

Video Article

Rapid Deletion Production in Fungi via *Agrobacterium* Mediated Transformation of OSCAR Deletion Constructs

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Abstract

Precise deletion of gene(s) of interest, while leaving the rest of the genome unchanged, provides the ideal product to determine that particular gene's function in the living organism. In this protocol the OSCAR method of precise and rapid deletion plasmid construction is described. OSCAR relies on the cloning system in which a single recombinase reaction is carried out containing the purified PCR-amplified 5' and 3' flanks of the gene of interest and two plasmids, pA-Hyg OSCAR (the marker vector) and pOSCAR (the assembly vector). Confirmation of the correctly assembled deletion vector is carried out by restriction digestion mapping followed by sequencing. *Agrobacterium tumefaciens* is then used to mediate introduction of the deletion construct into fungal spores (referred to as ATMT). Finally, a PCR assay is described to determine if the deletion construct integrated by homologous or non-homologous recombination, indicating gene deletion or ectopic integration, respectively. This approach has been successfully used for deletion of numerous genes in *Verticillium dahliae* and in *Fusarium verticillioides* among other species.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55239/>

Introduction

Genetic dissection is a powerful methodology for determining the functional importance of individual or combinations of genes. A standard approach to understand the role of specific genes is production of single gene mutants unaltered in any other gene. The most powerful and least potentially confounding approach is complete and precise deletion of a gene of interest's open reading frame (GOI ORF) without damage to any other gene function.

Because standard ligation approaches for deletion plasmid generation require multiple steps, the rationale for OSCAR¹ was to produce a more rapid *in vitro* approach. **Figure 1** depicts the assembly process in the OSCAR approach. The method described here has the advantage of combining rapid construction of individual gene deletion vectors in a single multipart reaction in combination with subsequent *Agrobacterium tumefaciens* mediated transformation (ATMT). OSCAR is very rapid and compares well with other strategies such as use of Gibson assembly in yeast². The OSCAR method has been used successfully with several Ascomycota species of fungi. These species include: *Fusarium verticillioides* (unpublished), *Verticillium dahliae*³, *Setosphaeria turcica*⁴, *Metarhizium robertsii*⁵, *Fusarium oxysporum f. sp. vasinfectum*⁶, *Pestalotiopsis microspora*⁷, *Colletotrichum higginsianum*⁸, and *Dothistroma septosporum*⁹ and *Sarocladium zeae* (unpublished).

This protocol provides step-by-step instruction for the method including primer design, flank PCR amplification, the OSCAR BP reaction, deletion construct structure confirmation, transformation of *Agrobacterium* with the construct followed by ATMT based transfer of the deletion construct into the fungal cells, and finally differentiating fungal deletion mutants from those with ectopically integrated deletion constructs.

Protocol

1. Primer Design for PCR Amplification of Gene Flanks

1. Download to a word processing file the genomic region of the gene of interest (GOI) including the open reading frame (ORF) and at least 2 kb flanking the gene on each side from FungiDB or other genomic data resource.
2. Highlight the ORF intended for deletion and label start and stop codons.
3. Identify and highlight adjacent ORFs within the downloaded sequence.

- Use the 2 kb 5' end of the GOI ORF and the primer design tool (see Materials List) to design PCR primer pair O1 and O2 to generate a minimum size product of 1 kb. Take care to not impact adjacent ORFs.
 - Paste the 2 kb 5' flank into the "Sequence Entry" window. Click on "Show Custom Parameters" box. Enter the following custom design parameters: primer TM 58 (min), 60 (opt) and 62 (max); Primer GC 40 (min), 50 (opt) and 60 (max); Primer Size 22 (min), 24 (opt) and 26 (max); Amplicon Size 1250 (min), 1500 (opt) and 2000 (max).
 - Choose the result that places the reverse primer (O2) closest to the ORF. Capture a screen shot of the assays and copy and paste the primer sequences into the word processing document.
 - Repeat the process for 3' flank to generate primers O3 and O4, this time choose the result placing the forward primer (O3) closest to the target ORF.
- Add appropriate attach sites to 5' ends of primers 1 through 4 (see Table 1).
- Also design and order ORF specific primers to be used for deletion confirmation in section 4 below. Parameters should be the same as in step 1.4.1 except use Amplicon Size 500 (min), 750 (opt) and 1000 (max) adjusting for length of ORF if necessary. Finally, for confirmation of homologous recombinants, generate 5' out and 3' out primers found just outside the flanks within the chromosome. These "out" primers will be used with *hygR* (210) and *hygF* (850), respectively (Fig 2).
- Order primers.

