Fungal Genetics Reports

Volume 59 Article 3

Efficient tools to target DNA to Podospora anserina

Michelle Déquard-Chablat
Institut de Génétique et Microbiologie

Tan-Trung Nguyen
Institut de Génétique et Microbiologie

Véronique Contamine
Institut de Génétique et Microbiologie

See next page for additional authors

Recommended Citation

Déquard-Chablat, M., T. Nguyen, V. Contamine, S. Hermann-Le Denmat, and F. Malagnac (2012) "Efficient tools to target DNA to Podospora anserina," *Fungal Genetics Reports*: Vol. 59, Article 3. https://doi.org/10.4148/1941-4765.1011

Efficient tools to target DNA to Podospora anserina

Abstract

Here we report the construction of two plasmids designed to target DNA sequences to two specific loci of *Podospora anserina*.

Authors

Michelle Déquard-Chablat, Tan-Trung Nguyen, Véronique Contamine, Sylvie Hermann-Le Denmat, and Fabienne Malagnac

Efficient tools to target DNA to *Podospora anserina*

Michelle Déquard-Chablat[1,2], Tan-Trung Nguyen[1,2], Véronique Contamine[1,2], Sylvie Hermann-Le Denmat[1,2,3] and Fabienne Malagnac[1,4]. [1] Univ Paris-Sud, Institut de Génétique et Microbiologie, UMR 8621, ORSAY, F-91405, France. [2] CNRS, ORSAY, F-91405, France. [3] Ecole Normale Supérieure, 75005 Paris, France. [4] Univ Paris Diderot, Sorbonne Paris Cité, UFR des Sciences du Vivant, 75205 Paris CEDEX 13, France

Fungal Genetics Reports 59: 21-25

Here we report the construction of two plasmids designed to target DNA sequences to two specific loci of *Podospora anserina*.

Because, in filamentous fungi, integration of transgenes proceeds mainly through non-homologous recombination, transformation results in a random distribution of these DNA sequences. Moreover, they are often found as multiple copies clustered at a single locus. Therefore, for a given transgene, expression may be highly variable among independent transformants. To get rid of both multiple copies and position effects, due to the impact of the chromatin structure upon transgene expression, we constructed tools to specifically target single copies at two well-defined loci, Pa_2_3690 and Pa_4_5450 (Espagne et al., 2008). These genes have been chosen for the following reasons: (1) they have a relatively stable expression during the entire Podospora's life cycle, (2) complete deletion of their ORF does not lead to an abnormal phenotype (Bidard et al., 2011), (3) they are located on two different chromosomes and (4) their second division segregation (SDS) frequency is 80% and 90%, respectively. Besides, a highly efficient homologous recombination ΔPaKu70 strain is available (El-Khoury et al., 2008), which makes gene replacement fairly easy. Taking advantage of this strain, we designed two plasmid tools to target genomic integration of any DNA fragment.

pCIB2 and pCIB4 plasmid constructions

As for *Neurospora crassa*, we set up a yeast recombinational cloning strategy using the pRS426 shuttle plasmid (Colot et al., 2006). For selectable marker, we chose the nourseothricine resistance gene (NouR) from pAPI508 (El-Khoury et al., 2008). First, we PCR-amplified from plasmid genomic libraries (Espagne et al., 2008) the flanking 5-prime and 3-prime regions of Pa_2_3690 (VeA5f2/NoS5r2 and NoP3f2/VeA3r2) and Pa_4_5450 (VeA5f4/NoS5r4 and NoP3f4/VeA3r4) respectively (about 1 kb each). Primers NoS5r2 and NoS5r4 contain a SwaI restriction site, whereas primers NoP3f2 and NoP3f4 contain a PacI restriction site. These two enzymes were chosen for restriction cloning because they recognize eight-base sequences, which are very infrequent in the Podospora genome. The flanking regions do not encompass coding sequence of the neighboring genes. Primers were designed to overlap either with pRS426 (VeA5f2, VeA3r2 and VeA5f4, VeA3r4) or with the NouR cassette (NoS5r2, NoP3f2 and NoS5r4, NoP3f4). Meanwhile, the NouR cassette was amplified using primers No5f, No3r. All PCR were done using the high fidelity Pfu DNA polymerase (Promega) for 25 cycles of amplification.

