

GENETIC STUDY OF A TRIADIMEFON-RESISTANT MUTANT OF *Aspergillus nidulans*

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ABSTRACT

The *trdA9* gene was identified in *Aspergillus nidulans* which confers resistance to the fungicides triadimefon and imazalil. This gene mapped in linkage group VII, is probably an allele to *imaA4*, and behaved as a dominant character. Although the degree of resistance shown by the mutant strain is relatively low, it was suggested that the mutant may be useful for the development of a DNA transformation system in *A. nidulans*. Also it seems that triadimefon does not induce point mutations, in the methionine system of *A. nidulans*.

INTRODUCTION

Triadimefon is a wide-spectrum systemic fungicide in which the basic structural unit is an azole. Azoles, which contain two or three nitrogen atoms in their five-membered rings, represent the structural nucleus of several drugs systemically utilized in human therapy of fungal infections and also against phytopathogenic fungi (Saag and Dismukes, 1988). These fungicides have a similar mechanism of action, i.e., they inhibit ergosterol biosynthesis by disrupting the plasmalemma and the intracellular membrane system of the fungi and they also affect a number of cellular events (Buchenauer, 1977; Stiers *et al.*, 1980; Hippe, 1984a,b; Hippe and Niedermeyer, 1984; Richmond, 1984; Burden *et al.*, 1989). Polyene antibiotics, which act on the membrane by complexing sterols, and fenarimol (an azole fungicide) significantly induce mitotic nondisjunction in a diploid strain of *Aspergillus nidulans* (Bellincampi *et al.*, 1980). Although selection of mutants resistant to fungicides that inhibit ergosterol synthesis has been difficult, it has been possible to identify *in vitro* the involvement of a multigenic system in the resistance of some of these fungicides. In *A. nidulans*, at least

10 loci are responsible for resistance to imazalil, a systemic fungicide with an azole nucleus. These different loci confer different resistance levels as well as cross-resistance to other fungicides that have the same mechanism of action (Tuyl, 1977). Because azole compounds are used in agriculture and in medicine, in addition to determining their mechanism of action, it is important to study their mutagenic potential and the genetic mechanisms involved in the resistance to these fungicides. With this in mind, mutants of *A. nidulans* resistant to triadimefon were selected and assayed for the potential of this fungicide in inducing point mutations in the methionine system of *A. nidulans*. The *trdA9* gene was identified which confers resistance to triadimefon and imazalil fungicides and shows dominance in relation to its wild allele. It appears that triadimefon does not induce point mutations in the genetic system assayed.

MATERIALS AND METHODS

Strains

The following *A. nidulans* strains were used: *proA1 pabaA6 yA2* to select resistant mutants, Master Strain E, which carries markers on all eight linkage groups (McCully and Forbes, 1965), for genetic analysis, *biA1 methG1* for point-mutation induction assays, and FGSC 715 (*biA1 acrA1 actB1 oliC1 benC28 cbx1 imaA4*) to carry out allelism tests. Strain FGSC 715 was obtained from the Fungal Genetic Stock Center, Dept. of Microbiology, University of Kansas Medical Center, Kansas City, USA, and the others were derived from the Glasgow Stock. The mutant alleles used in the present work were the following: *yA2*, yellow conidia; *biA1*, *methG1*, *pabaA6*, *proA1*, having growth requirements for biotin, methionine, *p*-aminobenzoic acid and proline, respectively; *acrA1*, *actB1*, *oliC1*, *benC28*, *cbx1* and *imaA4* are resistance markers for acriflavine, actidione, oligomycin, benomyl, carboxin and imazalil, respectively. All incubations were carried out at 37°C.

Media and fungicides

Minimal medium (MM) and complete medium (CM) were those described by Pontecorvo *et al.* (1953). Triadimefon (1-(4 chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)-2-butanone) and benomyl (methyl-1-butylcarbamoil-2-benzimidazole carbamate) are commercial fungicides from Bayer AG (Germany) and E.I. Du Pont de Neumors & Co. (USA), respectively. Imazalil (1-(β -allyloxy-2,4-dichlorophenethyl) imidazole nitrate) was kindly provided by Janssen Pharmaceutica (Belgium).

