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Identification of genes involved in terbinafine resistance in *Aspergillus nidulans*

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Aims: To determine the pattern and the genetic basis of resistance to terbinafine, a drug extensively used for the treatment of fungal infections in humans.

Methods and Results: Four resistant mutants from *Aspergillus nidulans* isolated after irradiation with ultraviolet light were crossed with the master strain F (MSF). Genetic analysis revealed that a single gene, located on chromosome IV, is responsible for resistance to terbinafine and that the alleles responsible for this resistance in these mutants are of a codominant or dominant nature at high terbinafine concentrations. Furthermore, the interaction of this mutation with another one identified on chromosome II causes the double mutant to be highly resistant.

Conclusions: Periodic surveillance of antimycotic susceptibility would be an important measure in detecting the emergence and spread of resistance. Mutation in a single gene could be responsible for resistance to terbinafine and a genic interaction may be responsible for a higher level of antimycotic resistance.

Significance and Impact of the Study: The understanding of the mechanisms that lead to changes in the sensitivity of a fungus to a given antifungal agent is important both in order to define strategies for the use of such agent and to guide the development of new antifungal agents.

INTRODUCTION

Terbinafine as an antifungal drug is an effective and well tolerated treatment of a wide range of superficial dermatophyte infections. This antimycotic agent, belonging to the allylamine group, acts by blocking the biosynthesis of ergosterol, the major esterol of fungi, by inhibiting the enzyme squalene epoxidase, a complex membrane-bound enzyme system that is not part of the cytochrome P-450 superfamily (Ryder 1992). In fungi this results in ergosterol deficiency of the cell wall and intracellular squalene accumulation. The latter may be the major mechanism responsible for the *in vitro* fungicidal action of terbinafine against most fungal pathogens (Petranyi *et al.* 1987; Gupta and Shear 1997).

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One of the major problems in treatment of fungal infections is the occurrence of strains resistant to antifungal agents and developing an understanding the mechanisms of resistance would be of great practical importance. Resistance to terbinafine in *Candida albicans* can be mediated by multidrug efflux transporters coded by the CDR2 gene (Candida drug resistance) (Sanglard *et al.* 1997). However, the terbinafine-resistant mutants of *Nectria haematococca*, a phytopathogenic fungus, contain at least 10 times more squalene than the wild-type strain, possibly suggesting the presence of a modified squalene epoxidase in these mutants. Thus, a reduced affinity of the squalene epoxide for both the substrate and the fungicide could explain terbinafine resistance in these mutants (Lasseron-De Falandre *et al.* 1999).

In the present study we analysed the genetic basis of resistance in *A. nidulans*, a convenient model for this purpose, and described four mutations at the *tsbA* locus of this fungus conferring resistance to terbinafine. We also

identified another gene (*tebB*) involved in this resistance that increased the level of terbinafine resistance. Furthermore, we discuss the effect of terbinafine on mitotic instability and cleistothecium production in this fungus.

MATERIALS AND METHODS

Fungal strains

The following strains of *A. nidulans* were used: *pabaA1*, used to select resistant mutants; *frA1 pabaB22 pyroA4 inoB2*, all markers on the linkage group IV of *A. nidulans*, and Master Strain F (MSF), which carries markers on all eight linkage groups (*adE20, suA1adE20, yA2, acrA1, galA1, pyroA4, facA303, sB3, nicB8 and riboB2*). The mutant alleles used in the present study were: yA2, yellow conidia; *facA303*, *frA1* and *galA1* inability to grow on acetate, fructose and galactose as sole carbon source, respectively; *adE20*, *inoB2*, *nicB8*, *pabaA1*, *pabaB22*, *pyroA4*, *riboB2* and *sB3*, growth requirements for adenine, inositol, nicotinic acid, *p*-aminobenzoic, *p*-aminobenzoic, pyridoxine, riboflavin and thiosulphate, respectively; *suA1adE20*, a suppressor of *adE20*; and *acrA1*, a marker for resistance to acriflavine.

Fungicide and media

Terbinafine ((E)-N-(6,6-Dimethyl-2-hepten-4-yn-yl)-N-methyl-1-naphthalene methanamine) ($C_{21}H_{25}N$) purchased from Sandoz (Sandoz S.A., Brazil) was dissolved in dimethylsulfoxide (DMSO) and added to the solid medium to isolate and characterize the strains. Minimal medium (MM) and complete medium (CM) were those described by Pontecorvo *et al.* (1953). The incubation temperature was 37 °C throughout.

Isolation of mutants and dosage response

Spores from the *pabaA1* strain were treated with UV light (survival rate of about 5%) (Cuadros *et al.* 1999) and incubated on CM dishes containing the terbinafine antimycotic at a concentration that inhibited growth (1.0–2.5 $\mu g\ mL^{-1}$). Resistant colonies were isolated on the 7th day of incubation and characterized by measuring the diameters of colonies grown for 72 h in CM dishes containing several terbinafine concentrations. Relative toxicity was expressed as ED50, i.e. the concentration reducing the colony radial growth by 50% (Martinez-Rossi and Azevedo 1989; Andrade-Monteiro *et al.* 1994).

