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Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi

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Abstract

Gene replacement via homologous double crossover in filamentous fungi requires relatively long (preferentially >0.5 kb) flanking regions of the target gene. For this reason, gene replacement cassettes are usually constructed through multiple cloning steps. To facilitate gene function studies in filamentous fungi avoiding tedious cloning steps, we have developed a PCR-assisted DNA assembly procedure and applied it to delete genes in filamentous fungi. While the principle of this procedure is essentially the same as other recently reported PCR-based tools, our technique has been effectively used to delete 31 genes in three fungal species. Moreover, this PCR-based method was used to fuse more than 10 genes to a controllable promoter. In this report, a detailed protocol for this easy to follow procedure and examples of genes deleted or over-expressed are presented. In conjunction with the availability of genome sequences, the application of this technique should facilitate functional characterization of genes in filamentous fungi. To stream line the analysis of the transformants a relatively simple procedure for genomic DNA or total RNA isolation achieving ~100 samples/person/day is also presented.

Keywords: PCR; Filamentous fungi; Gene replacement; Overexpression; Reporter fusion

1. Introduction

The availability of whole genome sequences for a number of filamentous fungi including the model organisms *Aspergillus nidulans* and *Neurospora crassa* as well as plant and human pathogens opens new research avenues. The first step of understanding gene functions generally involves disruption and/or over-expression of individual genes. Unlike in yeast where only about 50 bp of homologous DNA sequences are required for targeted integration, gene disruption by homologous replacement in filamentous fungi usually requires longer DNA sequences (preferentially 500 bp or more). Thus, construction of gene-disruption cassettes in filamentous fungi is usually accomplished through several tedious and time-consuming cloning steps. A more efficient functional characterization of genes in filamentous fungi requires a simple and fast procedure to build gene-disruption constructs.

The method of Chaveroche et al. (2000) ensures the presence of very long flanking sequences, but involves a laborious step of in vivo recombination in *Escherichia coli*. In contrast, PCR-based fusion techniques obviate cloning steps (Davidson et al., 2002; Kolkman and

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Stemmer, 2001; Shevchuk et al., 2004; Stemmer, 1994a,b) and PCR-assisted gene manipulations were carried out in the pathogenic fungus *Cryptococcus neoformans* (Davidson et al., 2002). Independently from these studies, we have developed a PCR-assisted technique that can be used to construct recombinant DNA molecules combining any two or three DNA fragments. Following this technique, deletion of 31 genes was carried out. The final product of each deletion construct composed of the chosen selective maker with 0.5–3.0 kb upstream and downstream flanking regions. In this report, we present a detailed protocol to carry out this technique, which was effectively used for gene manipulations in three filamentous fungal species, *A. nidulans*, *Aspergillus fumigatus*, and *Fusarium graminearum*. In addition, simple and fast methods to isolate filamentous fungal genomic DNA or total RNA are described.

2. Materials and methods: protocols

2.1. Strains, culture conditions, genetics, and transformation

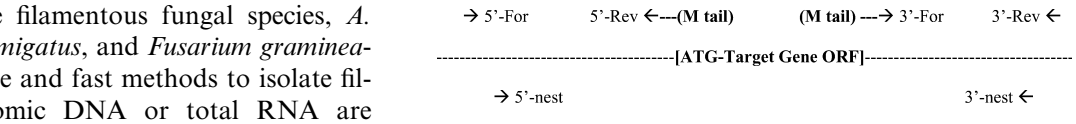
All *A. nidulans* strains derived from the standard laboratory Glasgow strain or Fungal Genetics Stock Center and carried auxotrophic markers appropriate for each transformation experiment. Transformation recipient strains include RMS011 (*pabaA1*, *yA2*; Δ*argB*::*trpC*$^{+}$; *trpC801*), PW1 (*biA1*; *argB2*; *methG1*), FGSC237 (*pabaA1*, *yA2*; *trpC801*), CS2902 (*biA1*, *pyrG89*; *pyroA4*; *riboB2*), CS2903 (*biA1*; *wA3*; *pyroA4*; *riboB2*), CS2904 (*pabaA1*, *pyrG89*; *pantoB100*), CS2905 (*yA2*, *pantoB100*, *riboB2*), CS2906 (*pyrG89*, *yA2*; *argB2*; *pantoB100*, *riboB2*). Genetic markers as in Clutterbuck (1994). The *A. fumigatus* strain AF293.1 (*pyrG*$^{-}$) was from Greg May at the University of Texas, MD. Anderson Cancer Center. *F. graminearum* (R-5317, equal to MRC1781 and NRRL5908) strains were from the *Fusarium* Research Center at the Penn State University. *A. nidulans* media are described in Martinelli and Kinghorn (1994). Transformation of *A. nidulans* and *A. fumigatus* was carried out as described by Tilburn et al. (1983) or Han et al. (2004a). Transformation of *F. graminearum* was performed as previously described (Lee et al., 2002).

