RESEARCH NOTE

Mutants of *Aspergillus nidulans* affected in asexual development

JOSY FRACCARO MARINS and MARIALBA AVEZUM ALVES CASTRO-PRADO*

State University of Maringá, Department of Cell Biology and Genetics, Av. Colombo 5790, Bloco H67, Maringá-PR, Brazil 87020-900

Introduction

The asexual cycle of *Aspergillus nidulans* is characterized by conidiophore formation, a multicell structure formed by four cell types: foot-cell, stalk, vesicle and sterigmata made up of metullae and phyalides. Conidia, or asexual spores, are formed mitotically by repeated subdivisions of the phyalides (Timberlake 1990; Mirabito and Osmani 1994).

Conidiophore morphogenesis depends on a strict control of specific conidiation genes. Control is mainly established by the activity and interactivity of genes *brlA* (bristle), *abaA* (abacus) and *wetA* (wet-white conidia). Whereas *brlA* expression occurs at the beginning of development, close to the period of vesicle formation, *abaA* is expressed almost at the end, approximately close to the period of phyalides formation. Expression of *wet* occurs after that of *aba*, and is seen as specific accumulation of its mRNA in the conidia (Boylan *et al*. 1987; Mirabito and Osmani 1994).

Expression of *brlA* is preceded by expression of *fluG (fluffy)*, *flbA (fluffy low bristle)*, *flbB*, *flbC*, *flbD* and *flbE*. Mutation in these genes gives rise to colonies that are unable to convert vegetative growth into conidiogenesis or asexual cycle. Consequently, fluffy mutants form colonies characterized by a large mass of undifferentiated, cottonized hyphae (Wieser *et al*. 1994; Wieser and Adams 1995).

The parasexual cycle of *A. nidulans* constitutes a powerful system of genetic analysis and is widely used in mapping new alleles or genes in the different chromosomes. The process produces diploid nuclei with possible recombinant haploid segregants through haploidization processes and mitotic exchanges (Azevedo 1989).

If diploid nuclei undergo mitotic exchange and haploidization within the heterokaryotic hyphae, haploid mitotic recombinants may be obtained directly from heterokaryons, without recovery of the diploid phase. In fact, the recombinants will be identified as homogeneous and vigorous growth sectors. The process, named parameiosis, is a variation of the parasexual cycle and has been described in several deuteromycetes, such as *Metarhizium anisopliae* (Bergeron and Messing-Al-Aidroos 1982), *Beauveria bassiana* (Bello and Pacolla-Meirelles 1998), *Trichoderma pseudokoningii* (Bagagli *et al*. 1995), and more recently in *A. nidulans* (Baptista *et al*. 2003).

5-Azacytidine (5AC) is an analogue of cytosine with a nitrogen atom at position 5 of the pyrimidic ring. The drug is phosphorylated in mammalian cells (5-azacytidine 5-phosphate) and incorporated in different RNA species and in DNA. 5AC incorporation into DNA may allow formation of a covalent complex between the cytosine-specific methyltransferase enzyme and the modified DNA; this inhibits DNA replication and methylation processes (Fergusson *et al*. 1997; Bender *et al*. 1998; Fernandez *et al*. 1998).

5AC has been developed as a chemotherapy helper in the control of certain types of cancer (Glover *et al*. 1986). 5AC and its alternative 5-aza-2′-desoxycytidine, *in vivo* and *in vitro*, revert epigenetic silencing of tumour suppressor genes in cancer cells (Bender *et al*. 1998; Bovenzi *et al*. 1999).

In the present study, 5AC was employed to obtain *A. nidulans* mutants by inducing base transitions and transversions (Spencer *et al*. 1996; Jackson-Grusby *et al*. 1997). The treated strain produced two mitotic clones which bore alterations typified by smaller and greater effects on conidiogenesis.

Materials and methods

Strains and culture media

Table 1 shows the strains used. Minimal medium (MM) consisted of Czapek-Dox with 1% (w/v) glucose. Complete medium (CM) was made by adding (in g/l) glucose 10, peptone 2, yeast extract 2, hydrolysed casein 1, and (in μg/l) inositol 4000, choline chloride 2000, pantothenic acid 2000,

* For correspondence. E-mail: maacprado@uem.br.

