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One Step Construction of *Agrobacterium*-Recombination-ready-plasmids (OSCAR), an efficient and robust tool for ATMT based gene deletion construction in fungi

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ABSTRACT

Increasing availability of genomic data and sophistication of analytical methodology in fungi has elevated the need for functional genomics tools in these organisms. Previously we reported a method called DelsGate for rapid preparation of deletion constructs for protoplast-mediated fungal transformation systems, which is based on Gateway® technology. However, over the past several years *Agrobacterium tumefaciens*-mediated transformation (ATMT) has become the preferred genetic transformation method for an increasing number of fungi. Therefore, we developed a method for One Step Construction of *Agrobacterium*-Recombination-ready-plasmids (OSCAR), to rapidly create deletion constructs for ATMT systems. The OSCAR methodology involves PCR amplification of the upstream and downstream flanks of the gene of interest, using gene specific primers each with a 5′ extension containing one of four different *att*B recombination sites, modified from the Invitrogen MultiSite Gateway® system. Amplified gene flanks are then mixed with specifically designed marker and binary vectors and treated with BP clonase, generating the deletion construct in a single cloning step. The entire process of deletion construct preparation can be accomplished in just 2 days. Using OSCAR we generated eight targeted deletion constructs and used two of them to generate deletion mutants in *Verticillium dahliae* by ATMT. In summary, OSCAR methodology combines PCR and Gateway® technology to rapidly and robustly generate precise deletion constructs for fungal ATMT and homologous gene replacement.

1. Introduction

The increasing availability of fungal genome sequences and genome wide examination tools, such as global transcriptome and multispecies comparative analyses (Axelson-Fisk and Sunnerhagen, 2006; Breakspear and Momany, 2007; Soanes et al., 2008), is leading to the identification of large sets of genes whose roles are worthy of functional exploration. This situation elevates the need for fast, highly efficient systems for gene deletion, a critical tool for functional analyses. The targeted deletion of genes requires both a suitable method for the transfer of foreign DNA to fungal cells and the generation of deletion constructs. The deletion constructs should contain the regions flanking the gene of interest, while the ORF is replaced by a DNA fragment harboring a marker that allows selecting cells transformed with this foreign DNA. Deletion mutants are produced upon transformation by integration of this construct in the fungal genome by homologous recombination. Traditional methods to generate deletion constructs were labor intensive, generally including several cloning steps. They were therefore not suitable for high-throughput approaches to gene deletion. To address the increasing need for methods that can be applied to the functional analysis of genes at a larger scale, in recent years efforts have been directed to speed up the generation of deletion constructs. These improvements include the use of overlap PCR (Davidson et al., 2002), the similar split-marker deletion method (Catlett et al., 2002) or PCR and restriction enzyme digestion and ligation (Kämper, 2004). The use of *Saccharomyces cerevisiae* based recombination methods for plasmid based deletion construction has also proven very effective and been used in some large scale approaches with filamentous fungi (Oldenburg et al., 1997; Raymond et al., 1999; Colot et al., 2006). Recently, we reported development of DelsGate, a rapid and efficient method for generating gene deletion plasmid constructs (García-Pedrajas et al., 2008, 2010). DelsGate employs Gateway® technology in combination with the homing endonuclease I-*Sce*I allowing a universal methodology for generation of deletion constructs for any gene in the fungal genome.

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The deletion constructs produced by the methodologies above described are compatible with protoplast-mediated transformation (PMT). Since the first report on successful PMT of *S. cerevisiae* (Hinnen et al., 1978), the use of protoplasts as starting material for genetic transformation has been widely used in fungal species (reviewed by Fincham (1989) and Ruiz-Diez (2002)). However, numerous fungal species have proven to be recalcitrant to PMT because of fungal isolate specific difficulties with viable protoplast production and/or low transformation frequencies. Alternative methods for DNA transfer to fungi have consequently been investigated, such as electroporation and biolistic transformation (reviewed by Ruiz-Diez (2002)), or a method which uses a mutant strain exhibiting increased frequencies of homologous recombination coupled with the use of compressed air to allow direct transformation of fungal hyphae (Levy et al., 2008).

A major breakthrough to allow avoidance of the difficulties presented by PMT was achieved with the application of *Agrobacterium tumefaciens*-mediated transformation (ATMT) to fungi. Bundock et al. (1995) were the first to show that the method based on the ability of *A. tumefaciens* to transfer part of its DNA, the so-called transfer DNA or T-DNA, normally used for plant transformation, could be applied to *S. cerevisiae*. In subsequent years ATMT was proven successful for a number of fungal species (reviewed by Michielse et al. (2005a)). The T-DNA cannot only integrate randomly in the fungal genome, but it is also an efficient substrate for homologous recombination (Bundock et al., 1999; Gouka et al., 1999; Michielse et al., 2005b), a feature that allows use of ATMT for targeted gene deletion. A major advantage of ATMT is that intact cells are used as starting material for transformation. Conidia, germinating conidia, vegetative hyphae and fruiting body mycelia have all been successfully transformed with *A. tumefaciens* (reviewed by Michielse et al. (2005a)). Additionally, for a number of species it has been shown that ATMT increases the frequency of single-copy integration events as compared with PMT (reviewed by Meyer (2008)). Absence of secondary mutations due to multiple integration events is a very desirable feature. For random mutagenesis it facilitates the recovery of tagged genes. When generating deletion mutants it is also important to avoid secondary integration events, in genes others than the gene of interest, which could have phenotypic effects. Due to these advantages ATMT is now an established method of choice for both targeted as well as random gene mutation in an increasing number of fungal species, including plant pathogens such as *Magnaporthe grisea*, *Fusarium oxysporum* (Khang et al., 2005) and *Verticillium dahliae* (Dobinson et al., 2004).

