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MECHANISMS OF RESISTANCE

A CTG Clade *Candida* Yeast Genetically Engineered for the Genotype-Phenotype Characterization of Azole Antifungal Resistance in Human-Pathogenic Yeasts

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ABSTRACT A strain of the opportunistic pathogenic yeast *Candida lusitaniae* was genetically modified for use as a cellular model for assessing by allele replacement the impact of lanosterol C14α-demethylase *ERG11* mutations on azole resistance. *Candida lusitaniae* was chosen because it is susceptible to azole antifungals, it belongs to the CTG clade of yeast, which includes most of the *Candida* species pathogenic for humans, and it is haploid and easily amenable to genetic transformation and molecular modeling. In this work, allelic replacement is targeted at the *ERG11* locus by the reconstitution of a functional auxotrophic marker in the 3′ intergenic region of *ERG11*. Homologous and heterologous *ERG11* alleles are expressed from the resident *ERG11* promoter of *C. lusitaniae*, allowing accurate comparison of the phenotypic change in azole susceptibility. As a proof of concept, we successfully expressed in *C. lusitaniae* different *ERG11* alleles, either bearing or not bearing mutations retrieved from a clinical context, from two phylogenetically distant yeasts, *C. albicans* and *Kluyveromyces marxianus*. *Candida lusitaniae* constitutes a high-fidelity expression system, giving specific Erg11p-dependent fluconazole MICs very close to those observed with the *ERG11* donor strain. This work led us to characterize the phenotypic effect of two kinds of mutation: mutation conferring decreased fluconazole susceptibility in a species-specific manner and mutation conferring fluconazole resistance in several yeast species. In particular, a missense mutation affecting amino acid K143 of Erg11p in *Candida* species, and the equivalent position K151 in *K. marxianus*, plays a critical role in fluconazole resistance.

KEYWORDS *ERG11* mutation, fluconazole resistance, heterologous expression, *Candida*, *Kluyveromyces*, *Candida lusitaniae*

Azole antifungals are the most widely used antimicrobial agents for the treatment of superficial and invasive fungal infections and for prophylaxis and empirical treatment in clinical wards, notably in oncohematology units (1). Frequent exposure to azole antifungals may contribute to the selection of resistance, both in filamentous fungi (2) and in *Candida* yeasts (3). The cellular target of azole antifungals is Erg11p (synonym CYP51 for the filamentous fungi), the lanosterol 14α-demethylase of the ergosterol biosynthetic pathway. By considering their frequency, three main mechanisms generally support resistance to azoles in *Candida* yeast (3, 4): point mutations of the *ERG11* gene, which decrease the binding affinity of the target protein to azole antifungals; overexpression of *ERG11*, which increases the intracellular concentration of the target protein; and overexpression of efflux membrane transporters, which lowers the intracellular concentration of the antifungal drug. In *C. albicans*, the two last mechanisms may arise from gene duplication by aneuploidization (5) or from point mutations in the transcription factors *UPC2* and *TAC1*, which regulate expression of the relevant gene

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ERG11 and *CDR* efflux pumps, respectively (6, 7). In addition, mutations arising by single-nucleotide polymorphism (SNP) of the *ERG11* gene are expected to occur frequently. More than 150 missense mutations within the coding region of *ERG11* were described so far in different yeast species (8), but very few of them were demonstrated to be directly responsible for the resistance. The impact of a missense mutation on azole resistance can be demonstrated either by purifying the mutated Erg11 protein and measuring the kinetic parameters of the enzymatic activity in the presence and in the absence of azole inhibitors (9, 10) or by heterologous expression of the *ERG11* allele in a yeast host model, generally *Saccharomyces cerevisiae* (9, 10, 11). However, while most *Candida* species are susceptible to azole antifungals, *S. cerevisiae* is much less susceptible, particularly to fluconazole. A genetically modified *S. cerevisiae* strain lacking the Pdr5p ABC efflux transporter was developed to enhance its susceptibility to fluconazole for heterologous expression experiments (12). Other issues may arise from the use of a 2μ-derived multicopy plasmid for expressing *ERG11* alleles in *S. cerevisiae* and from driving its expression with strong promoters, such as GAL10 or ADH1 (12), which can contribute to overexpress *ERG11* and to decrease azole susceptibility (13). Finally, *S. cerevisiae* is very distant from the CTG clade of yeasts that decode the CUG codon as a serine instead of a leucine (14, 15, 16), sometimes making site-directed mutagenesis necessary to render the *Candida ERG11* allele compatible with the genetic code of *S. cerevisiae* (10).

In this work, we created a genetically engineered strain of *Candida lusitaniae* (teleomorph *Clavispora lusitaniae*) to characterize the effect of missense point mutations of the *ERG11* gene at the phenotypic level. Compared to other biological systems, *C. lusitaniae* has the advantages of belonging to the CTG clade of *Candida* species, of being haploid, and of being susceptible to azole antifungals with no trailing growth, allowing detection of very small MIC variations. Conceptually, the system relies on the replacement of the resident *ERG11* allele of *C. lusitaniae* by any other *ERG11* allele. Replacement is targeted at the *ERG11* locus by the reconstitution of a functional auxotrophic marker in the 3′-untranslated region of the *ERG11* gene, and expression of the new allele is driven by the *C. lusitaniae* resident *ERG11* promoter. Our system was challenged by expression in *C. lusitaniae*-susceptible and -resistant *ERG11* alleles from *C. lusitaniae* and *C. albicans* and from a clinical isolate of *Kluyveromyces marxianus* cross-resistant to all azole antifungals and which bore two missense mutations leading to E123Q and K151E amino acid substitutions (17). Both homologous and heterologous expression in *C. lusitaniae* allowed us to resolve unequivocally the link between *ERG11* mutations and azole resistance.

RESULTS

Phylogenetic analysis of fungal pathogens based on lanosterol C14α-demethylase. Before using *Candida lusitaniae* as an alternative yeast species for heterologous expression of *ERG11*, we verified its position in a phylogenetic tree made with a total of 52 protein sequences identified as fungal lanosterol C14α-demethylase and that of the oomycete *Saprolegnia parasitica*, a parasite of fish close to the fungal kingdom, used to root the tree. The selection included 25 *Ascomycota* yeasts (2 *Taphrinomycota*, 23 *Saccharomycotina*), 21 *Pezizomycotina* hyphomycetes, of which 17 were *Eurotiomycetes* and 4 were *Sordariomycetes*, 4 *Basidiomycota* yeasts, and 2 *Mucormycota*. This selection contains species pathogenic for plants and animals, including humans, except 13 ascomycetous yeast species that are nonpathogenic. The tree thus obtained (Fig. 1) overlaps the phylogeny of the fungal species generally observed with other genetic markers (18, 19). Most of the human pathogens, including opportunistic fungi, of the phylum *Ascomycota* are distributed over two subphyla, the *Pezizomycotina* and the *Saccharomycotina*. The *Pezizomycotina* branch separates in two lineages of pathogens corresponding to the *Sordariomycetes* (*Fusarium* species) and the *Eurotiomycetes* (*Aspergillus*; dermatophytes and dimorphic fungi). The *Saccharomycotina* branch supports two lineages. One, also known as the CTG clade, contains the higher number of opportunistic pathogenic yeast of the genus *Candida*, including *C. albicans* and *C. lusitaniae*. These yeasts decode the CUG codon as a serine instead of a leucine. The

