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Homologous recombination and allele replacement in transformants of *Fusarium fujikuroi*

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Abstract The ascomycete *Fusarium fujikuroi* could be transformed stably to hygromycin resistance only when the transforming plasmid contained a fragment of DNA from the fungus. The transformation frequencies were roughly independent of the sequence of the particular fungal DNA fragment used, of its size (1.8 or 6 kb), and of whether this DNA was present only once in the fungal genome or about forty times (the genes for ribosomal RNA). The plasmid was integrated into the fungal genome by homologous recombination in the eighteen transformants tested; ectopic integration was never observed. The *carB* gene of *F. fujikuroi* was cloned and shown to complement unpigmented mutants deficient in phytoene dehydrogenase. A mutant *carB* allele was prepared in vitro and used to transform wild-type protoplasts; the transformants contained a genomic duplication and were heterozygous for *carB*; the mutant allele replaced the original wild-type allele when this was spontaneously lost in the transformants. This loss was due to gene conversion in some cases and to recombination between repeated sequences in others.

Key words Integrative transformation · Gene replacement · Carotene synthesis · *Fusarium fujikuroi* · *Gibberella fujikuroi*

Introduction

Fusarium fujikuroi (previously called *Fusarium moniliforme* and *Gibberella fujikuroi*) is a genetic species of ascomycete fungi (Xu et al. 1995; O'Donnell et al. 1998), that is well known as the industrial source of gibberellins (Jefferys 1970; Brueckner et al. 1989) and as the causative agent of the "bakanae" disease of rice, *Oryza sativa* (Phinney 1983). The fungus is an attractive subject for the investigation of secondary metabolism, particularly the gibberellin biosynthetic pathway (Bearder 1983; Avalos et al. 1999), and lends itself easily to the isolation of various kinds of mutants, but its genetical manipulation is subject to many constraints (Cerdá-Olmedo et al. 1994).

Low-frequency transformation of *F. fujikuroi* has been achieved using several heterologous selective markers (Sánchez-Fernández et al. 1991; Brückner et al. 1992). Plasmid pAN7-1, which carries a bacterial *hph* gene for resistance to hygromycin B between a promoter and a terminator derived from *Aspergillus nidulans* (Punt et al. 1987), has been used to transform many fungi (Punt and van den Hondel 1992), but transforms *F. fujikuroi* with very low efficiency, if at all. Reliable transformation of a *niaD* mutant of *F. fujikuroi*, that is unable to use nitrate as a nitrogen source, was obtained when the wild-type *niaD* gene of this fungus was inserted into the pAN7-1 plasmid; about half of the transformants were produced by integration of the plasmid into the *niaD* gene of the recipient (Tudzynski et al. 1996).

We have investigated the effect on *F. fujikuroi* transformation of the presence of various DNA inserts in a vector carrying a marker for hygromycin resistance. We have also studied the nature of the transformants, and describe a two-step procedure for allele replacement.

Materials and methods

Strains, plasmids, and culture conditions

IMI58289 is a wild-type strain of *F. fujikuroi* (O'Donnell et al. 1998), the genetic species previously known as *Gibberella fujikuroi* mating population C. Its mycelia are whitish to pale orange, depending on the illumination during growth, and contain small amounts of neurosporaxanthin and other pigmented carotenoids. SG22, a mutant of IMI58289, has bright-orange mycelia because it

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accumulates pigmented carotenoids (Avalos and Cerdá-Olmedo 1987). SG207 is a white mutant of SG22 (Fernández-Martín et al. 1995).

Plasmid pAN7-1, which carries the *hph* gene for resistance to hygromycin B from *Escherichia coli* between the *gpd* promoter and the *trpC* terminator from *A. nidulans* (Punt et al. 1987), was a kind gift of Dr. Peter Punt. Plasmid pHJA2 is a derivative of pBluescript KS+ (Stratagene, La Jolla, Calif.) formed by inserting, at the *Nae*I site of this plasmid, a 4-kb DNA fragment that contains the *hph* gene and was obtained by complete *Xba*I digestion and partial *Eco*RI digestion of pAN7-1. Other plasmids were named with the prefix p and the significant DNA fragments inserted in them. *E. coli* DH5α (Hanahan 1983) was used for transformation and plasmid maintenance.

Minimal medium (Avalos et al. 1985) and nutrient medium (minimal medium with 1 g/l yeast extract) were inoculated with spores (microconidia) collected from cultures grown on sporulation agar (Cerdá-Olmedo et al. 1994) at 28 °C under white light (about 15 W/m^2). Mycelia were subcultured by transfer of mycelial pieces with sterile toothpicks. Selective medium was nutrient medium supplemented with 100 mg/l hygromycin B. Solid media contained agar (16 g/l). All incubations were done at 30 °C, unless otherwise stated.