ATMT requires the generation of deletion constructs in binary vectors. For homologous recombination based approaches the vector construct is designed to contain, between the T-DNA borders, the flanking regions of the gene of interest with the intervening ORF replaced by a selectable marker. Methodologies reliant on PCR alone as described above to produce deletion constructs for PMT are therefore not directly applicable to ATMT. In this work we expand the use of Gateway® technology for gene deletion approaches with the development of a method to rapidly produce deletion constructs for use with ATMT in fungi. We named this method "OSCAR" or One Step Construction of *Agrobacterium*-Recombination-ready-plasmids. Gateway® technology has an advantage over the yeast recombination plasmid construction methodology in that it relies only on *Escherichia coli* transformation following an *in vitro* recombinase step, thus eliminating the steps involving yeast. We have tested OSCAR's efficiency by producing deletion constructs for eight genes in the agronomically important plant pathogen *V. dahliae*, a causal agent of Verticillium wilt. Additionally, for two of the targeted genes, we report the complete process of fungal deletion mutant production. We have therefore demonstrated that OSCAR provides a very rapid, robust and cost-effective approach for generation of gene deletion constructs in a binary vector. OSCAR should be widely applicable to fungal species in which ATMT has become the preferred genetic transformation method.

2. Materials and methods

2.1. Fungal and bacterial strains, culture media and growth conditions

E. coli strain DB3.1 (Invitrogen, Carlsbad, CA) was used to propagate plasmids pDONR™201, pDONR™P2R-P3 and pDONR™P4-P1R (Invitrogen), which harbor the *ccd*B gene, lethal for most *E. coli* strains. For other manipulations *E. coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) was used. *E. coli* was grown in or on Luria Bertani (LB) medium amended with appropriate antibiotics. *A. tumefaciens* strain AGL-1 (Hellens et al., 2000) was used in this study and maintained on LB. *V. dahliae* strain VdLs.17 (sequenced strain) isolated from lettuce (*Lactuca sativa* L.) and strain Dvd-T5 isolated from tomato (Dobinson et al., 1996), were maintained on Potato Dextrose Broth (Sigma, St. Louis, MO), supplemented to 2% agar (2PDA) and grown at room temperature.

2.2. Construction of binary and marker vectors

To construct a binary vector for OSCAR, the 2.5 kb Gateway cassette *att*P2R-*ccd*B-*cm*R-*att*P3 was amplified by PCR with the Expand High Fidelity PCR System (Roche, Mannheim, Germany), using plasmid pDONR™P2R-P3 (Invitrogen) as template and the following primers: M13F-*Kpn*I and M13R-*Hind*III (Table 1). The 2518 bp PCR product was gel-purified and cloned into pCR2.1-TOPO (Invitrogen) generating plasmid pTOPO-*att*P2R-*ccd*B-*att*P3. The P2R-*ccd*B-*cm*R-P3 cassette was then excised from pTOPO-*att*P2R-*ccd*B-*att*P3 by digestion with *Hind*III and *Kpn*I and inserted in the binary vector pPZP-RCS2 (Goderis et al., 2002), also digested with these enzymes. The resulting plasmid was named pOSCAR (GenBank Accession no. HM623914).

To produce the marker vector, in which the *E. coli* hygromycin B phosphotransferase (*hph*) resistance gene under the regulation of the *Aspergillus nidulans trp*C promoter (A-HygR cassette) is flanked by the *att*P1r and *att*P4 recombination sites, multiple cloning steps were required. See Supplementary Fig. S1 for an illustration of the construction steps. pDONR™P4-P1R (Invitrogen) was the source of *att*P1r and *att*P4 recombination sites, and pDONR-A-Hyg (García-Pedrajas et al., 2008, Accession no. EU360963) was the source of the A-*HygR* cassette. First, each *att*P recombination site and the A-*HygR* cassette were separately cloned in pBluescript II KS(+) (Stratagene, La Jolla, CA). The 276 bp fragment containing the *att*P1r recombination site was excised from pDONR™P4-P1R by digestion with *Not*I and *Sal*I and cloned in pBluescript II KS(+),

Table 1
Primers used in this study for the generation of the OSCAR plasmids, and construct verification.

Primer name	Primer sequence[a], 5′ → 3′
M13F-*Kpn*I	GGTACCGTAAAACGACGGCCAG
M13R-*Hind*III	AAGCTTCAGGAAACAGCTATGAC
OSC-F	CGCCAATATATCCTGTCAAACACT
OSC-R	CTAGAGGCGCGCCGATATCCT
Hyg-F (850)	AGAGCTTGGTTGACGGCAATTTCG
Hyg-R(210)	GCCGATGCAAAGTGCCGATAAACA
Vd02161F	TGGACCAGGTTGTCAGTCGAACTT
Vd02161R	GTGCAGGTGCCGAACGATGATTGA
Vd09930F	GACACCGACGACCTTGCTAC
Vd09930R	CGTACTCTTTCCTGTCTGTC

[a] The underlined text in the sequence of primers M13F-*Kpn*I and M13R-*Hind*III represent the recognition site for restriction enzymes *Kpn*I and *Hind*III, respectively.