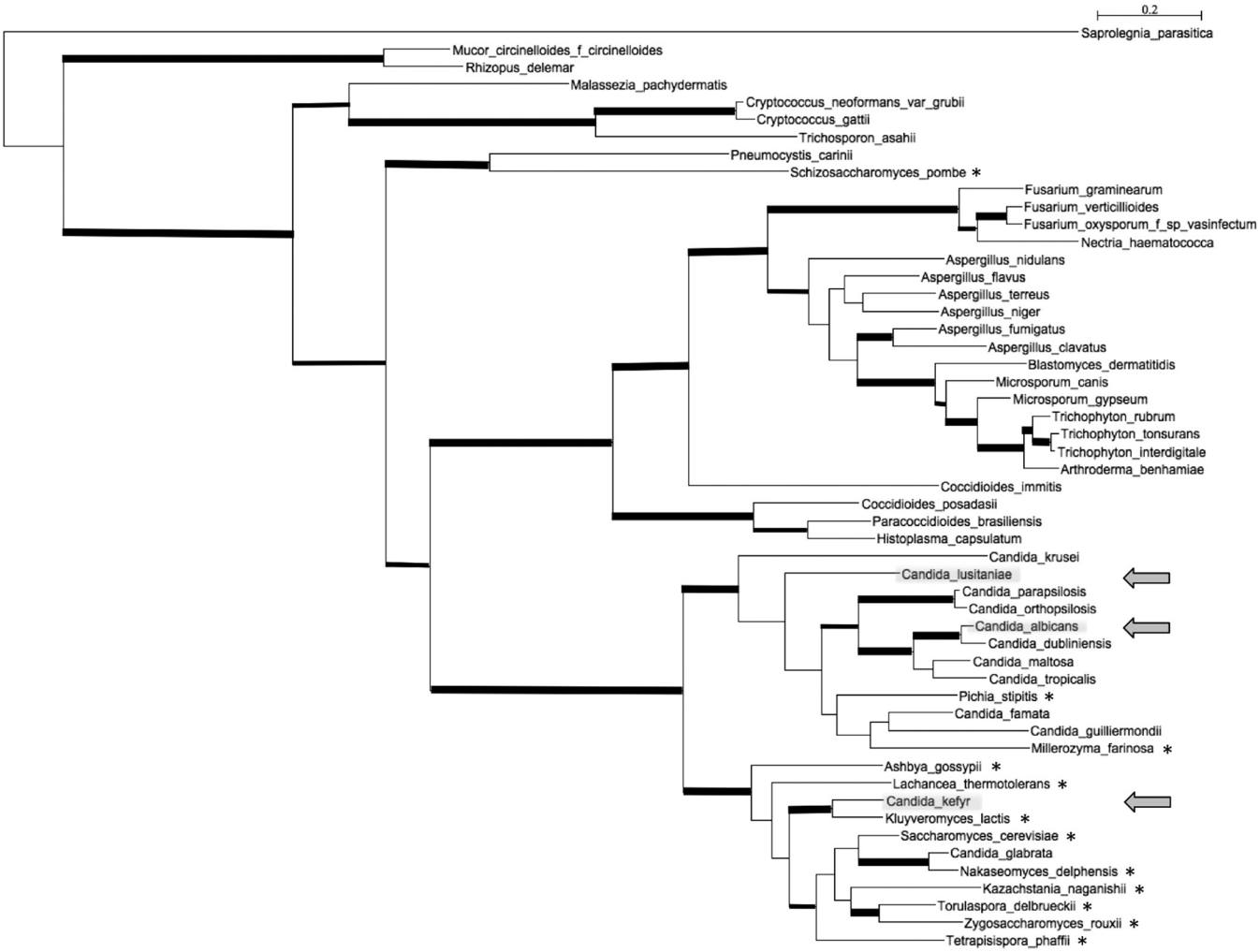

FIG 1 Maximum likelihood tree of pathogenic filamentous fungi and pathogenic and nonpathogenic yeasts using a Clustal Omega alignment of amino acid sequences of lanosterol 14-alpha-demethylase. Bootstrap values: thick branch, 90 to 100%; medium branch, 70 to 89%; thin branch, lower than 70%. Asterisks indicate species that are not considered to be pathogenic. Arrows show the position of the yeast species used in this study.

other lineage contains almost nonpathogenic yeasts, except *C. glabrata* and *K. marxianus*, and is represented by *S. cerevisiae*. The phylogenetic position of *C. lusitaniae* close to the node that separates the two lineages of yeasts makes it attractive for use as a versatile heterologous expression system among pathogenic yeasts. An EMBOSS Needle (EMBL-EBI) pairwise alignment was performed to evaluate the distance between the Erg11 protein of *C. lusitaniae* and those of *C. albicans* (70.5% identity) and *K. marxianus* (64.2% identity), which were further used in this study.

Engineering a *C. lusitaniae* strain for *ERG11* allele replacement. The *ERG11* gene (CLUG_04932) was located on the supercontig 6 of the genome of the *C. lusitaniae* strain ATCC 42720 (GenBank accession number NW_003101573). The intronless open reading frame of 1,578 nucleotides encodes a predicted protein of 525 amino acids. Synteny with *C. albicans* SC5314 was conserved. At 1,055 bp downstream of *C. lusitaniae ERG11* is a gene encoding a putative homoserine kinase (*THR1* gene), and at 2,040 bp upstream an open reading frame (ORF) with unknown function is present. The intergenic region between *ERG11* and *THR1* was large enough to integrate a genetic marker. As a recipient strain we used *C. lusitaniae* CBS 6936 *ura3*Δ, a mutant strain bearing a deletion of 990 bp covering the totality of the *URA3* gene. CBS 6936 is the type strain of the species *Clavispora lusitaniae*, the teleomorph of *C. lusitaniae*. The strain is susceptible to all antifungals, and our team developed several molecular tools

