

Duplications created by transformation in *Sordaria macrospora* are not inactivated during meiosis

Landry Le Chevanton, Gérard Leblon, and Suzanne Lebilcot

Laboratoire I.M.G., Université Paris-Sud, Centre d'Orsay, Bat. 400, F-91405, Orsay Cedex, France

Summary. We present here the first report of a transformation system developed for the filamentous fungus *Sordaria macrospora*. Protoplasts from a *ura5* strain were transformed using the cloned *Sordaria* gene at a frequency of 2×10^{-5} transformants per viable protoplast (10 per microgram of DNA). Transformation occurred by integration of the donor sequences in the chromosomes of the recipient strain. In 71 cases out of 74, integration occurred outside the *ura5* locus; frequently several (two to four) copies were found at a unique integration site. Using the advantage of the spore colour phenotype of the *ura5-1* marker, we have shown that the transformed phenotype is stable through mitosis and meiosis in all transformants analysed. No methylation of the duplicated sequences could be observed during meiotic divisions in the transformants.

Key words: *Sordaria macrospora* – Transformation – Filamentous fungi – Orotate phosphoribosyl transferase

Introduction

Transformation systems have been developed and characterized in several fungi. In the large majority of those organisms, transformants are mainly obtained by integration of the donor DNA into the chromosomes of the recipient strain. The relative frequency of homologous integration is highly variable from one organism to another: 100% in *Saccharomyces cerevisiae* (Struhl 1983), about 80% in *Aspergillus nidulans* (Yelton et al. 1984), and only 5% to 1% in *Neurospora crassa* (Case 1986) or *Coprinus cinereus* (Binnering et al. 1987). Differences in the stability of the transformed phenotype are also observed in the different systems. In general, transformants are stable through mitosis, but in a significant number of systems they are unstable through meiosis (review in Rambossek and Leach 1987). The meiotic instability has been observed with heterothallic organisms.

In order to obtain more information about the mechanism of integration of the donor DNA and the behaviour of the transformants through meiosis in a homothallic fungus, we developed a transformation system for *Sordaria macrospora*. This ascomycete has been used as a model system for cytological and genetic studies of meiotic pairing and recombination (Zickler et al. 1985; Moreau et al. 1985; Huynh et al. 1986) and the experimental methodologies de-

veloped to facilitate such studies were used here for an extensive analysis of a large number of transformants. This transformation system will also be important for the extension of previous studies to the molecular level and for example will be used to clone the genes corresponding to the meiotic mutants isolated.

Interestingly, comparison of the general organisation of the genetic map in *S. macrospora* and *N. crassa* (Perkins 1985; Leblon et al. 1987) and of the sequences of conserved domains of the rDNA gene (Y. Brygoo, personal communication) indicate that the two organisms are closely related although belonging to two different genera of the Sordariaceae. However an important difference between these two species is that *S. macrospora* is a homothallic, whereas *N. crassa* is heterothallic. In that respect, it was interesting to compare the characteristics of the two transformation systems and especially to check if the inactivation of the duplicated sequences demonstrated for *N. crassa* is also observed in *Sordaria*.

Materials and methods

Stock, media and general methods. All the mutant strains used in this work were derived from the *S. macrospora* wild-type isolate "St Ismier" strain FGSC 4818, ATCC 60255.

Routine media, mineral concentrate solution, methods of culture and genetic manipulations have been described previously (Zickler et al. 1984).

Genetic markers. *Yas1* is an ascospore colour mutation (Zickler et al. 1984). In the *ura5-1* mutant (isolated after N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis, Arnais et al. 1984) all asci formed after selfing contain eight underpigmented spores, frequently smaller than the wild-type spores and with reduced germination rate. This character is recessive and not spore autonomous: crossed to wild type, all hybrid asci contain eight regularly pigmented spores. The mutant shows a reduced rate of growth compared with the wild type. *Ura5-1* has been found to be auxotrophic and to require uracil or uridine for growth on minimal media (for composition see Zickler et al. 1984). This requirement cannot be supplemented with either dihydroorotate or orotate. Enzymatic assays with cell-free extract have shown that the *ura5* mutant lacks orotate phosphoribosyl transferase (OPRTase) activity (L. Le Chevanton, unpublished results).