Protoplast preparation

Mycelia were obtained from 10^6 spores incubated for 40 h in 50 ml of medium (275 g/l D-glucose, 1 g/l yeast extract, 1 g/l peptone) in a 500-ml flask shaken at 250 rpm. Mycelia were collected on filter paper, washed with water and CPM buffer (Harris 1982), and resuspended in 20 ml of CPM buffer. The suspension was sonicated in a Sonifier (Model 250, Branson Ultrasonics, Danbury, Conn.) for 30 s at 50 W, and centrifuged. Centrifugations to separate cells were carried out at 1200 ×*g* for 10 min at 4 °C. The precipitate was resuspended in 5 ml of CMP buffer, mixed with 5 ml of a solution (10 g/l) of Novozyme 245 (Novo Biolabs, Bagsvaerd, Denmark) in 0.9 M $MgSO_4$, and incubated for 4 h with gentle agitation. The protoplasts were separated from mycelial debris by filtration through a nylon membrane (average pore diameter 50 μm), washed three times with sorbitol solution (1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM TRIS-HCl pH 7.5) by centrifugation and resuspended (at 5×10^8 protoplasts/ml) in sorbitol solution.

Transformation

A mixture of 100 μl of protoplast suspension (5×10^7 protoplasts), 12.5 μl of PEG solution (500 g/l polyethyleneglycol 4 kDa, 50 mM $CaCl_2$, 10 mM TRIS-HCl pH 7.5), and up to 10 μl of plasmid solution was incubated for 20 min on ice, and mixed first with 1 ml of PEG solution and immediately thereafter with 2 ml of sorbitol solution. The protoplasts were recovered by centrifugation, incubated for 12 to 15 h in 0.5 ml of regeneration medium (minimal medium with 4 g/l yeast extract, 8 g/l peptone, 90 g/l KCl), mixed with 3 ml of melted (48 °C) selective agar and plated immediately on selective agar.

DNA manipulations and cloning

Genomic DNA was isolated (Giordano et al. 1999) from mycelia incubated for 48 h in nutrient broth (selective broth in the case of the transformants). After transfer to nylon membranes (Hybond-N, Amersham, Little Chalfont, Bucks., UK) DNA was hybridised with probes labelled with digoxigenin (Boehringer-Mannheim, Mannheim, Germany) and coupled for fluorescent detection on X-ray film (X OMAT S, Kodak). Band intensities were quantified with the NIH Image program (version 1.61, National Institutes of Health, Bethesda, Md.).

PCR was performed in a volume of 25 or 50 μl that contained about 5 ng of genomic DNA or about 10 pg of plasmid DNA, 0.2 mM of each of the four deoxynucleoside triphosphates, 1 μM of each primer, and the Expand PCR System (Boehringer-Mannheim). The reaction mixture was overlaid with light mineral oil, heated at 94 °C for 5 min and subjected to 35 cycles of denaturation (94 °C, 0.5 min), annealing (55 °C, 0.5 min) and polymerisation (72 °C, 1 min) and to a final polymerisation step at 72 °C for 10 min in a programmable thermocycler (Perkin-Elmer Cetus 480). For the first isolation of the *carB* gene fragment the annealing temperature was 45 °C. For the identification of *carB* alleles, the polymerisation cycles were carried out at 68 °C for 3 min.

Primers NS1 and NS8, based on conserved sequences of the genes for 18S rRNA, were described by White et al. (1990). The universal primers T3 and T7 (Stratagene) flank the polylinker sequence of pBluescript KS+ and pHJA2. Conserved motifs of gene *carB* were identified by comparison of homologous protein sequences from the fungi *Neurospora crassa* (Schmidhauser et al. 1990), *Cercospora nicotianae* (Ehrenshaft and Daub 1994) and *Phycomyces blakesleeanus* (Ruiz-Hidalgo et al. 1997). The conserved sequences AEGIWYP and HPGTGVP of the *al-1* gene product from *N. crassa* were chosen for the design of primers cBF and cBR, whose sequences are 5′-GC(TC)GA(AG)GG-TAT(CT)TGGTA(CT)CC-3′ and 5′-GG(AG)ACACC(AG)GT-ACC(AG)GG(AG)TG-3′, respectively. Many variations of the oligonucleotide sequences were excluded by taking into account the preference of *F. fujikuroi* for pyrimidines in the third codon position. Primers cBF and cBR were used for the PCR amplification of fragment UB of the *F. fujikuroi carB* gene. Primers cSF (5′-AGGTGGATTCCACAGGTTAG-3′) and cSR (5′-ACT-TCTCTTGCCACGTGAAG-3′) were based on the sequence of fragment UB. Primer cEF (5′-TCCGGCGCATTTCCTATC-3′) corresponds to an end of the 6.7-kb CB fragment, which contains gene *carB*, and was used, together with primer cSR, for the identification of the *carB* alleles.

