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DNA-mediated transformation of the basidiomycete *Coprinus cinereus*

D.M.Binninger[1,2], C.Skrzynia[1], P.J.Pukkila[1,2] and L.A.Casselton[3]

[1]Department of Biology 010A and [2]Curriculum in Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, USA, and [3]School of Biological Sciences, Queen Mary College, Mile End Road, London EL 4NS, UK

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We have developed a simple and efficient transformation system for the agaric fungus, *Coprinus cinereus*. Protoplasts were prepared from asexual spores that harbor one or two mutations in the structural gene for tryptophan synthetase. The protoplasts can be stably transformed using the cloned *Coprinus* gene at a frequency of 1 in 10^4 viable protoplasts. A variety of molecular events accompanies the formation of stable transformants, including insertion of the transforming DNA at the homologous locus. The transforming DNA is stable through cell division, mating, fruiting body formation, and meiosis.

Key words: Coprinus cinereus/gene transfer/homologous integration

Introduction

The basidiomycete fungus *Coprinus cinereus* provides an excellent model system for studies of gene expression at several levels of differentiation. Mating between two genetically compatible monokaryons (uninucleate haploid cells) results in formation of a dikaryotic mycelium in which one nucleus from each parent is maintained in every cell. Genetic compatibility in *Coprinus* is determined by a tetrapolar system involving two unlinked genes, *A* and *B*. The developmental steps controlled by these genes have been described in detail (for review see Casselton, 1978; Casselton and Economou, 1985). The unusual and significant aspect of this regulatory system is that many pairs of dissimilar alleles of each gene can interact to trigger the morphogenetic changes controlled by that gene. The comparatively simple mycelium of the dikaryon can be induced to differentiate into the morphologically complex fruit body (Moore *et al.*, 1979) by regulating the temperature and periodicity of the light (Lu, 1974; Morimoto and Oda, 1974; Kamada *et al.*, 1978). The fruit body has a typical mushroom appearance with ~ 100 double-sided gills that form the support structure for the meiocytes (basidia), the specialized cells that undergo karyogamy (nuclear fusion) and meiosis. A remarkable feature of *Coprinus* is its highly synchronous meiotic cycle; 65–75% of the 10^7 basidia on a single fruit body are in the same stage of meiosis (Raju and Lu, 1973). The duration and synchronous nature of the meiotic cycle allow discrete phases of meiosis to be examined. These stages of meiosis can be identified cytologically by using simple methods for visualising the nuclear contents of basidiospores with both the light and electron microscope (Lu and Raju, 1970; Pukkila and Lu, 1985).

A DNA-mediated transformation (or gene transfer) system for *Coprinus* would provide a powerful tool for molecular analysis of the incompatibility genes and the meiotic process. We were expecially interested in learning whether transforming DNA would be targeted to the homologous chromosomal locus in this higher basidiomycete. *Schizophyllum commune* is the only basidiomycete for which a stable transformation system has been reported (Munoz-Rivas *et al.*, 1986). However, the frequency of integration of the transforming DNA into the homologous genetic locus was not discussed in this initial report. The frequency of homologous integration in Ascomycete fungi is highly variable. In *Neurospora crassa*, a recent study revealed only a single insertion into the corresponding chromosomal location in 32 examples studied (Case, 1986), although higher frequencies of gene replacement events (6 out of 14) have been detected previously (Case *et al.*, 1979). In *Aspergillus nidulans*, the frequency of homologous integration is ~ 80% (Yelton *et al.*, 1984) while in *Saccharomyces cerevisiae*, the frequency is effectively 100% (Kingsman *et al.*, 1981). However, homologous integration is a much rarer event during transformation of organisms with more complex genomes. When cloned DNA is introduced into cultured mamalian cells by the calcium phosphate method, which is comparable to that usually used with fungi, the frequency of targeting the cloned DNA to homologous sequences residing in the chromosome is approximately 10^{-4}–10^{-5} (Smith and Berg, 1984; Lin *et al.*, 1985). This frequency is several orders of magnitude lower than that observed with fungi.

Here, we have investigated whether transformation can be achieved in *Coprinus*, whether the transforming DNA integrates with an appreciable frequency at the homologous chromosomal locus, and whether the transformants are stable in the absence of selection.

