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A new variant of self-excising β-recombinase/*six* cassette for repetitive gene deletion and homokaryon purification in *Neurospora crassa*

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ABSTRACT

In a previous study, we developed a cassette employing a bacterial β-recombinase acting on *six* recognition sequences (β-rec/*six*), which allowed repetitive site-specific gene deletion and marker recycling in *Neurospora crassa*. However, only one positive selection marker was used in the cassette. A tedious subsequent procedure was needed to purify homokaryons due to the lack of a negative selection after cassette eviction. Additionally, the endoxylanase *xylP* promoter from *Penicillium chrysogenum* used in the construct was not strongly regulated in *N. crassa*, which led to low efficiency in cassette eviction. Herein we report an improved variant of the self-excising β-recombinase/*six* cassette for repetitive gene deletions in *N. crassa* using a native endoxylanase *gh10-2* promoter from *N. crassa*, plus the introduction of a bidirectional selection marker to facilitate homokaryon selection using a thymidine kinase (*tk*) gene (negative selection) in addition to the phosphinothricin resistance gene (*bar*^{r}) (positive selection).

1. Introduction

Neurospora crassa has long been a model microorganism for studying biological processes and phenomena relevant to higher eukaryotes (Borkovich et al., 2004; Davis, 2000; Davis and Perkins, 2002). Over the past 70 years, various tools have been developed for manipulating genes in *N. crassa*. These include various auxotrophy-complementing markers and dominant selective markers conferring resistance to antibiotics or herbicides (Austin et al., 1990; Avalos et al., 1989; Case et al., 1979; Kinsey and Rambosek, 1984; Low and Jedd, 2008; Staben et al., 1989; Straubinger et al., 1992; Turner et al., 1997), various constitutive and inducible promoters (Aronson et al., 1994; Hurley et al., 2012; Ohrnberger and Akins, 1995; Pietschmann et al., 1991), and *mus-51*, *mus-52*, or *mus-53* deletion strains defective in the non-homologous end-joining (NHEJ) mechanism facilitating highly efficient homologous gene integration (Ishibashi et al., 2006; Ninomiya et al., 2004).

One method for producing multiple gene deletions in *N. crassa* involves combining the different gene deletions by genetic crossing, which takes advantage of the existing single knockout library (Dunlap et al., 2007; Wu et al., 2013a,b; Znameroski et al., 2012). However, this classical genetic approach is laborious to combine genetically linked genes, and the work load increases as the number of gene deletions to be combined increases. Another possible method is sequential targeted gene deletions with marker recycling (Krappmann, 2014). This approach was already successfully used in *N. crassa* and other filamentous fungi. The most established Cre/*loxP* recombination system was adopted for sequential gene deletions in *N. crassa* (Honda and Selker, 2009), and has been extensively used in *Aspergillus fumigatus* (Krappmann et al., 2005), *Aspergillus nidulans* (Forment et al., 2006), *Aspergillus oryzae* (Mizutani et al., 2012), *Trichoderma reesei* (Steiger et al., 2011), and other ascomycetes (Florea et al., 2009). However, this method requires a prior introduction of the Cre recombinase encoding gene into the *N. crassa* genome, which requires additional round of transformation and analysis of transformants (Honda and Selker, 2009). Moreover, when the *cre* recombinase gene is left in the genome, this system presents a risk of chromosomal rearrangements between *loxP* sites remaining in the genome after multiple rounds of deletions. Hence, it is preferable to delete the *cre* gene and to recover the original genotype after the desired gene deletions have been completed, which requires yet additional round of transformation and analysis of transformants (Honda and Selker, 2009).

