

Duplicate gene inactivation affects ascospore viability in *Aspergillus nidulans*

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ABSTRACT

A gene inactivation system (GIS) alters duplicated genes in the *Aspergillus nidulans* genome. GIS activity only occurs in the sexual cycle of the strain bearing the *Dp(II-I)* duplication. A reduction of the number of ascospores per cleistothecium and of their viability was observed in meiotic crosses, heterozygous for the *Dp(II-I)* duplication. In the present work a gene inactivation system that acts in the *A. nidulans* genome is characterized. It influences ascospore viability among the meiotic segregants of the first and second generations (F1 and F2) and reoccurrence of the process after 5-azacytidine treatment of the segregants.

INTRODUCTION

In Ascomycetes, methylation of duplicated genes is related to the gene inactivation process (Fincham, 1989; Goyon and Faugeron, 1989; Castro-Prado and Zucchi, 1993). In *Neurospora crassa*, meiotic instability of transformant DNA segments, present in more than one copy in the receptor genome, allows the characterization of RIP (repeat induced point mutation), a phenomenon which permits recognition of duplicated sequences, inactivating the expression of genes included on them (Selker *et al.*, 1987; Selker, 1990). RIP alters both copies of the duplication, promoting GC-AT transitions. Such transitions are consequences of deamination of the 5-methyl-cytosine

residues (Cambareri *et al.*, 1989). These alterations have only been observed in the sexual cycle of *N. crassa*, during the period from fertilization to karyogamy.

Although RIP activity results in base methylation and transitions, the cells can survive the inactivation of an essential duplicate gene, because RIP activity is limited to the nucleus, acting only in cells presenting one nucleus of each parent (dikaryon) (Selker *et al.*, 1987; Selker, 1990). However, a drastic reduction in the number of ascospores is observed among the progeny of crosses involving strains bearing duplicated genes, when compared to those involving normal ones (Cambareri *et al.*, 1989; Glass and Lee, 1992).

Linked duplications are invariably altered by RIP, while non-linked ones are altered at frequencies ranging from 10 to 50% (Foss and Selker, 1991). This process is repeated in the first meiotic generation (F1) strains, i.e., in strains already submitted to the RIP phenomenon (Okamoto *et al.*, 1993). Apparently RIP can avoid the rough chromosomal rearrangements commonly generated by crossing-over between homologous duplicated sequences, since these

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sequences would be submitted to the RIP process until they became highly divergent (Cambareri *et al.*, 1991; Rand, 1992).

Among the Ascomycetes, gene inactivation systems similar to RIP have been described in *Podospora anserina* (Debuchy *et al.*, 1988), *Ascobolus immersus* (Faugeron *et al.*, 1990) and *Aspergillus nidulans* (Castro-Prado and Zucchi, 1993). The main characteristic separating them from RIP is that inactivation of duplicated genes was observed to occur only by methylation of the cytosine residues. Spontaneous and 5-azacytidine-induced reactivation of these genes have also been observed (Goyon and Faugeron, 1989). Previous studies revealed the strong effect of gene inactivation system (GIS) on the sexual cycle of strains bearing the *Dp*(II-I) duplication, reducing ascospore number per cleistothecium and affecting the number of both parental segregant classes (Castro-Prado and Zucchi, 1993). The low efficiency of GIS in the non-linked *Dp*(II-I) duplication has also been demonstrated by means of the isolation of segregants bearing duplicated genes (that had not been affected by GIS), among progenies of crosses heterozygous for the duplication (Castro-Prado and Zucchi, 1996). Mutants bearing in tandem duplication should provide additional support to this observation.

MATERIAL AND METHODS

Strains

The UT448 and UT196 strains were derived from Utrecht stocks and the others originated from our laboratory. Figure 1 presents the genotypes and phenotypes of the strains used, invariably kept in complete medium, at 5°C. The DWM, D3 and 3⁷ strains bear a *Dp*(II-I) duplication that includes the genetic markers *Acr*, *w* and *meth*⁺ in the duplicated segment. D3 is a mitotic segregant of the Z1//UT196 diploid (Castro-Prado and Zucchi, 1991) that presents duplicated genes in heterozygosis, not affected by the inactivating mechanism (Figure 1).

Media

Minimum medium (MM) was Czapeck-Dox with 1% (w/v) glucose. Complete medium (CM) contained yeast extract, hydrolyzed casein, hydrolyzed nucleic acids, vitamins, etc. (Van de Vate and Jansen, 1978; Zucchi, 1990). Solid media (MM or CM) contained 1.5% agar.