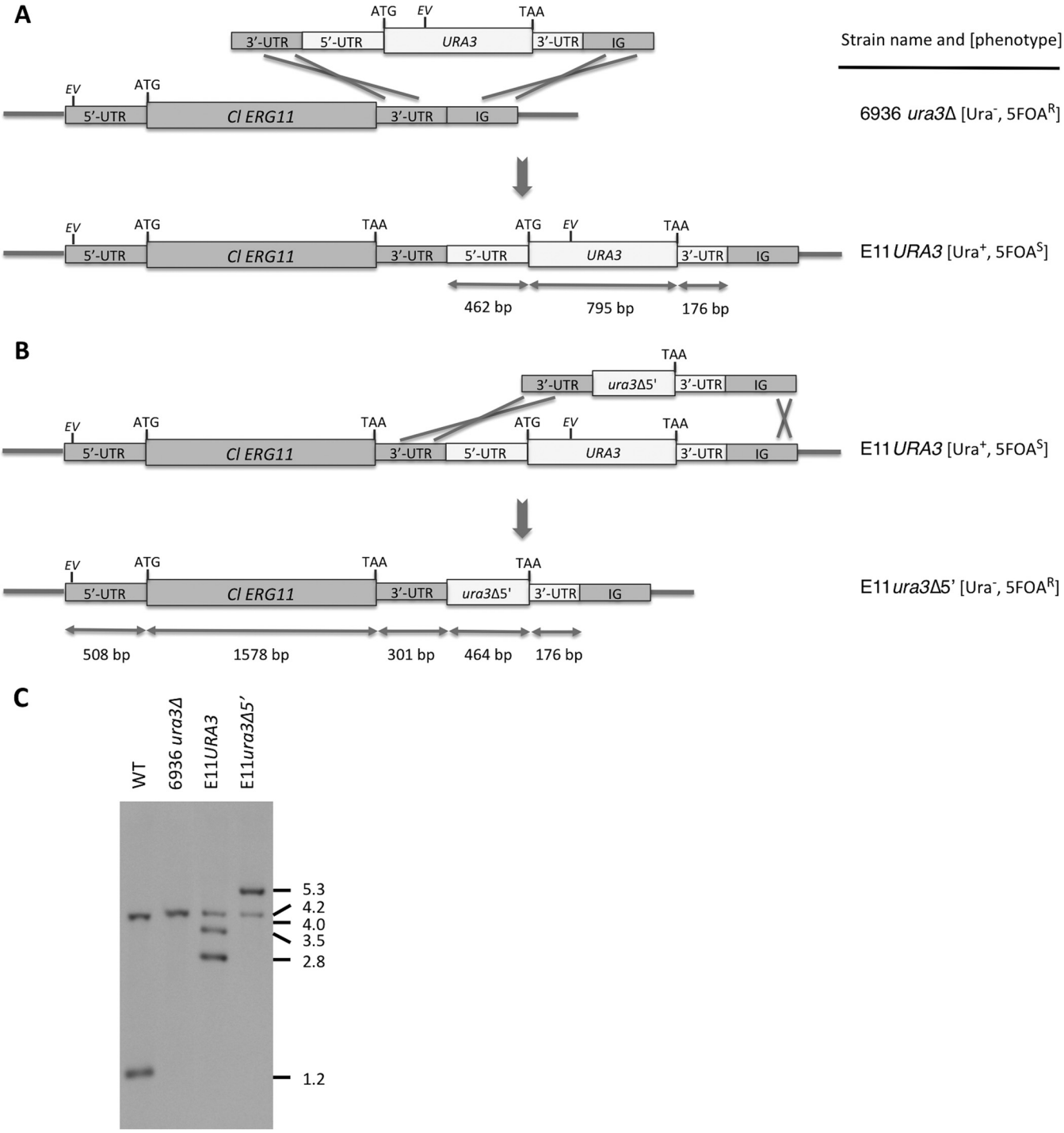

FIG 2 Molecular modifications sequentially achieved to obtain the recipient strain E11*ura3Δ5′*. (A) Transformation of the 6936 *ura3Δ* strain with a DNA cassette bearing the genetic marker *URA3* (white box) flanked by DNA sequences homologous to the 3′-UTR region of the *ERG11* locus (gray boxes). Crossing lines symbolize recombination events that allow the incoming linear DNA cassette to be integrated into the recipient chromosome to give the E11*URA3* strain. (B) Transformation of the E11*URA3* strain with a DNA cassette bearing a nonfunctional *URA3* truncated of its promoter and of the NH2 part of the ORF (white box), resulting after recombination in the strain E11*ura3Δ5′*. (C) Southern blot analysis of the different strains used and constructed in this study after digestion of the genomic DNA with EcoRV and hybridization with *URA3* probes. DNA fragment size is given in kilobase pairs. WT, wild-type strain 6936; *EV*, EcoRV site; ATG, initiation codon; TAA, stop codon; UTR, untranslated regulatory region; IG, intergenic region.

to modify its genome. The release of the genome of the strain CBS 6936 (GenBank accession number LYUB02000000 [20]) allowed us to verify that the genomic map surrounding *ERG11* (GenBank accession number OVF10151.1) was comparable to that of ATCC 42720. In a first step, *URA3* was integrated within the *ERG11-THR1* intergenic

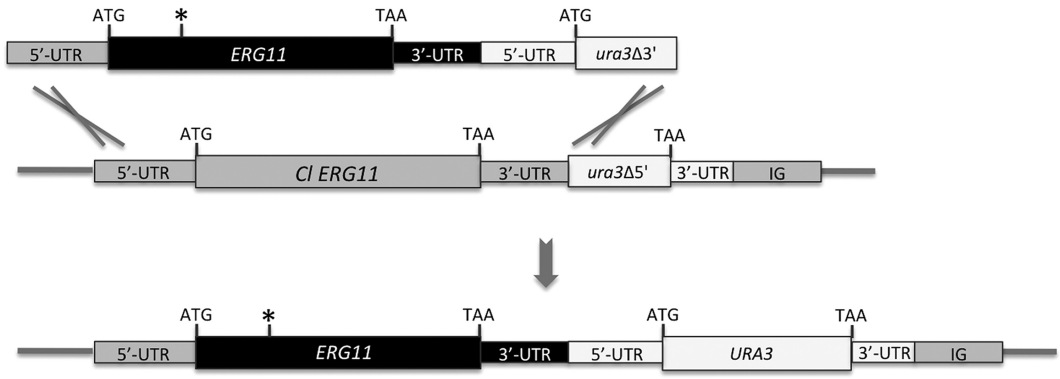

FIG 3 Strategy used to replace the resident *ERG11* allele of *C. lusitaniae* by any other *ERG11* allele bearing or not bearing a mutation. The E11*ura3Δ5′* recipient strain was transformed by a DNA cassette made of the promoter region of the *C. lusitaniae ERG11* gene (gray box, 5′-UTR), the ORF, and the 3′-UTR region of any new *ERG11* allele (black box) and the *URA3* complementary information (white box), allowing reconstitution and expression of a functional *URA3* allele downstream of *ERG11*. *, mutation; abbreviations are defined in the legend to Fig. 2.

region (Fig. 2A). For that, we used a construction made of the *URA3* gene with its own 5′- and 3′-untranslated region (UTR) flanked on each side by large DNA fragments (around 500 bp) homologous to the *ERG11-THR1* intergenic region of integration. The linear DNA cassette was transformed by electroporation into the *ura3*Δ recipient strain, and the selected prototroph colonies were verified for correct targeted integration of the cassette by PCR and nucleotide sequencing, which occurred in 8 transformants over 12 analyzed (75% of targeted integration events). One transformant was selected (strain E11*URA3*), and in a second step, the promoter and two-thirds of the 5′ part of the *URA3* coding region were removed from strain E11*URA3*. For that, we constructed a deletion cassette made of the 3′-UTR *ERG11* region directly fused to the 3′ coding part of *URA3* (Fig. 2B) to transform strain E11*URA3*. Replacement of the wild *URA3* copy by integration of the truncated *ura3* cassette occurred in 100% of the clones analyzed, conferring resistance to the toxic analog 5-fluoroorotic acid (5FOA) (21). The molecular organization was verified by nucleotide sequencing and Southern blot analysis of EcoRV-digested genomic DNA with a *URA3* probe (Fig. 2C). One strain harboring a nonfunctional part of the *URA3* gene in its *ERG11-THR1* intergenic region was selected and named E11*ura3Δ5′* (deleted in the 5′ end of *URA3*) and was further used in this study for all *ERG11* allele replacement experiments. We verified that the molecular modifications performed in strain E11*ura3Δ5′* did not change its susceptibility to antifungals: in particular, the MIC of fluconazole was 0.5 μg/ml, identical to that of wild-type strain 6936.