2.2. Double joint PCR (DJ-PCR) to build a gene replacement construct

A typical reaction assembling three components using the *argB* gene of *A. nidulans* as a selective marker is described as an example. In general, sufficient amount of maker DNA is pre-amplified, cleaned, and stored as stocks. Template DNA for marker gene amplification can be genomic or plasmid DNA. The *argB* gene of *A. nidulans* is amplified with the following primers: *argB-For*: 5′-gac cag ttt aga ggc ctc-3′, *argB-Rev*: 5′-gtg tta ggc ctg gat cta-3′.

2.2.1. First round PCR: amplification of 5′- and 3′-flanking regions of the gene(s) of interest

(a) Design the following six primers for the 1st round PCR: 5′-For, 5′-Rev +marker tail, 3′-For +marker tail, 3′-Rev, 5′-nest and 3′-nest (see below and Fig. 1)



Note 1. Add common marker (e.g., *argB*) tail (M tail) sequences at the *5′-upstream* of the 5′-Rev +marker tail and 3′-For +marker tail primers: e.g., *argB tail for 5′-Rev*: 5′*-agt caa atg agg cct cta aac tgg tc a-* (put your 5′-Rev primer here)-3′; *argB tail for 3′-For*: 5′*-agc caa ggt aga tcc agg cct aac ac a-* (put your 3′-For primer here)-3′.

Note 2. Design a nested primer pair starting just after a 'T' so that no mismatch mutations are introduced at the 3′ ends of PCR product in the event that the *Taq* or Long Expand polymerases add an additional 'A' at the 3′-ends (Clark, 1988).

(b) After designing primers, perform two separate conventional PCR reactions using 5′-For and 5′-Rev +marker tail, and 3′-For and 3′-Rev +marker tail, respectively.

(c) Check PCR products by agarose gel-electrophoresis and clean up using any PCR-cleanup kit.

Note 3. The procedure described above is used in the Yu laboratory. Routinely, at the Orsay laboratory the chimeric primers are used to amplify the selective marker, not the flanking sequences. Both procedures work with equal efficiency.

2.2.2. Second round PCR: fusion of three fragments

Mix following ingredients and perform reaction *without primers:*

PCR mixture (final 25 μl)	PCR
1 μl of purified 5′-flanking amplicon	94 °C 2 min
1 μl of purified 3′-flanking amplicon	94 °C 30 s —⊤
3 μl of purified *argB* amplicon	58 °C *10 min* × 10–15 cycles
2 μl of dNTP (2.5 mM each)	72 °C 5 min —⊥
2.5 μl of 10× PCR buffer	72 °C 10 min
15.25 μl of sterile distilled water	
0.25 μl of *Taq* polymerase	