Genetic analysis

Genetic analysis of these mutants was that usually employed for *A. nidulans* (Pontecorvo *et al.* 1953). Mutants were assigned to their chromosome by mitotic analysis and diploids were constructed as described by Roper (1952). Crosses between two resistant strains were performed to carry out the allelism test.

RESULTS

Four terbinafine-resistant colonies were randomly selected from the survivors of the *pabaA1* strain after mutagenic treatment (Terb7, Terb8, Terb9 and Terb10). The relative terbinafine resistance of these resistant mutants, expressed as the terbinafine concentration that inhibited 50% colony growth, was about 80–140 times higher than that of the original *pabaA1* strain (Table 1).

Each resistant strain was crossed with the MSF strain, which is sensitive to terbinafine. In all crosses the resistant and sensitive phenotypes segregated at a 1 : 1 ratio, indicating inheritance of a single gene mutation. The resistance phenotype segregated against the *pyroA* marker located on chromosome IV.

Crosses between the resistant segregants (Terb7 X Terb8; Terb7 X Terb9; Terb7 X Terb10 and Terb8 X Terb9) showed allelism of the *tebA7, tebA8, tebA9 and tebA10* mutations, since ascospores from hybrid cleistothecia produced only resistant colonies. At least 250 meiotic segregants from each cross were analysed. The *tebA* gene was mapped about 25.0 units from the *pyroA* gene, which is also located on chromosome IV of *A. nidulans*. Crosses between Terb7 and *frA1 pabaB22 pyroA4* strains, and Terb7 and *inoB2* strains revealed the relative position of gene *tebA* in the genome of *A. nidulans* (Fig. 1). The *inoB2* mutation located on chromosome IV segregated independently of *tebA* gene. The dose–response curves for the Terb7 mutant in the presence of terbinafine

Table 1 Relative toxicity (ED_{50}) of sensitive, resistant and hyper-resistant haploid and heterozygous diploid strains of *A. nidulans* to terbinafine

Strains	Relevant Genotype	ED_{50} ($\mu g\ ml^{-1}$)
pabaA1	*tebA*$^+$	0.01
MSF	*tebA*$^+$ *tebB1**	0.02
Terb7	*tebA7*	1.4
Terb8	*tebA8*	0.9
Terb9	*tebA9*	0.8
Terb10	*tebA10*	1.3
MSF// Terb7	*tebA*$^+$ *tebB1*// *tebA7 tebB*$^+$	0.4
MSF// Terb8	*tebA*$^+$ *tebB1*// *tebA8 tebB*$^+$	0.8
MSF// Terb9	*tebA*$^+$ *tebB1*// *tebA9 tebB*$^+$	0.9
MSF// Terb10	*tebA*$^+$ *tebB1*// *tebA10 tebB*$^+$	0.4
Segregant 1	*tebA7 tebB1**	3.8
Segregant 2	*tebA10 tebB1**	3.4

*Probable genotype.
//diploid strains.

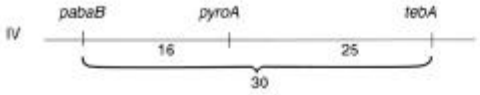

Fig. 1 Mapping of the locus conferring terbinafine resistance (*tebA*) in *Aspergillus nidulans*. The locus was mapped meiotically on chromosome IV in relation to other markers in the same chromosome

and the ED50 values for the heterozygous diploids when compared to the haploid parents (Fig. 2 and Table 1) indicate that resistance to terbinafine represents a codominant character. This pattern is the same for the *tebA10* mutation but the *tebA8* and *tebA9* mutations presented mostly a dominant character (Table 1).

Among the resistant segregants from crosses between each resistant strain and MSF, 50% showed higher resistance to terbinafine than the four original isolates. This observation raised the possibility of a second mutation, not linked to the *tebA* gene that may be responsible for increasing the level of terbinafine resistance. In fact, this mutation (named *tebB1* here) was located on chromosome II based on the mitotic segregation of terbinafine resistance with genetic markers located on that chromosome. The level of resistance to terbinafine of two of these hyper-resistant segregants (Segregants 1 and 2) (about 340–380 times higher than *pabaA1* strain) is shown in Table 1. A typical result of the meiotic segregation from crosses between Terb7 (and other) strain and MSF indicating that *tebB* gene is close to *acrA* gene, a marker located on chromosome II, is shown in Table 2.

Table 2 Meiotic analysis of the terbinafine-resistant mutants (Terb7 X MSF)

Sensitive	Resistant			
	Resistant		Hyper-resistant	
	acrA$^+$	*acrA1*	*acrA*$^+$	*acrA1*
137	46	15	7	59

Furthermore, we observed that a subinhibitory concentration of terbinafine stimulated the production of cleistothecia in haploid strains resistant or sensitive to this fungicide. Also, terbinafine induced sectored growth with normal morphology in haploid or diploid strains cultivated on solid CM.