Results

Characterization of the double-mutant recipient and transforming DNA

We have used a *Coprinus* genomic clone containing the tryptophan synthetase gene (*TRP 1*) to transform a tryptophan auxotroph to prototrophy. Tryptophan synthetase (EC 4.2.1.20) catalyzes the conversion of indoleglycerol phosphate to tryptophan in a two-step reaction (Umbarger, 1978). A non-reverting tryptophan auxotroph was constructed by independently selecting for two mutations that block each of these partial reactions. The first partial reaction converts indoleglycerol phosphate to indole and glyceraldehyde-3-phosphate and is blocked by the *trp 1-1* mutation. This mutation does not affect the second part of the reaction, the condensation of indole with serine to form tryptophan (Tilby, 1976). The second mutation in the gene was selected by screening for the loss of the ability to utilize indole (see Materials and methods). Both the *trp 1-1* mutation and the newly created *trp 1-6* mutation that blocks the conversion of indole to tryptophan mapped to the same genetic locus. Further confirmation that both mutations are in the same gene was obtained from complementation tests. The interallelic complementation of *trp 1-1* with *trp 1-3* (Tilby, 1976) was abolished by the *trp 1-6* mutation. These genetic data showed that the *trp1-1* and *trp 1-6* mutations were

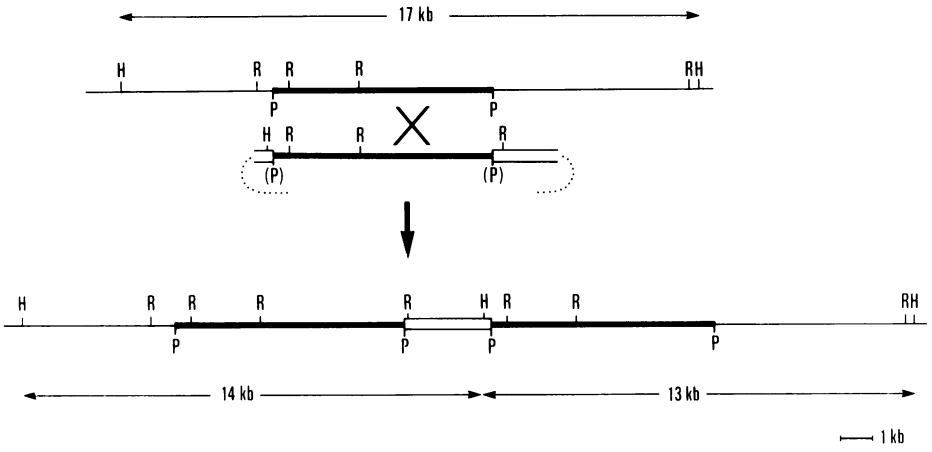

Fig. 1. Schematic diagram showing integration of PCc1001 into the homologous *TRP 1* genetic locus. The 6.5-kb *Pst*I genomic fragment (thick solid line) which contains the *TRP 1* gene of *Coprinus* was cloned into pUC9 (open box) to yield recombinant plasmid pCc1001. For pCc1002, both *Pst*I sites were deleted (indicated by parentheses). The dotted line indicates that circular plasmid DNA was used for transformation. Flanking genomic DNA is represented by the thin line. The *TRP I* gene is located on a 17-kb *Hin*dIII fragement in the untransformed recipient. Integration of pCc1001 into the resident *TRP I* locus by a single homologous recombination event leads to a duplication of the *TRP I* sequences separated by pUC9 DNA. The 17-kb *Hin*dIII fragment is replaced by two new *Hin*dIII fragments of 13 kb and 14 kb. Restriction sites are designated: H, *Hin*dIII; P, *PstI* and R, *Eco*RI.

both within the *TRP 1* gene. When tested individually, the reversion frequency of each mutation was $< 1 \times 10^{-6}$. Therefore, the chance of recovering a spontaneous Trp^+ colony was less than 1 in 10^{12}. We have never recovered a revertant from strains having the double mutation.

The plasmid DNA used in the initial transformation experiments was pCc1001 that contained the *TRP 1* gene on a 6.5-kb *PstI* genomic DNA fragment. The *TRP 1* gene was cloned by heterologous DNA hybridization using a subclone of the tryptophan synthetase gene (*TRP5*) from yeast (Walz *et al.*, 1978; Zalkin and Yanofsky, 1982) as a probe. A restriction map of pCc1001 is shown in Figure 1. DNA sequence analysis revealed significant homology to the yeast *TRP5* gene (data not shown). The complete nucleotide sequence of the *Coprinus cinereus* tryptophan synthetase gene will be reported elsewhere.