The CB fragment was isolated from a partial genomic library of 6–8 kb *Xba*I fragments cloned at the *Xba*I site of pHJA2. One of the plasmids, called pCB, contained the *carB* gene, as shown by hybridisation with fragment UB and PCR amplification with the specific primers cSF and cSR.

Fragments U1–U4 and R1 were isolated from a similar genomic library cloned at the *Xba*I site of plasmid pAN7-1. The DNA fragments UB and R2 were cloned at the *Eco*RV site of pBluescript KS+; the resulting plasmids were cut with *Xba*I and *Hin*dIII and the larger fragment was inserted in pAN7-1 cut with the same restriction enzymes.

To produce the mutant *carB* allele, plasmid pCB was partially digested with *Kpn*I, end-filled with T4 polymerase, self-ligated, and transformed into *E. coli*. Restriction with *Kpn*I and *Xba*I confirmed that the desired mutant allele had lost the *Kpn*I site in *carB*. DNA sequencing was carried out by Medigene (Martinsried, Germany) using a Model ABI377 automatic sequencer (Perkin-Elmer Applied Biosystems, Norwalk, Conn.). For other DNA manipulations see Sambrook et al. (1989).

Carotene analyses

Mycelia were grown for 4 days on nutrient agar either in the dark or under white light (about 15 W/m^2). Carotenoids were extracted and quantified as described by Avalos and Cerdá-Olmedo (1986); the concentration of neurosporaxanthin was estimated from the absorption of the acetone extract at 475 nm and the concentrations of intermediate carotenes eluted from the column.

Results

Cloning of single-copy and repeated DNA sequences

A fragment of repeated DNA was obtained from the most abundant fragment size class produced by

digestion of genomic DNA with a restriction enzyme. Electrophoretic separation of *Xba*I-digested genomic DNA produced a broad smear with two prominent bands of 2 and 6 kb, respectively. DNA from the 6-kb band was eluted and inserted into pAN7-1. Ten of the inserts exhibited identical restriction patterns; one of these, called R1, was shown by partial sequencing to be a fragment of the gene for 28S rRNA. The restriction patterns of 17 other inserts were all different. Four of them, called U1–U4, were chosen for further work. The plasmids that contain these inserts are called pR1 and pU1–pU4, respectively.

An 1.8-kb fragment of the gene for 18S rRNA was obtained by PCR amplification of wild-type *F. fujikuroi* DNA with primers based on conserved segments of that gene. The fragment, called R2, was inserted in plasmid pAN7-1 to form plasmid pR2.

Loss of function of the gene *carB*, which codes for phytoene dehydrogenase, results in an albino phenotype and in the intracellular accumulation of the colourless phytoene (Avalos and Cerdá-Olmedo 1987). In contrast, a functional *carB* allele allows the production of neurosporaxanthin and other coloured carotenoids and leads to the pale orange pigmentation of the wild type when grown in the light, and to the bright orange pigmentation of certain overproducing mutants. PCR amplification of genomic DNA with primers based on conserved segments of genes for phytoene dehydrogenase led to the production of an 820-bp DNA fragment called UB; this was confirmed to be a fragment of the *carB* gene by sequencing. Fragment UB was inserted into pAN7-1 to form plasmid pUB and used to screen a partial library of genomic DNA. The 6.7-kb DNA fragment called CB, present in the plasmid pCB, contained the whole *carB* gene (Fig. 1). The single bands in Fig. 1A and the frequent isolation of albino *carB* mutants deficient in phytoene dehydrogenase (Avalos and Cerdá-Olmedo 1987) indicate that there is a single copy of gene *carB* in the genome.

The presence of a functional *carB* gene in plasmid pCB was confirmed by using it to transform protoplasts of the albino *carB* mutant strain SG207 to hygromycin resistance. Transformation restored the carotenoid content and the bright orange pigmentation of strain SG22, from which the albino mutant SG207 was originally obtained (Table 1).

A null mutant allele of *carB* was prepared in vitro by inserting 4 bp at the *Kpn*I restriction site present in the gene *carB* in plasmid pCB. The plasmid that carries the mutant allele was called pCB1.