***ERG11* allele replacement.** *ERG11* allele replacement was targeted by the reconstitution of a functional *URA3* marker in the *ERG11-THR1* intergenic region. The promoter region of the *C. lusitaniae ERG11* gene, the ORF of the new allele *ERG11* to be tested with its own 3′ UTR region, and the promoter and 5′ coding part of *URA3* were fused *in vitro* as a single fragment and transformed into the E11*ura3Δ5′* strain (Fig. 3). The 5′ coding part of the *URA3* gene was designed to be redundant over 300 bp with the resident 3′ coding part, so that a crossing over can take place allowing integration of the DNA cassette and the concomitant reconstitution of a functional *URA3* marker. Recovery of prototroph transformant yeast cells indicated that the DNA cassette had integrated in the *ERG11* region. Molecular verifications by nucleotide sequencing showed that the second recombination event necessary for integration of the cassette took place in the promoter region of the *C. lusitaniae ERG11* gene in 100% of the transformants tested. Before measuring the fluconazole MIC by the CLSI microplate method in RPMI medium lacking uracil, we verified that the strain E11*ura3Δ5′*, expressing different *ERG11* alleles, had no growth defect attributable to decreased expression of the *URA3* gene compared to the growth of the reference strain 6936.

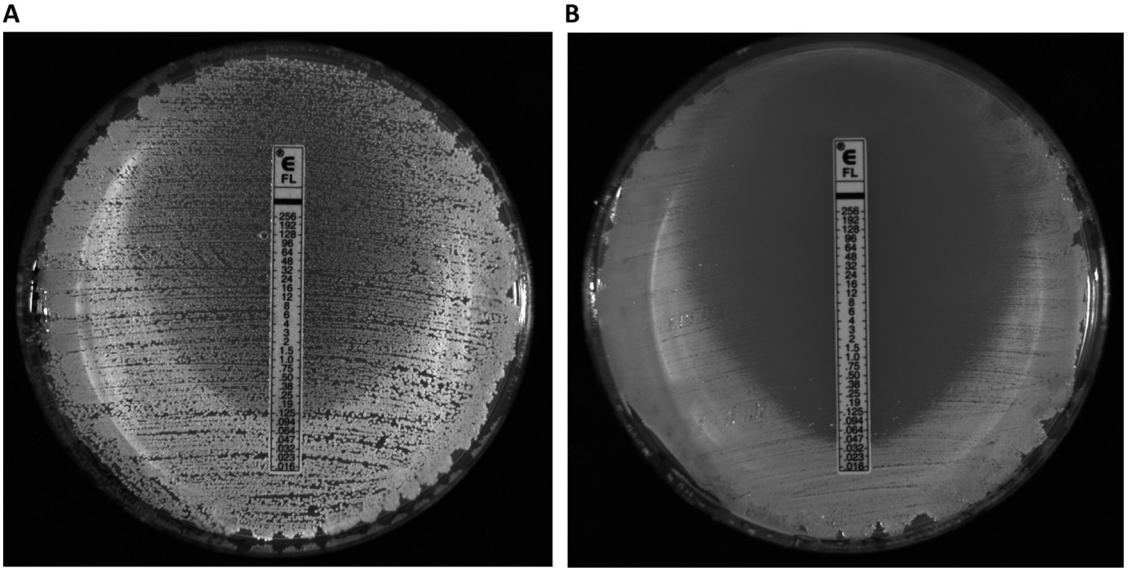

FIG 4 Etest determination of fluconazole susceptibility after 24 h of incubation on RPMI medium. (A) *C. albicans* SC5314. (B) *C. lusitaniae* E11*ura3*Δ*5′* strain expressing the *ERG11* allele *A* of *C. albicans* SC5314.

Heterologous expression of *C. albicans ERG11* allele in *C. lusitaniae*. Our genetic model was first challenged with the *ERG11* wild-type (*CaERG11wt*) alleles *A* and *B* of the diploid *C. albicans* SC5314. This strain, commonly used in research laboratories, is susceptible to fluconazole but exhibits a strong trailing growth that can make MIC determination difficult (Fig. 4A). Using Etest, the fluconazole MIC inhibiting 50% of the growth of *C. albicans* SC5314 was 0.094 μg/ml. When expressed in the E11*ura3*Δ*5′* strain of *C. lusitaniae*, both *CaERG11wt* alleles *A* and *B* conferred susceptibility with a MIC of 0.047 μg/ml (as shown for allele *A* in Fig. 4B), that is to say a MIC comparable to that observed for *C. albicans* SC5314 and 10 times lower than that measured for *C. lusitaniae* E11*ura3*Δ*5′* (0.5 μg/ml). This result shows the tight correlation between the Erg11 protein expressed and the fluconazole MIC conferred to *C. lusitaniae* E11*ura3*Δ*5′* and suggests that susceptibility to fluconazole mainly depends on Erg11p. Moreover, while a strong residual growth of *C. albicans* SC5314 could be observed within the inhibition ellipse, growth of *C. lusitaniae* E11*ura3*Δ*5′* expressing *CaERG11wt* was fully inhibited within the ellipse zone. As already mentioned, this growth inhibition was not the consequence of a growth defect of the E11*ura3*Δ*5′* strain in RPMI lacking uracil but rather the result of the susceptibility of *C. albicans* Erg11p to fluconazole inhibition. The CLSI method was preferred to Etest in the rest of the study because of the occurrence of fluconazole hypersusceptibility with some *ERG11* alleles. With the CLSI method, the *CaERG11wt* alleles *A* and *B* conferred susceptibility comparable to that of *C. lusitaniae* E11*ura3*Δ*5′* with a MIC of 0.0625 μg/ml (Table 1).

Our model was further challenged with three different *ERG11* alleles of *C. albicans* SC5314 in which nonsynonymous mutations were introduced *in vitro*. We chose two mutations previously described in *C. albicans* to support fluconazole MIC increase. The mutation Y132H (tyrosine at position 132 replaced by histidine) is located in hot spot I of the protein, a helix-rich segment important for substrate entry, and the mutation G464S (glycine at position 464 replaced by a serine) in the hot spot III region, important for heme fixation (22).