Transformation efficiency

Two methods were used to make oidia competent for transformation. Oidia are haploid asexual spores which differentiate at the tips of the monokaryotic mycelium. Treatment of intact oidia with lithium acetate yielded one transformant per 10^7 viable cells. When Onozuka R-10 cellulase and chitinase were used to convert the oidia to protoplasts, the transformation efficiency was increased 1000-fold to one transformant per 10^4 viable protoplasts. For the experiments described here, protoplasts were prepared from germinated oidia. However, the efficiency of transformation and regeneration was similar if protoplasts were prepared from ungerminated oidia (Table I). The transformation efficiency does not appear to be strain-dependent since the frequency was similar for each of three strains containing the *trp 1-1,1-6* mutations and one strain having only the *trp 1-1* allele (Table I). As little as 3 μg of pCc1001 DNA per $10^7 - 10^8$ total protoplasts was saturating for the transformation system. No inhibitory effect was observed when the amount of DNA was increased to 50 μg. For each stable Trp^+ transformant, there were approximately 100 abortive colonies that initially grew to a limited extent on the selective medium. The abortive colonies were not observed in the absence of the transforming DNA.

Physical analysis of transformants

To verify that the Trp^+ colonies were transformants, genomic

Table I. Transformation frequencies using mycelial and oidial protoplasts

Strain	Mutations	Source of protoplasts	Transformants per 10^4 viable cells
218	*trp 1-1,1-6*	Mycelium	1.08
LT4	*trp 1-1,1-6*	Mycelium	1.12
LT5	*trp 1-1,1-6*	Mycelium	1.40
218	*trp 1-1,1-6*	Oidia	0.90
HT14.01	*trp 1-1*	Oidia	0.71

DNAs from 28 stable transformants and 3 abortive transformants were digested with *Eco*RI and analyzed by Southern blot hybridization using a probe made from the cloning vector, pUC9. Since the recipient strain does not have detectable homology with pUC9 (Figure 2, lane b), the presence of these exogenous sequences would confirm that the Trp^+ phenotype was the result of transformation, not reversion of the *trp1-1,1-6* mutations. Twenty (71%) of the Trp^+ isolates had a fragment that hybridized to pUC9 and comigrated with the 3.3-kb *Eco*RI fragment of pCc1001. A representative example is shown in lane a of Figure 2. Six (21%) transformants had one or two fragments of altered size that hybridized to pUC9 with (Figure 2, lane e) or without (Figure 2, lane c) the 3.3-kb fragment. Such patterns would arise if the integration event occurred within the pUC9 sequences. Two (8%) stable transformants had no detectable homology with pUC9, although each had additional fragments, not present in the recipient strain, that hybridized to *TRP 1* DNA (data not shown). The abortive transformants also lacked homology to pUC9 (Figure 2, lane d). These unstable transformants failed to grow on minimal medium after they had been cultured in the non-selective medium.

To determine the frequency of integration of pCc1001 DNA into the homologous *trp 1-1,1-6* locus, blots of *Hin*dIII digested genomic DNAs from these 28 stable transformants were hybridized with a probe made from pCc1001. The *TRP 1* gene lies within a 17-kb *Hin*dIII genomic fragment in the untransformed recipient. Integration of pCc1001 into the resident *TRP 1* locus would lead to a duplication of the *TRP 1* sequences separated by pUC9 DNA. This type of integration event would

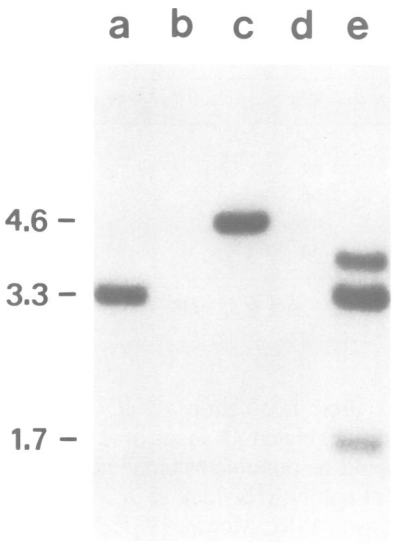

Fig. 2. Detection of pUC9 DNA in genomic DNA from transformants using autoradiography. Genomic DNAs from the untransformed recipient (**lane b**), an abortive transformant (**lane d**) and three stable transformants, T1 (**lane c**), T3 (**lane e**) and T5 (**lane a**), were digested with *Eco*RI and analyzed by Southern hybridization using ^{32}P-labeled, nick-translated pUC9 DNA as a hybridization probe. Size markers (in kb) are shown on the left side.