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nicotinic acid 1000, riboflavin 1000, 4-aminobenzoic acid 100, folic acid 500, pyridoxine 500, thiamine 200 and biotin 2 to MM. Selective medium (SM) consisted of MM supplemented with nutritional requirements for each strain. Sorbitol medium was Czapek-Dox with 1% (w/v) sorbitol. Acriflavine medium, cycloheximide medium and sulphanilamide medium were made by adding (in μg/ml) acriflavine 25.0, or (in mg/ml) cycloheximide 1.0 or sulphanilamide 1.0. Solid medium contained 1.5% agar. Incubation temperature was 37°C.

5-Azacytidine treatment

Petri dishes containing MM + pyridoxine + methionine + 100 μM 5AC were inoculated with conidia of master strain A757 and incubated for 5 days at 37°C. Treated colonies yielded visible mitotic sectors with morphologies different from that of the original strain.

Genetic techniques

General methodology has been described by Pontecorvo *et al*. (1953). Heterokaryons were obtained in liquid MM + 2.0% liquid CM and were inoculated in Petri dishes containing MM. Cleistothecia were obtained from heterokaryons, in sealed plates, after 21 days of incubation. Diploid strains were prepared according to Roper (1952).

Mapping of mutant alleles by parasexual cycle

BF6//A783 and BW1//A783 diploid strains were obtained in MM and haploidized in CM + benomyl (0.5 μg/ml). Mitotic segregants isolated from each diploid strain were transferred to distinct selective media to determine their phenotypes (Hastie 1970).

Phenotypic analysis of parameiotic segregants

Parasexual segregants were isolated from BF6//A783 heterokaryons in MM. Segregants were purified in CM and transferred to appropriate selective media to determine their phenotypes. Mitotic stability of segregants was tested in CM + benomyl (0.5 μg/ml). Mitotically stable recombinants underwent sexual cycle.

Cytological analyses

Conidia of BF6 strain were inoculated over dialysis membranes supported by solidified CM. Plates were incubated at 37°C and samples, stained with lacto-phenol cotton blue, were examined under light microscope after 24–36 h.

Results and discussion

Two stable developmental mutants of *A. nidulans* were isolated after treatment of master strain A757 with 5AC (100 μM). Mutants, named BW1 and BF6, had phenotypic characteristics distinct from the original strain: white conidia (BW1) and fluffy morphology (BF6). To determine whether both mutations behave as single Mendelian alleles, mutant strains BW1 and BF6 were meiotically crossed to A610 and A783 master strains, respectively. Mutant alleles, provisionally called *wA5* and *flu6*, in both crosses, segregated themselves from their respective wild-type alleles in a 1:1 ratio. This fact showed that a single Mendelian gene was involved in the determination of each mutant phenotype (table 2).

The assignment of a mutant allele to a particular linkage group is made by the parasexual cycle (Azevedo 1989) and by mitotic haploidization (McCully and Forbes 1965; Hastie 1970). Mitotic analysis may be simplified by the

Table 1. Genotypes of *Aspergillus nidulans* strains used.

Strain	Genotype*	Origin
A783	*SulA1* (I)*; AcrA1* (II)*; ActA* (III)*; pabaB22* (IV)*; nicA2* (V)*; sbA3* (VI)*; choA1* (VII)*; riboB2, chaA1* (VIII)	FGSC
A757	*yA2* (I), *methA17* (II)*; pyroA4* (IV)	FGSC
UT448	*riboA1, pabaA124, biA1* (I)*; AcrA1, wA2* (II)	Utrecht Stock
B520	*pabaA124, biA1* (I)*; methA17* (II)	LGM
G1101	*AcuM301* (I)*; wA3* (II)*; pyroA4* (IV)	Glasgow
A610	*yA2, pabaA1* (I)	FGSC
B211	*yA2, biA1* (I)*; AcrA1, wA2, methA17* (II)*; uvsH77, pyroA4* (IV)*; chaA1* (VIII)	LGM
BF6	*yA2* (I)*; methA17* (II)*; pyroA4* (IV)*; flu6* (VIII)	LGM
BW1	*yA2* (I), *wA5*, *methA17* (II)*; pyroA4* (IV)	LGM