Both mutations were introduced separately and in combination in the *CaERG11wt* allele *A*, and the modified alleles were expressed in *C. lusitaniae* E11*ura3*Δ*5′*. The Y132H replacement did not result in any fluconazole MIC increase, with the strain being as susceptible to fluconazole (MIC of 0.0625 μg/ml) as the one expressing the unmodified *CaERG11wt* allele. The G464S replacement gave a 4-fold increase in fluconazole MIC, and the combination of both mutations Y132H and G464S yielded the same MIC as that

TABLE 1 Susceptibility of *C. lusitaniae* E11*ura3*Δ*5′* strains expressing different *ERG11* alleles to fluconazole

ERG11 donor strain	*ERG11* allele/mutation	FLC MIC[a] (μg/ml)	MIC variation factor[b]	S/R[c]
C. albicans SC5314	WT allele *A*	0.0625	Ref	S
C. albicans SC5314	WT allele *B*	0.0625	1	S
C. albicans SC5314	G464S	0.25	4	S
C. albicans SC5314	Y132H	0.0625	1	S
C. albicans SC5314	G464S + Y132H	0.25	4	S
C. lusitaniae 6936	G460S	0.5	1	S
C. lusitaniae 6936	Y132H	0.5	1	S
K. marxianus PAZ	E123Q + K151E	64	Ref	R
K. marxianus PAZ	E123Q + K151	0.0078	1/8,200	S
K. marxianus PAZ	E123 + K151E	32	0.5	R
K. marxianus PAZ	E123 + K151	0.0078	1/8,200	S
C. lusitaniae 6936	E115 + K143 (WT)	0.5	Ref	S
C. lusitaniae 6936	E115Q + K143	0.5	1	S
C. lusitaniae 6936	E115 + K143E	8	16	R
C. lusitaniae 6936	E115Q + K143E	16	32	R
C. albicans SC5314	E115 + K143E	8	128	R

[a]MICs were measured by the microplate dilution CLSI method and defined as the minimal concentration of FLC inhibiting 90% of the growth of the strain compared to its growth in drug-free medium.
[b]Ref corresponds to the FLC MIC inhibiting growth of the *C. lusitaniae* strain E11*ura3*Δ*5′* expressing the unmodified *ERG11* wild-type allele *A* of *C. albicans* SC51314, the clinical allele of *K. marxianus* PAZ, or the wild-type allele of *C. lusitaniae*. MIC variation is expressed as a multiplication factor corresponding to the MIC conferred by a modified allele divided by the MIC of the reference allele from the same donor strain.
[c]S, susceptible (MIC of ≤2 μg/ml); R, resistant (MIC of >4 μg/ml).

for the single G464S replacement. As a control, we introduced the mutations Y132H and G464S separately in a *C. lusitaniae ERG11* wild-type allele (*ClERG11wt*) and expressed them in E11*ura3*Δ*5′*, but no change of the fluconazole MIC was observed compared to that of the wild-type allele (fluconazole MIC of 0.5 μg/ml). The expression level of the wild-type and mutated *ERG11* alleles in E11*ura3*Δ*5′* was verified by reverse transcription-quantitative PCR (RT-qPCR) (Fig. 5A and C). In the case of *C. albicans*, the amount of mRNA of each mutated allele varied significantly ($P < 0.05$) compared to that of the wild-type allele, but the amplitude of variation did not exceed a factor of 2.5 (e.g., for the Y132H and G464S alleles). Moreover, a 4.3-fold difference in mRNA amounts between the *CaERG11* G464S allele and the *CaERG11* Y123H + G464S allele has no effect on the fluconazole MIC (0.5 μg/ml for both alleles). We therefore considered that the wild-type and mutated *ERG11* alleles were expressed at a similar level in *C. lusitaniae* E11*ura3*Δ*5′* and that small mRNA variations had no influence on fluconazole MIC.

Heterologous expression of *K. marxianus ERG11* alleles from a clinical fluconazole-resistant isolate in *C. lusitaniae*. *Kluyveromyces marxianus* (anamorph *Candida kefyr*) is a yeast species emerging in oncohematology. We recently described a *K. marxianus* clinical isolate which was resistant to azole antifungals (17). Nucleotide sequencing of *ERG11* revealed two missense mutations leading to the amino acid substitutions E123Q and K151E compared to the Erg11p sequence of the fluconazole-susceptible *K. marxianus* reference strain CBS 6556. Expression in *C. lusitaniae* E11*ura3*Δ*5′* of the *K. marxianus ERG11* allele from the resistant clinical isolate (*KmERG11ci*) bearing both E123Q and K151E mutations conferred fluconazole resistance with a MIC as high as 64 μg/ml (Table 1). We then engineered different *KmERG11ci* alleles by removing each of the mutations separately and then both at the same time. Expression of these alleles in *C. lusitaniae* E11*ura3*Δ*5′* showed that the sole mutation K151E was necessary to confer fluconazole resistance of up to 32 μg/ml. Surprisingly, expression of a *KmERG11ci* allele bearing only the E123Q mutation or no mutation conferred hypersusceptibility to fluconazole (fluconazole MIC of 0.0078 μg/ml), that is to say a MIC nearly 8,200 times lower than that conferred by the *KmERG11ci* allele bearing the two mutations and 64 times lower than that observed for *C. lusitaniae* E11*ura3*Δ*5′* expressing its own wild-type *ERG11* allele (MIC of 0.5 μg/ml).