Plasmids and phages. The *Escherichia coli* vectors used were pBR329 (Covarrubias and Bolivar 1982), pUC18 and pUC19 (Yanisch-Perron et al. 1985). Lambda λ LLC100, pLLC600, pLLC900, pLLC500 and pLLC510 have been described elsewhere (Le Chevanton and Leblon 1989). pLLC800 is an *XbaI-KpnI* subclone of λ LLC100 in a pUC18 vector. pLLC810, pLLC820, pLLC830 and pLLC840 were obtained from pLLC800.

Bacterial and DNA manipulations. General methods for bacterial and phage manipulations were those described by Maniatis et al. (1982). Large-scale isolation of plasmids from *E. coli* cultures was according to Holmes and Quigley (1981). The method for preparing total DNA from *S. macrospora* has been described elsewhere (Le Chevanton and Leblon 1989). Restriction enzyme digestion, transfert of DNA from agarose gels to nylon membranes (PALL BIO-DYNE A), 32 P-labelling of DNA by nick translation, and membrane hybridization were performed by standard procedures (Maniatis et al. 1982). Titration of the number of integrated copies of donor DNA was performed by dot blotting (Anderson and Young 1985).

Preparation of *Sordaria macrospora* protoplasts. Mycelium grown for 3 days in liquid minimal medium (55.5 mM glucose, 1.8 mM KH_2PO_4 , 1.7 mM K_2HPO_4 , 8.3 mM Urea, 1 mM MgSO_4 , 5 μM biotin and 0.1 ml/l mineral concentrate, with 0.4 mM uridine) was fragmented and then spread on a cellophane disk on the top of minimal medium plus 15 g/l Bacto-agar supplemented with 0.4 mM uridine. After 24 h incubation at 23° C, the mycelium was harvested and suspended in phosphate buffer (13 mM Na_2HPO_4 , 45 mM KH_2PO_4 , 600 mM KCL, pH 6). Then 20 mg/ml Glucanex (Novo-Ferments, Basel, Switzerland) was added and the preparation incubated at 37° C with gentle agitation for 3 h. Protoplasts were separated from mycelial fragments by filtration, pelleted by centrifugation, suspended in stabilizer (40 mM TRIS-HCl pH 9.3, 0.6 M sucrose), repelleted and suspended in stabilizer plus 10 mM CaCl_2 . Protoplasts were frozen at -80° C at this stage without decrease in competence.

Transformation of *Sordaria macrospora*. For transformation, 200 μl of protoplast suspension (5×10^6 protoplasts/ml) was mixed with 10 μg of DNA in stabilizer plus 10 mM CaCl_2 . Then 2.2 ml of 40 mM TRIS-HCl pH 9.3, 60% polyethylene glycol 4000, 10 mM CaCl_2 was added in three steps (200, 500 and 1500 μl). After 15 min, complete regeneration medium (3 g/l yeast-extract, 0.6 M sucrose, 3.6 mM KH_2PO_4 , 3.7 mM K_2HPO_4 , 2 mM MgSO_4 and 0.05 ml/l mineral concentrate) was added and the mixture was kept at 23° C for 1 h without agitation. Protoplasts were finally pelleted, resuspended in minimal regeneration medium (1.8 mM KH_2PO_4 , 1.7 mM K_2HPO_4 , 8.3 mM Urea, 1 mM MgSO_4 , 5 μM biotin, 0.6 M sucrose, 33 mM sorbose, 0.3 mM glucose, 0.05 ml/l mineral concentrate) and plated on minimal regeneration medium plus 15 g/l bacto-agar. Plates were incubated at 23° C.