To estimate the redundancy of the newly isolated DNA fragments in the genome of *F. fujikuroi*, we hybridised them with various amounts of *Fusarium* genomic DNA and compared the threshold hybridisations with those of the single-copy sequence UB (Fig. 2). We concluded that the *F. fujikuroi* genome contains about 40 copies of the 28S rRNA gene and probably a single copy of the U DNA fragments. These values are approximate, because they are dependent on the sequence and the size of the probes and the efficiency of labelling.

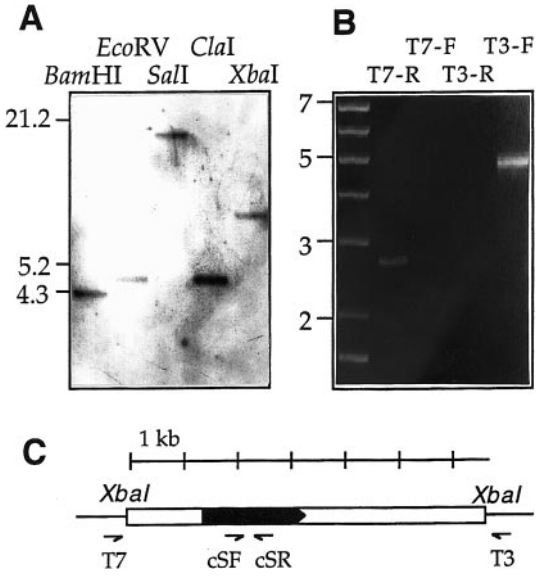


Fig. 1A–C Cloning of the *carB* gene. **A** Hybridisation of genomic DNA samples of *F. fujikuroi* with fragment UB of the *carB* gene. DNA fragments obtained by digestion with the restriction enzymes indicated *above* each lane were separated by electrophoresis and probed with the UB fragment of the *carB* gene. The 6.7-kb *Xba*I restriction fragment that hybridises with the probe fragment UB was termed fragment CB. **B** DNA products obtained by PCR replication of plasmid pCB, which carries fragment CB. The primer combinations are shown *above* each lane. **C** Relative locations of the primers and the *carB* gene in plasmid pCB; cSF and cSR are oligonucleotides internal to fragment UB; fragment CB is indicated as a *box* and the *carB* gene as a *filled arrow*. Fragment sizes are given in kb

Transformation of *F. fujikuroi*

Attempts to transform *F. fujikuroi* protoplasts with plasmid pAN7-1 resulted in slight background growth on hygromycin plates. A few small distinct colonies were eventually obtained, but their failure to grow when transferred to fresh hygromycin plates suggested that they were abortive transformants: presumably the ex-

Table 1 Carotenoid content (μg/g dry mass) of mycelia grown for 4 days at 22 °C in the dark

Strain[a]	Compound(s)[b]			Total
	Phytoene	Intermediate	Neurosporaxanthin	
Wild type	–	3	4	7
SG22	–	145	463	608
SG207	631	–	–	631
Tr	–	123	425	548

[a] SG22 is a bright-orange mutant derived from the wild type, SG207 is an albino mutant of SG22, and Tr is a hygromycin-resistant transformant of SG207 obtained with plasmid pCB

[b] Phytoene is colourless. The colour of mycelium is due to lycopene, γ-carotene, β-carotene, and torulene (collectively called "intermediate" carotenes) and neurosporaxanthin; –, undetectable (< 5 μg/g for phytoene, < 1 μg/g for coloured carotenoids)