Genetic analysis

The general methodology followed those described by Pontecorvo *et al.* (1953) and by Van de Vate and Jansen (1978). Heterokaria were prepared in liquid MM plus CM (2%). Cleistothecia were obtained from the heterokarya, after 21 days of incubation at 37°C, in sealed Petri dishes containing solid MM, supplemented according to the requirements of the crossed strains.

Gene reactivation

Reactivation of the *meth*⁺ and *Acr* genes (inactivated by GIS) was accomplished by seeding conidia of the strains presenting *meth* and *Acr*⁺ phenotypes in Petri dishes containing supplemented MM, according to the strain requirements, plus 300 µM 5-azacytidine (Bull and Wooton, 1984). The strains were incubated during

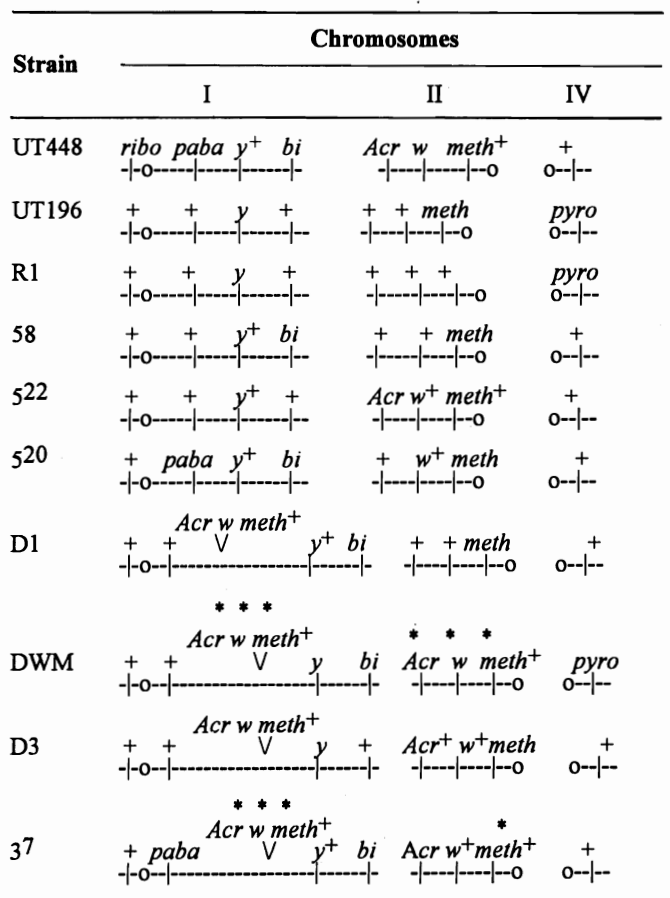


Figure 1 - Schematic diagram of chromosomes I, II and IV of the *Aspergillus nidulans* strains used.

Asterisks (*) indicate the inactivated genes; V represents a duplicated segment, transposed from chromosome II to I.

five days at 37°C, and afterwards conidia were collected from the colonies in saline solution and seeded on two sets of media: a) MM supplemented with the strain requirements plus acriflavine (62.5 µg/ml) and b) supplemented medium lacking methionine. After 48 h of incubation at 37°C the revertants were observed.

Ascospore viability

The number of ascospores, inside each analyzed cleistothecium, was determined in a Neubauer chamber. Samples of ascospore suspensions at appropriate dilutions were seeded in CM and the developed colonies were observed after 48 h of incubation at 37°C.

Statistical analyses

The results were analyzed with the Kruskal-Wallis test.

RESULTS AND DISCUSSION

Ascospore viability among the first generation of meiotic segregants

A drastic reduction in the number and viability of ascospores was observed among the progeny of the D3 x 58 cross, in the cleistothecia (Table I). The segregation analyses of the markers of chromosomes I and II, among the viable progeny from six cleistothecia, presenting ascospore concentrations lower than 10⁴ ascospore/ml (minimum number for scoring in the Neubauer chamber), revealed that the parental class, bearer of the *Dp*(II-I), was selectively and systematically eliminated (Table II).

These results are a consequence of GIS activity in the nucleus of the D3 strain during the sexual cycle. As D3 is a mitotic segregant, and considering that GIS (as far as it is known) does not act in mitosis, the lethal effect of its activity was evident in this cross because D3 had not been affected by GIS before. The predominance of *y*, *bio*⁺, *meth*⁺ segregants, observed among the progeny of the cleistothecium number 7 (Table II), suggests that some nuclei can escape from GIS activity maintaining normal expression of the duplicated genes.