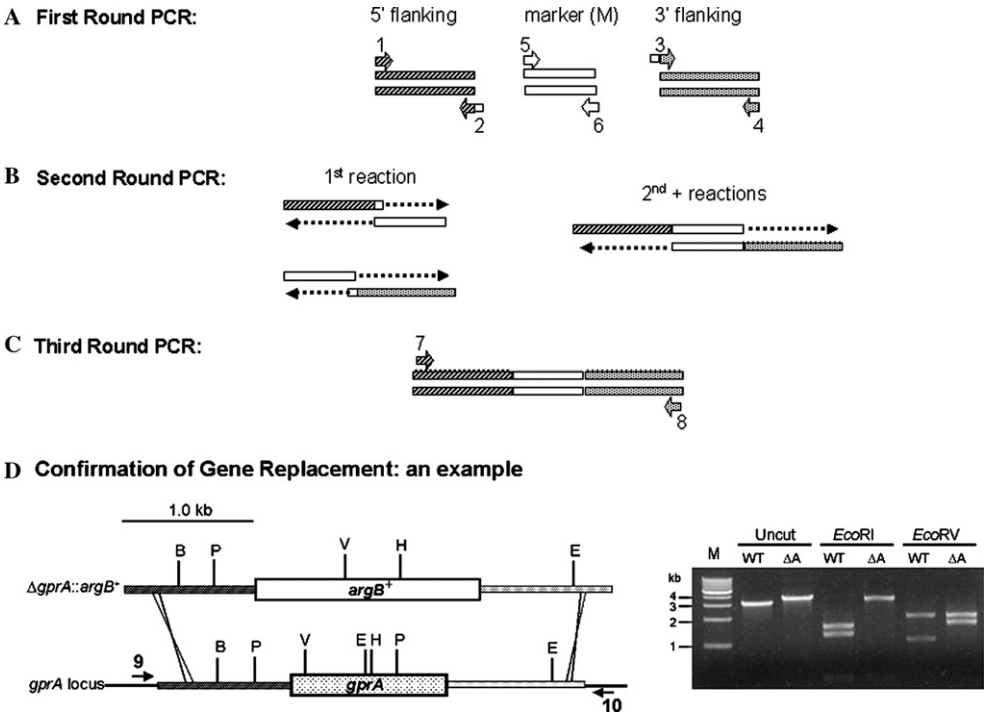


Fig. 1. Schematic representation of the construction of a gene replacement cassette. A typical reaction will fuse DNA fragments of a 5′ flanking sequence, a 3′ flanking sequence and a marker (M). Primers 2 and 3 should carry 25–30 bases of homologous sequence overlapping with the ends of the selectable marker of choice. The arrows numbered from 1 to 10 represent primers for the PCRs and primers 2 and 3 are 45–60 bases long chimeric primers. If a polymerase that incorporates an A to the 3′ end of the PCR product is used, it is strongly suggested that nested primers (7 and 8) are designed after a T in the genomic sequence. (A) First round PCR: amplification of the components using the specific and chimeric primers. (B) Second round PCR: the assembly reaction is carried out without using any specific primers, as the overhanging chimeric extensions act as primers. The first two cycles are shown in detail. (C) Third round PCR: amplification of the final product using nested primers. (D) Confirmation of gene replacement: an example of deletion confirmation of the *gprA* gene encoding a putative G protein coupled receptor (Seo et al., 2004) is shown. The *gprA* deletion cassette was transformed into RMS011 (carrying the Δ*argB*::*trpC*$^{+}$ allele) selecting for arginine prototrophy. Transformants were randomly picked and examined for double crossover-mediated gene replacement pattern by PCR amplification of the *gprA* locus using a primer pair beyond the flanking regions included in the cassette (primers 9 and 10). As shown, amplicons of wild type (3.2 kb) and deletion (3.8 kb) alleles of *gprA* differ in size (uncut). In addition, restriction enzyme digestion patterns of the individual amplicons further distinguish the wild type and null alleles. When digested with *Eco*RI, while the wild type amplicon is cut into three fragments (note the ~300 bp faint band) due to the presence of an *Eco*RI site in the *gprA* ORF, the Δ*gprA* amplicon gave rise to two bands. Similarly, the different positions of the *Eco*RV sites in the *gprA* and *argB* ORFs resulted in varying digestion patterns. Restriction enzyme abbreviations are for *Bam*HI (B), *Eco*RI (E), *Eco*RV (V), *Hin*dIII (H), and *Pst*I (P).

Note. Use 1:3:1 molar ratio for 5′-flanking:*argB*:3′-flanking amplicons. The total DNA amount of the three components should be between 100 and 1000 ng. The annealing time was routinely 10 min in the procedure carried out at the Yu lab. However, the experience of the Orsay laboratory showed that this may be reduced to 2 min. The latter was used routinely by the Orsay group. This product can be used directly without further purification.

For long constructs, a Long Expand polymerase (Roche, etc.) should be used in preference to the *Taq* polymerase.

2.2.3. Third round PCR: amplification of the fused knockout construct

(a) Design a nested primer pair preferentially starting just after a 'T' as explained in Section 2.2.1.

(b) Perform a conventional PCR in 100 μl with less than 1 μl (usually 0.5 μl) of the second round product by using nested primers.

(c) Confirm the size of the product and cut with selected restriction enzymes to verify the PCR product.

(d) Purify the double-jointed final PCR product and directly use it for transformation.

2.2.4. Conditions for long (>5 kb) recombinant DNA

The recommended conditions for second round PCR for relatively long (>5 kb) recombinant DNA molecules are (with Long Expand polymerase) 2 min at 94 °C; 15 cycles of (45 s at 93 °C, 2 min at 62 °C and 12 min at 68 °C) and 15 min post-polymerization. The third round PCR conditions are (using Long Expand polymerase) 2 min at 94 °C; 35 cycles of (45 s at 93 °C, 45 s at 62 °C, and 12 min at 68 °C) and 15 min post-polymerization.