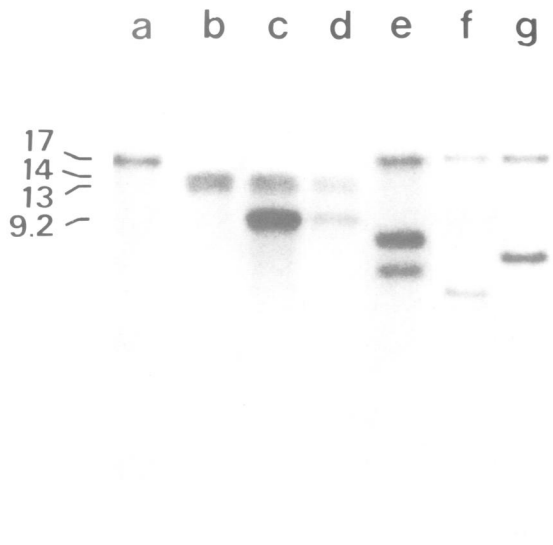

Fig. 3. Southern analysis of transformants obtained with pCc1001 DNA. *Hin*dIII digested genomic DNA was analyzed by Southern hybridization using ^{32}P-labeled, nick-translated pCc1001 as a probe. **Lane a** shows the untransformed recipient (218). **Lanes b** (T24), **c** (T30), and **d** (T31) are transformed strains with pCc1001 integrated into the *TRP 1* locus. **Lanes e** (T28), **f** (T5), and **g** (T6) are transformants containing pCc1001 integrated elsewhere in the genome. Size markers (in kb) are shown at the left edge.

disrupt the endogenous 17-kb fragment and thereby create two new *Hin*dIII fragments of 13 kb and 14 kb. A schematic representation of this process is shown in Figure 1. The hybridization patterns for 3 of the 28 transformants were consistent with this model (Figure 3, lanes b–d). Two of these transformants probably contain tandem duplications of pCc1001 since each had a 9.2-kb *Hin*dIII fragment that comigrated with linear pCc1001. The remaining transformants had the endogenous 17-kb *Hin*dIII fragment in addition to at least two new fragments. There was a wide variation in the sizes of the new fragments with no apparent common subset of fragments among the 28 transformants examined. Three representative examples of integration of pCc1001 DNA at genomic sites other than the *TRP 1* locus are shown in Figure 3, lanes e–g.

The identification of transformants arising by homologous integration at the *TRP 1* locus was simplified by using plasmid pCc1002 which was constructed by deleting the *Pst*I sites from pCc1001. Integration of pCc1002 at the *TRP 1* locus results in replacement of the endogenous 6.5-kb *Pst*I fragment by a single new fragment of 15.7 kb. A total of 64 Trp$^+$ colonies that had been transformed with pCc1002 were examined by Southern analysis. As illustrated in Figure 4A, lane b, homologous insertion into the *TRP 1* locus was also observed with this plasmid. For this transformant, the new fragment is larger than the predicted 15.7 kb since it contains a tandem duplication of the 9.2-kb pCc1002 DNA sequences. When the genomic DNA from this transformant was digested with *Hin*dIII and hybridized with pCc1002 DNA, the hybridization pattern was similar to that expected for a homologous integration with a tandem duplication; compare Figure 4B, lane b to Figure 3, lane d. Sixty (94%) of the transformants contained a single new *Pst*I fragment along with the endogenous 6.5-kb *Pst*I fragment which indicated that a single integration event had occurred at a site other than the *TRP 1* locus (Figure 4A, lanes c–d). As predicted, the endogenous 17-kb *Hin*dIII fragment that contains the *TRP 1* gene was not disrupted in these transformants (Figure 4B, lanes c–d). Multiple insertions were also observed since three (5%) of the transformants contained more than one new *Pst*I fragment (data not shown). Although most of the transformants contained a single copy of pCc1002 at each site of integration, nine isolates contained tandem duplications. These transformants contained a *Pst*I fragment larger than the predicted 15.7 kb that hybridized strongly with pCc1002. When the genomic DNA was digested with *Hin*dIII, a fragment that comigrated with linear pCc1002 was observed (data not shown).