Results

Characteristics of the donor DNA and ability to transform the *ura5-1* mutant

Figure 1 indicates the restriction fragments of the *ura5* gene of *Sordaria macrospora* used for the different transforma-

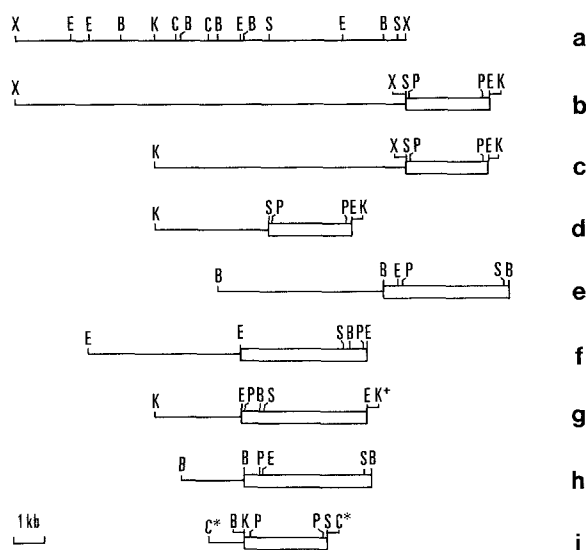


Fig. 1a-i. Restriction mapping of the plasmids used for transformation. **a** Partial map of the insert of λ LLC100 containing the *ura5* gene from *Sordaria macrospora* and surrounding sequences; **b** pLLC600; **c** pLLC800; **d** pLLC810; **e** pLLC900; **f** pLLC100; **g** pLLC820; **h** pLLC830; **i** pLLC840. Vector in **b-d**, **i**, pUC18; in **e-h**, pBR329. The open boxes in the plasmid maps indicate the vector sequences. Lines refer to the corresponding fragment from **a**. The coding sequence of the *ura5* gene is contained in the 0.9 kb *Bam*HI fragment present in pLLC840 and therefore is present in all these plasmids. Restriction enzyme abbreviations: X, *Xba*I; E, *Eco*RI; B, *Bam*HI; K, *Kpn*I; C, *Cla*I; S, *Sph*I; P, *Pvu*II; K⁺ is a *Kpn*I site from pUC18; C* is a *Cla*I site destroyed during the construction of the plasmid

Table 1. Transformation of the *ura5-1* mutant by plasmid DNAs

Expt.	Trans-forming DNA	Number of <i>ura</i> ⁺ colonies		Number of transformants per μg of DNA
		Primary	Secondary	
1	pUC18	0	—	0.6
	pLLC600	5	4	
	pLLC900	39	10	
2	pBR329	0	—	12
	pLLC810	104	18	
	pLLC830	14	6	
3	pUC19	0	—	6
	pLLC810	62	15	
	pLLC820	42	7	
	pLLC830	74	14	
4	pUC18	0	—	10
	pLLC810	201	Not isolated	

tion experiments. Uracil independent colonies were only observed if protoplasts were treated with plasmids containing the *ura5* gene and never with pUC18, pUC19 or pBR329 DNAs (Table 1). The colonies which grew after transfer onto fresh minimal media were called primary transformants. They represented 50% of the transferred colonies. Primary transformants correspond to stable transformants (non-abortive) described in other systems. Transformation frequencies (i.e. number of the primary transformants per microgram of DNA) ranged from 0.6 to 11.6. As these large variations in transformation frequency were observed

in different experiments even when the same plasmid was used (e.g. with pLLC830, 0.8 and 7 transformants per microgram of DNA, see Table 1), they cannot be attributed to the characteristics of the individual donor DNA.

Purification of the primary transformants

Protoplasts produced from the wild-type *Sordaria* strain contain an average of four nuclei (Huynh et al. 1986). Thus primary transformants may be heterokaryotic if only one of the nuclei is transformed. Primary transformants were tentatively purified after meiosis by selfing on selective medium. Only a fraction of the primary transformants was able to produce asci with eight pigmented spores of wild-type size and successive transfers on selective media did not modify this frequency. Eight-spored asci from each primary transformant were dissected and their spores were analysed for their *ura5* phenotype. The strains derived from *ura5*⁺ spores were called secondary transformants (Table 1). They were purified from asci with eight *ura*⁺ spores.