The annealing temperature depends on the actual primers. The elongation time is determined based on the length of the desired final product. In most cases, 15–30 μl of the final PCR product can be used for transformation.

2.3. Single-joint PCR (SJ-PCR): construction of the alcA(p)::ORF or fusion of any two DNA fragments

2.3.1. First round PCR

(a) Amplification of the *alcA* promoter is carried out with the primer pair 5′ For primer-5′ ccg ttc tgc tta ggg ta3′ and 3′ Rev primer-5′ttt gag gcg agg tga tag gat tgg a3′ using wild type genomic DNA as a template.
(b) The target gene ORF with the (probable) poly(A) binding site (AATAAA in most cases) is amplified with a primer pair where the 5′ forward primer carries about 15–25 bases overlapping sequences with the *alcA* (p) 3′ end, e.g., 5′-tcc aat cct atc acc tcg cct caa a-ATG-target gene ORF sequences-3′.

2.3.2. Second round PCR

Both amplicons are purified as described above and mixed in 1:1 molar ratio. These fragments are joined by PCR with no primers. Reaction mixture is the same as above. The thermo-cycling conditions are 94 °C for 3 min, 10 cycles of (94 °C for 30 s, 58 °C for 10 min, 72 °C for 5 min) and 72 °C for 10 min.

2.3.3. Third round PCR

In this third round PCR, the joined product will be amplified with nested primers carrying restriction enzyme sites (if necessary) to be used for cloning into vectors.

2.3.4. Cloning and sequencing

(a) Clean-up the final PCR product, cut with appropriate restriction enzyme(s) or blunt-ended and clone it to a vector of choice, e.g., pSH96 (Wieser and Adams, 1995).
(b) Pick three to five clones, establish the direction of the insert and sequence the clones to ensure no mutations are introduced by the PCR.

2.4. Fungal genomic DNA isolation (adapted and modified from a commonly used rapid yeast genomic DNA isolation protocol)

These methods enable one person to isolate more than 100 genomic DNA samples a day and thus improve the efficiency of analysis of genetic alterations. These methods can be used to monitor large number of transformants for correct integration of the construction and expression of the relevant mRNA.

(a) Inoculate 2 ml of liquid minimal medium + 5 g/L yeast extract + supplements in an 8 ml test tube with $\sim 10^5$ spores and grow ~16 h (no longer than 20 h) at 37 °C (stationary culture).
(b) Collect the mycelial mat, squeeze excessive medium using paper towel and transfer the squeeze-dried sample into a 2 ml screw cup tube with an O-ring cap.
(c) Add 500 μl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA, pH 8.0), 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1) and 300 μl of 0.5 mm zirconia/silica beads (BioSpec).
(d) Tightly close the cap and insert up to eight tubes in Mini-BeadBeater-8 (BioSpec) in a cold room and homogenize at the highest speed for 2 min.
(e) Take out the tubes and centrifuge at 16,000*g* for 10 min at room temperature or 4 °C.
(f) Gently transfer the aqueous (upper) phase into a new 1.5 ml microcentrifuge tube.
(g) Add two volumes of ice cold absolute ethanol (kept at −20 °C), mix well and centrifuge at 16,000*g* for 10 min to pellet DNA.
(h) Decant the supernatant and fill the tubes with the DNA pellets with 70% ethanol.
(i) Decant 70% ethanol as much as possible and dry the DNA pellets.
(j) Resuspend isolated genomic DNA in 50 μl of TE (pH 8.0) with RNase A (10 μg/ml final concentration).
(k) Use 0.5–1 μl of genomic DNA solution for 20–50 μl PCR. Alternatively, 5–10 μl of DNA solution can be used for a restriction enzyme digestion for Southern blot analyses.

2.5. Total RNA isolation

(a) Place pieces of mycelia (about 0.1 g and no more than 0.2 g) into a pre-labelled 2 ml screw cup tube with an O-ring cap. Quick-freeze in liquid nitrogen and store at −80 °C until all samples are ready.
(b) Remove up to eight tubes with mycelia from −80 °C, keep on ice, and add 1 ml Trizol (Invitrogen) and add 400 μl 0.5 mm zirconia/silica beads (BioSpec) to each sample.
(c) Insert up to eight tubes into a Mini-BeadBeater-8 (in a cold room) and homogenize for 2.5 min at max speed (homogenize).
(d) Let tubes sit at RT for 5 min and add 200 μl of chloroform. Shake tubes vigorously by hand for 15 s. Incubate at RT for 3 min.
(e) Microcentrifuge at 12,000*g* for 15 min at 4 °C or RT.

