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Yeast recombination-based cloning as an efficient way of constructing vectors for *Zymoseptoria tritici*

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ABSTRACT

Many pathogenic fungi are genetically tractable. Analysis of their cellular organization and invasion mechanisms underpinning virulence determinants profits from exploiting such molecular tools as fluorescent fusion proteins or conditional mutant protein alleles. Generation of these tools requires efficient cloning methods, as vector construction is often a rate-limiting step. Here, we introduce an efficient yeast recombination-based cloning (YRBC) method to construct vectors for the fungus *Zymoseptoria tritici*. This method is of low cost and avoids dependency on the availability of restriction enzyme sites in the DNA sequence, as needed in more conventional restriction/ligation-based cloning procedures. Furthermore, YRBC avoids modification of the DNA of interest, indeed this potential risk limits the use of site-specific recombination systems, such as Gateway cloning. Instead, in YRBC, multiple DNA fragments, with 30 bp overlap sequences, are transformed into *Saccharomyces cerevisiae*, whereupon homologous recombination generates the vector in a single step. Here, we provide a detailed experimental protocol and four vectors, each encoding a different dominant selectable marker cassette, that enable YRBC of constructs to be used in the wheat pathogen *Z. tritici*.

1. Introduction

Zymoseptoria tritici is a dimorphic ascomycete fungus, which ranges amongst the most wheat pathogens in Europe (Dean et al., 2012; Gurr and Fones, 2015). Developing new strategies to control this pathogen requires in-depth knowledge of its invasion strategy and insight into crucial cellular processes required for growth and proliferation. Such progress is strongly dependent on development of molecular tools and techniques. Previous work provided transformation protocols, vectors with different dominant selectable markers, conditional promoter analysis, GFP reporter system, virulence assays and high-throughput automated image analysis for *Z. tritici* (Bowler et al., 2010; Kema et al., 2000; Perez-Nadales et al., 2014; Rohel et al., 2001; Rudd et al., 2008; Skinner et al., 1998; Stewart and McDonald, 2014; Zwiers and De Waard, 2001). However, to further accelerate progress and extend the repertoire of molecular tools, efficient cloning methods are needed.

The majority of vectors for manipulation of *Z. tritici* have been generated using conventional cloning methods, including the use of restriction enzymes and *in vitro* ligation protocols (Adachi et al., 2002; Choi and Goodwin, 2011; Marshall et al., 2011; Motteram et al., 2009, 2011; Roohparvar et al., 2007; Zwiers and De Waard, 2001; Zwiers et al., 2007). However, these procedures carry numerous limitations. Firstly, they depend on the availability of unique and compatible restriction sites in the vector and the DNA fragment(s) to be cloned. Indeed, searching for the availability of such restriction sites or introducing new restriction sites in the DNA is time and labour-intensive (Benoit et al., 2006). Furthermore, the various manipulations could modify the primary sequence of the encoded gene product (Andersen, 2011), with the downstream risk of affecting the function of the gene products (Kilaru et al., 2009).

Recently, Gateway recombination technology was used to generate vectors for *Z. tritici* (Bowler et al., 2010; Mirzadi Gohari et al., 2014; Scalliet et al., 2012). The Gateway cloning method is based on the site-specific recombination properties of the bacteriophage lambda and provides a highly efficient way to clone DNA fragments of interest (Hartley et al., 2000; Landy, 1989). Whilst this is a

Abbreviations: YRBC, yeast recombination-based cloning; *sdi1*, succinate dehydrogenase; *hph*, hygromycin phosphotransferase; *nptII*, neomycin phosphotransferase; *bar*, bialaphos resistant gene; GFP, green-fluorescent protein; Zt, *Zymoseptoria tritici*; RB and LB, right and left border; bp, base pairs.

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powerful method for molecular cloning, the Gateway technology introduces 25 bp long "attachment sites" that results in an introduction of 8–11 additional amino acids. Such modification of the primary sequence, couple with the relatively high costs of the Gateway site-specific recombination kits, limit use of this cloning method (Engler et al., 2008).

An alternative cloning approach makes use of the ability of *Saccharomyces cerevisiae* to recombine DNA fragments *in vivo* by homologous recombination (Ma et al., 1987; Raymond et al., 1999). Here, DNA fragments, with overlapping sequences, are transformed into *S. cerevisiae* for *in vivo* recombination (Ma et al., 1987). Such overhangs can be as short as 30 bp (Kilaru et al., 2006; Oldenburg et al., 1997; Schuster et al., 2011a) and are added using commercially synthesized primers. This method circumvents both the need for restriction enzymes and expensive commercial kits. Most importantly, yeast recombination-based cloning (YRBC) avoids changes in the primary DNA sequence. Instead, this method allows precise cloning of multiple overlapping DNA fragments in a single step, thereby rapidly generating complex vectors (Andersen, 2011; Shanks et al., 2006). This powerful cloning method, YRBC has been used to construct viral and bacterial vectors (Shanks et al., 2006; Youssef et al., 2011), and indeed, to assemble the entire genome of the prokaryote *Mycoplasma genitalium* d from 25 overlapping DNA fragments (Gibson et al., 2008). In fungi, YRBC has been used in *Coprinopsis cinerea* (Kilaru et al., 2006), and subsequently, to investigate the corn pathogen *Ustilago maydis* (Schuster et al., 2011a) and the rice blast fungus *Magnaporthe oryzae* (Dagdas et al., 2012; Lu et al., 2014).