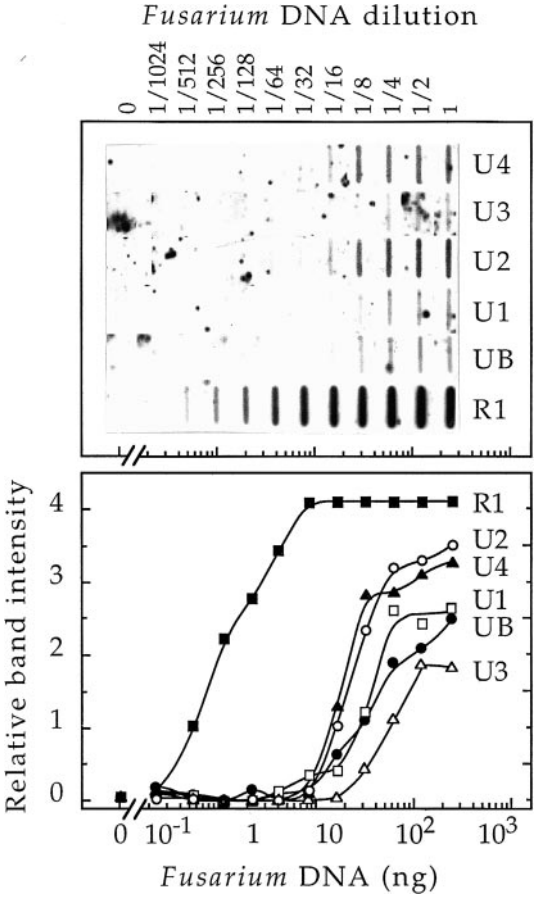


Fig. 2 Relative abundance of various sequences in the *F. fujikuroi* genome. *Upper panel* Mixtures of DNA from *Fusarium* and phage lambda were prepared and 0.25 μg aliquots were hybridised with an excess of the DNA probes R2, U1, U2, U3, U4 and UB. The fractions (*top*) refer to the proportion of *Fusarium* DNA in the mixtures. *Lower panel* Plot of the relative band densities in the blot shown in the *upper panel* against the amount of *Fusarium* DNA in the mixtures

ogenous DNA was expressed in the recipient cells, but could not be maintained indefinitely in them.

Insertion of DNA fragments from *F. fujikuroi* into plasmid pAN7-1 led to the appearance of stable hygromycin-resistant transformants (Table 2). The transformation frequency was independent both of the size of the insert (1.8 to 6.9 kb) and of the number of homologous sequences in the recipient genome (one or about 40). No transformants were obtained with plasmids that contained smaller inserts, such as the 0.8-kb UB fragment of *carB*.

Because the protoplasts contain several nuclei, the colonies formed by transformed protoplasts on hygromycin agar (“primary” transformants) could be heterokaryons containing both transformed and untransformed nuclei. Since the microconidia of *F. fujikuroi* are uninucleate (Avalos et al. 1985), the microconidia of primary transformants should produce homokaryotic “secondary” transformants.

All hygromycin-resistant colonies tested (31, from 14 independent transformation experiments) contained

Table 2 Transformation of *F. fujikuroi* with plasmids containing various DNA inserts

Plasmid	Inserted DNA		Transformants/μg DNA[a]		
	Size (kb)	Copy number[b]	Experiment 1	Experiment 2	Experiment 3
None	–	–	0	0	0
pAN7-1	None	0	0	0	0
pUB	0.8	1	0	0	0
pU1	6.0	≈1	8.3	4.6	0.4
pU2	6.8	≈1	4.3	4.2	0.6
pU3	6.9	≈1	4.5	7.2	0.8
pU4	6.1	≈1	9.5	9.0	0.4
pR1	6.1	≈40	0.8	6.6	0.8
pR2	1.8	≈40	7.0	5.0	0.4

[a] Transformation frequencies obtained in three independent experiments with different batches of protoplasts are given. In each experiment aliquots from the same batch of protoplasts were exposed to 5 μg of DNA (3 μg of pU1, 0.7 μg of pU2, 2 μg of pU3, and 2 μg of pU4 were used in Experiment 1)
[b] Estimated number of copies of the inserted DNA present in the fungal genome

DNA that was detectable by hybridisation with linearised plasmid pAN7-1, while the wild-type strain did not. This was expected, because *F. fujikuroi* does not mutate spontaneously to hygromycin resistance under the conditions of our experiments.

The exogenous DNA had been integrated into the recipient genome in all secondary transformants tested (27, from 11 independent transformation experiments). The size of the hybridising DNA in the secondary transformants differed from that of the original plasmid. In the absence of prior restriction, the hybridising DNA had, as expected, the same slow electrophoretic mobility as bulk genomic DNA.

The plasmid was integrated by recombination between the homologous DNA sequences of plasmid and fungus in all secondary transformants tested (18, obtained in 11 independent transformation experiments using four different plasmids). The U1 DNA is present in a single 12-kb *Eco*RI fragment of the fungal genome and a single 10-kb *Eco*RI DNA restriction fragment of plasmid pU1. Insertion of pU1 by recombination with the resident U1 led to the replacement of the 12-kb fragment by two new fragments (transformants T3 and T4 in Fig. 3A). These fragments have a combined size of 22 kb, as expected. Transformant T5 exhibited the wild-type band, in addition to the two bands produced by plasmid integration; this could be due to excision of the integrated plasmid in some nuclei of the multinucleated mycelium or to chromosome rearrangements (Perkins et al. 1993). The U2 DNA has a *Sal*I restriction site and is part of two *Sal*I fragments, 3.7 and 4.4 kb long, found in the fungal genome; plasmid pAN7-1 itself has another *Sal*I restriction site. The *Sal*I restriction fragments of the transformants were those expected from the integration of plasmid pU2 by recombination with the resident U2 sequence: the two fragments that contained pAN7-1 sequences added up to 13.5 kb, the total size of plasmid