A great advance in serial deletion of genes in filamentous fungi was achieved by Kopke et al. (2010) and Hartmann et al. (2010). They combined the recombinase-encoding sequence within the marker module to be excised. Such flipper cassettes can regulate the expression of the recombinase gene enabling one-step marker excision. The method of Kopke et al. (2010) utilized the *Saccharomyces cerevisiae* FLP/FRT system, which was first introduced and extensively used in functional studies of yeast *Candida albicans* (Morschhäuser et al., 1999, Reuss et al., 2004, Morschhäuser et al., 2005) and recently in the filamentous

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fungus *Ustilago maydis* (Khrunyk et al., 2010). Still, the disadvantage of the *trans*-acting FLP/FRT system is a possible recombination between the FRT sites left in the genome after each round of FLP-mediated marker removal, creating a potential problem of undesired chromosomal rearrangements. The method of Hartmann et al. (2010) employed the strictly *cis*-acting prokaryotic small β serine recombinase (β-rec) acting on *six* recognition sequences (β-rec/*six*), which was previously demonstrated to operate in *Bacillus subtilis*, plants and mammalian cells (Canosa et al., 1996; Díaz et al., 1999; Grønlund et al., 2007). The synthetic, codon-optimized β-recombinase proved to be functional in the filamentous fungus *A. fumigatus* (Amich et al., 2013; Hartmann et al., 2010; Jimenez-Ortigosa et al., 2012; Kieler et al., 2013; Lee et al., 2014; Mouyna et al., 2013).

In our previous study we introduced the prokaryotic β serine recombinase-based gene deletion and marker recycling system into *N. crassa* (Szewczyk et al., 2013). The knock-in cassette is composed of β-recombinase and the phosphinothricin (PPT) resistance gene (*bar*^{r}) flanked by the *six* recognition sequences. Only one transformation is needed to introduce the knock-in cassette into the target gene locus and to simultaneously delete the target gene. Upon the induction of β-recombinase, the whole cassette (including the recombinase) is evicted, and only a single *six* sequence is left in the genome. Hence, only one transformation is needed for one deletion and eviction cycle. We used the previously constructed cassette to successfully delete two genes which are located on the same chromosome only 2.65 Mbp apart, as another big advantage of this system is the strict *cis* action of β-recombinase, which eliminates the danger of undesired and uncontrolled chromosomal rearrangements (Hartmann et al., 2010; Szewczyk et al., 2013).

However, the endoxylanase *xylP* promoter from *Penicillium chrysogenum* used in the construct was found to be weakly regulated by xylose in *N. crassa*, which led to low eviction efficiency. Additionally, no positive selection method was available to select for the evictants directly. Typically the evictants still contain the original knock-in cassette, and a subsequent purification step is needed to obtain the homokaryotic strains. In this paper we report on creating an improved self-excising β-recombinase/*six* cassette employing a new native promoter, plus the introduction of bidirectional selection using the herpes virus thymidine kinase gene (*tk*) to facilitate evictant selection and homokaryon purification (Sachs et al., 1997). Thymidine kinases phosphorylate nucleoside analogs such as 5-fluoro-2′-deoxyuridine (FdU), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine (FIAU) or trifluorothymidine (TFT), which results in their conversion into toxic forms capable of inhibiting cell growth or viral infection (Machover, 1991; McKenzie et al., 1995). *N. crassa*, like other fungi, lacks detectable thymidine kinase activity, making it a convenient negative selection marker in this organism conferring sensitivity to nucleoside analogs activated by this enzyme (Grivell and Jackson, 1968).

2. Materials and methods

2.1. Strains and media

Genetic manipulations using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA) and GENEART Seamless Cloning and Assembly Kit (Invitrogen) were performed in One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen). Media for *E. coli* growth were as described in Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and GENEART Seamless Cloning and Assembly Kit (Invitrogen). Kanamycin or ampicillin was added as required, to final concentrations of 50 μg/ml or 100 μg/ml, respectively.

The *N. crassa* strains used in this research are listed in Table 1. For general cultivation of *N. crassa*, Vogel's medium (Vogel, 1956) supplemented with 2% sucrose and solidified with 1.5% agar was used. For selective plates after *N. crassa* transformation, Vogel's-N (nitrogen free) medium, supplemented with 0.5% proline as a nitrogen source and solidified with 1.5% agar, was used. Additionally, 2% sorbose, 0.05% glucose, and 0.05% fructose were added to restrict colonial growth (Brockman and de Serres, 1963; Perkins, 2006). Selection of the *bar*^{r} gene was performed using 400 μg/ml of phosphinothricin (PPT) (GoldBio, St. Louis, MO). To select against the *tk* gene as a positive screening for the excision event, 10 μM 5-fluoro-2′-deoxyuridine (FdU; Matrix Scientific, Columbia, SC) was used (Buxton and Radford, 1982; Sachs et al., 1997). Vogel's medium with 2% glucose, 2% xylose, or 2% (w/v) beechwood xylan (Sigma) was used to test induction of the *gh10-2* (NCU08189) promoter. The beechwood xylan powder was dry steam-sterilized in aluminum foil packets to avoid hydrolysis during autoclaving and then added to medium cooled to 65 °C (Zhao and Chen, 2012).