**ribo, paba, bio, meth, pyro, nic, cho,* Requirements for riboflavin, *p*-aminobenzoic acid, biotin, methionine, pyridoxine, nicotinic acid and choline, respectively; *y* (yellow); *w* (white); *cha* (chartreuse) are mutations for conidia colour; *Sul*, *Acr* and *Act*, resistance to sulphanilamide, acriflavine and cycloheximide, respectively; *sb* and *acu*, inability to use sorbitol and ammonium acetate, respectively, as carbon sources; *flu*, fluffy mutation; *uvsH*, sensitivity to UV radiation.
Gene symbols according to Clutterbuck (1994).
FGSC, Fungal Genetics Stock Center, University of Missouri, Kansas City, USA; LGM, Laboratory of Genetics of Microorganisms of the State University of Maringá, Brazil.

availability of mapping strains, such as A783, with markers on each of the eight chromosomes of *A. nidulans*. Diploids A783//BW1 and A783//BF6 were formed and haploidized in CM + benomyl, which facilitated the isolation of haploids from diploid strains (Hastie 1970). The resulting haploid segregants were analysed phenotypically.

Table 2. Meiotic segregation of markers from chromosomes I, II, IV and VIII in the A610 × BW1 and A783 × BF6 crosses.

Cross	Genetic marker	Number of segregants
A610 × BW1	*paba*$^+$	77
	paba	55
	w$^+$	70
	w	62
	meth$^+$	73
	meth	59
A783 × BF6	*meth*$^+$	59
	meth	38
	pyro$^+$	46
	pyro	51
	flu$^+$	43
	flu	54

Sixtyeight haploid segregants derived from diploid A783//BW1 were chosen. Phenotypic analysis showed that genes *wA* and *AcrA* recombined with all markers of the mapping strain (A783). However, no recombinant between *Acr* and *w* was obtained. Complete absence of the two recombinant classes, *w*$^+$*Acr*$^+$ and *wAcr*, mapped the mutant allele *wA5* to chromosome II (table 3). Table 3 also shows that mutant allele *flu6* assorted independently from all markers of the mapping strain, with the exception of linkage group VIII marker (*cha*). The data mapped *flu6* to chromosome VIII.

BW1//A783 and BF6//A783 diploid strains produced green conidia and were indistinguishable from the homozygous wild-type control A783//G1101 (results not shown), indicating that *wA5* and *flu6* mutations are recessives to their wild-type alleles.

BF6 had the same characteristics as other *A. nidulans* mutants, such as rapid growth, abnormal differentiation and invasiveness (Timberlake 1990). The mutant showed a delay of 10 hours in development of mature conidiophores compared to the control strain (A757) (table 4 and figure 2).

Parameiosis, a variation of the parasexual cycle, has been recently described in *A. nidulans* (Baptista *et al*. 2003).

Table 3. Linkage analysis of BW1 and BF6 mutants.

BW1//A783	*Sul* (I)		*Acr* (II)		*Act* (III)		*paba* (IV)		*nic* (V)		*sb* (VI)		*cho* (VII)		*ribo* (VIII)	
	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
w$^+$	38	13	0	51	34	17	32	19	23	28	34	17	47	04	32	19
w	10	07	17	0	10	07	13	04	16	01	07	10	16	01	14	03
BF6//A783	*y* (I)		*Acr* (II)		*Act* (III)		*paba* (IV)		*nic* (V)		*sb* (VI)		*cho* (VII)		*cha* (VIII)	
	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
flu$^+$	28	16	18	26	23	21	03	41	37	07	19	25	23	21	20	24
flu	12	03	07	08	10	05	14	01	13	02	10	05	12	03	15	0

