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Fluorescent and bimolecular-fluorescent protein tagging of genes at their native loci in *Neurospora crassa* using specialized double-joint PCR plasmids

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ABSTRACT

The double-joint polymerase chain reaction (DJ-PCR) is a technique that can be used to construct vectors for targeted genome integration without laborious subcloning steps. Here we report the availability of plasmids that facilitate DJ-PCR-based construction of *Neurospora crassa* tagging vectors. These plasmids allow the creation of green or red fluorescent protein (GFP or RFP) tagging vectors for protein localization studies, as well as split-yellow fluorescent protein (YFP) tagging vectors for bimolecular fluorescence complementation (BiFC) analyses. We have demonstrated the utility of each plasmid with the tagging of known meiotic silencing proteins. Microscopic analysis of the tagged strains indicates that SMS-2 and QIP form macromolecular complexes in the perinuclear region during meiosis.

1. Introduction

The study of molecular genetics often involves inserting or removing DNA from a genome. The efficiency of this process can be improved by reducing the time and labor involved in the construction of transformation vectors, which are fragments of DNA designed to facilitate the genomic change, and in screening for individuals with the desired outcome. The finding that disrupting the *Neurospora crassa* non-homologous end-joining (NHEJ) pathway (e.g. by mutating *mus-51* or *mus-52*) eliminates random integration of transgenes has led to a dramatic increase in screening efficiency (Ninomiya et al., 2004). This major technological advance has prompted several groups to construct tools to improve the efficiency of transformation vector construction. For example, the Neurospora Genome Project group has designed a strategy for semi-automated construction of genome-wide gene-replacement vectors (Colot et al., 2006), while Honda and Selker (2009) have developed a system for efficiently generating vectors and tagging a gene at its native locus with FLAG, HA, Myc, GFP, or HAT–FLAG.

The tagging of genes at their native loci with fluorescent protein constructs is particularly desirable for our work on meiotic silencing by unpaired DNA (MSUD), a mechanism that suppresses the expression of unpaired genes during meiosis (Shiu et al., 2001). In *N. crassa*, meiosis occurs in a relatively small number of asynchronously developing spore sacs (asci), which are encased in a hard-shelled fruiting body (perithecium). The limited amount of isolatable tissue has thus made microscopic techniques more feasible than biochemical ones, and as a result, our current model for MSUD is largely supported by a combination of genetic experimentation and fluorescent microscopy studies. In this model, the presence of unpaired DNA triggers the production of an aberrant RNA molecule that is made double-stranded (ds) by the SAD-1 RNA-directed RNA polymerase (RdRP) (Shiu and Metzenberg, 2002). The DCL-1 Dicer processes this dsRNA into small interfering (si) RNA (Alexander et al., 2008), which are then incorporated into a protein complex including the SMS-2 Argonaute (Lee et al., 2003) and possibly an exonuclease known as QIP. QIP colocalizes with SMS-2 in the perinuclear region, is required for MSUD, and interacts with an SMS-2 paralog (QDE-2) in the vegetative phase of the fungus (Maiti et al., 2007; Xiao et al., 2010; Lee et al., 2010). A fifth MSUD protein, SAD-2, is required for the perinuclear localization of SAD-1 (Shiu et al., 2006).

Previous work with MSUD fluorescent protein tags relied predominantly on "first generation" tagging plasmids (Freitag et al., 2004; Freitag and Selker, 2005; Bardiya et al., 2008). Although these plasmids allow transformation-ready vectors to

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be constructed for most genes in a single cloning step, their utilization limits the targeting of a transgene to a specific locus (i.e., *his-3*). For studies requiring more than one transgene to be expressed in a strain, an efficient way to construct a vector, designed to deliver a transgene anywhere in the genome, is highly desirable. This is especially true for MSUD studies, where we rely on fluorescent colocalization studies and split-*yfp* complementation assays to investigate the relationships among various proteins.

The vector construction tools created by the Neurospora Genome Project group as well as Honda and Selker rely on yeast recombinatorial cloning as an improved method over conventional restriction fragment ligation and cloning (Colot et al., 2006; Honda and Selker, 2009). To create vectors for native locus tagging of Neurospora genes, we developed a series of plasmids that allow the use of a technique previously demonstrated in Aspergillus fungi: the double-joint polymerase chain reaction (DJ-PCR) (Yang et al., 2004; Yu et al., 2004). With DJ-PCR, oligonucleotide primers are used to create artificial overlaps in three DNA fragments necessary for a complete transformation vector, which are then fused and amplified without the need for ligation or cloning. The three DNA fragments include a center fragment, containing the desired transgene sequences, and two flanking fragments, containing sequences necessary to target the transgene to a specific genomic location.

In a recent report, we used a *qip-gfp* native locus tag to show that QIP and SMS-2 colocalize in the perinuclear region (Xiao et al., 2010). Here we provide a full description of the steps we undertook to create this tag (as well as others) by DJ-PCR. In total, we have developed seven plasmids that facilitate DJ-PCR-based construction of *N. crassa gfp*, *rfp*, and split-*yfp* transformation vectors. We have demonstrated the utility of these plasmids by tagging *qip*, *sms-2*, and *sad-2* at their native loci, visualizing their protein localization during meiosis, and further investigating the possibility of an interaction between QIP and SMS-2. The seven plasmids described in this work (GenBank accession numbers JF749198–749204) are available through the Fungal Genetics Stock Center (FGSC; McCluskey et al., 2010).