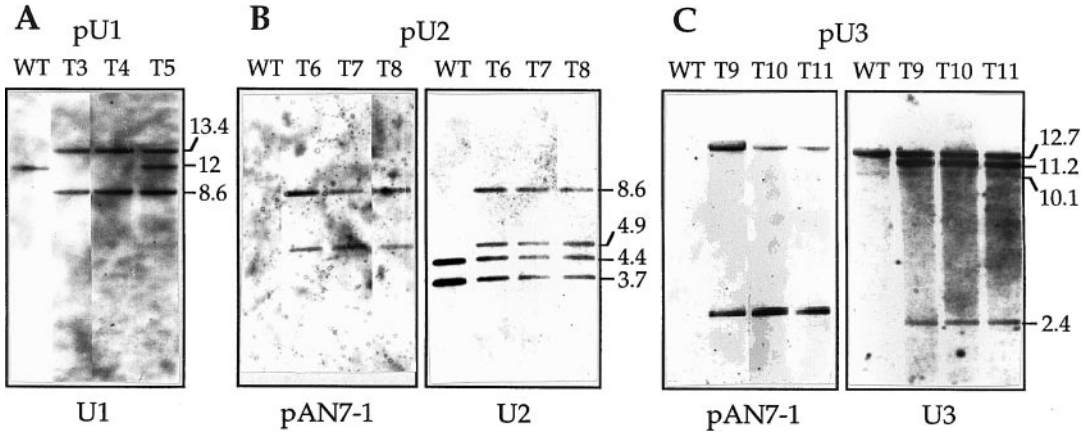


Fig. 3A–C Homologous integration of transforming DNA in the *F. fujikuroi* genome. Genomic DNA from the wild-type strain (WT) and transformants (T) was digested with *Eco*RI (**A**) or *Sal*I (**B** and **C**), fractionated by electrophoresis in agarose, and hybridised with the DNA probes indicated *below* the hybridisation profiles. The transformants were obtained with the plasmids indicated *above* the profiles; plasmid pUx was constructed by inserting the DNA fragment Ux into plasmid pAN7-1. Fragment sizes are given in kb

pU2. The U2 sequences were present in the same two fragments and in two fragments of the same sizes as those found in the wild type (Fig. 3B). The U3 DNA has a *Sal*I restriction site 0.3 kb from one of its ends. The results with pU3 are in agreement with the ones of pU2 except for the expected quantitative changes (Fig. 3C).

The transformants were remarkably stable. After one vegetative growth cycle on sporulation agar without hygromycin, microconidia were harvested and plated on nutrient agar to produce isolated colonies. Most of these colonies (94 ± 4% for transformants obtained with plasmids pR1, pR2, pU1, pU2, pU3, and pCB1) retained the resistant phenotype, as judged by their ability to grow upon transfer to fresh hygromycin agar. This fraction was only 45 ± 9% for the transformants obtained with pU4, which were thus the most unstable.

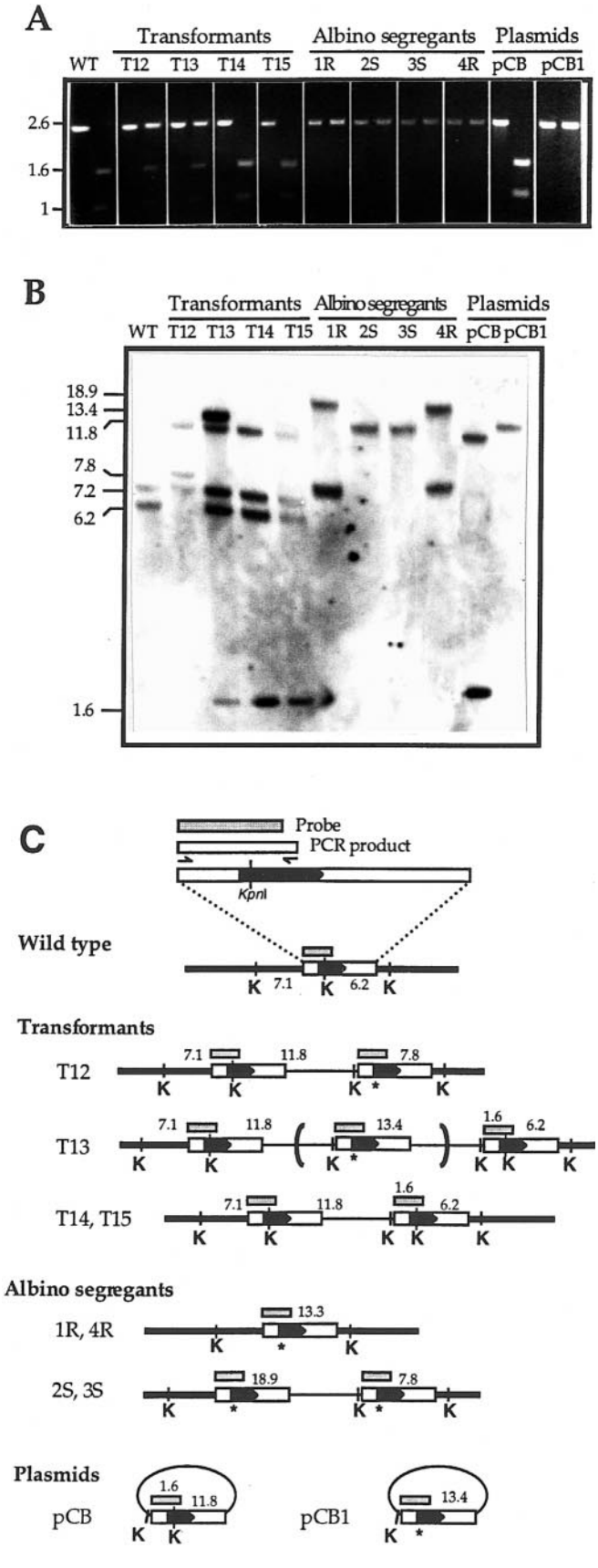
The presence of free plasmids in the secondary transformants was confirmed by transformation of *E. coli* to ampicillin resistance with DNA from secondary transformants. The low transformation frequencies (about 10^{-7} times those obtained with pure plasmid DNA) indicated that there were very few free plasmids in the transformants. The plasmids recovered from the *E. coli* transformants had the same restriction patterns as the plasmids (pR1, pR2, and pU4) initially incubated with the fungal protoplasts.