Test system

The test system used was the induction of methionine suppressors *SumethA1* (thinly growing colonies), *sumethB1* (brown pigment colonies) and *SumethC1* (thick colonies with hyaline edges) (Siddiqi, 1962; Lilly, 1965). The fungicides were dissolved in sterile distilled water and immediately added in minimal volumes to the autoclaved medium cooled to 45°C, to give the desired concentrations. 10⁸ conidia of the *biA1 methG1* strain were plated for mutant selection onto methionineless medium containing biotin, supplemented with the fungicide to be tested. Plates containing no fungicide were used as control. Also, suitable conidial dilutions were plated onto a medium supplemented with methionine and biotin for viable counts and onto the same medium plus the fungicide for survival determinations. All mutant colonies were scored after five days of incubation.

Isolation of resistant mutants and fungitoxicity test

Triadimefon resistant mutants were induced with benomyl as described by Speakman and Nirenberg (1981), using concentrations of the fungicide which reduced the wild type colony radial growth by 25% and 50%. About 10⁷ conidia of the *proA1 pabaA6 yA2* strain were plated on 12.5 ml of CM containing an appropriate concentration of benomyl. The plates were incubated for 8 h at 37°C to permit germination of inoculated conidia. All plates were then overlaid with 12.5 ml of CM containing an inhibitory concentration of triadimefon (500 µg/ml). Incubation was continued for a further 12 days before resistant colonies could be isolated. The toxicity of triadimefon was estimated by the reduction of colony size after incubation of the strains for four days in solid-medium plates containing different concentrations of the fungicide. The relative toxicity was expressed as percentage of growth in the presence of the fungicide or ED₅₀, which is the concentration that causes 50% reduction in radial growth.

Genetic analysis

General techniques were those of Pontecorvo *et al.* (1953). Diploids were obtained according to the technique of Roper (1952) and mitotic analysis was done by haploidization (Forbes, 1959) induced by *p*-fluorophenylalanine (Lhoas, 1961; Morpurgo, 1961). The allelism test was carried out by crosses between the two resistant strains.

RESULTS AND DISCUSSION

Table I shows that the fungicide triadimefon is not effective in inducing point mutations in the methionine suppressors in *A. nidulans*. Indeed, it causes total inhibition of *SumethC1* revertants and decreased frequency of the *SumethA1* and *sumethB1* revertants at doses of 3 μg and 5 μg fungicide per ml medium, respectively. Even though the frequency of reversion at the *sumethB1* locus was increased at the triadimefon concentration that permitted 82% conidial survival (10 $\mu\text{g}/\text{ml}$), this increase was not sufficient for the fungicide to be considered a mutagen (Gabridge and Legator, 1969). This increase may have been a consequence of a morphological change in the original *biA1 methG1* strain (data not shown) leading to an overestimate of *sumethB1* revertants in relation to the other two revertants scored. As also shown in Table I, the frequency of the *SumethA1* locus was also greatly decreased (about 40 times) when the fungicide was used at a concentration of 10 μg per ml medium. A decrease in the frequency of reversions at concentrations that have little or no effect on the survival of the original strain may indicate, as also reported for cercobin fungicide (Martinez-Rossi and Azevedo, 1987), that the methionine-independent mutants are more sensitive to triadimefon than the original strain. If the revertants are more sensitive than the original strain, the different types of methionine-independent mutants may be underestimated, thus representing a false non-mutagenic effect of the fungicide. Therefore, we cannot exclude the effect of triadimefon, although indirect, on the DNA of *A. nidulans*, a fact that makes it necessary to analyse the mutagenic potential of triadimefon and other azole compounds on other test systems.

Table I - Effect of triadimefon on reversion of the strain *biA1 methG1* to methionine independence.

| Triadimefon concentration ($\mu\text{g}/\text{ml}$) | Percent survival | Frequency of revertants/ 10^6 survivors | | |
|---|---------------------|---|-----------------|-----------------|
| | | <i>SumethA1</i> | <i>sumethB1</i> | <i>SumethC1</i> |
| 0 | 100 | 1.3 | 1.3 | 0.1 |
| 1 | 100 | 0.9 | 0.8 | 0.1 |
| 3 | 88 | 1.1 | 1.8 | 0 |
| 5 | 85 | 0.9 | 0.9 | 0 |
| 10 | 82 | 0.03 | 1.7 | 0 |