Mitotic stability of the transformants

Whereas the primary transformants showed variations in their growth rate (from almost no growth to wild-type growth), the secondary transformants showed a stable wild-type rate of growth even after serial transfer on non-selective medium. However, even if some nuclei had lost their *ura*⁺ sequences, the phenotype of the coenocytic mycelium would not be modified. In order to detect the presence of possible *ura*⁻ nuclei, 16 secondary transformants (including transformants 810.20, 810.32, 820.1 and 900.3.1 in which linkage between the transforming gene and the *ura5* locus was detected, see below) were selfed after five successive serial passages on non selective medium and checked for the production of asci with eight underpigmented spores. Such asci were produced at low frequency (10^{-2} to 10^{-4}) and the genetic analysis of 38 of them showed that they nevertheless contained *ura5*⁺ spores. Moreover, no asci with *ura*⁻ spores were observed after selfing of the 74 secondary transformants on selective medium. Therefore, we conclude that the secondary transformants were mitotically stable.

Meiotic stability and genetical analysis of the transformants

Seventy-four secondary transformants isolated in experiments 1 to 3 (Table 1) were crossed with the double mutant strain *ura5-1, yas1*. In each cross, about ten hybrid asci (recognized by the fact that they displayed four black spores and four yellow spores) were dissected and their spores analysed for their *ura5* genotype. In 73 cases, a 4:4 segregation of the *ura*⁺ marker was observed. In 1 case (transformant 900.3), 6⁺:2⁻ and 8⁺:0⁻ segregation was also observed, with frequencies indicating that two independent loci determined the *ura*⁺ phenotype (Table 2). These two loci were separately isolated in the progeny of a 900.3 to *yas1* cross. The fact that, in all cases, Mendelian segregation of the *ura*⁺ marker was observed indicates that the transforming sequence has a chromosomal location and that the *ura*⁺ phenotype is stably transmitted through meiosis.

The 74 secondary transformants and strains bearing each of the two *ura*⁺ components of the transformant 900.3 were crossed with a *ura5*⁺, *yas1* strain. In each case, the

Table 2. Genetic analysis of 74 secondary transformants

Transformant ^a	Crossed with the <i>ura5-1</i> allele			Crossed with the <i>ura5</i> ⁺ allele		
	4 <i>ura</i> ⁺ 4 <i>ura</i> ⁻	6 <i>ura</i> ⁺ 2 <i>ura</i> ⁻	8 <i>ura</i> ⁺	8 <i>ura</i> ⁺	6 <i>ura</i> ⁺ 2 <i>ura</i> ⁻	4 <i>ura</i> ⁺ 4 <i>ura</i> ⁻
810.20	6	0	0	43	0	0
810.32	4	0	0	44	0	0
820.1	6	0	0	48	0	0
900.3	5	11	4	14	0	0
900.3.1 ^b	9	0	0	8	2	0
900.3.2 ^b	5	0	0	2	6	2
Others ^c	586	1	1	112	443	98

^a Transformants are enumerated in order to show the transforming plasmid involved. Each transformant of independent origin is identified by an arabic number following the corresponding plasmid number, e.g. 820.1 means first secondary transformant isolated after transformation of the *ura5-1* mutant by pLLC820

^b The two *ura5*⁺ components which were simultaneously present in transformant 900.3 were separately isolated in the progeny with strain 900.3.1 and 900.3.2

^c Pooled results of the analysis of 70 transformants in which the transforming gene is not linked to the *ura5* locus

spores of ten hybrid asci were analysed for their *ura5* phenotype (Table 2). In 71 cases, segregation indicated the independent location of the transforming gene with respect to the *ura5* locus. In 4 cases, linkage between the transforming gene and the *ura5* locus was detected and for 3 of them, no *ura*⁻ offspring were observed even after analysis of an increased number of hybrid asci, indicating strong linkage.

Molecular analysis of the transformants

The number of sites of integration, the number of integrated copies of the plasmid and the nature of the recombination events were investigated by Southern blotting on DNA from the secondary transformants.

The probes used in this analysis were an electroeluted 0.9 kb *Bam*HI fragment encompassing the *ura5* gene (the *ura5* probe) and the vector present in the donor DNA (pBR329 or pUC18, the vector probe).