A W303 yeast strain was transformed with the 5-prime and 3-prime flanking regions as well as the NouR cassette and the pRS426 plasmid digested with XhoI and EcoRI for each construction.

Because of homologous ends, the four fragments were ligated, generating the pCIB2 (Pa_2_3690) and pCIB4 (Pa_4_5450) plasmids. For each construction, four yeast colonies showing restored prototrophy to Uracil were selected. Plasmid DNA was extracted and used as template for PCR amplification (primers PU/NouR Int Rev and RP/NouR Int) to check the constructions. Once verified, the plasmids were transformed into *Escherichia coli*. Ten clones were picked for each transformation and checked by colony PCR with the primers mentioned above. pCIB2 (Genbank accession number JX262692) and pCIB4 (Genbank accession number JX262693) plasmid DNA was purified from two large cultures, inoculated with a single clone and sequenced (see Tables 1 and 2 for primers and Figure 1 for plasmid graphic maps). pCIB2 and pCIB4 are available from the Fungal Genetics Stock Center (Kansas City, MO).

Table1: Primers used to amplify the flanking regions of the target genes and the NouR cassette

name	5-prime -> 3-prime sequence
No5f	tcttcccttccacttcttcacacagaccac
No3r	ggcacaaagcatcaagaaggcaaacagaac
Pa_2_3690 locus	
VeA5f2	gagcgcgcgtaatacgactcactatagggcg*CGCC*accggtgttctcggcatctttctg
NoS5r2	gtggtctgtgtgaagaagtggaagggaaga*TTTAAAT*cgtctgacccgatcaaagcatgag
NoP3f2	gttctgtttgccttcttgatgctttgtgcc*TTAATTA*accgcatgtttggtgttggc
VeA3r2	gaacaaaagctggagctccaccgcggtggcg*CGCC*gcagacagggaaccaaggac
Pa_4_5450 locus	
VeA5f4	gagcgcgcgtaatacgactcactatagggcg*CGCC*gtgtgcgctaaacgttgggtaatg
NoS5r4	gtggtctgtgtgaagaagtggaagggaaga*TTTAAA*tggtaccttatctggcctgtcc
NoP3f4	gttctgtttgccttcttgatgctttgtgcc*TTAATTAA*ggtcacaccgaactgagaagg
VeA3r4	gaacaaaagctggagctccaccgcggtggcg*CGC*cacaccttcgcagacctagc

Sequences in bold, regular and underlined correspond respectively to the pRS426 vector, the ends of the resistance cassette and the ends of the flanking region of one of each target gene. Bases in italic uppercase were added to create appropriate restriction sites (ggcgcgcc = *Asc*I, atttaaat = *Swa*I, ttaattaa = *Pac*I).

Table 2: Primers used to check the constructions and the integration in Podospora genomic DNA.

name	5-prime -> 3-prime sequence	position
Ext 5'2	gttctgcctcaccttcatcg	Upstream of Pa_2_3690 5'UTR
Ext 3'2 rev	acgtggccctgacatcatc	Downstream of Pa_2_3690 3'UTR
Ext 5'4	tccacagcctacgacaggtg	Upstream of Pa_4_5450 5'UTR
Ext 3'4 rev	cccttcggtgaactacctg	Downstream of Pa_4_5450 3'UTR
NouR Int	ttcgtggtcgtctcgtactc	Inside *Nat1* gene
NouR Int Rev	ggtgcgttgacgttggtgac	Inside *Nat1* gene

Cloning DNA fragments into pCIB2 and pCIB4

To clone alleles of interest into either pCIB2 or pCIB4, blunt-ended DNA fragments can be ligated to the SwaI site, whereas DNA fragments with cohesive ends generated by PvuI or AsiSI

https://newprairiepress.org/fgr/vol59/iss1/3
DOI: 10.4148/1941-4765.1011

digestions can be ligated to PacI site. Prior to transformation, the ligations were hydrolyzed using SwaI or PacI, in order to eliminate self-ligated vectors.
Once alleles of interest are introduced into pCIB2 and pCIB4, and prior to transformation into Podospora, these plasmids have to be digested to generate linear fragments to favor homologous recombination at the corresponding target locus. For this purpose AscI sites have been introduced into primers VeA5f2, VeA3r2 and VeA5f4, VeA3r4 (see Figure 1).