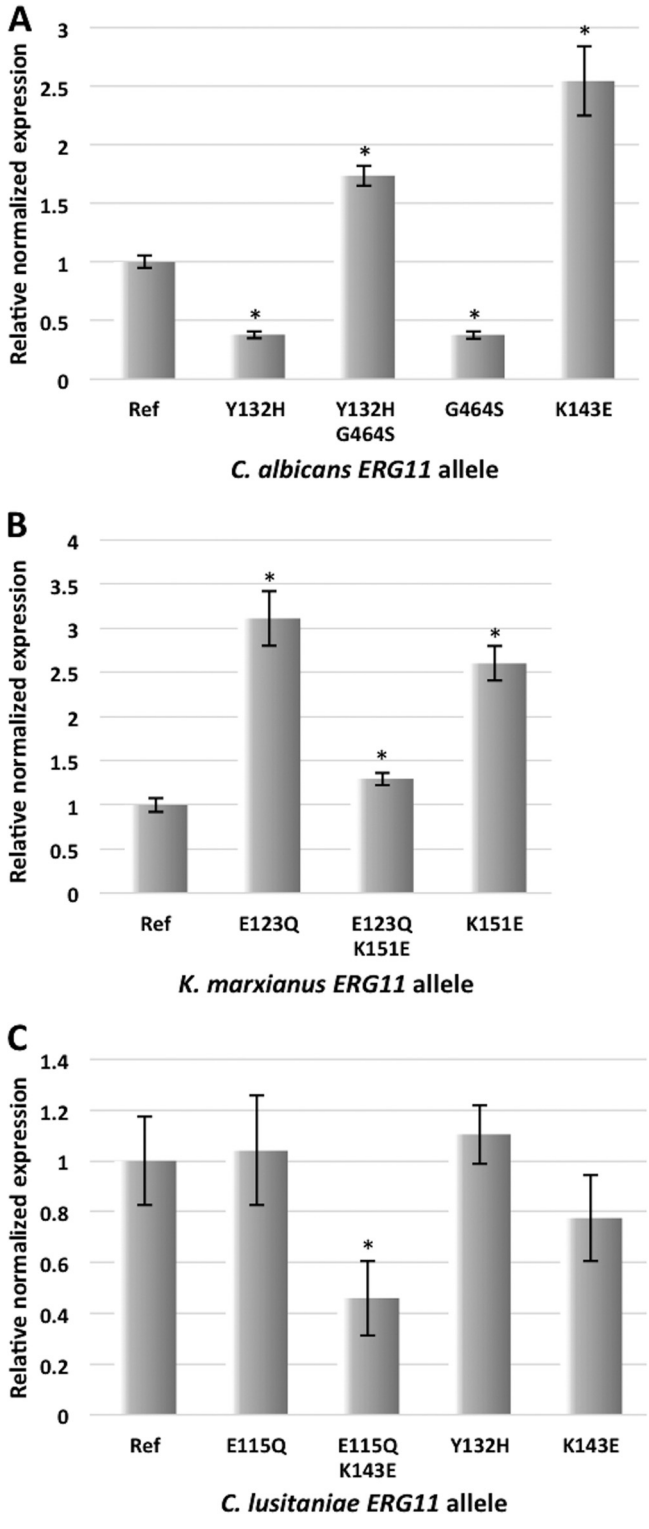

FIG 5 Normalized expression levels of different *ERG11* alleles in the *C. lusitaniae* E11*ura3*Δ*5′* strain grown in RPMI medium. (A) *C. albicans ERG11* allele expression in fold variation relative to the expression level of the reference SC5314 WT *ERG11* allele *A* in *C. lusitaniae*. (B) *K. marxianus ERG11* allele expression in fold variation relative to the expression level of the reference *K. marxianus* PAZ clinical resistant *ERG11* allele in *C. lusitaniae*. (C) *C. lusitaniae ERG11* allele expression in fold variation relative to the expression level of the reference 6936 WT *ERG11* allele in *C. lusitaniae*. Expression of reference alleles (described in Table 1) in the *C. lusitaniae* E11*ura3*Δ*5′* strain was set to 1. *ERG11* allele expression levels were quantified and normalized relative to the housekeeping gene *ACT1*. Values are presented with SEM (standard errors of the means). *, $P < 0.05$ compared to the respective reference *ERG11* allele.

The E123Q and K151E mutations of *KmERG11ci* would be equivalent to E115Q and K143E in *ERG11* alleles of *Candida* species. As a control, the mutations E115Q and K143E were introduced in combination and separately in the *C. lusitaniae ERG11* wild-type allele and expressed in the E11*ura3*Δ*5′* strain. This confirmed that the K143E mutation supported a 16-fold fluconazole MIC increase from 0.5 to 8 μg/ml and that the mutation E115Q did not influence the MIC compared to the wild-type allele (MIC of 0.5 μg/ml). However, we observed repeatedly that when K151/143E was combined with E123/115Q in the *ERG11* alleles of both *K. marxianus* and *C. lusitaniae*, the resulting *ERG11* allele conferred a 2-fold higher fluconazole MIC to the *C. lusitaniae* E11*ura3*Δ*5′* strain than the allele bearing the sole K151/143E mutation. Finally, we introduced the K143E mutation in the *CaERG11wt* allele and showed that it conferred a 128-fold increase of fluconazole MIC (from 0.0625 to 8 μg/ml) when expressed in *C. lusitaniae* E11*ura3*Δ*5′*. We verified that wild-type and mutated *ERG11* alleles were expressed at a similar level in *C. lusitaniae* by reverse transcription-quantitative PCR (Fig. 5A, B, and C).

DISCUSSION

Allelic variability and missense mutations of *ERG11* constitute one of the molecular mechanisms by which fungi can resist the cellular action of azoles, i.e., by decreasing the binding affinity of the enzyme to the antifungal drugs (8, 9, 22, 23). In this work, we genetically engineered a strain of *C. lusitaniae*, a haploid opportunistic pathogenic yeast belonging to the CTG clade of *Candida* yeasts and susceptible to all antifungals (24), for the homologous and heterologous expression of different wild-type and mutated *ERG11* alleles. As the growth of an *erg11* null mutant of *C. lusitaniae* is strongly impaired, the recipient strain used in our experiments harbors its own resident *ERG11* allele until it is replaced by the incoming new *ERG11* allele. Both the selection of yeast transformants and the expected targeting of the new *ERG11* allele are achieved by reconstituting an entire functional metabolic gene, *URA3* here, in the 3′ intergenic region of *ERG11*. Owing to this strategy, *ERG11* allelic replacement occurred in 100% of the prototroph Ura^+ yeast transformants. This efficiency was expected because events leading to nonhomologous recombination cannot be selected, as the genetic marker used in transformation experiments is nonfunctional until it recombines with its complementary part integrated near *ERG11*. The system ensures that any introduced *ERG11* allele is expressed from the same wild-type resident *C. lusitaniae ERG11* promoter.

The *C. lusitaniae* E11*ura3*Δ*5′* strain thus obtained was first challenged by the successful heterologous expression of wild-type *ERG11* alleles of *C. albicans* SC5314. This clinical isolate is susceptible to fluconazole but exhibits a strong trailing growth in the presence of fluconazole concentrations above the MIC. Assembly 22 of the *C. albicans* SC5314 genome sequence now makes it possible to distinguish the nucleotide sequence of both alleles at any particular genetic locus (25). In the SC5314 strain, the *A* and *B* alleles of *ERG11* display 10 SNP, of which two are nonsynonymous (T348A and A383C), resulting in D116E and K128T amino acid changes. As already reported in other isolates (11, 26, 27), these changes were not associated with azole resistance. When expressed individually in *C. lusitaniae* E11*ura3*Δ*5′*, both *CaERG11* alleles conferred full susceptibility to fluconazole with the same MIC as that observed with the SC5314 strain (0.0625 μg/ml). Surprisingly, this fluconazole MIC was about 10 times lower than that conferred by the allele of *C. lusitaniae* (MIC of 0.5 μg/ml), suggesting that the CaErg11 protein is intrinsically more susceptible to fluconazole inhibition. Hence, *C. lusitaniae* can be considered a performing expression system for the genotype-phenotype characterization of *ERG11* mutations, as fluconazole MIC is only dependent on the Erg11 protein expressed and not on the cellular environment in which it is expressed. Trailing growth, interpreted as tolerance to fluconazole, was not observed in *C. lusitaniae* E11*ura3*Δ*5′* expressing *CaERG11*. This was not surprising as, in our experience, trailing growth in the presence of fluconazole was not observed with *C. lusitaniae* wild-type and clinical isolates. This shows that tolerance is not dependent on Erg11 protein but

rather on other factors, such as pH, Hsp90 acetylation, or Vps21-dependent membrane trafficking, as previously reported for *C. albicans* (for a review, see reference 28).