Undigested DNA from the 18 transformants obtained with pLLC810 and pLLC820 and the recipient strain tested with the *ura5* probe, gave a signal only in the region of high molecular weight DNA. No signal was found when DNA from the recipient strain was tested with the vector probe, and again the only signal revealed by probing the DNA from transformants with this second probe was in the high molecular weight DNA (data not shown).

Prior to electrophoresis and blotting, the DNA extracted from the transformants was digested with a restriction enzyme (*Xba*I) which has no site within the transforming plasmid. In each case the vector probe revealed only one band (Fig. 2, B2 and C2) and no hybridization occurred with the DNA from the *ura5-1* strain. The size of this band was different in all the transformants, except for 810.20 and 810.32 in which the bands were of the same size.

Using the *ura5* probe with the recipient strain DNA, a 12 kb fragment was revealed (Fig. 2, A1). In 15 transformants, this 12 kb band was present in addition to another band (Fig. 2, C1) of the same size as the band revealed by the vector probe. In the 3 remaining strains (810.20,

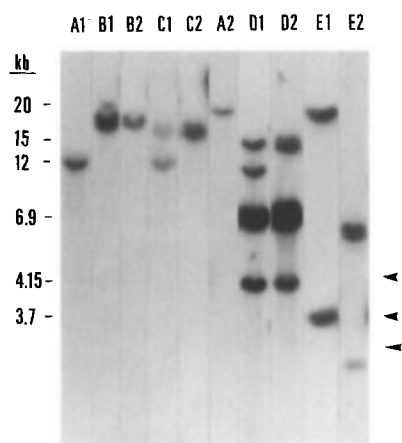


Fig. 2. Molecular analysis of the transformants. Digested genomic DNA was analyzed by Southern hybridization using ^{32}P -labelled, nick-translated probes. Lanes A1, B1, B2, C1, C2 *Xba*I-digested DNA; lanes A2, D1, D2, E1, E2, *Pvu*II-digested DNA. Lanes A1, A2, strain *ura5-1* DNA; lanes B1, B2, transformant 810.20 DNA; lanes D1, D2, transformant 820.1 DNA; lanes C1, C2, E1, E2, transformant 810.28 DNA. The probes used were: lanes A1, B1, C1, A2, D1, E1, 0.9 kb *Bam*HI fragment; lanes B2, C2, D2, E2, vector sequences. The arrow heads on the right indicate the size of the vector pBR329 (4.15 kb), the size of the insert in pLLC810 (3.7 kb) and the size of the vector pUC18 (2.7 kb)

810.32 and 820.1), the 12 kb band was absent (Fig. 2, B1) and a unique *Xba*I fragment of 19 kb was observed in 810.32 and 810.20 (Fig. 2, B1) and a fragment larger than 22 kb in 820.1. The sizes of these fragments were identical to those of the fragments revealed by the vector probe (compare lane B1 with B2 and lane C1 with C2 in Figure 2).

These results indicated that in all cases the transforming DNA was integrated within a unique *Xba*I fragment and that the integration event had not removed the vector part of the plasmid. The absence of the resident fragment in 810.20, 810.32 and 820.1 DNA suggested the occurrence of an homologous integration event in these three transformants. The size of the *Xba*I fragment indicated that one copy of the plasmid was integrated in 810.20 and 810.32 and more than one copy in 820.1 (in that case the number of copies was estimated as three or four).

Analysis of the homologous insertions

The pattern of hybridization 810.20 and 810.32 DNA digested by *Kpn*I or *Pvu*II with both probes corresponds to the structure expected of an homologous insertion of the plasmid at the resident locus as shown in Fig. 3.