Table 1
Neurospora crassa strains used in this publication.

Strain name	Genotype	Reference
WT	FGSC #2489	Mylyk et al. (1974)
mus-53::*hph*	FGSC #10139	Ishibashi et al. (2006)
adh-1::*gh10-2-KO*	*mus-53*::*hph adh-1*::*gh10-2*(*p*) *β-rec/six*(*bar-tk*)	This work
adh-1::*xylP-KO*	*mus-53*::*hph adh-1*::*xylP*(*p*) *β-rec/six*(*bar-tk*)	This work
adh-1::*six* #1	*mus53*::*hph adh-1*::*six*	This work
adh-1::*six* #4	*mus53*::*hph adh-1*::*six*	This work

2.2. Plasmids and PCR constructs

First, the novel cassette variant was created with the herpes virus thymidine kinase gene (*tk*) (Sachs et al., 1997), fused in-frame to the 3′ end of the *bar*^{r} gene via a five Gly-Ala repeat $(GA)_5$ linker (Szewczyk et al., 2013, 2006). The *tk* gene and $(GA)_5$ linker were amplified from plasmid pSK397 (Krappmann et al., 2005, 2006) using primers ES050JF (ACTTGTTTAGAGGTAATCCTTCTTAGATTCAGTTAGCCTCCCCC ATC) and ES023JF (TGCCCGTCACCGAGATCGGAGCTGGTGCAGGC). This fragment was fused via the fusion PCR technology with two other fragments: one fragment containing the first *six* site, β-recombinase under the control of *xylP* promoter from *P. chrysogenum* (Zadra et al., 2000) and transcriptional terminator *trpC* from *A. nidulans*, and the other fragment containing the *bar*^{r} gene and the second *six* site. Plasmid pβrec(bar)TOPO1 was used as the template for the amplification of the fragments carrying β-recombinase and *bar*^{r} gene (Szewczyk et al., 2013). Unique restriction *Dra*I sites were introduced at the ends of the cassette to facilitate future release of the cassette from the vector. The resulting fusion PCR product was cloned into pCR-Blunt II-TOPO vector using Zero Blunt TOPO PCR vector Cloning Kit (Invitrogen). The plasmid was named pβ(bar-tk)TOPO7 and for future reference, it contains the cassette *xylP*(p)β(*bar-tk*) (Fig. 1A).

Second, we replaced the *xylP* promoter from *P. chrysogenum* with a native promoter from *N. crassa* gene *gh10-2* (NCU08189). Using 5′ phosphorylated primers ES088JF (P-ATTGAGAGACTCTTATAGATACG) and ES089JF (P-GGTCGTGCCTGGTTGTGG), 2200 bp of *gh10-2* (NCU08189) promoter were amplified from genomic DNA of *N. crassa* WT strain FGSC #2489 (McCluskey et al., 2010; Mylyk et al., 1974). The resulting phosphorylated *gh10-2* (NCU08189) promoter fragment was ligated into an inverted PCR product of the pβ(bar-tk)TOPO7 vector lacking the *xylP* promoter, using T4 DNA ligase (Fermentas, Vilnius, Lithuania). The plasmid was named pgh10-2β(bar-tk)TOPO15, which contains the cassette *gh10-2*(p)β(*bar-tk*) (Fig. 1B). The full sequences of the described plasmids carrying the new variants of self-excising β-recombinase/*six* cassettes are available in Supplementary data. In order to test the functionality of the new variants of the self-excising β-recombinase/*six* cassettes, we attempted to delete the alcohol dehydrogenase gene *adh-1* (NCU01754) (Park et al., 2007). The replacement cassettes containing the deletion cassette *xylP*(p)β(*bar-tk*) or *gh10-2*(p)β(*bar-tk*) flanked by 5′ and 3′