcription polymerase chain reaction.

Data analyses

The Livak method was chosen to normalize expression ratios in strains grown in the presence of antifungals by comparison with control cultures because both target and reference transcripts were amplified with efficiencies near 100% and within 5% of each other.¹³

Statistical analyses

The Friedman test (nonparametric statistical test) in paired samples was used to compare expression ratios of *CYP51A* in the presence of antifungals in each generation. Because of the multiplicity of tests, the alpha risk was 1.6% for all analyses. These statistical analyses were performed using SAS 9.4 software (SAS Institute).

Results

Constitutive *CYP51A* expression

Constitutive expression of *CYP51A* was observed for all *F. solani* strains cultivated without antifungals. Moreover,

even if the relative quantification of *CYP51A* was different depending on the strains, no association could be made between *CYP51A* expression levels and MIC values ($r^2=0.05$).

CYP51A expression after exposure to voriconazole

Nine strains (isolates described in Table 1, except CBS 124896) were cultured with different concentrations of voriconazole over 1, 2, or 3 generations. All demonstrated overexpression of the *CYP51A* gene, with a ratio ranging from 3 to 2,165. For each experimental condition in the presence of voriconazole, the mean ratios shown in Fig. 2 are significantly different from 1; the control ratio was obtained from strains grown in culture medium without voriconazole (Table 3). Both concentrations tested induced overexpression even if the *CYP51A* expression is independent of the voriconazole dose ($p=0.045$ for a voriconazole concentration at 1/2 MIC and $p=0.236$ for a voriconazole concentration at 1/4 MIC). Moreover, no significant differences were observed between the three generations ($p=1.000$, $p=0.470$, and $p=0.631$ for generations 1, 2, and 3, respectively). In control conditions, without antifungals, no modification of the expression is observed over time and generations. Thus, after exposure to voriconazole, an overinduction of *CYP51A* was observed irrespective of the concentration of antifungal used and the generation studied.

CYP51A expression after exposure to azole antifungals

Four isolates (CBS 124899, CBS 118931, CBS 124896, and CBS 124629) were cultivated with other antifungals (posaconazole, tebuconazole, or amphotericin B) using the same protocol as above. After exposure to posaconazole and tebuconazole, overinduction was observed with p -values <0.0001 and 0.006 , respectively, but not after amphotericin B exposure (Table 4 and Fig. 3).

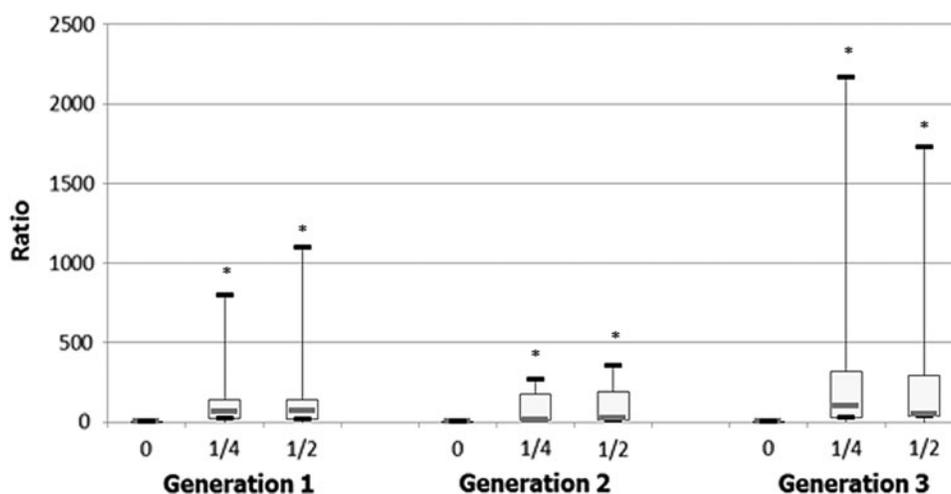


FIG. 2. *CYP51A* expression in the presence of voriconazole ($*p < 0.016$). For each generation cultivated in the presence of one-quarter or one-half the voriconazole MIC, the relative expression of *CYP51A* was determined by RT-qPCR. The signals obtained were expressed relative to corresponding controls (set to 1) grown without voriconazole. Strains used were CBS 115660, CBS 124630, CBS 124899, CBS 124895, CBS 118931, CBS 124889, CBS 224.34, CBS 102824, and CBS 117608. The comparison is statistically significant according to the Friedman test if p -value <0.016 . MIC, minimum inhibitory concentration.