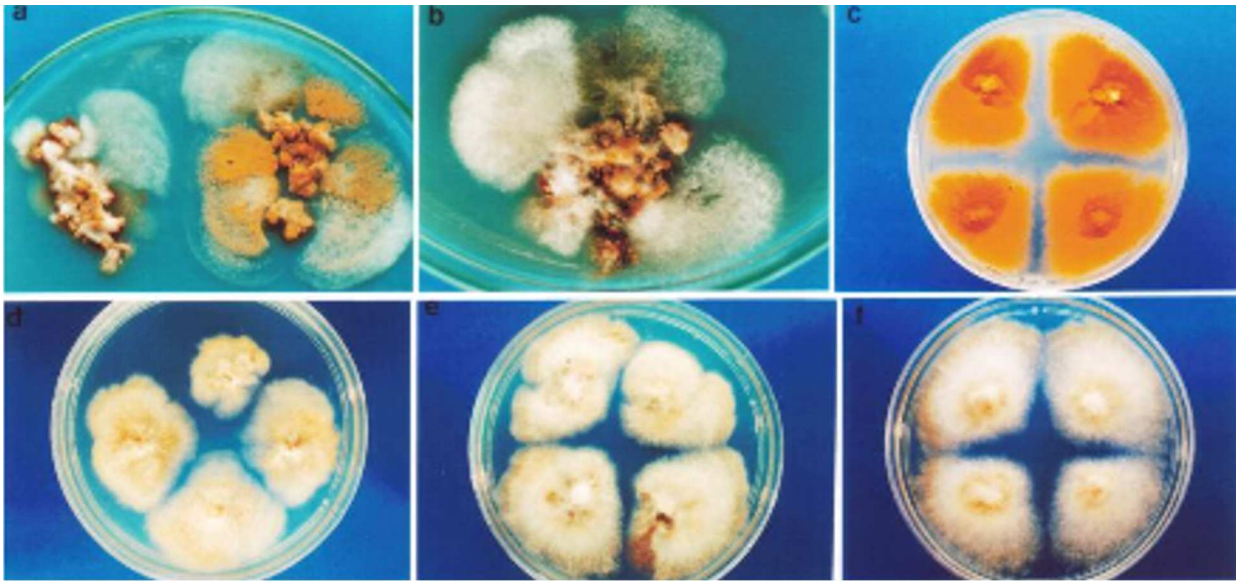

Figure 1. (a and b) Parasexual segregants (fluffy sectors) obtained from BF6//A783 heterokaryons. (c and e) Parasexual aneuploid, and (f) haploid segregant derived from BF6//A783 heterokaryons growing in CM + benomyl.

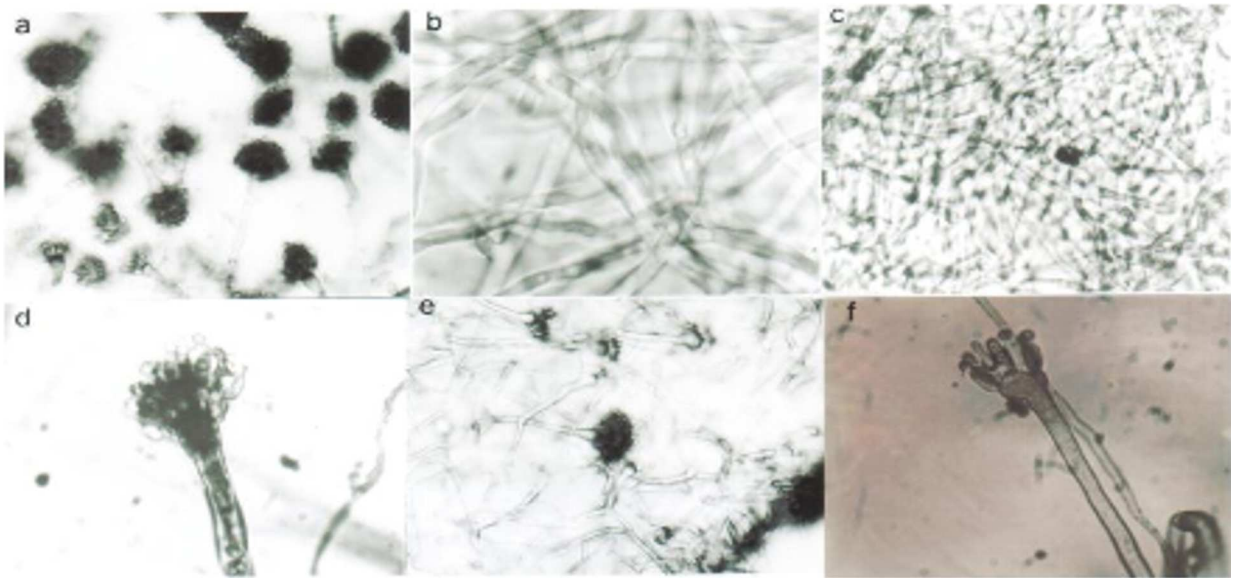

Figure 2. Cytological analysis of mutant BF6. (a) Conidiophores of strain A757 strain after 24 h incubation at 37°C. Mycelial growth of mutant BF6 after 24 h (b) and 30 h (c) incubation at 37°C. Conidiophores of mutant BF6 formed after 34 h (d) and 36 h (e) incubation. (f) Conidiophore of mutant BF6 with abnormal vesicle and reduced number of metullae and phyalides.