Table I - Total number and percent of viable ascospores per cleistothecium obtained from the DWM x 5²², UT448 x UT196 (control), and D3 x 58 crosses.

N	DWM x 5 ²²		UT448 x UT196 (control cross)		D3 x 58	
	Total ascospores	Viability (%)	Total ascospores	Viability (%)	Total ascospores	Viability (%)
1	207,050	39.0	575,000	53.5	45,000	20.0
2	8,510	70.5	545,000	72.0	23,500	6.5
3	75,000	42.4	450,000	35.5	65,000	9.0
4	54,979	71.3	430,000	90.7	7,500	1.1
5	107,963	65.3	400,000	100.0	140,000	3.6
6	115,010	92.6	450,000	25.6	156,000	2.0
7	97,560	73.8	323,130	29.4		
8	65,000	100.0	325,000	36.9		
9	107,500	14.0	237,000	18.4		
10	126,000	13.2	450,000	32.2		
11	8,750	27.6	322,500	30.0		
12	101,246	13.4	422,500	15.0		

Ascospore viability among the second generation of meiotic segregants

The DWM strain is a first generation segregant obtained among the progeny of the D1 x 20² cross (Castro-Prado and Zucchi, 1993) and had already been submitted to GIS. This strain presents the *Acr*⁺, *w*, *meth* phenotype, but the *Acr*⁺ and *meth* markers change to *Acr* and *meth*⁺, respectively, when this strain grows in the presence of 5-azacytidine (Castro-Prado and Zucchi, 1996). Such data indicate that those genes must be present in the DWM strain, but are methylated and inactivated. This observation was confirmed from the analysis of the DWM x 5²² cross, in which no significant difference in ascospore viability was found, when compared to the control cross (Kruskal-Wallis test, *P* < 0.05, Tables I and III). Apparently the DWM strain became resistant to the lethal effect of GIS when submitted to a new sexual cycle. Although GIS can still act in the nucleus of DWM strain, the alterations were probably less intense and did not even affect ascospore viability.

Table II - Meiotic segregation of the *y*, *bio*, *w* and *meth* markers in the D3 x 58 cross. (P1) = D3; (P2) = 58.

Cleistothecium	<i>y</i> , <i>bi</i> ⁺ , <i>meth</i> ⁺ (P1)	<i>y</i> ⁺ , <i>bi</i> , <i>meth</i> (P2)	<i>w</i> , <i>bi</i> ⁺ , <i>meth</i> ⁺
7	15	1	0
8	11	11	0
9	0	24	0
10	5	41	1
11	2	57	0
12	36	85	0

Table III - Total number and percent of viable ascospores per cleistothecium obtained from DWM x 5²², UT448 x UT196 (control) and D3 x 58 crosses. The demethylated DWM strain genotype is *bio*(I); *Acr*, *w*, *meth*^(II); *pyro*(IV).

Cleistothecium number	DWM (demethylated) x 5 ²²	
	Total ascospores	Viability (%)
1	0	0
2	62,011	17.9
3	0	0
4	31,250	45.2
5	0	0
6	22,500	38.4
7	0	0
8	0	0
9	65,000	59.4
10	35,000	17.1
11	0	0
12	0	0

The same cross was performed after 5-azacytidine treatment of the DWM strain (five days at 37°C). The phenotype of DWM changed to *Acr meth*⁺. The results allowed us to conclude that, once demethylated, the DWM strain becomes a target for drastic alterations (consequence of GIS), reducing ascospore viability per cleistothecium (Table III).

GIS reoccurrence in a meiotic segregant from the DWM (demethylated) x 5²² cross

Meiotic *meth* segregants were recovered from several cleistothecia from the DWM (demethylated) x 5²² (*meth*⁺ x *meth*⁺) cross (Table IV). GIS activity in the nucleus of the DWM strain was confirmed not only by the reduction of the viable progeny, but also by the appearance of *meth* segregants among the progeny. Segregation of the parental classes among the progenies of the DWM x 5²⁰ and DWM (demethylated) x 5²² crosses (Tables V and VI) was observed. A strong

Table IV - Segregation of *meth* ascospores in the cross DWM (demethylated) x 5²², among the viable progeny of eight cleistothecia.