(f) Remove upper aqueous phase to a new tube and add 1 volume of isopropanol (approximately 500 μl) to aqueous phase. Mix thoroughly by inverting tube several times.
(g) Let sit at RT for 10 min and centrifuge at 9200*g* at RT for 10 min.
(h) Remove all supernatant carefully, gently add 1.5 ml of 70% ethanol (made with DEPC-treated water) and wait for 1 min.
(i) Remove nearly all of supernatant by leaving the tubes upside down.
(j) Air dry the pellet at room temperature for 5–10 min.
(k) Resuspend the total RNA samples in 50–100 μl of DEPC-treated sterile H_2O or other appropriate buffer. Vortex and flick tube with fingers to release the pellet from the wall of the tube. Pipette up and down gently and briefly heat to 65 °C and repeat pipetting until the entire RNA pellet is dissolved. Spin briefly to collect sample at the bottom of the tube. From this point on keep tube on ice or frozen at −80 °C. Quality of total RNA isolated by this procedure is high enough to be used in reverse transcription of the transcriptome.

2.6. Primers and polymerases

PCRs were run on a MJ Research Gradient Cycler PTC-225 and PTC-100 or an Applied Biosystems GeneAmp PCR system 2700 with heated lids. Primers were purchased from Integrated DNA Technologies, or from Sigma Genosys. Amplifications were carried out using Long Expand Polymerase (Roche) or Triplemaster (Brinkmann). PCR products were purified by High Pure PCR Product Purification Kit (Roche) or Qiagen PCR purification kit.

2.7. Cloning vectors and Escherichia coli strains

The pGEM-Teasy (Promega) and pSH96 (Wieser and Adams, 1995) vectors were used. Plasmids were prepared in *E. coli* DH10B or DH5α.

3. Results and discussion

Fig. 1 summarizes the whole procedure of our PCR-assisted technique. The individual components are separately amplified by a conventional PCR. In the protocol used by the Yu group (see below for an alternative method) the primers used to amplify the 3′ end of the 5′ flanking sequence and the 5′ end of the 3′ flanking sequence of the target gene carried 25–30 bases complementary to 5′ and 3′ of the selective marker, e.g., *argB*$^+$, respectively. The three, 5′ flanking region, marker and 3′ flanking region, amplicons are mixed in 1:3:1 molar ratio and the second round of thermo-cycling is carried out. These three DNA fragments will be specifically joined together during the second round PCR. The elongation time is according to the final size of the desired construct (∼1 min/kb). After each round of PCR, the amplified components are purified using a commercially available PCR clean-up kit. In the third round of PCR, the double-joint product is amplified with a nested primer pair (primers 7 and 8 in Fig. 1). We found that using nested primers gave almost 100% success (single product with no artefacts) whereas using the first round primer pairs (primers 1 and 4) generated extremely low success levels with high chance of getting PCR artefacts.

The final amplicon is cleaned and directly used for transformation. While the construction of cassettes is an extremely rapid procedure and many independent cassettes can be constructed in a short time, the limiting step is the identification of transformants carrying the desired insertion and/or deletion without any extraneous events. A useful way to identify the desired homologous recombination event in a primary screening is to amplify the DNA of relevant transformants with a primer pair complementary to sequences outside the deletion construct (for example, primers 9 and 10 in Fig. 1). Fig. 1D shows how the deletion mutant of *gprA*

