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Tools and Techniques

Establishing a versatile Golden Gate cloning system for genetic engineering in fungi

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ABSTRACT

The corn pathogen *Ustilago maydis* is a well-studied fungal model organism. Along with a broad set of experimental tools, versatile strategies for the generation of gene replacement mutants by homologous recombination in *U. maydis* have been developed. Nevertheless, the production of corresponding linear DNA constructs still constitutes a time-limiting step. To overcome this bottleneck, various resistance cassette modules were adopted for use with the so-called Golden Gate cloning strategy. These modules allow not only simple gene deletions but also more sophisticated genetic manipulations like inserting sequences for C-terminal protein tagging. The type IIs restriction enzyme *Bsa*I was selected for this novel approach as its recognition sites are comparatively rare in the *U. maydis* genome. To test the efficiency of the new strategy it was used to test the influence of varying flank lengths as well as the effect of non-homologous flank ends on homologous recombination. Importantly, to proof a broad applicability in other fungi the same strategy was used to generate mutants in the filamentous ascomycete *Aspergillus nidulans*. Hence, we present a highly efficient and economic cloning strategy that speeds up reverse genetic approaches in fungi.

1. Introduction

Ascomycete and basidiomycete fungi serve as important eukaryotic models in fundamental and applied research. Within the Ascomycota well-known examples include the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* (Osmani and Mirabito, 2004; Davis and Perkins, 2002; Borkovich et al., 2004; Galagan et al., 2005). Within the Basidiomycota, *Ustilago maydis*, a plant pathogen causing corn smut disease, is currently gaining increasing momentum as a simple eukaryotic model organism (Bölker, 2001; Brefort et al., 2009; Dean et al., 2012; Vollmeister et al., 2012; Feldbrügge et al., 2013). This pathogen shows a typical dimorphism and is thus able to grow either in a yeast or in a filamentous form. The yeast form is genetically tractable by homologous recombination yielding mitotically stable mutants. A large set of genetic tools is available, including integrative and self-replicating plasmids, targeted insertion at defined genomic loci and constitutive and inducible promoters (Spellig et al., 1996; Stock et al., 2012; Brachmann et al., 2001; Zarnack et al., 2006). The manually refined genome sequence of *U. maydis* was released in 2006 (Kämper et al., 2006; accessible at http://mips.helmholtz-muenchen.de/genre/proj/ustilago) facilitating reverse genetic approaches.

Several years ago, a straightforward gene replacement strategy based on a vector library containing resistance cassette modules that allow different types of genetic manipulations was established (Brachmann et al., 2004; Kämper, 2004; Fig. 1A). At that time the collection contained 32 plasmids harbouring four different types (I–IV) of gene replacement modules (Brachmann et al., 2004; see below). Each plasmid is available with four different selectable markers, HygR, CbxR, NatR and PhleoR, mediating resistance against hygromycin, carboxin, nourseothricin and phleomycin, respectively (Brachmann et al., 2004). As the most basic application, type I modules allow for simple gene replacements by one of the four resistance cassettes (Fig. 1B and C; Brachmann et al., 2004). As a second option, type II modules effect the expression of the gene of interest rather than deleting it (*e.g.*, to render it conditional) by transcriptional fusion to different heterologous promoters. To this end, respective promoter cassettes, mediating inducible or constitutive expression of the target gene, were introduced upstream of the different resistance cassettes (Fig. 1). Furthermore, the collection provides type III modules to examine the expression of target genes by

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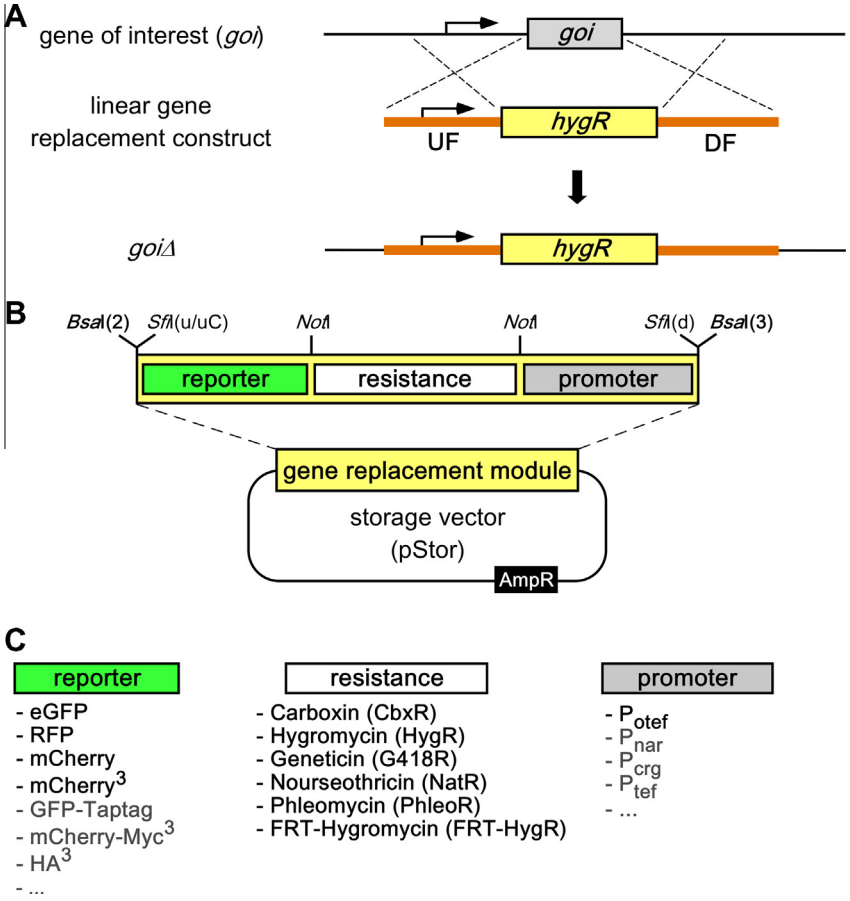