The DNA from 820.1 was digested with *Eco*RI and *Pvu*II. The *Pvu*II digest probed with the *ura5* sequence also shows the loss of the 19.6 kb fragment corresponding to the resident gene (compare lane A2 with D1 in Figure 2). Fragments of 15, 11.5, 6.9 and 4.1 kb were observed, the 6.9 kb fragment being more intense than the others (6.9 kb corresponds to the size of pLLC820) (see Fig. 2, D1). The same pattern was observed with the vector probe, except that the band at 11.5 kb had disappeared (compare lane D1 with D2 in Figure 2). Such a pattern is compatible with the hypothesis of homologous insertion of multiple copies of the plasmid. The 4.1 kb band corresponds to a truncated copy which could have been generated by a non-homologous recombination event either before or after the

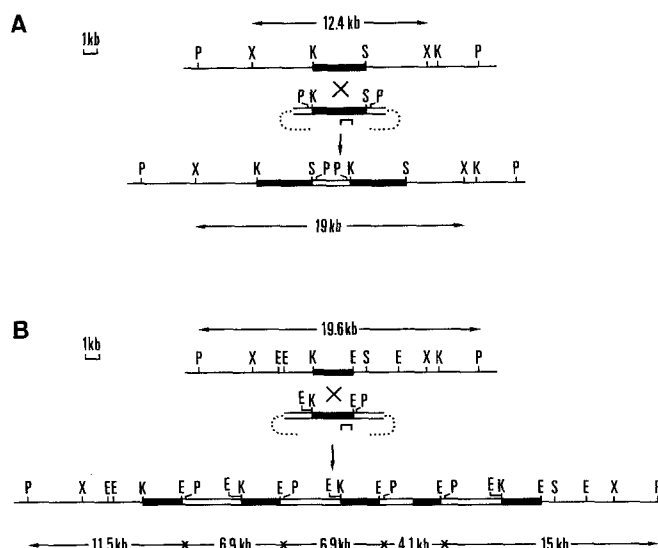


Fig. 3A and B. Diagram showing two cases of integration of a transforming plasmid into the homologous *ura5* genetic locus. The genomic fragment (thick solid line) which contains the *ura5* gene was cloned into pUC18 or pBR329 to yield respectively plasmid pLLC810 and pLLC820. Flanking genomic DNA is represented by the thin line, and the dotted line indicates that circular plasmid DNA was used for transformation. The first line represents the structure of the *ura5* region in the untransformed strain, the second the structure of the plasmid and the third line the result of the integration deduced from the molecular analysis. **A** Schema corresponding to transformants 810.20 and 810.32. **B** Schema corresponding to transformant 820.1. The number of tandem repeats and the location of the truncated unit could be different from those indicated. Restriction enzyme abbreviations: P, *Pvu*II; X, *Xba*I; K, *Kpn*I; S, *Sph*I; E, *Eco*RI. The horizontal bracket indicates the extent of the 0.9 *Bam*HI fragment referred to as the *ura5* probe

integration (Fig. 3). This structure fits with the results obtained by the analysis of the *Eco*RI digested DNA.

Analysis of the non-homologous insertions

In order to investigate the number of integrated copies and the arrangement of these copies, DNA from the transformants was digested with an enzyme that cuts only once in the transforming plasmid (*Kpn*I for pLLC810 and *Pvu*II for pLLC820). For five transformants (810.15, 810.28, 820.2, 820.3, 820.4), the analysis was consistent with an insertion of only one copy of the plasmid. Further analysis by *Pvu*II for 12 "810" transformants and *Eco*RI for 3 "820" transformants (sites at the borderline between insert and vector in both cases) confirmed this hypothesis and allowed us to establish the sequence involved in the insertion: the lower band revealed by the *ura5* probe in the *Pvu*II digestion of 810.28 DNA corresponds to the size of the insert in pLLC810 (3.7 kb, see Fig. 2, lane E1) whereas no band corresponding to the size of the vector pUC18 (2.7 kb) was observed in the same digest probed with the vector sequence (see Fig. 2, lane E2). This indicates that the recombination event involved the vector sequence. This type of event was also observed to have occurred in transformants 810.15, 820.3 and 820.4. In 820.2, the recombination seems to have taken place in the insert sequence. For the other transformants (see Table 3), more than one copy had integrated. For some of them (810.6, 810.11, 810.17, 810.29), the presence after hybridization with both probes

