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Short communication

Seamless deletion of a large DNA fragment in the taxol-producing fungus *Pestalotiopsis microspora*

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ABSTRACT

To reduce the unnecessary gene clusters in the taxol-producing fungus *Pestalotiopsis microspora*, we report the development of an effective DNA deletion method that relies on a deletion cassette constructed with the Gateway-technique and overlap extension PCR, using the orotidine 5′-phosphate decarboxylase as recyclable marker for selection. By this approach, two adjacent DNA sequences can be sequentially deleted in a single transformation mediated by *Agrobacterium tumefaciens*, resulting in the deletion of a large DNA fragment. Additionally, the selection marker is spontaneously eliminated in this process. We used this method to successfully remove the *mus53* locus of *P. microspora*.

Pestalotiopsis fungi are plant pathogens and endophytes in a variety of forest hosts and known for their capacity of production of a variety of secondary metabolites (Yang et al. 2012), for instance, the diterpenoid derivative paclitaxel (taxol) that has been used to treat several types of solid cancer in clinic (Strobel et al. 1996; Fu et al. 2009). The discovery of taxol motivates efforts to establish large-scale fermentation process to make the drug. We previously reported the isolation of *P. microspora* strain NK17, which produces taxol and a polyketide derivative pestalotiollide B, a promising inhibitor of cholesterol ester transfer proteins (Bi et al. 2011; Niu et al. 2015). To improve the yield of taxol in NK17 requires effective molecular tools for this non-model fungus. Despite that we previously established a positive selection marker with hygromycin B resistance gene *hygR* (Hao et al. 2014; Yu et al. 2015), we found that this marker could not be used repeatedly, which is desperately needed in repeated genetic manipulations of *P. microspora*.

Generally, recyclable selection markers have been developed in other organisms via either an endogenous mechanism

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or the activity of heterogeneous recombinases. For example, artificially created homologous sequences, such as the HisG sequence from *Escherichia coli* (Alani et al. 1987) or the CYCTT sequence (Yang et al. 2009), are flanked to the selection marker and will be inserted into the target locus. After the pop-out process, residual HisG/CYCTT sequence is left at the locus and can be accumulated in the genome after multiple rounds of manipulation, which may promote chromosomal rearrangement or other genomic changes. As such, the ideal strategy in genetic manipulation for a drug-producing fungus should be residual-free. Several seamless gene deletion methods have been developed for *Aspergillus niger*, *Talaromyces versatilis* (Delmas et al. 2014), *A. aculeatus* (Tani et al. 2013) and *Saccharomyces cerevisiae* (Akada et al. 2006). However, none of the methods seems suitable for *P. microspora*, since they require transformation by protoplasts, which do not regenerate to form mycelia in this species. The orotidine 5′-phosphate decarboxylase (ODCase) has been used as a recyclable marker in several fungi such as *A. aculeatus* (Tani et al. 2013) and *S. cerevisiae* (Akada et al. 2006). We found that with minor modification, this marker equivalent gene (*pm-ura3*) found in *P. microspora* can be used as recyclable selection marker in multiple genome manipulations in a seamless way. We designed a DNA construct for sequential deletion of two adjacent DNA fragments in a single transformation, which could in principle double the deletion efficiency, and in the meanwhile, the ODCase marker was eliminated. With this approach, we deleted a locus encoding DNA ligase IV (*mus3*) in *P. microspora* without leaving any marker sequences or heterologous DNA sequences after manipulation.

Strains and plasmids

Pestalotiopsis microspora NK17 was isolated and stored by our laboratory (Niu et al. 2015) and maintained in potato lactose broth (PLB) or PLB derivatives supplemented with 2% (w/v) agar (PLA) at 25 °C. We used an ODCase-deficient strain of *P. microspora*, designated as *ura3Δ-1* (Chen et al. 2015), as a recipient in this study. *E. coli* strain DB3.1 (Invitrogen, Carlsbad, CA, USA) was used to propagate the T-DNA-containing vector pOSCAR (Paz et al. 2011) and strain DH5α was used to propagate other plasmids. The construction of plasmid pB-Ura-OSCAR is detailed in the Supplementary Fig. S1. *Agrobacterium tumefaciens* strain LBA4404 was used for the conjugation and transformation of *P. microspora*. The minimal medium for *P. microspora* consisted of 1.34% (w/v) yeast nitrogen base supplemented with 2% (w/v) lactose.