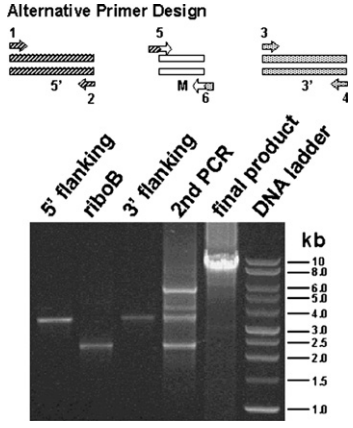


Fig. 2. An alternative primer design and construction of a deletion cassette for the *hhoA* gene. As mentioned in the text, the chimeric primers (primers 5 and 6) can be used to amplify the selective marker (M), not the flanking sequences. Many genes including *hhoA* were deleted employing this method. The photograph of the agarose gel electrophoresis shows the resulting products of each PCR step in constructing a deletion cassette of the *hhoA*. The first three lanes show the purified PCR products of the 5′ flanking (5′), the chimeric *riboB* gene (M amplified with an alternative chimeric primer set), and the 3′ flanking (3′) regions, which are 3.3, 2.2, and 3.5 kb, respectively. The fourth lane shows the resulting products (10 μl loaded) of the second round PCR where the 5′, M, and 3′ components were optimised to the 1:3:1 molar ratio, i.e., ∼200, ∼450, and ∼210 ng, respectively. The final 8.3 kb product was then amplified with a nested primer pair using 0.5 μl of the second round PCR product as the template. Although the assembled product can be 9.0 kb, the use of the nested primers resulted in a final product of 8.3 kb. A 1 kb DNA ladder (Promega) is shown. The final product was used to transform a *riboB2* mutant strain.

Table 1
Summary of gene manipulations in filamentous fungi

Gene/annotation number[a] (Reference)	Predicted product/function	Types of manipulation	Length of flanking sequence	% Correct gene replacement (# correct/# screened)
AN0660	Transporter of the uracil/uridine/allantoin family	Replacement with *riboB* (2.2 kb) of *A. nidulans*	3 kb, 3 kb	34% (11/32)
AN4152	The same as above	The same as above	3 kb, 3 kb	12% (1/8)
AN3352	The same as above	The same as above	3 kb, 3 kb	12% (1/8)
AN8416	The same as above	The same as above	3 kb, 3 kb	14% (5/35)
AN9326	The same as above	The same as above	3 kb, 3 kb	8% (2/26)
AN7955	The same as above	The same as above	3 kb, 3 kb	15% (3/20)
AN2053	Putative ENT1 transporter	The same as above	3 kb, 3 kb	20% (4/20)
AN5493	Putative CNT1 transporter	The same as above	3 kb, 3 kb	15% (3/20)
AN6118	Agta (aspartate/glutamate transporter)	The same as above	4.3 kb, 4.2 kb	28% (7/25)
AN4998	Putative GAP protein	Replacement with *pyrG* (1.8 kb) of *A. fumigatus*	3 kb, 3 kb	11% (3/27)
AN2278	Putative *S. cerevisiae* SWI2 homologue	The same as above	2 kb, 2 kb	0% (0/20)[c]
AN3621	Putative *S. cerevisiae* GCN5 homologue	The same as above	2 kb, 2 kb	40% (8/20)
AN1777	Putative *S. cerevisae* CTI6 homologue	Replacement with the *riboB* homologue of *N. crassa*	2 kb, 2 kb	15% (3/20)
hhoA	Histone H1	Replacement with *riboB* of *A. nidulans*	3 kb, 3 kb	30% (12/40)
H3	Histone H3	*alcA* promoter-GFP-fusion, heterologous integration	N/A[b]	N/A
AN6201	Putative *S. cerevisiae* SSN6 homologue	Replacement with *pyrG* of *A. fumigatus*	2 kb, 2 kb	20% 4/20
gprA (Seo et al., 2004)	Putative GPCR (PreB) similar to Ste2p	Replacement with *argB* (1.8 kb) of *A. nidulans*	966 bp, 984 bp	8% (6/76)
gprB (Seo et al., 2004)	Putative GPCR (PreA) similar to Ste3p	The same as above	1017 bp, 1096 bp	4% (2/50)
gprC (Han et al., 2004a)	Putative GPCR (unknown) similar to Gir3p (*S. pombe*)	The same as above	891 bp, 815 bp	5% (1/20)
gprD (Han et al., 2004a)	Putative GPCR (negatively controls sexual development)	The same as above	768 bp, 504 bp	2% (2/100)
gprG (Han et al., 2004a)	Putative GPCR (unknown function)	The same as above	719 bp, 480 bp	3% (1/30)
gprH (Han et al., 2004a)	Putative GPCR (unknown function)	The same as above	711 bp, 523 bp	3% (1/30)
gprK AN7795.2	Putative GPCR with 7-TM and an RGS domain	The same as above	807 bp, 1189 bp	17% (6/36)
pkaB AN4717.2	The second PKA catalytic subunit	The same as above	591 bp, 804 bp	10% (2/20)
rgsA (Han et al., 2004b)	RGS (inhibits GanB)	The same as above	911 bp, 939 bp	40% (20/50)
rgsB (Han et al., 2004b)	RGS (unknown function)	The same as above	616 bp, 775 bp	4% (2/50)
sfgA AN8129	A C6 factor, a suppressor of *fluG* (Seo et al., 2003)	The same as above	1085 bp, 963 bp	12% (4/32)
AN6437	Filamentous fungal-specific hypothetical protein	The same as above	870 bp, 794 bp	15% (3/20)
gprA	GPCR	Fused with *alcA(p)*	N/A	N/A
gprD	GPCR	The same as above	N/A	N/A
fadA (Yu et al., 1996)	G protein α subunit	The same as above	N/A	N/A
sfaD (Rosén et al., 1999)	G protein β subunit	The same as above	N/A	N/A
gpgA AN2742.2	G protein γ subunit	The same as above	N/A	N/A
phnA AN0082.2	A gene encoding a phosducin like protein	The same as above	N/A	N/A
pkaB	The second PKA catalytic subunit	The same as above	N/A	N/A
rgsA (Han et al., 2004b)	RGS	The same as above	N/A	N/A
sfgA	C6 factor	The same as above	N/A	N/A
nsdD (Han et al., 2001)	A GATA type sexual developmental activator	The same as above	N/A	N/A
Af-*flbA*	An *A. fumigatus* RGS protein: the homolog of *A. nidulans* FlbA (Lee and Adams, 1994a)	Replacement with *pyrG* of *A. fumigatus*	782 bp, 865 bp	10% (2/20)
Af-*fluG*	*A. fumigatus* FluG: homolog of *A. nidulans* FluG(Lee and Adams, 1994b)	Replacement with *pyrG* of *A. fumigatus*	911 bp, 905 bp	15% (3/20)
Fg-*flbA* (RGS1)	A *F. graminearum* RGS protein: homolog of*A. nidulans* FlbA	Replacement with HygB	822 bp, 1075 bp	1/1