Meiotic transmission of transforming DNAs

The physical analysis indicated that the cloned *TRP 1* gene had inserted into the resident *TRP 1* locus in 4 of the 92 Trp$^+$ transformants examined. The three transformants in this class that had been obtained using pCc1001 DNA were crossed to a Trp$^+$ strain (JV6) in order to confirm the location of the integrated transforming DNA. JV6 was also crossed to the recipient strain, 218, and to a transformant (T5) in which the pCc1001 DNA had not integrated at the *TRP 1* locus. Table II summarizes the results of random spore analysis for each cross. The transforming *TRP 1* DNA in T5 appeared to be loosely linked to the *TRP 1* locus, which is consistent with the physical analysis that showed that pCc1001 DNA had not integrated into the *trp 1-1,1-6* locus of the recipient strain (Figure 3, lane f). Southern hybridization data showed that pCc1001 had integrated into the *TRP 1* locus in T24, T30 and T31 (Figure 3, lanes b–d). When these transformants were crossed to the Trp$^+$ strain (JV6), 99–100% of the progeny were Trp$^+$. These genetic results confirmed that the pCc1001 DNA sequences in these transformants were very tightly linked to the *TRP 1* locus. Lack of recovery of Trp$^-$ progeny was not due to failure of the spores to germinate since crosses of the Trp$^-$ recipient (218) to the TRP$^+$ strain (JV6) yielded the expected 1:1 segregation of Trp$^-$ progeny. Meiotic transmission of the transforming DNA was also demonstrated by crossing T24 to a *trp 1-1* strain (HT14.01). This cross allow-

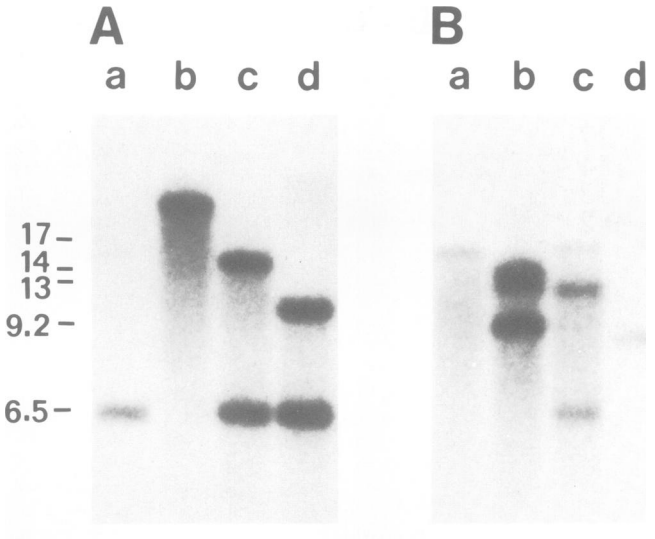

Fig. 4. Southern analysis of transformants obtained with pCc1002 DNA. Genomic DNA from the untransformed recipient (**lane a**) and transformants that harbor pCc1002 DNA integrated into the *TRP 1* locus (**lane b**) or at another site in the genome (**lanes c** and **d**) was digested with *Pst*I (**Panel A**) or *Hin*dIII (**Panel B**). Following gel electrophoresis and blotting to a nylon membrane, the samples were hybridized with pCc1002 labeled with ^{32}P by nick translation. Size markers (in kb) are shown at the left edge.

ed us to distinguish between Trp^- progeny carrying the *trp 1-1,1-6* double mutation (resulting from loss of the transforming DNA), and those that carried the *trp 1-1* allele, since *trp 1-1* strains can utilize indole (Tilby, 1976) whereas the *trp 1-1,1-6* strains cannot. Fifty of 96 random spores from the T24 × HT14.01 cross were Trp^+. All 46 of the Trp^- spores were able to grow on indole, indicating that none of them had arisen from loss of the plasmid DNA.