Fig. 1. Gene deletion in *U. maydis* and rationale of the new library. (A) Schematic representation of the gene deletion strategy applied in *U. maydis*. Flanking regions are amplified by PCR and ligated to a resistance cassette (herein *hygR* mediating hygromycin B resistance). The resulting strain carries the resistance cassette instead of the gene of interest (*goi*). (B) Architecture of the gene replacement modules that are stored in storage vectors carrying the ampicillin resistance gene for selection in *E. coli* (AmpR). Up to three components can be contained in the module: The presence of the resistance cassette is crucial for selection. Optionally, a transcriptional reporter and a promoter cassette can be added. *Bsa*I sites (2) and (3) were introduced at the ends of the modules while *Sfi*(u/uC) and *Sfi*(d) as well as internal unique cloning sites from the traditional system were kept. (C) Lists of available variants. Black font, cassettes are available for both the traditional and the novel Golden Gate based system. Grey font, cassettes are only available in the traditional system but could easily be adopted.

replacing the *ORF* with a reporter gene such as *gfp* or *mcherry*. For this purpose vectors were generated, in which a reporter gene cassette was placed downstream of the resistance cassettes. In the most complex variants, the type IV modules, all three cassettes were combined, e.g., to study expression of an essential gene (Fig. 1B and C; Brachmann et al., 2004).

Recently, additional compatible modules were developed that added increasing value to the existing library: the reporter cassette can now be inserted in a way that leads to translational fusions with the C-terminus of the encoded protein (type V modules; Fig. 1B and C; Becht et al., 2006). Different reporter genes encoding mCherry, eGFP, pGFP or RFP and tags like the Tap Tag, HA or Myc epitope have been established within the set of type V plasmids (Becht et al., 2006; König et al., 2009; Baumann et al., 2012). In order to delete several genes in one mutant, a HygR-cassette module, which can be removed using the FLP recombinase to allow marker recycling, was designed as a variant for simple gene deletions (HygR-FRT; Khrunyk et al., 2010). Moreover, geneticin was established as a fifth dominant selectable marker for *U. maydis* (G418R; Kojic and Holloman, 2000; Baumann et al., 2012; Fig. 1C). The latter innovations now allow i.a. analysing multi-gene families with members of redundant function or introduction of several reporter genes to co-localise multiple proteins (Khrunyk et al., 2010). In summary, the actual library includes five different types of replacement cassettes available with up to five selectable markers.

To generate a gene replacement construct, the module of choice is fused to PCR-amplified flanks of about 1 kb using the restriction enzyme *Sfi*I (Brachmann et al., 2004; Kämper, 2004; Fig. 1A and B). The advantage of this enzyme is that the five core nucleotides between its two identical recognition sequences can be chosen freely (5′-GGCCNNNN↓NGGCC-3′). Thus, distinct core sequences (here referred to as *Sfi*I(u/uC) and *Sfi*I(d); (Brachmann et al., 2004)) were designed to enable directional cloning with a single enzyme (Kämper, 2004). Generation of the gene replacement construct is preferentially achieved by subcloning PCR-generated flanks into appropriate vectors. In a second step these are combined with the cassette of choice to result in a vector containing the final construct (for details see Brachmann et al., 2004). For the subsequent generation of the linear transformation constructs, the respective part was usually excised from the plasmid using restriction sites that are located close to the insertion sites. This led to short non-homologous sequences at each end of the two flanks. Alternatively, the deletion construct could be amplified from this vector or the ligation reaction by PCR.

'Golden Gate' cloning is a recently described, very efficient high-throughput cloning procedure (Engler et al., 2008). The strategy is based on type IIs restriction enzymes that do not cleave within their recognition sequence but a few basepairs downstream. (Szybalski et al., 1991; Engler et al., 2008). This allows seamless joining of DNA fragments with inverse oriented sites without introducing foreign nucleotides (see below). Thus, a one-pot