[a] Most of the genes are of *A. nidulans* except the last three. References are described when available.
[b] Not applicable.
[c] Although five out of 20 transformants carried exact deletions, these also carried extraneous insertion events.

was identified and confirmed by PCR amplification followed by restriction enzyme digestions. However, this method is not practical when a long deletion construct was employed for gene replacement. In this case, transformants can be pre-screened for the absence of the DNA fragment amplification between the deleted gene and its flanking sequences. Transformants without amplicon(s) may carry the deletion and are then checked by genomic DNA Southern blot analysis.

As mentioned in the Materials and methods section, DJ-PCR can be accomplished by amplifying the selective marker with chimeric primers (Fig. 2). This method has been routinely used at the Orsay laboratory and 14 genes were deleted employing this alternative approach. An example of a final product (5′ flanking region-*riboB*$^{+}$–3′ flanking region) is shown in the photograph of an agarose gel electrophoresis (Fig. 2).

Table 1 shows genes that were manipulated in three different fungal species. In an organism like *A. nidulans*, where hundred of different auxotrophic mutations are known, virtually any such mutation present in a given strain can be used as a complementation marker, as the corresponding gene can usually be identified in the genomic sequence and subsequently amplified. Moreover, to minimise homologous recombination at the locus of the selective marker, the marker can be amplified from an organism different from the one in which the selection is carried out. This was shown previously by the use of the *pyr4* gene of *N. crassa* or the *pyrG* gene of *A. fumigatus* to complement the *pyrG 89* mutation *of A. nidulans*. In Table 1 we also demonstrate that the *N. crassa* homologue of *riboB* of *A. nidulans* can be used to complement the *riboB2* mutation. The availability of several fungal genome sequences increases the range of heterologous markers that can be used.

Correct deletion mutant not carrying other extraneous events are obtained at frequencies ranging from 2 to over 40% (Table 1). The rate seems to be primarily determined by the gene to be replaced. For instance, while only two out of 100 transformants screened carried the correct *gprD* deletion. (Han et al., 2004a), the *gprK* deletion rate was 17%. Moreover, about 40% of the transformants were precise *rgsA* deletion mutants (Han et al., 2004b). In general, correct gene replacement rates are found to be 2–15% when 0.5–1.0 kb flanking regions were used to promote homologous double cross-over. We found that increasing the length of the flanking regions to 2–3 kb resulted in higher, 8–20%, homologous double cross-over (gene replacement) events (Table 1). While long flanking sequences are useful in promoting homologous recombination, the high gene density of most filamentous fungi (about 1 gene every 3 kb) may harbour a risk of introducing an unwanted PCR generated mutation(s) in the genes adjacent to the one of interest. To prevent this, it is essential to use the high fidelity Long Expand polymerase when long flanking regions including overlapping adjacent genes have to be used to promote homologous recombination.