Table 3. Summary of the molecular analysis of the transformants

Name	Number of regions of insertion	Insertion in the <i>ura5</i> locus	Number of inserted copies	Presence of tandem structure	Recombination
810.20 ^a , 810.32	1	Yes	1	—	Homologous
820.1 ^a	1	Yes	4(3) ^b	Yes	Homologous
810.15 ^a , 820.3, 820.4 ^a	1	No	1	—	Plasmid
810.28 ^a	1	No	1 ^b	—	Plasmid
820.2 ^a	1	No	1	—	Insert
810.6 ^a , 810.17 ^a	1	No	2	Yes	Complex
810.11, 810.29 ^a	1	No	2(3) ^b	Yes	Complex
810.27 ^a , 810.19	1	No	2	No	Complex
810.21 ^a , 810.4	1	No	3	No	Complex
810.22 ^a , 810.23	1	No	3 ^b	No	Complex

^a Checked for possible methylation

^b Analysed by dot-blots. Numbers in parentheses are estimates from dot-blots when these differ from previous estimation

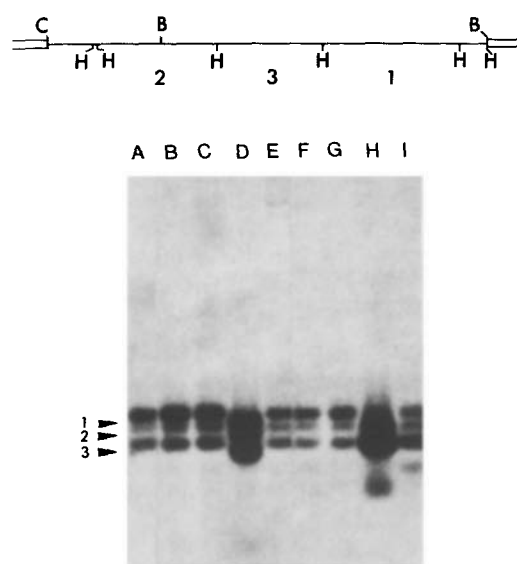


Fig. 4. Test for methylation in secondary transformants. Upper part represents the restriction map of the insert in pLLC840. *Solid line* represents the *Sordaria* sequence and the *open box* the pUC18 sequences. Restriction sites are: C, *ClaI*; B, *BamHI*; H, *HpaII*. The *ClaI* site is present in the genomic sequence but is destroyed during the construction of pLLC840. The 0.9 kb *BamHI* fragment including the *HpaII* fragments numbered 1,3 and part of 2 is referred to in the text as the *ura5* fragment: this fragment contains the open reading frame of the *ura5* gene. The sizes of the *HpaII* fragments 1 to 3 are respectively 385, 344 and 300 bp. Lower part shows the Southern blotting analysis of secondary transformants. *HpaII* digests of DNA from the *ura5-1* strain (lane a) and from eight secondary transformants (lanes b to i) were fractionated in a 2.4% agarose gel and the gel blot was probed with the ³²P labelled 0.9 kb *BamHI* fragment electroeluted from pLLC840. The numbers on the left indicate *HpaII* fragments 1–3. Lane a, *ura5-1* strain; b, 810.27; c, 810.17; d, 810.21; e, 820.1; f, 820.4; g, 810.20; h, 810.22; i, 810.28

of an intense *KpnI* fragment showing the same size as pLLC810 indicated the presence of tandem repeats. Digestion with *PvuII* confirmed this observation. In these cases, the presence of extra bands distinct from the repeat sequence did not allow the localization of the integration event.

In the six remaining transformants (810.4, 810.19, 810.21, 810.22, 810.23 and 810.27), no tandem repeats were observed and the pattern of bands was complex. For some of these transformants, the number of integrated copies was analysed by dot-blotting titration with the *ura5* probe (see Table 3). In all cases, the number of integrated copies did not exceed three.

Every transformant analysed showed a unique pattern of bands in the different digests, except for 810.20 and 810.32 which represent cases of homologous integration of one copy of the plasmid. Therefore each non homologous integration event was unique.

Analysis of possible methylation in the secondary transformants

In order to investigate if methylation of cytosine occurred in the duplicated sequences in the secondary transformants, the DNA from 12 of them (marked ^a in Table 3) and from the recipient strain was digested with *HpaII*. This enzyme recognizes the sequence CCGG and is able to cut only when the two Cs are not methylated (McClelland and Nelson 1988). After blotting, these digests were probed with the *ura5* probe. The differences in the pattern of hybridization between the recipient strain and the transformants could be explained by the integration of the transforming plasmid and no methylation of cytosine seems to have occurred in the duplicated sequences of the transformants (Fig. 4).