Cleistothecium	<i>meth</i> ⁺	<i>meth</i>
1	4	3
2	0	2
3	21	3
4	16	0
5	50	11
6	24	41
7	50	43
8	182	0

selection against the parental class, bearer of the *Dp*(II-I) duplication when GIS acts, was verified. So, when DWM was demethylated by 5-azacytidine there was reoccurrence of GIS activity during meiosis. These results support those presented in Tables I and IV.

The 3⁷ strain was isolated among the *w*⁺ *meth* *Acr* segregants of cleistothecium number 7 from the DWM (demethylated) x 5²² cross. The conidia of the 3⁷ strain, treated with 5-azacytidine, presented the *meth*⁺ phenotype. Meiotic analysis of the 3⁷ x R1 cross showed, in several analyzed cleistothecia, a reduction of the total number of ascospores, and low viability (Table VII). The statistical analyses gave support to the hypothesis of GIS reoccurrence in segregants of the second generation, showing a difference (Wilcoxon, *P* < 0.05) between the control cross (UT448 x UT196) and the 3⁷ x R1 cross. The analysis of the *y paba bio Acr* and *meth* segregants, in one of the cleistothecia, again showed strong selection against the parental class, bearer of the *Dp*(II-I) (Table VIII).

Among eukaryotes the DNA methylation process acts as a DNA post-replicative modifying system, and has been related to the control of gene expression (Doerfler, 1983; Razin and Cedar, 1991). Actively transcribing chromosomal regions of eukaryotes are normally non-methylated, and inactive regions are intensively methylated (Holliday, 1987; Cedar, 1988). The inactivation of structural gene

Table V - Meiotic segregation of some markers of chromosomes I and III in the DWM x 5²⁰ cross. A sample of 72 colonies were analyzed among the progenies. P1 = DWM, P2 = 5²⁰.

Genetic markers analyzed	Number of segregants	
	<i>w</i> (34)	<i>w</i> (38)
<i>pa</i> ⁺ <i>meth</i> <i>pyro</i>	10 (P1)	10
<i>pa</i> <i>meth</i> <i>pyro</i>	11	10
<i>pa</i> ⁺ <i>meth</i> <i>pyro</i> ⁺	7	7
<i>pa</i> <i>meth</i> <i>pyro</i> ⁺	6	11 (P2)

P1, P2 = Parental classes.

Table VI - Meiotic segregation of some markers of chromosomes I and II in the DWM (demethylated) x 5²² cross. P1 = DWM (demethylated), P2 = 5²².

Genetic markers analyzed	Number of segregants	
	<i>w</i> (1)	<i>w</i> ⁺ (43)
<i>bio</i> ⁺ <i>meth</i> ⁺ <i>pyro</i> <i>Acr</i>	0	0
<i>bio</i> <i>meth</i> ⁺ <i>pyro</i> <i>Acr</i>	0 (P1)	0
<i>bio</i> ⁺ <i>meth</i> ⁺ <i>pyro</i> ⁺ <i>Acr</i>	1	43 (P2)
<i>bio</i> <i>meth</i> ⁺ <i>pyro</i> ⁺ <i>Acr</i>	0	0

P1, P2 = Parental classes.

Table VII - Total number and viability of ascospores from the 3⁷ x R1 cross.

Cleistothecium number	Total ascospores	Viability (%)
1	11,250	10.8
2	10,000	1.4
3	8,750	26.5
4	7,500	23.5
5	45,000	0.0
6	27,500	0.0
7	41,250	5.8
8	62,500	0.0

Table VIII - Meiotic segregation of the parental class normal (P1) and the bearer of the duplication *Dp*(II,I) (P2), in the 3⁷ x R1 cross.

	<i>y</i> ⁺ <i>paba</i>	<i>y paba</i> ⁺ <i>bi</i> ⁺
<i>Acr meth</i>	2 (P2)	4
<i>Acr</i> ⁺ <i>meth</i> ⁺	3	22 (P1)

expression, after RIP or GIS processes, demonstrates the regulatory role of DNA methylation.

Considering that one of the proposed mechanisms to trigger the RIP effect in *N. crassa* is precocious synapsis involving both copies of a gene duplication (Fincham, 1989), DNA methylation could play an important role in recombination control between duplicated segments, an event potentially capable of generating chromosomal rearrangements (Selker, 1990). The involvement of DNA methylating processes and point mutations in the maintenance of the stability of the genome give support to this hypothesis (Krickler *et al.*, 1992).