TABLE 3. COMPARISON OF THE EXPRESSION RATIOS OF *CYP51A* IN THE PRESENCE OF VORICONAZOLE

		VRZ = 0		VRZ = ¼		VRZ = ½		p
		n	Ratio mean	n	Ratio mean	n	Ratio mean	
G 1	VRZ=0 vs. ¼	9	1	9	97.0	8	94.9	0.0001
	VRZ=0 vs. ½	9	1					0.0002
G 2	VRZ=0 vs. ¼	9	1	9	87.9	8	138.5	0.0001
	VRZ=0 vs. ½	9	1					0.0002
G 3	VRZ=0 vs. ¼	7	1	6	569.3	6	439.7	0.0011
	VRZ=0 vs. ½	7	1					0.0011

For each generation cultivated in the presence of one-quarter or one-half the voriconazole MIC, the relative expression of *CYP51A* was determined by RT-qPCR. The signals obtained were expressed relative to corresponding controls (set to 1) grown without voriconazole. Strains used were CBS 115660, CBS 124630, CBS 124899, CBS 124895, CBS 118931, CBS 124889, CBS 224.34, CBS 102824, and CBS 117608. The comparison is statistically significant according to the Friedman test if *p*-value <0.016.

G, generation; VRZ, voriconazole.

Discussion

Different mechanisms of resistance to antifungal agents have been described, such as the lack of molecule import into the cell, lack of activation in the case of a prodrug, overproduction of the antifungal target, modification or disappearance of the target, metabolic derivation, or active efflux.¹⁴ In the *Candida* genus, most of these mechanisms are involved in azole resistance: upregulation of drug transporters, numerous point mutations in *ERG11*, overexpression of *ERG11*, and loss of function of *ERG3*.^{14,15}

Resistance to azole antifungals in *A. fumigatus* is a major and increasing problem in many countries.^{7,16–18} *A. fumigatus* isolates may be resistant to a single azole such as itraconazole or more rarely voriconazole, two azoles such as itraconazole and posaconazole, or multiazole resistant.¹⁶ The major resistance mechanism in clinical isolates is a substitution of leucine for histidine at position 98 associated with the presence of multiple copies of a 34-base pair sequence repeated in tandem in the promoter of the *CYP51* gene, designated TR₃₄/L98H.⁷ The origin of this azole resistance is double. On the one hand, an environmental source is suggested and explained by agricultural use of triazole fungicides that are highly similar to the antifungal triazoles used in medicine.^{19–21} On the other hand, a secondary acquisition of resistance can be observed associated with minority resistant populations harboring mutations in *CYP51A*, mainly in patients receiving long-term azoles.²²

FSSC is a fungal complex with high MICs for major antifungal agents. The results of this study show that cultivation

in the presence of subinhibitory doses of voriconazole induces an overexpression of *CYP51A*. A positive trend of this expression seems to be observed over the generations, although it is not significant. Other data are therefore necessary to support the hypothesis of an acquired resistance mechanism following exposure to antifungal molecules. An adaptation to the drug environment may also be hypothesized as similar global upregulation mechanisms implying *ERG* genes have already been described in several *Candida* species in response to azoles or other sterol biosynthesis inhibitors.²³ This overexpression is also observed with other molecules of the azole class such as posaconazole, used in clinical practice, or tebuconazole, used in agriculture. On the other hand, we noted that no modification of *CYP51A* expression is induced by amphotericin B, which presents another mechanism of action. Polyene causes the formation of concentration-dependant channels that kill cells by allowing ions and other cellular components to escape.¹⁴

Because of the ecological and clinical characteristics of fusariosis, these observations may contribute to explaining the lower susceptibility to antifungal agents. Azole compounds, such as voriconazole or posaconazole, are used in prophylactic or pre-emptive treatments in immunocompromised patients at risk for invasive fungal infections. Recently, the primary cutaneous portal of entry has been considered as a reservoir for invasive fusariosis.²⁴ Consequently, isolates hosted by patients could develop resistance in response to the selection pressure generated by administration of various antifungals.