Discussion

We present here the first report of the transformation of the homothallic species *S. macrospora*. Both genetic and molecular analysis of the transformants have shown: firstly, that it is possible to transform protoplasts from the *ura5-1* mutant of *Sordaria macrospora* with a plasmid containing the corresponding gene; secondly that integration of the transforming plasmid can occur by different molecular events; and finally that this system allows easy study of the mitotic and meiotic stability of the transformants.

Evidence for transformation includes the appearance of new *ura5* copies in all the transformants and the presence of exogenous vector sequences in the DNA of the transformed strains. Our observations on the rules of transfor-

mation in *S. macrospora* confirm and extend the earlier descriptions of efficiency and integration events in filamentous fungi (review in Rambossek and Leach 1987) (i.e. low efficiency of transformation and integration of the transforming DNA in the genome of the recipient strain). The frequency of homologous insertion observed for *Sordaria macrospora* (4%) is low compared with the 33% obtained in the analogous system for *Podospora anserina*. However, low frequencies of homologous integration are also observed for most filamentous fungi and seem to be more determined by the mutant/gene pair used than to be a characteristic of the organism (for example in *N. crassa*, 90% homologous insertion is observed using the *trp-1* system, Kim and Marzluf 1988, but only 15% with the *qa2* system, Dhawale and Marzluf 1985). A consequence of the low number of transformants resulting from homologous integration in *Sordaria* could be the absence of gene replacement in our experiments.

Another interesting point is that multiple integrations at a unique site are observed in 12 out of 18 transformants, although integrations in multiple sites are scarce here (1 out of 74). In cases of multiple integration, the number of copies remains low (two to four) and tandem repeat are observed.

These two facts have been observed in most systems (Rambossek and Leach 1987). Multiple integration could result either from amplification of one integrated copy or by integration of multiple copies, independently or as the result of recombination events prior to the integration. As cotransformation has been observed in several systems (e.g. *C. cinereus*, Mellon et al. 1987) including *Sordaria*, (L. Le Chevanton and G. Leblon, unpublished results), it appears likely that at least two different plasmids can be integrated in one nucleus. This supports the hypothesis of integration of plasmids in multiple copies.

The results obtained with *Sordaria macrospora* are especially relevant to questions on the nature and sequence of events leading to meiotic stability in transformants. In the closely related species, *N. crassa*, extensive genetic and molecular studies have shown that transformants are unstable through meiosis (Case 1986; Fehér et al. 1986). The mechanism of this instability appears in most cases to be the inactivation of duplicated sequences by methylation of the cytosine residues in these sequences (Selker et al. 1987; Selker and Garrett 1988). The same mechanism is involved in the transformation of *Ascobolus immersus* (Goyon and Faugeron 1989). The spore colour phenotype of the mutant *ura5-1* and the homothallic nature of *S. macrospora* gave us an easy and powerful way to check for the stability of the transformants. Our observations indicated that although the *ura5* gene is duplicated in all of them, the 74 transformants analysed were meiotically stable and that no methylation of the *ura5* sequences could be detected in 12 secondary transformants tested. No sequence specificity has been reported in the inactivation of duplication in *Neurospora* and *Ascobolus*. This suggests that the absence of inactivation of duplicated *ura5* sequence is not specific to this gene, but is a general feature in *Sordaria*. Thus, the differences in stability of the transformants in these different systems could result in differences in a general regulation system (i.e. inactivation of the duplicated sequences) rather than in the transformation mechanism per se.

As the inactivation of duplicated sequences takes place just before meiosis (Selker et al. 1987) we suggest that such

inactivation may act in heterothallic species as a regulatory mechanism associated with the coordinated expression of the two nuclei of different mating type. Such a mechanism would not be required in a homothallic species such as *S. macrospora* in which the two nuclei are most likely derived from the same parental nucleus.

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