A. nidulans strains bearing the *Dp*(II-I) duplication yield severely reduced progeny, when submitted to the sexual cycle (Castro-Prado and Zucchi, 1993). The *Dp*(II-I) consists of a large segment of chromosome II, including the *Acr*, *w* and *meth*⁺ genes duplicated and transposed to the chromosome I (Castro-Prado and Zucchi, 1991). A gene inactivating system (GIS) useful to detect and inactivate duplicated sequences has been identified in *A. nidulans* and acts through methylation of cytosine residues (Castro-Prado and Zucchi, 1996). The process is nucleus limited, acting only in cells containing one nucleus from each parent. Although both copies of the duplication could be methylated, GIS has shown to be less efficient than non-linked duplication *Dp*(II-I) (Castro-Prado and Zucchi, 1993). Even so, this mechanism results in low ascospore viability, as observed in meiotic crosses between normal and duplicated *Dp*(II-I) strains (Tables I and II).

Transformed strains of *A. nidulans* containing several copies of transformant DNA also presented meiotic instability when submitted to the sexual cycle, generating cleistothecia presenting low fertility (Tilburn *et al.*, 1990) and additional extra gene inactivation from both homologous and heterologous origins.

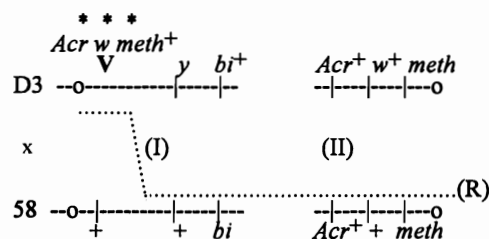
It was observed here that the gene inactivating mechanism promotes low fertility in strains bearing genic duplication. Another characteristic of the GIS mechanism is the reduction of the parental class bearing the *Dp*(II-I) (Tables II, VI and VIII). However the appearance of small white (*w*) sectors in colonies of *y*⁺, *bio*, *w*⁺, *meth* derived from the D3 x 58 cross (Table II) indicates that some recombinant classes bearing the *Dp*(II-I) in the chromosome I were included among the parental classes, because their *Acr*, *w*, and *meth*⁺ genes (included in the duplication) were inactivated (Figure 2).

Comparing the crosses: DWM (demethylated) x 5²², DWM x 5²⁰ and 3⁷ x R1 (Tables V, VI, and VIII), it is possible to verify that ascospore viability and the parental class, bearer of the *Dp*(II-I), are altered only after GIS activity (Tables VI and VIII).

Normal meiotic behavior is recovered when the strain DWM become refractory to the GIS process, as shown in (Table VI). Refractoriness may be a consequence of complete methylation of the cytosine residues present in both duplicated segments. But reactivation of the GIS-inactivated genes may spontaneously occur during mitosis and in this way, the DWM strain could not be refractory to GIS if the methylation level of its duplicated genes were found altered, at the moment of fertilization (Castro-Prado and Zucchi, 1996).

Analysis of the 3⁷ x R1 cross (Tables VII, and VIII) showed that although the 3⁷ strain is a first generation segregant (as well as DWM), it does not present GIS refractoriness. This clearly indicates that the duplication was still a substrate for the methylation process.

This work describes the features of a gene inactivating system in *A. nidulans*, specific for homologous duplicated DNA sequences that acts

**Figure 2** - Origin of the recombinants *y*⁺, *bio*, *w*⁺, *meth*, in the D3 x 58 cross.

through methylation, more like that described in *Ascobolus immersus* (Goyon and Faugeron, 1989) than to RIP of *N. crassa* (Selker *et al.*, 1987).

Although the real biological function of the inactivation systems described in several organisms until now remains unknown (Faugeron *et al.*, 1990; Selker, 1990; Krickler *et al.*, 1992; Castro-Prado and Zucchi, 1993), the participation of these systems in gene dynamics is a consensual observation. Investigations on the particularities of each system would help to clarify the biological and evolutionary meanings of the inactivation of duplicate genes.

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RESUMO

Descrevemos aqui a ação de um sistema de inativação gênica (GIS) que altera genes presentes em duplicata no genoma de *Aspergillus nidulans*. A atividade de GIS ocorre apenas no ciclo sexual da linhagem carregando a duplicação *Dp*(II-I). Verificou-se também a redução da viabilidade e do número de ascósporos por cleistotécio, em cruzamentos meióticos, heterozigotos para a duplicação *Dp*(II-I). Neste trabalho caracterizamos um sistema de inativação gênica que atua no genoma de *A. nidulans*. Ele influencia a viabilidade dos ascósporos, entre os segregantes meióticos de primeira e de segunda (F1 e F2) gerações, além da reincidência do processo depois do tratamento daqueles segregantes com 5-azacitidina.

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