Discussion

There are three important observations from our results. First, Trp^- protoplasts prepared from haploid asexual spores (oidia) of *Coprinus* can be transformed to Trp^+ with an efficiency of 1 in 10^4 viable protoplasts. Second, a variety of molecular events leads to stable transformants, including insertion of the cloned *TRP 1* gene into the homologous locus of the recipient. Finally, cloned DNA that has integrated into the *TRP 1* locus is stable through mitotic growth and development of the fruit body, even in rich medium supplemented with tryptophan, and these sequences are faithfully transmitted through meiosis.

Oidia provide an abundant and homogenous population of cells from which to prepare protoplasts for transformation. Almost 10^9 oidia can be harvested from a single Petri plate culture. Since the oidia are derived from the monokaryon, these haploid cells represent a clonal population and are genetically identical. Basidiospores are another abundant source of cells that could potentially be used for transformation. However, oidia may offer some experimental advantages since basidiospores, being immediate products of meiosis, are not genetically homogeneous.

The recovery of one *Coprinus* transformant in 10^4 viable protoplasts is comparable to the efficiency obtained with transformation systems for the filamentous Ascomycete fungi, *A. nidulans* (Yelton *et al.*, 1984; Tilburn *et al.*, 1983) and *N. crassa* (Case *et al.*, 1979) and the basidiomycete, *S. commune* (Munoz-Rivas *et al.*, 1986). The transformation efficiency should be sufficient to allow screening of a cosmid genomic library for genes that complement specific phenotypic mutations; a cosmid genomic library for *Coprinus* would contain about 2000 clones. This

Table II. Frequency of trp^- segregants among random spore progeny after crosses to a Trp^+ strain (JV6)

Strain[a]	Trp^+	Trp^-	% Trp^-
T5	70	11	14
T24	98	0	0
T30	99	1	1
T31	92	1	1
218	54	45	45

[a]Phenotype of each strain is Trp^+ except for 218 (the recipient) which is Trp^-.

approach was used by Yelton *et al.* (1985) to isolate the developmentally regulated *yA* locus of *A. nidulans*. In a similar manner, it should be possible to clone genes that rescue mutations that affect mating (Haylock *et al.*, 1980), morphogenesis (Uno and Ishikawa, 1982; Moore, 1981; Takemura and Kamada, 1972) and meiosis (Gibbins and Lu, 1982; Tani *et al.*, 1977; M.Zolan and P.Pukkila, in preparation) in *Coprinus*. The occurrence of tandem copies of the transforming DNA may facilitate recovery and recloning of such sequences (Turner and Ballance, 1985).

Our physical analysis of 92 Trp^+ transformants showed that stable transformants could result from a variety of molecular events. The most common type of transformation event was integration of the cloned *TRP 1* gene into genomic sites other than the *TRP 1* locus. It is clear that stable integration does not always utilize extensive sequence homology. This has been a common observation with transformants of Ascomycete fungi with the exception of yeast. Integrative transformation and complementation of a *pyrG* mutant of *A. nidulans* was obtained with a cloned copy of the *pyr4* gene from *N. crassa* even though the *pyr4* gene failed to hybridize to specific genomic fragments of *A. nidulans* (Ballance *et al.*, 1983; Ballance and Turner, 1985). Similarly, the *amdS* gene of *A. nidulans* can integrate into the *A. niger* genome despite limited sequence homology (Kelly and Hynes, 1985). Our Southern analysis indicated that for six transformants, the bacterial plasmid sequences of the donor DNA may have participated in the integration although an alternative interpretation would be sequence rearrangements. Case (1986) has shown that recombination of the donor DNA into the chromosome of *N. crassa* can occur within the plasmid vector sequences. Detailed DNA sequence analysis is need to evaluate the role that particular DNA sequences may play in integrative transformation in these systems.

One of our initial questions was whether the transforming DNA would be targeted to its homologous genetic locus at an appreciable frequency. For our experiments, the donor DNA was supercoiled although we do not know if it was nicked or broken before entry into the nucleus. Linearization has been shown to increase targeting in some systems (Orr-Weaver *et al.*, 1981; Thomas *et al.*, 1986) but not others (Yelton *et al.*, 1984). We are currently testing the effects of linearization in the *Coprinus* system. In the experiments reported here, insertion of the transforming DNA at the homologous locus occurred in 5% (4 out of 92) of the transformants examined. This frequency indicates that gene disruption techniques (Rothstein, 1983; Shortle *et al.*, 1982) are feasible with this higher basidiomycete. Yashar and Pukkila (1985) have identified genomic clones encoding genes whose transcriptional activity is altered during fruit body morphogenesis. Steady-state levels of some transcripts are 10–20 fold higher in the fruit body compared to the vegetative

mycelium. It is likely, although not proven, that some of these genes play important roles in fruit body development. Targeted gene disruption should provide a method for directly evaluating the function of these genes.