Table 4. Time of conidiphore vesicle and sterigmata appearance and conidia production of BF6 mutant in complete medium (A757 strain is control).

	Time (h)	
Conidiophore structures and conidia production	A757	BF6
Vesicles	21	31
Sterigmata	22	32
Conidia	24	34

The diploid nuclei are unstable in this process and undergo mitotic exchange and haploidization within the heterokaryotic hyphae. The formation of recombinant haploids directly from the heterokaryons is the result (Bagagli *et al*. 1995; Bello and Pacolla-Meirelles 1998; Baptista *et al*. 2003). Nine parasexual segregants (P1 to P9) were isolated as sectors of vigorous growth directly from BF6//A783 heterokaryons (figure 1). Segregants were submitted to the mitotic instability test in CM + benomyl. Segregants that either formed colonies with irregular boards, or showed reduced growth or frequently formed new mitotic sectors were considered aneuploids (P1, P2, P6, P7, P8 and P9). On the other side, the stable segregants, that is those that in presence of benomyl did not originate new mitotic sectors, but formed homogeneous colonies and maintained the recombinant phenotype (P3, P4 and P5), were considered probable parameiotic segregants (figure 1 and table 5).

Segregants P3, P4 and P5 were individually crossed with master strain G1101 for evaluation of their meiotic stability. In crosses P4 × G1101 and P5 × G1101, segregation of the genetic markers was approximately $1^+ : 1^-$. Although P3 segregant was stable in the presence of benomyl, segregation of markers *pyro* and *flu* was different from the expected $1^+ : 1^-$ (table 6). The results characterize P4 and P5 as parameiotic and P3 as aneuploid segregants.

Table 5. Phenotype of haploid P3, P4 and P5 segregants derived from A783//BF6 heterokaryon.

Parasexual segregant	Phenotype	Origin*
P3	*SulA1* (I)*; AcrA1* (II)*; ActA1* (III)*; flu6* (VIII)	IA
P4	*AcrA1* (II)*; pabaB22* (IV)*; sbA3* (VI)	IA
P5	*yA2* (I)*; pabaB22* (IV)*; chaA1* (VIII)	IA

* IA, Independent assortment.

Table 6. Segregation of nutritional markers from crosses between P3, P4 and P5 segregants with G1101 master strain.

	Meiotic cross		
Genetic marker	P3 × G1101	P4 × G1101	P5 × G1101
paba$^+$	–	59	57
paba	–	62	53
w$^+$	–	58	47
w	–	63	63
y$^+$	–		11
y	–		16
pyro$^+$	41*	66	64
pyro	84	55	46
flu$^+$	83*	–	–
flu	42	–	–
cha$^+$	–	–	27
cha	–	–	20

* Significantly different from 1+: 1–, Chi-square test, $P < 0.05$.
–, Not determined.

The mutagenic effect of 5AC, reported in several organisms such as *Escherichia coli, A. nidulans* and *Salmonella typhimurium*, was employed in the present study to isolate developmental mutants of *A. nidulans* (Watanabe *et al*. 1994; Kelecsényi *et al*. 2000; Ohta *et al*. 2000; Costa *et al*. 2001). These mutants may be useful in biochemical studies on mechanisms controlling differentiation and in molecular characterization of 5AC-induced changes in eukaryotic DNA.

References

Azevedo J. L. 1989 Melhoramento genético de fungos utilizados no controle biológico de insetos: utilização do processo parameiótico. *Mem. Inst. Osvaldo Cruz* **84**, 27–29.

Bagagli E., Furlaneto M. C., Pizzirani-Kleiner A. A. and Azevedo J. L. 1995 Genetic recombinants in *Trichoderma pseudokoningii* (RIFAI) without typical parasexuality. *Can. J. Microbiol*. **41**, 1132–1134.

Baptista F., Machado M. F. P. S. and Castro-Prado M. A. A. 2003 Alternative reproduction pathway in *Aspergillus nidulans. Folia Microbiol*. **48**, 597–604.

Bello V. A. and Pacolla-Meirelles L. D. 1998 Localization of auxotrophic and benomyl resistance markers through the parasexual cycle in *Beauveria bassiana* (Bals.) Vuill entomopathogen. *J. Invertebr. Pathol*. **72**, 119–125.

Bender C. M., Pao M. M. and Jones P. A. 1998 Inhibition of DNA methylation by 5-aza-2′-desoxycytidine suppresses the growth of human tumour cell lines. *Cancer Res*. **58**, 95–101.