Here, we introduce the detailed protocol to construct vectors using YRBC. We also provide four vectors, carrying different dominant selectable marker cassettes, suitable for yeast recombination-based construction of vectors for use in *Z. tritici*.

2. Materials and methods

2.1. Fungal growth conditions and genomic DNA isolation

Z. tritici was grown in YG broth (yeast extract, 10 g/l; glucose, 30 g/l) for 3 days at 18 °C with 200 rpm. Three ml of cells were harvested by centrifugation at 13,000 rpm for 2 min and followed by addition of 400 µl of lysis buffer (2% Triton X, 1% SDS, 100 mM NaCl, 10 mM Tris HCl pH-8.0, 1 mM EDTA), 500 µl phenol: chloroform (1:1) and a small scoop of acid washed glass beads (425–600 µm; Sigma–Aldrich, Gillingham, UK). The tubes were mixed for 10 min by using IKA Vibrax shaker (IKA, Staufen, Germany) and centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a fresh Eppendorf tube containing 1 ml of 100% ethanol. The tubes were centrifuged for 10 min at 13,000 rpm and the DNA was washed with 500 µl of 70% ethanol. The residual ethanol was removed by incubating the tubes at 55 °C for 5 min and DNA was suspended in 50 µl water/RNaseA solution. For PCR applications, the genomic DNA was diluted with water by 200 times.

2.2. Construction of vectors pCGEN-YR, pCHYG-YR and pCBAR-YR using conventional ligation method

The vectors pCGEN-YR, pCHYG-YR and pCBAR-YR were constructed using conventional restriction digestion and ligation cloning method. The yeast recombination cassette consists of URA3 and 2µ *ori* from plasmid pNEB-hyg-yeast (Schuster et al., 2012) was cloned into the vectors pCGEN (Motteram et al., 2011), pCHYG (Motteram et al., 2009) and pCAMB-BAR (Kramer et al., 2009) resulting in vectors pCGEN-YR, pCHYG-YR and pCBAR-YR respectively. For construction of vector pCGEN-YR, a 8257 bp of vector pCGEN (*Sac*II and *Psi*I fragment) was ligated with 2820 bp fragment of vector pNEB-hyg-yeast (*Sac*II and *Ssp*I fragment). For construction of vector pCHYG-YR, a 8117 bp of vector pCHYG (*Sac*II and *Psi*I fragment) was ligated with 2820 bp of fragment of vector pNEB-hyg-yeast (*Sac*II and *Ssp*I fragment). For construction of vector pCBAR-YR, a 7616 bp of vector pCAMB-BAR (*Bcl*I and *Psi*I fragment) was ligated with 2847 bp of fragment of vector pNEB-hyg-yeast (*Bcl*I and *Dra*I fragment). Primers SK-41 and SK-Sep-137 (Table 1) were used to identify the positive clones and the expected band sizes are 2728 bp, 2588 bp and 2090 bp for vectors pCGEN-YR, pCHYG-YR and pCBAR-YR respectively.

2.3. Construction of vector pCCBX-YR using yeast recombination-based cloning

Plasmid pCCBX-YR was constructed using *in vivo* recombination in the yeast *S. cerevisiae* DS94 (MATα, *ura3-52*, *trp1-1*, *leu2-3*, *his3-111*, and *lys2-801* (Tang et al., 1996) following published procedures (Raymond et al., 1999). For the recombination events, the fragments were amplified with 30 bp homologous sequences to the upstream and downstream of the fragments to be cloned. The detailed steps involved in the construction of this vector are described below.

2.4. Primer designing and PCR amplification of DNA fragments

Primer design is vital step in constructing the vectors using YRBC. The 30 bp overlapping sequences to the next DNA fragment needs to be incorporated in the 5′ end of the 20–25 bp primer sequence, which makes the total primer length to 50–55 bp. Likewise, primers SK-Sep-11, SK-Sep-12, SK-Sep-282 and SK-Sep-283 (Table 1) were synthesized and then the desired DNA fragments were amplified either from *Z. tritici* IPO323 (Goodwin et al., 2011; Kema and van Silfhout, 1997) genomic DNA using Phusion high-fidelity DNA polymerase (Thermo Scientific, Leicestershire, UK). PCR was performed by using 1 µl of template DNA with final concentration of 200 µM each dNTPS, 0.5 µM of each oligos, 1x HF buffer, 0.02 U/µl of Phusion DNA polymerase in a total volume of

Table 1
Primers used in this study.

Primer name	Direction	Sequence (5′ to 3′)[a]
SK-41	Sense	GTGGATGATGTGGTCTCTACAGG
SK-Sep-11	Antisense	*ATTCAGAATGGTGAGGCATCGGTACAAGCT*CATGCTGTTGTTGAGTGCGTCC
SK-Sep-12	Sense	*AGCTTGTACCGATGCCTCACCATTCTGAAT*TGCTCAAGGACCTGCCCCAAG
SK-Sep-137	Antisense	CCCGATCTAGTAACATAGATGACA
SK-Sep-282	Sense	*GCTTGACGACATTCCGAAACCCCCAATTTC*GCTACCGAGCGGCGAGCAGA
SK-Sep-283	Antisense	*GCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCC*CTTCCGTCGATTTCGAGACAGC

[a] *Italics* indicate part of the primer that is complementary with another DNA fragment, to be ligated by homologous recombination in *S. cerevisiae*.