Two amino acid substitutions, Y132H and G464S, then were introduced in the *C. albicans ERG11* allele and expressed in *C. lusitaniae* E11*ura3*Δ*5′*. Both mutations were previously shown to be responsible for a 4-fold increase in fluconazole MIC by heterologous expression of *C. albicans ERG11* alleles in *S. cerevisiae* (11). In our experiments, only the G464S substitution was confirmed to confer a 4-fold fluconazole MIC increase (from 0.0625 to 0.25 μg/ml). The Y132H substitution had no effect, and the lack of synergy between the Y132H and G464S mutation contrasts with a previous work (11). However, the expression systems cannot be compared: one used a *pdr5* mutant of *S. cerevisiae* as the host and a 2μ multicopy plasmid to express a heterologous *ERG11* allele under a galactose-inducible promoter (11), and the other, in this study, used a fluconazole-susceptible *Candida* species where the heterologous *ERG11* allele is introduced as a single copy expressed from the *C. lusitaniae ERG11* endogenous promoter. Surprisingly, in spite of using the same clonal genetic background of strain E11*ura3*Δ*5′*, RT-qPCR assays showed variations in amounts of *ERG11* mRNA detected, possibly due to different mRNA half-lives within the cell or to slight variations of *UPC2* expression, the transcription factor that regulates *ERG11* (29). However, the variations detected, less than 3-fold, are not expected to have a significant effect on fluconazole MIC or to be correlated to a specific resistance pattern, as previously reported in several studies with *C. albicans* (30, 31, 32, 33). Furthermore, both the Y132H and G464S *CaERG11* alleles were expressed at the same level in *C. lusitaniae* E11*ura3*Δ*5′*, indicating that the lack of phenotype with Y132H only was probably not derived from a decreased expression level of *CaERG11*. Alternatively, heterologous expression may lead to posttranslational modifications, enhanced degradation, or protein sequestration that can account for the lack of phenotype. However, when introduced in the *C. lusitaniae ERG11* allele, the mutations Y132H and G460S (equivalent to *C. albicans* G464S) had no effect on the fluconazole MIC, showing that the impact of a mutation on resistance cannot necessarily be extrapolated from one species to another. Most variations in susceptibility to azole antifungals are due to conformational changes of the protein, which themselves depend on specific interactions between several key amino acids that can differ from one species to another.

We then explored whether *C. lusitaniae* could be used to express *ERG11* alleles from non-CTG clade pathogenic yeasts, i.e., from the *Saccharomycetaceae*. We previously described a *Kluyveromyces marxianus* clinical isolate cross-resistant to all azole antifungals (17). The *ERG11* allele of this isolate harbored two nonsynonymous mutations leading to E123Q and K151E substitutions. Expression in *C. lusitaniae* E11*ura3*Δ*5′* of site-directed mutagenized *ERG11* alleles of *K. marxianus*, *C. lusitaniae*, and *C. albicans* provided a new demonstration that substitution at the K151 (*Saccharomycetaceae*)/K143 (CTG clade) position of Erg11p confers resistance to fluconazole (MIC increased by factors of 32, 16, and 128, respectively), as previously reported in K143R mutants of *C. albicans* (23) and *C. tropicalis* (34). Recently, crystal structures of the *S. cerevisiae* (35) and *C. albicans* (36) sterol C14α-demethylases have been resolved. The ionized lysine residue at position 151/143, located in the αC region of Erg11, forms an H-bond with the heme ring D of the catalytic pocket (35, 36). Alteration of this bond could be sufficient to hamper the fixation of azole antifungals to the sixth coordination site of iron. Interestingly, we show here that anionic lysine can be replaced by cationic glutamic acid and not only by anionic arginine, as previously reported (23, 34), to confer high resistance levels. In contrast, the E123Q substitution (E115Q for the CTG clade) had no role in resistance, although a synergistic effect (2× MIC) was always observed when combined with K143/151E. Surprisingly, expression of the *K. marxianus* allele without K151E substitution conferred hypersusceptibility to *C. lusitaniae* to 0.006 μg/ml, while fluconazole MICs reported for the clinical isolates of *C. lusitaniae* generally vary from 0.19 to 4 μg/ml (24).

Besides modifying susceptibility to azole antifungals, the impact of *ERG11* mutations on the biology of the fungal cell remains poorly investigated. At the Erg11p level, some

TABLE 2 Origin and susceptibility to fluconazole of the strains used in this study

Strain	Origin	Genotype	FLC MIC[a] (μg/ml)	S/R[b]	Reference or source
C. lusitaniae CBS 6936	Citrus peel	Wild type	0.5	S	20
C. lusitaniae 6936 *ura3*Δ	CBS 6936	*ura3*Δ	0.5	S	41
C. lusitaniae E11*ura3*Δ*5′*	6936 *ura3*Δ	*ura3*Δ	0.5	S	This study
C. albicans SC5314	Clinical	Wild type	0.0625	S	46
K. marxianus PAZ	Clinical	Wild type	>256	R	17

[a]MIC determined by the microplate dilution CLSI method.
[b]S, susceptible (MIC of ≤2 μg/ml); R, resistant (MIC of >4 μg/ml).

amino acid substitutions decrease the binding affinity of the enzyme to fluconazole (37), while others tend to increase binding affinity to lanosterol (38). In some cases, mutations can decrease the activity of fluconazole-resistant Erg11p, which then could contribute to slight accumulation of 14-methyl fecosterol in the yeast cell, as observed in some Y132F variants of *C. parapsilosis* (39). However, no correlation could be drawn between a specific sterol pattern, azole susceptibility, *ERG11* variability, and *ERG11* expression level in a collection of 39 fluconazole-susceptible and -resistant isolates of *C. parapsilosis* (39). On the other hand, considerable sterol variations were described in a *C. glabrata* clinical isolate harboring a G315D substitution in Erg11p. This mutation nullified the function of Erg11p and allowed viability of the cells in spite of massive accumulation of 14α-methylated sterols and complete lack of ergosterol, with concomitant acquisition of amphotericin B resistance (40). This type of mutation probably cannot be expressed in *C. lusitaniae*, since a null *erg11* mutant is unviable. In the present work, the MIC of amphotericin B for strain E11*ura3*Δ*5′* expressing either its own allele or different *ERG11* alleles is the same (1 μg/ml; data not shown), suggesting few variations, if any, in ergosterol content.

We demonstrated in this study that *C. lusitaniae* can be successfully used as a heterologous expression system to assess the impact of different mutations of the *ERG11* gene on fluconazole susceptibility. We showed that some mutations modulating susceptibility to fluconazole are species specific, while the K143/151E mutation has ubiquitous effects conferring high levels of resistance to fluconazole when present in *ERG11* alleles of different yeasts species. The expression system we have developed is close to the clinical context and MIC determination outcomes: *C. lusitaniae* is pathogenic and susceptible to antifungals, including azoles, and allele replacement is performed at the chromosomal level as a single copy expressed from the endogenous *UPC2*-regulated promoter (unpublished data) of *ERG11*. In this study, the versatility of *C. lusitaniae* as an expression system was tested with *ERG11* alleles of different yeast species, i.e., *C. albicans*, from the CTG clade, and *K. marxianus*, an emerging pathogen of the *Saccharomycetaceae* family. We believe that our biological system could facilitate structure-function studies, since the crystal structure of a fungal Erg11p has been very recently reported for *S. cerevisiae* (35) and *C. albicans* (36). This opens the way to the systematic functional characterization of the interactions between important amino acids of the protein and different substrates of the enzyme, i.e., sterols and cofactors, and antagonist molecules such as azoles.