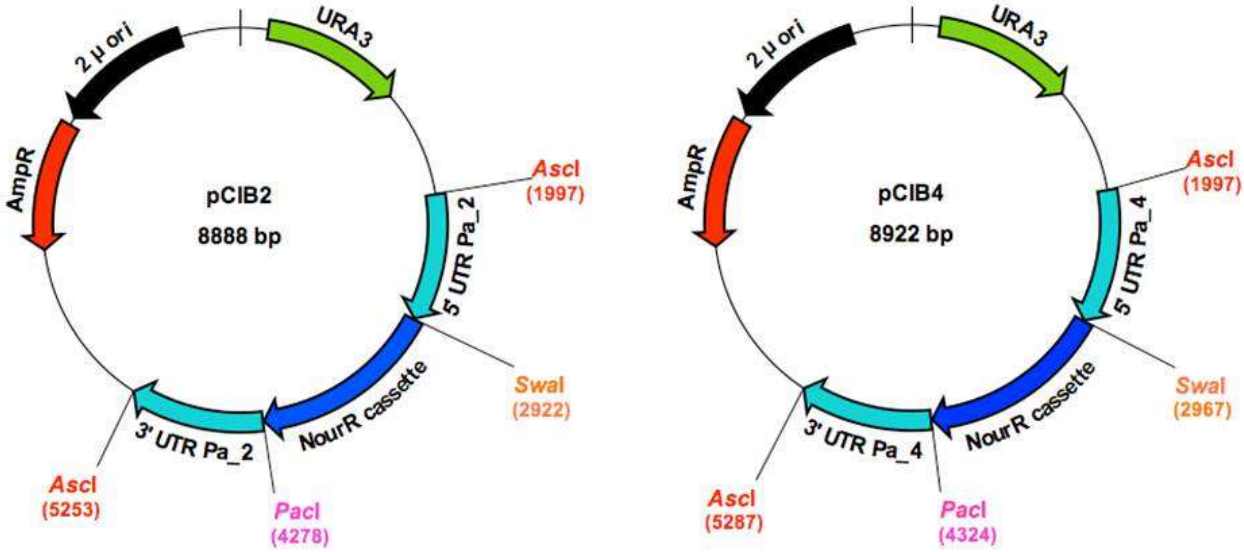

Figure 1. Physical maps of the pCIB vectors. The two vectors are derived from the pRS426 plasmid (Colot *et al.*, 2006). The NourR cassette includes the PaGPD promotor, the Nat1 ORF and the PaAS1 terminator (El-Khoury *et al.*, 2008). Unique cloning sites and the *Asc*I sites, which are used to obtain linear transformation fragments for integration targeting, are indicated. Pa_2 and Pa_4 correspond respectively to Pa_2_3690 and Pa_4_5450.

As a proof of principle, site-directed integration was tested. Alleles of four different genes were cloned into pCIB4, using either SwaI or PacI. After independent transformations of the ΔPaKu70 strain (El-Khoury et al., 2008) with five linear fragments, PCR analyses of a total of 30 transformants showed 100% of single-copy integration at the Pa_4_5450 targeted locus. We then checked the expression of the five transgenes. We first saw that introduction of wild-type allele of Pa_3_6770 restores viability of the corresponding deleted mutant, which proves efficient transcriptional gene expression at the Pa_4_5450 locus. When Pa_3_6770 was integrated at the same Pa_4_5450 locus but driven by the promoter of the highly expressed AS4 gene (Silar & Picard, 1994), it showed a 14-fold increase in expression (as measured by RT-qPCR). Western-blot analysis further confirmed this over-expression (Figure 2). Additional RT-qPCR experiments performed on the remaining three over-expressed alleles integrated at the Pa_4_5450 locus also showed clear increases, ranging from 6- to 50-fold.