Fungal transformation

Agrobacterium tumefaciens-mediated transformation (ATMT) of *P. microspora* was carried out as previously described (Chen et al. 2015). Briefly, *A. tumefaciens* strain harboring the binary vectors was grown in 5 mL LB containing antibiotics: rifampicin (25 μg/mL), spectinomycin (100 μg/mL) and streptomycin (100 μg/mL). Approximately 100 μL of the bacterium culture (10^8 CFU/mL) was mixed with an equal volume of *ura3Δ-1* conidia (10^7). The mixture was co-cultured for 48 h on the induction medium before being transferred to PLA for selection. Transformants were single-conidium purified.

Construction of the deletion construct

The deletion construct was generated using primers listed in the Supplementary Table S1 (their locations are shown in the Supplementary Fig. S2). Primers 1/2 and primers 3/4 were designed to amplify the 5′ and 3′ homologous flanking sequences of the target chromosome region, respectively. Primers 2 and 3 harbored complementary overlapping sequences at their 5′ ends so that the 5′ and 3′ flanking segments could be spliced by overlap extension (SOE) PCR. Primers 5/6 were designed to amplify the middle portion of the target sequence (middle fragment in the Supplementary Fig. S2 and other figures). Primers 1, 4, 5, and 6 contained *attB2r*, *attB1r*, *attB4*, and *attB3* sequences, respectively, at their 5′ ends.

The construction of the deletion cassette is illustrated in Fig. 1. The BP clonase reaction (5 μL) included 30 ng of purified SOE PCR products, 30 ng of the purified middle segment, 60 ng of pB-Ura-OSCAR, 60 ng of pOSCAR, and 1 μL of BP clonase II enzyme mix (Invitrogen). The BP reaction was carried out at 25 °C overnight, and terminated by incubating the reaction mixture with 0.5 μL of proteinase K (20 μg/μL) at 37 °C for 10 min. The reaction mixture was then used to transform competent *E. coli* cells by a standard heat shock method. The resultant vector, pOSCAR-53UM, contained a replacement cassette carrying the 5′ and 3′ flanking sequences and a middle fragment derived from the target region, with the gene encoding ODCase (*pm-ura3*) serving as a marker. This construct—referred to as the 53UM cassette—was loaded in the T-DNA region for delivery into *P. microspora* by ATMT.

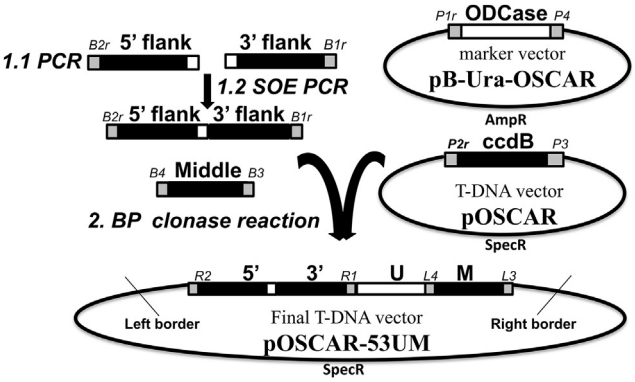

Fig. 1 – Schematic of the construction of the 53UM cassette with the T-DNA vector. PCR amplification of the three homologous DNA fragments from both ends and a middle part of the target locus was followed by splicing of the 5′ and 3′ flanking sequences by overlap extension PCR. A BP clonase reaction was carried out using a mixture of purified flanking, middle fragments, the binary vector pOSCAR and the marker vector pB-Ura-OSCAR with ODCase as a selection marker to obtain the final plasmid pOSCAR-53UM harboring the 53UM deletion cassette. The construction of pB-Ura-OSCAR was shown in the Supplementary Fig. S1. B1r, B2r, B3, B4, P1r, P2r, P3, P4, R1, R2, L3, and L4 represent lambda recombination sites (Gateway system, Invitrogen).

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