In order for transformation to be useful as a tool for molecular analysis of meiosis, the transforming DNA must be stable through the meiotic cycle. Meiotic instability has been observed with *A. nidulans*. Tilburn *et al.* (1983) observed that the amdS$^+$ phenotype was lost in a third of the progeny from a selfed cleistothecium. Yelton *et al.* (1984) also observed up to a 12% instability with some *trpC* transformants after selfing. In contrast, the three transformants we examined that contained *TRP 1* DNA integrated into the homologous locus were stable through cell differentiation and meiosis, even though selective pressure was not applied.

Materials and methods

Coprinus strains and cultures

The wild-type strain used was JV6, a derivative of ATCC 42722. The tryptophan synthetase mutants used were HT14 and HT14.01 which had the *trp 1-1* allele, and HT7, which contained *trp 1-3* (Tilby, 1976). A double mutant *trp1-1,1-6* was derived from *trp 1-1*, a strain that has partial tryptophan synthetase activity and can utilize indole for growth. Following mutagen treatment with u.v., oidia were subjected to filtration enrichment (Woodward *et al.*, 1954) in indole-supplemented medium. Two mutants no longer able to utilize indole were recovered. Genetic confirmation that the second mutation abolishing indole utilization was in the *TRP 1* gene was obtained from mapping and complementation tests. Strains 218, LT4, and LT5 were used for transformation and are each derivatives of one of the double mutants.

The life cycle and general techniques for using *Coprinus* have been described in detail (Lewis, 1961; Moore and Pukkila, 1985). The yeast−malt−glucose medium (Rao and Niederpruem, 1969) that was used for growth under non-selective conditions was supplemented with 100 μg/ml L-tryptophan. The minimal medium was that of Shahriari and Casselton (1974) except the ammonium tartrate was replaced with 28 mM ammonium chloride. L-tryptophan or indole supplements were added when appropriate at final concentrations of 500 μM and 10 μM respectively. For protoplast regeneration, the normal minimal medium was modified to contain a reduced concentration of glucose (5 mg/ml), 1.5% agar (Difco), 0.5 M sucrose and 0.5% soluble starch (Sigma S-2004). Tryptophan supplement was added for viability determinations.

Transforming DNA

Recombinant plasmid pCc1001 contains the tryptophan synthetase gene (*TRP 1*) of *C. cinereus* on a 6.5-kb *Pst*I genomic fragment cloned into the plasmid vector, pUC9 (Vieira and Messing, 1982). Details of cloning and sequencing of this gene will be reported elsewhere. pCc1002 was derived from pCc1001 by deleting both *Pst*I sites. pCc1001 was digested with *Pst*I (Bethesda Research Laboratories) followed by treatment with T4 DNA Polymerase (New England Biolabs) in the presence of four dNTPs to generate blunt-ends. The blunt-ended fragments were religated with T4 DNA ligase (New England Biolabs) and then used to transform *Escherichia coli* HB101 (Dagert and Ehrlich, 1979). The reaction conditions for each enzyme were those suggested by the manufacturer. Plasmid DNA was isolated by using an alkaline−SDS lysis procedure (Ish-Horowicz and Burke, 1981) including ethidium bromide−CsCl equilibrium gradient centrifugation.

Transformation

Our procedure for preparing competent cells from intact oidia was adapted from the method described by Dhawale *et al.* (1984). Oidia were harvested from a single plate culture (~ 10^9 cells) and germinated at 37°C for 48 h without shaking in 200 ml of YMG medium supplemented with tryptophan. Any aerial growth was discarded before the cells were harvested by centrifugation (750 *g* for 5 min at room temperature) and washed once by resuspension in 1 mM EDTA and 10 mM Tris−HCl, pH 7.5 (TE). The cells were then resuspended in 25 ml 100 mM lithium acetate and incubated at 37°C with gentle shaking for 30 min. The cells were collected by centrifugation and resuspended in 0.4 ml 100 mM lithium acetate before addition of 10−20 μg plasmid DNA in 50 μl TE. After incubating the cells for 30 min at 37°C with gentle shaking, 4 ml 40% polyethylene glycol (PEG) 4000 (J.T.Baker) in 100 mM lithium acetate was added and the incubation continued for an additional 60 min. The cells were washed once with water and resuspended in 1 ml water. Aliquots of 10^7 cells were spread onto the surface of minimal medium agar plates and incubated at 37°C for 3−10 days.