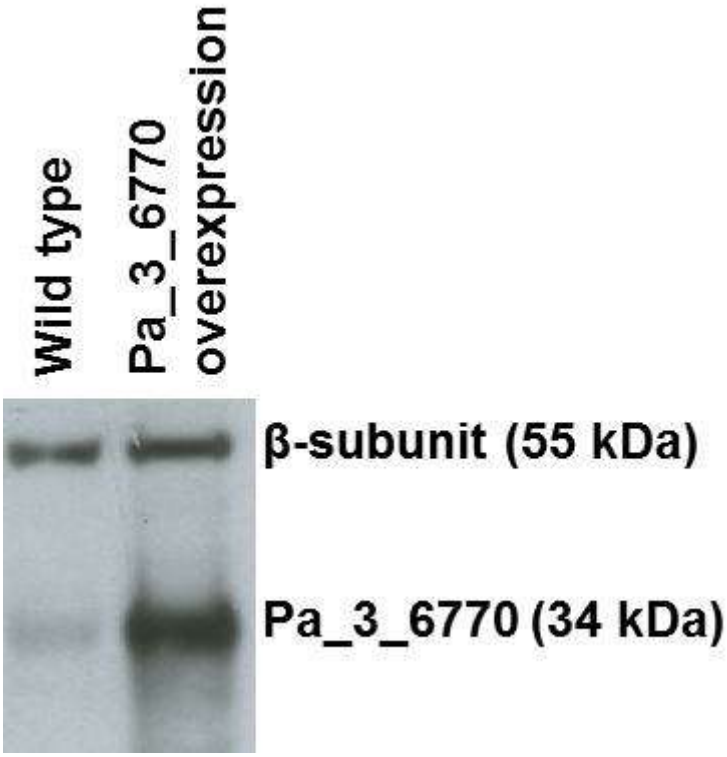

Figure 2. Mitochondrial extracts (80 µg) purified from the indicated strains were resolved on an SDS-polyacrylamide gel and subjected to immunoblotting. Product of the Pa_3_6770 gene and β-subunit of the mitochondrial ATPase (used as a loading control) were detected by *S. cerevisiae* anti-antibodies directed against the corresponding homologous proteins.

The Podospora community can now rely on an efficient targeting system to compare the expression of various alleles of a given gene.

Acknowledgments
We thank Robert Debuchy, Evelyne Coppin and Jinane Aït Benkhali for discussion and Robert Debuchy and Vinosa Yogarajah for technical assistance. We also thank Jean Velours (IBGC-CNRS, Bordeaux, France) and Geneviève Dujardin (CGM-CNRS, Gif/Yvette, France) for providing the *S. cerevisiae* anti-β-subunit antibody and Pasquale Scarcia and Ferdinando Palmieri (University of Bari, Italy) for providing antibodies directed against Pa_3_6770 *S. cerevisiae* homologous polypeptide.

https://newprairiepress.org/fgr/vol59/iss1/3
DOI: 10.4148/1941-4765.1011

References

Bidard F, Aït Benkhali J, Coppin E, Imbeaud S, Grognet P, Delacroix H and Debuchy R, 2011. Genome-wide gene expression profiling of fertilization competent mycelium in opposite mating types in the heterothallic fungus *Podospora anserina*. *PLoS ONE* 6: e21476.

Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA and Dunlap JC, 2006. A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 103: 10352-10357.

El-Khoury R, Sellem CH, Coppin E, Boivin A, Maas MFPM, Debuchy R and Sainsard-Chanet A, 2008. Gene deletion and allelic replacement in the filamentous fungus *Podospora anserina*. *Curr. Genet.* 53: 249-258.

Espagne E, Lespinet O, Malagnac F, Da Silva C, Jaillon O, Porcel BM, Couloux A, Aury J-M, Ségurens B, Poulain J, *et al.*, 2008. The genome sequence of the model ascomycete fungus *Podospora anserina*. *Genome Biol* 9: R77.

Silar P and Picard M, 1994. Increased longevity of EF-1 alpha high-fidelity mutants in *Podospora anserina*. *J. Mol. Biol.* 235: 231-236.