Bergeron D. and Messing-Al-Aidroos K. 1982 Haploidization analysis of heterozygous diploids of the entomogenous fungus *Metarhizium anisopliae. Can. J. Genet. Cytol*. **24**, 643–651.

Bovenzi V., Le N. L., Cote S., Sinnett D. and Momparler L. F. 1999 DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2′-deoxycytidine. *Anticancer Drugs* **10**, 471–476.

Boylan M. T., Mirabito P. M., Willett C. E., Zimmerman C. R. and Timberlake W. E. 1987 Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans. Mol. Cell. Biol*. **7**, 3113–3118.

Clutterbuck A. J. 1994 Linkage map and locus list. *Prog. Ind. Microbiol*. **29**, 791–824.

Costa M. A., Silva N. C. G. and Castro-Prado M. A. A. 2001 Genetic and cytological characterization of a developmental mutant of *Aspergillus nidulans* induced by 5-azacytidine. *Biol. Res.* **34**, 91–98.

Fergusson A. T., Vertino P. A., Spitzner J. R., Baylin S. B., Muller M. T. and Davidson N. E. 1997 Role of estrogen receptor gene demethylation and DNA methyltransferase DNA adduct formation in 5-aza-2′-deoxycytine-induced cytotoxicity in human breast cancer cells. *J. Biol. Chem*. **272**, 32260–32263.

Fernandez M., Olek J. W. and Sanchez J. 1998 Analysis of DNA methylation processes related to the inhibition of DNA synthesis by 5-azacytidine in *Streptomyces antibioticus* EHT 7451. *Biol. Chem*. **379**, 559–562.

Glover T. W., Coylemorris J., Pearcebirge L., Berger C. and Gemmill R. M. 1986 DNA demethylation induced by 5-azacytidine does not affect fragile-X expression. *Am. J. Hum. Genet*. **38**, 309–318.

Hastie A. C. 1970 Benlate-induced instability of Aspergillus diploids. *Nature* **226**, 771.

Jackson-Grusby L., Laird P. W., Magge S. N., Moeller B. J. and Jaenish R. 1997 Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl. Acad. Sci. USA* **94**, 4681–4685.

Kelecsényi Z., Spencer D. L. and Caspary W. J. 2000 Molecular analysis of 5-azacytidine-induced variants in mammalian cells. *Mutagenesis* **15**, 25–31.

McCully K. S. and Forbes F. 1965 The use of p-fluorophenylalanine with ‘master strains’ of *Aspergillus nidulans* for assigning genes to linkage groups. *Genet. Res*. **6**, 352–359.

Mirabito P. M. and Osmani S. A. 1994 Interaction between the developmental program and cell cycle regulation of *Aspergillus nidulans. Dev. Biol*. **5**, 139–145.

Ohta T., Watanabe-Akanuma M. and Yamagata H. 2000 A comparison of mutation spectra detected by the *Escherichia coli lac*(+) reversion assay and the *Salmonella typhimurium his*(+) reversion assay. *Mutagenesis* **15**, 317–323.

Pontecorvo G., Roper J. A., Hemmons L. M., McDonald K. D. and Bufton A. W. J. 1953 The genetics of *Aspergillus nidulans. Adv. Genet*. **5**, 141–238.

Roper J. A. 1952 Production of heterozygous diploids in filamentous fungi. *Experientia* **8**, 14–15.

Spencer D. L., Caspary W. J., Hines K. C. and Tindall K. R. 1996 5-Azacytidine-induced 6-thioguanine resistance at the *gpt* locus in AS52 cells: cellular response. *Environ. Mol. Mutagen*. **28**, 2100–2116.

Timberlake W. E. 1990 Molecular genetics of *Aspergillus* development. *Annu. Rev. Genet*. **24**, 5–36.

Watanabe M., Nohmi T. and Ohta T. 1994 Effects of the *umuDC, mucAB*, and *samAB* operons on the mutational specificity of chemical mutagenesis in *Escherichia coli*: II. Base substitution mutagenesis. *Mutat. Res*. **314**, 39–49.

Wieser J. and Adams T. H. 1995 *flbD* encodes a Myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development. *Genes Dev*. **15**, 491–502.

Wieser J., Lee B. N., Fondon J. W. III and Adams T. H. 1994 Genetic requirements for initiating asexual development in *Aspergillus nidulans. Curr. Genet*. **27**, 62–69.

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