The efficient action of azole fungicides as inhibitors of ergosterol biosynthesis and the consequent difficulty in the development of resistance to these fungicides under practical conditions prompted us to study the genetic principles that may lead to the development of resistance in *A. nidulans*. Using benomyl fungicide as a mutagen and triadimefon at a concentration of 500 µg per ml medium to select resistant strains, we obtained five mutant strains resistant to triadimefon from 10⁷ conidia that survived benomyl. None of these mutant strains presented any resistance to benomyl. As shown in Table II, the triadimefon ED₅₀ value for TRD 9 was about 1.5 times higher than that shown by the sensitive strains, indicating a relatively low level of resistance to this fungicide. Ascospores from hybrid cleistothecia from a cross between TRD 9 and MSE showed a segregation of one resistant to one non-resistant, which indicates a mutation in a single gene. This gene was allocated to linkage group VII by haploidization analysis and no genetic linkage was observed between the resistance marker and *nicB8*, which is also located in linkage group VII.

Table II - Relative toxicity of triadimefon and imazalil for various strains of the fungus *A. nidulans*.

| Strain | Relevant genotype | ED ₅₀ ^a (µg/ml) | |
|-------------------------|-------------------|---------------------------------------|------------|
| | | Triadimefon | Imazalil |
| MSE | - | 9.1 | 0.05 |
| <i>proA1 pabaA6 yA2</i> | - | 9.5 | Not tested |
| TRD 9 | <i>trdA9</i> | 15.8 | 0.37 |
| TRD9//MSE | <i>trdA9</i> | 19.0 | Not tested |
| FGSC 715 | <i>imaA4</i> | 25.1 | 0.66 |

^a These values represent the fungicide concentrations that caused 50% reduction in maximal colony growth.

Cross-resistance to inhibitors of ergosterol biosynthesis has been shown by some resistant mutants. Sherald and Sisler (1975) and Waard and Sisler (1976) selected triarimol-resistant mutants of *A. fumigatus* and *A. nidulans* that also showed resistance to triforine and fenarimol, respectively. A triadimefon-resistant strain of *Ustilago maydis* was resistant to triarimol (Leroux and Gredt, 1976). As shown in Table II, we also observed cross-resistance between triadimefon and imazalil in *A. nidulans*. The level of resistance to imazalil shown by the TRD 9 strain was about 7.4 times higher than that shown by the sensitive strain. Furthermore, the FGSC 715 strain which carries the *imaA4* mutation, one of the genes conferring resistance to imazalil,

also showed resistance to triadimefon (Table II). Crosses between these two strains (FGSC 715 and TRD 9) showed allelism or close linkage of *imaA4* and the *trdA* marker. The dose-response curve of the heterozygous diploid TRD 9//MSE (*trdA9*//+) to triadimefon (not shown) suggests that *trdA9* is dominant for triadimefon resistance (see ED₅₀ in Table II), whereas the dose-response curve of the heterozygous diploid *imaA4*//+ showed an intermediate degree of dominance for imazalil resistance, or a semi-dominant character (Tuyl, 1977). Because these mutant strains show distinct characteristics we could consider them as distinct mutations within the same gene (Erickson *et al.*, 1985). Furthermore, mutants with a dominant selectable phenotype are useful to develop a DNA transformation system. Most of the transformation systems available require specific auxotrophic recipient strains. A transformation system based on a dominant selectable marker gene would be much more advantageous, since non-auxotrophic strains could be transformed (Fernández-Larrea and Stahl, 1989). The benomyl-resistant gene of *Neurospora crassa* was used as a dominant selectable marker gene to transform *Podospora anserina* (Fernández-Larrea and Stahl, 1989). We believe that this *trdA9* gene which confers resistance to triadimefon is also useful to transform sensitive strains of *A. nidulans* or even other fungi.

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RESUMO

Foi identificado um gene denominado *trdA9* em *Aspergillus nidulans*, o qual confere resistência aos fungicidas triadimefon e imazalil. Este gene foi mapeado no grupo de ligação VII, provavelmente alelo ao *imaA4*, e apresentou um caráter dominante em diplóide heterozigoto. Embora o grau de resistência mostrado pela linhagem mutante seja relativamente baixo, sugeriu-se que ele possa ser usado para desenvolver um sistema de transformação. Além disto, foi mostrado que o triadimefon não induz mutação gênica no sistema metionina de *A. nidulans*.

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