The PCR-based fusion technique has also been used to obtain transcriptional fusions between the target ORFs with a given promoter (see examples using *alcA* (p) in Table 1). Similar to DJ-PCR, the *alcA* promoter was amplified with a specific and fixed primer pair (see the protocol). The target gene ORF is then amplified

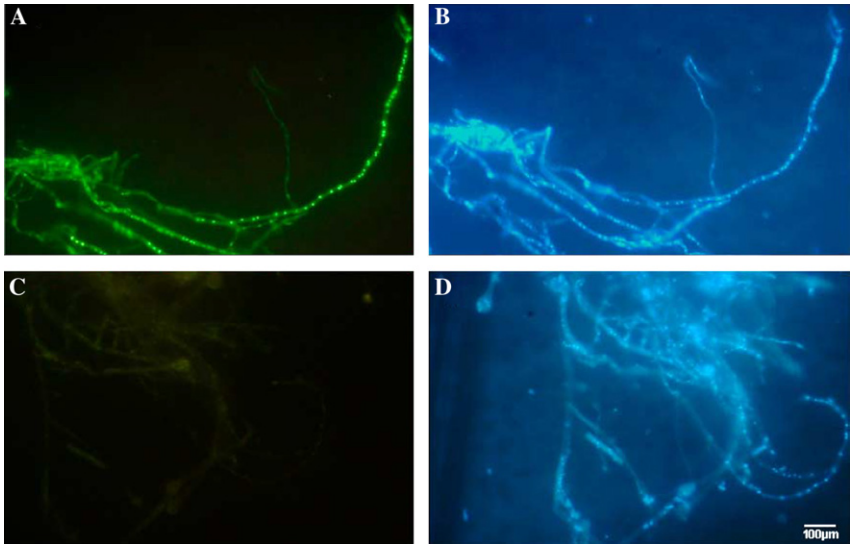


Fig. 3. GFP-H3 fusion protein under the control of the *alcA* promoter. This figure illustrates the use of an inducible GFP fusion, constructed by double joint PCR, to monitor the presence and intracellular localization of the H3 protein. The strain carries a single copy of the *alcA*(p)::H3::GFP fusion construct integrated ectopically. (A) and (B) A strain pre-grown in inducing liquid medium (1% ethanol). (C) and (D) The same strain pre-grown in non-inducing liquid medium (1% glucose). The samples were stained with DAPI. (A) and (C) A field observed and photographed through a filter suitable for the detection of GFP fluorescence and (B) and (D) display a field observed and photographed through a filter suitable to detect DAPI fluorescence (1000× magnification). For details see Materials and methods.

with a 5′ forward primer containing 25–30 overlapping homologous sequences with the 3′ end of the *alcA*(p), and 3′ reverse primer designed at downstream of the probable poly(A) binding site (AATAAA). The fusion PCR product is then cloned into an appropriate vector. We usually use plasmids that can confer direct integration at a given locus. For instance, pSH96 carries the 5′ half of the *trpC* gene and it directs the integration at the *trpC* locus if a *trpC801* mutant is used as a recipient (Wieser and Adams, 1995). Overexpression constructs of nine *A. nidulans* genes were generated employing this method. Essentially the same PCR-assisted method joining two DNA molecules can be used for reporter labelling or site-directed mutagenesis. The *alcA* promoter, histone-H3 and GFP were joined by DJ-PCR and the fusion reporter construct was directly integrated into the *Aspergillus* genome without any intermediate cloning steps. As shown in Fig. 3, the *alcA* (p)::H3::GFP construct responds to the *alcA* promoter and shows the expected nuclear localization pattern. This technique can also be used for site-directed mutagenesis, in this case, mutations are introduced in the overlapping 30 bp regions by appropriate chimeric primers carrying the desired mutation(s) (not shown).

Finally, in order to speed up the screening process, we also have developed and presented genomic DNA and total RNA isolation protocols for filamentous fungi. With this technique a single person can carry out more than 100 genomic DNA or total RNA isolations in a single day. We hope that application of the methods described in this report will contribute to gene function studies in various filamentous fungi.

Note added in proof

A highly similar method has been developed by the Osmani lab and will be published elsewhere (see below). Yang L., Ukil, L., Osmani, A., Nahm, F., Davies, J., DeSouza, C.P.C., Dou, X., Perez-Balaguer, A. Osmani, S.A. 2004. Rapid production of gene replacement constructs and generation of GFP-tagged centromeric marker in *Aspergillus nidulans*. Eukaryot. Cell, in press.

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