Allele replacement

We used homologous integration to replace the wild-type *carB* allele by a mutant allele constructed in vitro. Plasmid pCB1, which carries the mutant allele, was used to transform wild-type protoplasts to hygromycin resistance. Wild-type and mutant alleles were detected by electrophoretic separation of *Kpn*I restriction fragments (Fig. 4A). All transformants were produced by homologous integration (Fig. 4B). Transformant T12 was produced by homologous integration of a single copy of plasmid pCB1; both *carB* alleles were present in the genome, as shown by the similar amounts of cut and uncut DNA molecules. Transformants T14 and T15 contained two copies of the wild-type allele, presumably as a result of gene conversion. The results with transformant T13 are consistent with the tandem integration of about four copies of the plasmid at the same homologous genome site. The proposed structures of the transformant genomes are represented schematically in Fig. 4C.

The recessive nature of the mutant allele is manifest in the pale orange colour of wild-type and transformant colonies grown in the light. The colour was maintained in microconidial subcultures of transformants T13, T14, and T15, but about 1 out of every 600 colonies produced by microconidia from transformant T12 were albino in phenotype ($1.7 \pm 0.4 \times 10^{-3}$, mean and standard error of the mean for the microconidia of five secondary transformants). The complete lack of activity of the mutant *carB* allele and the homozygosity of the albino strains for this allele was confirmed by carotenoid analyses of 4-day-old mycelia grown in the light. The albino segregants contained no coloured carotenoids, but had considerable amounts of phytoene (631 and 707 μg/g dry mass in segregants 1R and 2S, respectively, of transformant T12); under the same conditions (28 °C, 15 W/m^2 white light), the wild type contained no phytoene, but its coloured derivatives (176 μg/g dry mass, 80 % of which was neurosporaxanthin).

Most of the albino segregants (28 out of 38) were hygromycin sensitive. Four albino segregants were analysed in detail (Fig. 4). Those that were sensitive to hygromycin contained a single copy of the mutant *carB* allele and were generated presumably by excision of the vector and the wild-type *carB* allele. The resistant segregants contained the vector and two copies of the mutant *carB* allele and resulted presumably from gene conversion of the wild-type to the mutant allele.



Discussion

The transformation of *F. fujikuroi* involved the integration of the exogenous DNA into the recipient genome and required sequence homology between them. Partial homology may suffice, as shown by the transformation of *F. fujikuroi* with a plasmid that contained a selectable gene (*niaD*) from *A. niger* (Sánchez-Fernández et al. 1991), but no *F. fujikuroi* DNA. The *A. niger* and *F. fujikuroi niaD* genes are not very closely related: they do not cross-hybridise under stringent conditions and their nucleotide sequences (EMBL Accession Nos. M77022 and X90699, respectively) are not very similar. The heterologous *niaD* transformants are very rare, grow very slowly and require much longer incubation times to appear than would be permissible with hygromycin selection.