Protoplasts were prepared by treatment of cells with Onozuka R-10 cellulase (Yakult Honsha, Japan) and chitinase (Sigma C-6137) using a procedure based on that described by Kiguchi and Yanagi (1985). Oidia were either germinated for 48−60 h in standing culture to provide mycelium for digestion or they were used directly. When mycelium was used, 400 mg mycelium was suspended in 2 ml MM (0.5 M mannitol in 50 mM maleate pH 5.5) containing 40 mg cellulase and 2 mg (4 units/mg) chitinase. The cells were digested for 2 h at 30 or 37°C without shaking except for brief vortexing at 30 min intervals to release protoplasts into suspension. Protoplasts were separated from mycelial debris by filtering through 100 μm pore nylon cloth, washed twice with MM, once with MMC (MM containing 50 mM $CaCl_2$) and resuspended in MMC to give 10^8 cells/ml. All centrifugations were at 750 *g* for 5 min at room temperature. When oidia were used directly for protoplast formation this procedure was modified slightly. Cells were harvested and washed in 5 ml MM before being resuspended in 1 ml MM containing 20 mg cellulase and 1 mg chitinase. Digestion was for 2.5 h at 37°C with gentle shaking. The protoplasts were diluted by addition of 4 ml MM and harvested by centrifugation. The protoplasts were washed twice in MM followed by resuspension in MMC at a density of $1-5 \times 10^8$ protoplasts/ml.

The transformation procedure was based on that of Ballance and Turner (1985). The protoplasts (0.2 ml aliquots) were mixed with plasmid DNA (3−50 μg in 20 μl TE) and 50 μl PEG (25% PEG 4000, 50 mM $CaCl_2$ in 10 mM Tris−HCl, pH 7.5) in 15 ml polypropylene conical (Corning) tubes. The tubes were placed in ice for 20 min and then 2 ml PEG was added. The protoplasts were incubated at room temperature for 5 min before addition of 4 ml MMC. To regenerate the protoplasts, 0.5 ml aliquots were mixed with 7.5 ml of regeneration medium (kept molten at 50−55°C) and poured over a layer of regeneration medium in 100 mm plastic Petri plates. The plates were inverted and incubated at 37°C. Colonies were examined after 3−5 days. Regeneration medium contained 0.5 M sucrose as the osmotic stabilizer. This could be replaced by 0.6 M KCl but the resulting regeneration efficiency was 10-fold lower. The protoplasts could also be regenerated by spreading them directly onto the surface of the regeneration medium. Protoplast viability ranged from 2−15% and was estimated by making appropriate dilutions into regeneration medium supplemented with tryptophan.

Filter hybridization of genomic DNA

Cultures for DNA extraction were grown in 30 ml liquid YMG medium as described previously (Cassidy *et al.*, 1984). DNA was isolated from the tissue samples using the rapid small-scale procedure described by Zolan and Pukkila (1986). Genomic DNA (0.5−1.0 μg) was digested for 2 h at 37°C in a 50 μl reaction containing 30 units of *Eco*RI, *Pst*I, or *Hin*dIII (Bethesda Research Labs) in the buffer supplied by the manufacturer. DNA samples were electrophoresed in 0.7% agarose (Seakem HGT) gels in 40 mM Tris−acetate, pH 7.8, 5 mM sodium acetate and 1 mM EDTA. The DNA fragments were transferred to Hybond−N nylon membranes (Amersham) and cross−linked to the filter by irradiation with u.v. light according to the manufacturer's directions. DNA hybridization probes were labeled with [^{32}P]dCTP (New England Nuclear) by nick translation to a specific activity > 10^8c.p.m./μg (Rigby *et al.*, 1977). Filters were hybridized for 24 h at 65°C in an aqueous buffer similar to that described by Maniatis *et al.* (1982) except 20 mM sodium pyrophosphate, pH 7.0 was included in all buffers. Autoradiographs were made with XAR-5 X-ray film (Kodak) and Cronex lightning plus intensifying screens (DuPont).

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