MATERIALS AND METHODS

Yeast strains and culture conditions. The *Candida lusitaniae* wild-type strain 6936 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was used for gene cloning and as a susceptible reference strain for antifungal agent susceptibility tests. The auxotrophic mutant 6936 *ura3*Δ (41), bearing a deletion of 990 bp in the orotidine 5′-phosphate decarboxylase gene, was used for transformation experiments. *Candida albicans* SC5314 (ATCC MYA-2876) and the *Kluyveromyces marxianus* (anamorph *Candida kefyr*) clinical isolate PAZ (17) were used as *ERG11* donor strains for allelic replacement (Table 2).

Yeasts were cultivated in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 35°C under agitation (250 rpm). In transformation experiments, selection was achieved on synthetic YNBS medium (0.67% yeast nitrogen base without amino acids [Difco Laboratories], 2% glucose, 1 M sorbitol) and, when needed, 25 μg/ml uracil and 0.8 mg/ml 5-fluoorootic acid (5FOA). For antifungal susceptibility tests, we used RPMI 1640 (Sigma). Solid media were obtained with 2% agar (Sigma).

Antifungal agents and susceptibility tests. Stock solutions of 2 mg/ml fluconazole (ICN Biomedicals Inc.) and 100 mg/ml 5-fluoroorotic acid were prepared in sterile distilled water and dimethyl sulfoxide (DMSO), respectively. Microdilution assays were performed according to CLSI standards (42) in RPMI 1640, pH 7.0, buffered with 0.165 M 4-morpholinepropanesulfonic acid (MOPS). Yeast suspensions were diluted with RPMI 1640 medium at a final cell suspension of 10^3 cells/ml. The 96-well plates were incubated for 48 h at 35°C, and growth was measured with an automated microtiter reader at 450 nm. All experiments were done at least in triplicate, and growth variations did not exceed 10%. For each *ERG11* allele expressed in *C. lusitaniae*, fluconazole MIC was determined for seven prototroph clones randomly selected after transformation. Fluconazole susceptibility was also determined using Etest (bioMérieux, France) with concentration ranges from 0.016 to 256 μg/ml. Etest strips were deposited onto solid RPMI medium inoculated with the yeast strains at the cellular density recommended by the supplier.

DNA extraction, PCR amplifications, and nucleotide sequencing. Genomic DNA was extracted by a glass bead method for yeast cell disruption as previously described (41). The high-fidelity DNA polymerase *Pfu* Turbo (Stratagene) was used to amplify DNA fragments by PCR for cloning steps and for overlapping PCR. Routine PCRs were performed with Hot-Start *Taq* DNA polymerase (Qiagen) as recommended by the supplier. All primers used in this study (see Table S1 in supplemental material) were synthesized by Eurofins MWG Operon (Europe). When required, PCR products were sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems) by Eurofins MWG Operon.

Molecular constructions. For the DNA cassette constructions, the different DNA fragments to be assembled were generated by PCR using primers having 15-bp overlap homology at their ends. The DNA fragments then were recombined *in vitro* and cloned in a pUC19 plasmid vector using the In-Fusion HD cloning kit (Clontech). Recombinant plasmid DNA was selected by transforming the Stellar competent *Escherichia coli* cells provided with the kit and plating on LB solid medium supplemented with 50 μg/ml ampicillin according to the supplier's recommendations. DNA cassettes were recovered from the recombinant plasmids by PCR amplification with the relevant primers, purified through QIAquick columns (Qiagen), and used for yeast transformation. All steps of the molecular constructions performed in this work are detailed in Fig. S1 in the supplemental material in connection to the oligonucleotide information presented in Table S1.

Yeast transformations. Auxotrophic strains were transformed by an electroporation procedure previously described (43), slightly modified by adding 1 M sorbitol to the lithium acetate buffer, using 1 to 2 μg of transforming DNA per experiment. Prototrophic transformants were selected on YNBS selective medium after 3 days of incubation at 35°C.

Southern blotting. Approximately 10 μg of *C. lusitaniae* DNA was digested with the appropriate restriction enzyme, separated by electrophoresis in a 1% agarose gel, and transferred onto nylon membranes (Hybond N+; Roche Molecular Biochemicals). Hybridization was carried out with digoxigenin (DIG)-labeled probes synthesized with a PCR DIG probe synthesis kit (Roche Molecular Biochemicals), as specified by the supplier.

RT-qPCR. Total RNA was extracted with a guanidinium thiocyanate-phenol-chloroform extraction procedure (44) using TRI Reagent (Molecular Research Center, Inc.) as described by the manufacturer. Briefly, 10^7 cells were treated with 1 ml TRI Reagent, transferred to a screw-cap 1.8-ml tube containing 450 μl of 0.5-mm acid-washed glass beads, and disrupted with 2 cycles of 40 s at 5 m/s (6,800 rpm) in a Precellys 24 tissue homogenizer at 4°C. The recovered aqueous phase was extracted successively with 5:1 (vol/vol) acidic phenol-chloroform, pH 5.1, and 24:1 (vol/vol) chloroform-isoamyl alcohol and precipitated with isopropanol. Total RNA was solubilized in RNase-free water and quantified with an Agilent RNA 6000 Nano chip.

Biological triplicates were prepared by growing yeast strains to mid-exponential growth phase (1.5×10^7 cells/ml) in 5 ml of RPMI medium. The expression of *ERG11* was determined by RT-qPCR in a one-step real-time PCR system (GoTaq 1-Step RT-qPCR; Promega) and the CFX96 real-time PCR detection system (Bio-Rad Laboratories). Fold changes in gene expression relative to that of control wild-type strain 6936 were determined from *ACT1*-normalized expression levels by the $2^{-\Delta\Delta CT}$ method. The primers used for this assay are described in the supplemental material (Table S1).

Databases and software used in this study. The amino acid sequences of the lanosterol C14α-demethylase proteins of different fungi were retrieved from the National Center for Biotechnology Information (NCBI), GenBank, and UniProt. The names of species, strains, and accession numbers are given in Table S2 in supplemental material. Multiple-sequence alignment and molecular phylogeny were performed using the programs embedded in Seaview v4.5.3 (45). Alignments were carried out with Clustal Omega, and phylogenetic trees were computed using the PhyML v3.1 algorithm (maximum likelihood) with a bootstrap repetition of 100. Fluorescence data from RT-qPCR were collected and analyzed, including statistical tests, with CFX Manager software, version 3.1 (Bio-Rad).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01483-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We have no conflicts of interest to declare.

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