The transforming plasmid was integrated in the fungal genome at the site of sequence homology in all transformants investigated (eighteen obtained with four independent plasmids). Our failure to detect ectopic integration in this sample indicates that homologous integration is largely predominant; for a significance level of 0.05, we estimate that homologous integration occurs in at least 85% of all transformants, and perhaps much more. Ectopic integration predominated (9 out of 15 cases) when *F. fujikuroi* was transformed with the homologous *niaD* gene (Tudzynski et al. 1996); the reason for this divergence may lie in differences in the methods and in the genetic backgrounds of the strains used.

Transformation required long homologous sequences, but did not seem to be affected by variations in length above the required minimum. Very short homologous sequences are sufficient in *Saccharomyces cerevisiae* (Wach et al. 1994) and *Candida albicans* (Wilson et al. 1999), where about 50 bp on each side of a selectable marker are enough for gene disruption with linear DNA. Homologous integration in other filamentous fungi re-

Fig. 4A–C Allele replacement. **A** Detection of *carB* alleles. Electrophoretic profiles of the products of PCR with primers cEF and cSR, using DNA from plasmids carrying the wild type and mutant alleles (pCB and pCB1, respectively) and genomic DNA from the wild type (WT), transformants obtained with pCB1, and albino segregants of transformant T12. The wild-type and mutant alleles differed in a *Kpn*I restriction site. The albino segregants were termed R or S according to whether or not they expressed the selectable marker (hygromycin resistance) borne by the transforming vector. Two samples were run in each case, undigested (*left lane*) or cleaved with *Kpn*I (*right lane*). **B** The genomic and plasmid DNA indicated *above* the lanes was digested with *Kpn*I, fractionated by electrophoresis in an agarose gel, and hybridised with the *carB* probe. **C** Schematic diagrams of the deduced genomic structures. The *filled arrow* represents the *carB* gene and the *box* that includes it represents the *F. fujikuroi* DNA inserted in the transforming vector. The *thick line* represents flanking *F. fujikuroi* genomic DNA. The *thin line* represents the vector. The *numbers* indicate the distances (in kb) between consecutive *Kpn*I sites (indicated by K). The *asterisk* represents the *carB* mutation. The *arrows* mark the positions of the PCR primers. Transformant T13 contained about four copies of the DNA in the region indicated in *parentheses*

quires longer homologous sequences and its frequency increases with their size. Thus, the threshold with *Neurospora crassa* is about 5 kb and a 9-kb sequence permitted homologous integration in 30% of the transformants (Asch and Kinsey 1990). In *A. nidulans*, homologous integration occurred in 43, 27, and 14% of the transformants obtained with *niaD* gene fragments 2.1 kb, 1.2 kb, and 0.9 kb long, respectively (Bird and Bradshaw 1997).

The low transformation frequencies obtained in our experiments are not unusual in fungi, but much higher frequencies are found in some species (Lemke and Peng 1995). The scarcity of targets for homologous integration in genomic DNA, in comparison with all possible targets for ectopic integration, may be a limiting factor. This, however, is at variance with the observation that DNA sequences present once and about 40 times (the genes for ribosomal RNA) in the genome gave the same transformation frequencies. Similar results were obtained in *N. crassa* which contains about 200 copies of the genes for ribosomal RNA in its genome (Russell et al. 1989). The competence of protoplasts, i.e, the degree of access of exogenous DNA to the nucleus, is likely to be the limiting factor for the transformation of *F. fujikuroi*.

The transformants were not completely stable, as judged by the loss of the resistance phenotype in the spores of transformants. We recovered intact plasmids from some of the transformants, suggesting that they had been excised from the chromosome by a second homologous recombination event.

The integration and excision of homologous DNA facilitates allele replacement (Scherer and Davis 1979; Miller et al. 1985). Homologous integration of a vector carrying a mutant *carB* allele in a wild-type genome led to a heterozygous duplication. Loss of the wild-type allele allowed segregants to express the mutant allele. The uninucleate nature of *F. fujikuroi* spores facilitates the detection of recessive segregants.

The heterozygous duplication could be resolved by two separate mechanisms: gene conversion (which leads to a homozygous duplication) and recombination between the repeated sequences (which leads to the excision of the vector and one of the alleles). The transformants with two copies of the wild-type allele and the hygromycin-resistant albino segregants provide evidence for the first mechanism; the hygromycin-sensitive albino segregants provide evidence for the second. The two mechanisms are not exclusive, since gene conversion could have preceded recombination.

Detection of gene conversion in our experiments was relatively easy because the *carB* gene permits the detection of allele replacement by visual screening of colonies. Genes and mutants for the synthesis of carotenoid pigments could therefore provide useful markers for future transformation experiments in *F. fujikuroi*.

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