2. Materials and methods

2.1. Plasmid construction

The final plasmids for this study are depicted in Fig. 1. The primer sets used are described below and in Table 1.

pTH1067.9 (-*gfp*). The *gfp*-coding region was (PCR-)amplified from plasmid pMF272 (Freitag et al., 2004) using "primer set A" and the *Aspergillus nidulans trpC* terminator [*trpC*(T)] (Mullaney et al., 1985) was amplified from *A. nidulans* genomic DNA using "primer set B". The resulting DNA fragments were joined by fusion PCR, amplified with "primer set C", and inserted into pCRII-TOPO (Invitrogen, Carlsbad, CA). A hygromycin resistance gene (*hph*, encoding a hygromycin B phosphotransferase) was then released from plasmid pCB1004 (Carroll et al., 1994) with the restriction enzyme *Hpa*I and inserted into the *Hpa*I restriction site of the $(GA)_4$::*gfp*::*trpC*(T)-pCRII-TOPO plasmid to create pTH1067.9. $(GA)_4$ or "GA-linker" refers to a 24-bp DNA fragment encoding four repeats of the amino acids Glycine and Alanine.

pTH1111.1 (-*rfp*). The *A. nidulans pyrG* terminator [*pyrG*(T)] (Oakley et al., 1987) was amplified from *A. nidulans* genomic DNA using "primer set D" and inserted into pCRII-TOPO to create plasmid pTH1083.1. A *Spe*I/*Not*I restriction fragment containing *pyrG*(T) was then released from pTH1083.1 and inserted into the identical restriction sites in the *rfp*-containing plasmid pMF334 (Freitag and Selker, 2005). The resulting *rfp*::*pyrG*(T) plasmid served as a template for the amplification of fragment $(GA)_4$::*rfp*::*pyrG*(T) with "primer set F", and the PCR product was inserted into pCRII-TOPO. As described above, *hph* was inserted into the *Hpa*I site of the resulting plasmid to create pTH1111.1. Since the dimeric *rfp* construct used here is subject to repeat-induced point (RIP) mutation in subsequent crosses, the inclusion of a *rid* (RIP defective; Freitag et al., 2002) allele in the target strain is recommended.

pTH1112.8 (-*yfpn*). *Apa*I restriction sites were placed on both sides of *pyrG*(T) by the amplification of said terminator using "primer set I" with pTH1083.1 as a template, and the PCR product was inserted into pCRII-TOPO. The *Apa*I-bracketed terminator was then inserted into the *Apa*I site of the pYFPN plasmid (Bardiya et al., 2008). This modified plasmid was then used as a template for the amplification of $(GA)_4$::*yfpn*::*pyrG*(T) with "primer set J", and the PCR product was inserted into pCRII-TOPO. *hph* was inserted into the *Hpa*I site of the resulting plasmid to create pTH1112.8.

pTH1108.2 (-*yfpc*). *Apa*I restriction sites were placed on both sides of *trpC*(T) by the amplification of said terminator using "primer set G" with pTH1067.9 as a template, and the PCR product was inserted into pCRII-TOPO. The *Apa*I-bracketed terminator was then inserted into the *Apa*I site of the pYFPC plasmid (Bardiya et al., 2008). This modified plasmid was then used as a template for the amplification of $(GA)_4$::*yfpc*::*trpC*(T) with "primer set H", and the PCR product was inserted into pCRII-TOPO. *hph* was then inserted into the *Hpa*I site of the resulting plasmid to create pTH1108.2.

pTH1117.12 (*gfp*-). The *N. crassa ccg-1* promoter region [*ccg-1*(P)] (Loros et al., 1989) was amplified from *N. crassa* genomic DNA using "primer set K" and the *gfp*-coding region was amplified from pMF272 using "primer set L". The resulting DNA fragments were joined by fusion PCR, amplified with "primer set M", and inserted into pCRII-TOPO. *hph* was inserted into the *Eco*RV site of the *ccg-1*(P)::*gfp*::$(GA)_4$-containing plasmid to create pTH1117.12.

pTH1123.1 (*yfpn*-). *ccg-1*(P) was amplified from *N. crassa* genomic DNA using "primer set K" and the *yfpn*-coding region was amplified from pYFPN using "primer set L". The resulting DNA fragments were joined by fusion PCR, amplified with "primer set N", and inserted into pCRII-TOPO. *hph* was inserted into the *Eco*RV site of the *ccg-1*(P)::*yfpn*::$(GA)_4$-containing plasmid to create pTH1123.1.

pTH1124.1 (*yfpc*-). *ccg-1*(P) was amplified from *N. crassa* genomic DNA using "primer set O" and the *yfpc*-coding region was amplified from pYFPC using "primer set P". The resulting DNA fragments were joined by fusion PCR, amplified with "primer set Q", and inserted into pCRII-TOPO. *hph* was inserted into the *Eco*RV site of the *ccg-1*(P)::*yfpc*::$(GA)_4$-containing plasmid to create pTH1124.1.

2.2. Double-joint polymerase chain reaction (DJ-PCR)

Extensive technical details of DJ-PCR were described by Yang et al. (2004) and Yu et al. (2004). Our three-step version of the technique is similar to that of Yu et al. (2004) and a graphical description is provided in Fig. 2.

2.2.1. Step 1

The center and the flanking DNA fragments (Fig. 2) were amplified with AccuPrime Pfx Polymerase (Invitrogen) under standard PCR-cycling conditions. Center fragments were amplified using the plasmid templates, primer sets, and annealing temperatures described in Tables 2 and 3. Flanking DNA was amplified from *N. crassa* genomic DNA using the primer sets described in Table 3. 5′ overhangs of 25–27 nucleotides were included in the forward primer for the right flank and the reverse primer for the left flank to allow fusion of the flanks and the center fragments in step 2.