TABLE 4. COMPARISON OF THE EXPRESSION RATIOS OF *CYP51A* IN THE PRESENCE OF OTHER MOLECULES (POSACONAZOLE, TEBUCONAZOLE, AND AMPHOTERICIN B)

		n	Ratio mean	P
Posaconazole	Control	12	1	<0.0001
	Culture with molecule	20	118.4	
Tebuconazole	Control	4	1	0.006
	Culture with molecule	8	138.1	
Amphotericin B	Control	10	1	0.023
	Culture with molecule	20	1.25	

Relative expression of *CYP51A* determined by RT-qPCR after culture of strains CBS 124899, CBS 118931, CBS 124896, and CBS 124629 in the presence of posaconazole, tebuconazole, or amphotericin B (1/2 MIC). The signals obtained were expressed relative to corresponding controls (set to 1) grown without antifungals. The comparison is statistically significant according to the Friedman test if *p*-value <0.016.

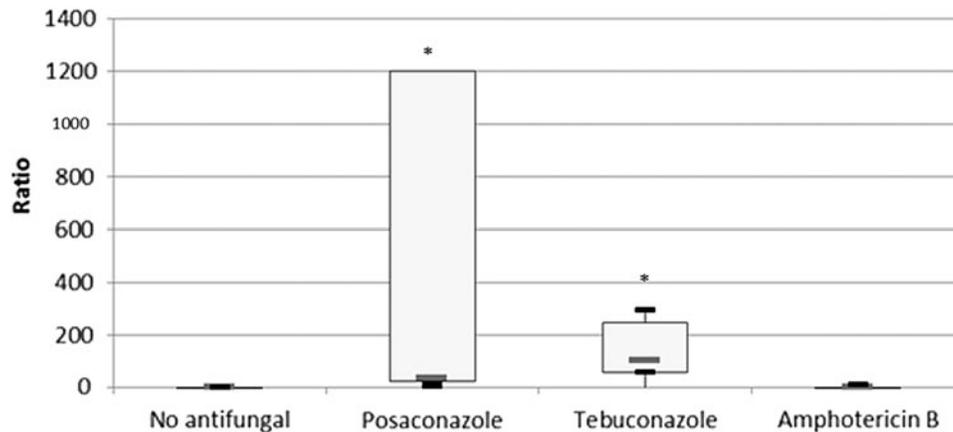


FIG. 3. *CYP51A* expression in the presence of other antifungal agents (* $p < 0.016$). Relative expression of *CYP51A* determined by RT-qPCR after culture of strains CBS 124899, CBS 118931, CBS 124896, and CBS 124629 in the presence of posaconazole, tebuconazole, or amphotericin B (1/2 MIC). The signals obtained were expressed relative to corresponding controls (set to 1) grown without antifungals. The comparison is statistically significant according to the Friedman test if p -value < 0.016 .

On the agricultural side, *Fusarium* is considered as one of the main phytopathogenic genera, and the overexpression of *CYP51A* in *F. graminearum* in the presence of tebuconazole has already been described.^{10,25}

Together, these observations highlight the risk of the emergence of antifungal-resistant species due to environmental pressures.

To conclude, in this work, an overexpression of *CYP* genes has been shown in the presence of azole antifungals. Being observed over several generations, this mechanism could be involved in the decreased sensitivity of the *Fusarium* genus to antifungals. However, additional data, especially phenotypic (MIC), are still needed to definitively prove this hypothesis.

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Disclosure Statement

No competing financial interests exist.

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