DISCUSSION

In the present study we isolated terbinafine-resistant mutants of *A. nidulans* to analyse the pattern and the genetics basis of resistance to terbinafine. We found that four resistant mutants selected after UV treatment of the *pabaA1* strain showed mutation in the same gene, denominated *tebA*. The role of this gene in the wild type strain was not determined but all mutants grew and conidiated as well as the wild type strain on complete medium. Terbinafine-resistant mutants in *Nectria haematococca*, induced by UV light, also resulted from mutation in only one gene, a modified squalene epoxidase with a reduced affinity for terbinafine has been suggested to

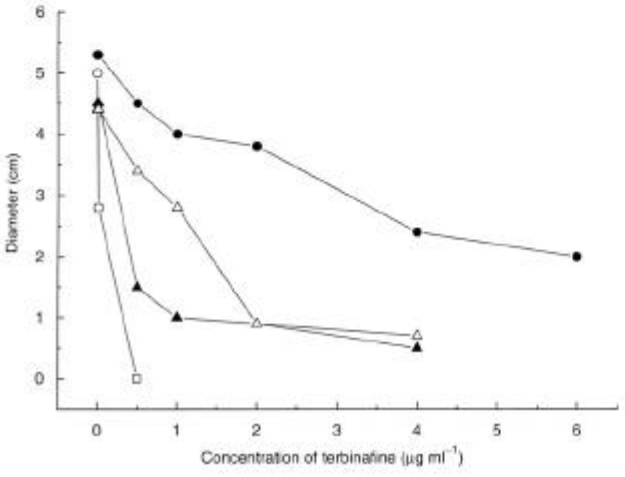

Fig. 2 Dose-response curves for the terbinafine-resistant strain Terb7 (△), the hyper-resistant strains (segregant 1) (●), the diploid strains Terb7//MSF (▲), the *pabaA1* strain (×), and the Master strain F(○) of *Aspergillus nidulans*

explain resistance in these mutants (Lasseron-De Falandre *et al.* 1999). The majority of these resistant mutants were not affected in their growth rate, sporulation or pathogenicity (Lasseron-De Falandre *et al.* 1991). However, mutants that alter the expression of ERG9, a gene encoding the squalene synthase of *Saccharomyces cerevisiae*, show altered susceptibility to terbinafine (Kennedy and Bard 2001). These mutations altered transcriptional regulation of the ERG9 gene affecting flux through the ergosterol biosynthetic pathway. The authors proposed that when the expression of squalene synthase increases this in turn increases the flux through the pathway by increasing the intracellular concentration of enzymes in the pathway. This would explain the decreased susceptibility to terbinafine and other antifungal compounds that target enzymes in the ergosterol biosynthetic pathway (Kennedy and Bard 2001). Another possibility that should be considered is genes involved in the transcriptional regulation of expression of the gene that encodes the squalene epoxidase (Leber *et al.* 2001). Mutation in these genes should alter the susceptibility to terbinafine.

We also found a second mutation (named *tebB1*), probably carried by MSF, which, in combination with *tebA* mutations, causes the double mutant to become highly resistant to terbinafine. Curiously, MSF by itself is sensitive to terbinafine probably because the *tebA*$^{+}$ allele is epistatic to gene *tebB* (or at least epistatic to the *tebB1* allele). The role of the *acrA1* mutation, located on chromosome II of MSF, in this hyper-resistance phenotype was ruled out because some of the terbinafine hyper-resistant meiotic segregants were *acrA*$^{+}$, i.e. sensitive to acriflavine.

This gene interaction was not observed in diploid strains since their dose–response curves in the presence of terbinafine were similar to or below the dose–response curves presented by the resistant strains. This suggests that the mutation carried by the *tebB* gene has a recessive character. In contrast, all mutations in the *tebA* gene showed a codominant or dominant character. It is known that there is considerable variation in the degree of dominance depending on the mutations, the antimycotic and the concentration tested. Here, the mutation and the role of each gene seems to be the determinant factors in the difference in the degree of dominance between the *tebA* and *tebB* mutations. The nature of this interaction will be better understood by molecular analysis of our mutants.

Ergosterol is involved in chromosome distribution during mitosis and inhibitors of ergosterol biosynthesis such as azoles may interfere with this process, causing chromosome nondisjunction in *A. nidulans* (Bellicampi 1980). The phenomenon of sectorization in several strains of this fungus in the presence of subinhibitory concentrations of terbinafine suggests that this antimycotic agent has a similar effect. In fact, the sectorization phenomenon occurs much more effectively in diploid strains. Furthermore, the presence of terbinafine stimulated clestothecium formation in terbinafine-sensitive and -resistant strains. This phenomenon, which may be affected by nutritional and other chemical factors, could be a response to the cellular stress caused by terbinafine and thus represent another form of terbinafine resistance in this fungus.

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