2. Production of OSCAR Constructs

- Using a hi-fidelity *Taq*, carry out two reactions, one each for the 5' and 3' flanks, using primers (100 μ M) O1 and O2 in one reaction and primers O3 and O4 in the second. Reaction mixture contains the following: 29.5 μ L sterile distilled water (SDW), 5 μ L 10x LA PCR buffer, 8 μ L 2.5 mM dNTP mix, 5 μ L 25 mM $MgCl_2$, 1 μ L template (50 ng/ μ L), 0.5 μ L hi-fidelity *Taq*, 0.5 μ L Primer O1 and 0.5 μ L Primer O2 for 5' flank (or 0.5 μ L Primer O3 and 0.5 μ L Primer O4 for 3' flank) Use reaction conditions are as follows: 94 °C for 1 min, then 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, then final steps of 72 °C for 5 min and then hold indefinitely at 10 °C.
- Run 0.8% agarose 1x TAE (40 mM Tris acetate pH 8.3, 1 mM EDTA) gel electrophoresis for approximately 125 Vh in a standard minigel apparatus to determine product size and relative concentration. Post stain the gel with non-ethidium stain according to the manufacturer instructions. Visualize on a UV illuminator.
- If 5' and 3' flank products are in roughly even concentration, combine them and use an affinity column kit (or PEG precipitation 90 μ L combined PCR products, 240 μ L TE buffer pH 7.5, 160 μ L of 30% polyethylene glycol PEG8000 30 mM $MgCl_2$) to co-purify the PCR products according to the manufacturer's protocol. If they are not of equal concentration combine all of low concentration product with an estimated equal amount of product from the higher yield reaction.
- Use a spectrophotometer to measure purified DNA concentration.
- Carry out BP reaction by mixing the following: 1 μ L of 60 ng/ μ L pOSCAR, 2 μ L of 60 ng/ μ L pA-Hyg-OSCAR, 1 μ L of 60 ng/ μ L combined flanks, 1 μ L of clonase. Incubate for 16 h at 25 °C. Terminate the reaction by adding 0.5 μ L proteinase K and incubate for 10 min at 37 °C.
- Transform high competence *E. coli* cells. Both commercially available competent cells and home-made DH5 α $CaCl_2$ competent cells were used successfully as recipients as described by the manufacturer's instructions. Plate on low sodium (0.5 g/L NaCl) LB containing 100 μ g/mL spectinomycin (Spec).
- Identify the correct construct by performing DNA minipreps¹⁰ of colonies generated above. Perform double digestion with *HindIII* and *KpnI* as follows: pipette 5 μ L DNA, 2 U of each enzyme, 2 μ L appropriate 10x buffer with SDW to a 20 μ L total volume. This releases the insert (approximately 4.5 kb with 1.5 kb gene flanks) from the vector (ca. 7 kb) and cuts any sites in flanks which can give predictable band sizes.
- Sequence¹¹ select clones showing an appropriate banding pattern.

3. *Agrobacterium tumefaciens* Mediated Transformation (ATMT) of Fungi

- Transform *Agrobacterium tumefaciens* strain AGL1 with OSCAR deletion construct¹².
- Transform fungus with AGL1 containing the GOI OSCAR deletion plasmid. To do this carry out the following steps:
 - Prepare a fungal spore suspension with sterile water at 2×10^6 spores/mL. Quantify spores on a hemocytometer or automated cell counter. Place 500 μ L of this in a 1.5 mL microcentrifuge tube. This set up is for 4 transformation plates.
 - Use a blue sterile disposable loop to add an easily visible gob (should cover about 20% of the loop diameter) of the deletion plasmid containing AGL1 from 2-3 day old LB-Spec 100 medium (see Materials List for composition) containing plates and mix into the spore suspension. Vortex till bacterial cells are well dispersed (ca. 5 min medium speed).
 - Pipette 100 μ L of the conidia-Agro suspension onto the center of a cellulose membrane filter placed on each of four 6 cm Petri dishes containing co-cultivation medium¹².
 - Spread the suspension with sterile glass beads (about 4) to cover the whole surface of the membrane filter. Alternatively spread the suspension using a traditional spreader tool. Allow the membrane filter to dry in a hood for approximately 10 min, wrap with paraffin film. Repeat steps 3.2.2 and 3.2.3 to set up multiple transformations.
 - Incubate the plate for 2 days at room temperature, inverted.
 - Transfer the membrane filter onto 6 cm *Aspergillus* selection medium¹² plates containing hygromycin B (Hyg) 150 μ g/mL, 200 mM cefotaxime and 100 μ g/mL moxalactam. Incubate at room temperature for 5-7 days before isolating Hyg resistant colonies.
 - With sterile toothpicks, transfer putative transformants onto 6 cm Potato Dextrose Agar (PDA) plates containing 150 μ g/mL Hyg, 100 μ g/mL kanamycin.

4. Deletion Mutant Identification by PCR

- Extract DNA for PCR from transformants by thermolysis¹³.

- Once transformants form colonies on the PDA from step 3.2.7, use a sterile toothpick to transfer a small amount (2 mm x 2 mm) of mycelium or yeast cells from the colony into 100 μ L of lysis solution (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol) in a microcentrifuge tube.
- Incubate the mixture at 85-90 $^{\circ}$ C for 20-30 min. Store the crude extract containing genomic DNA at -20 $^{\circ}$ C until use in step 4.2.
- Carry out 4 PCR reactions per transformant, one each to detect homologous recombination of the 5' and 3' flanks, respectively, one for the selectable marker (hygromycin phosphotransferase in this case) and one for the GOI ORF.
NOTE: We generally use inexpensive low-fidelity *Taq* for these reactions. Reaction conditions are as described in step 2.1 above and contain SDW 20 μ L, 10x *Taq* buffer 2.5 μ L, dNTPS (10 mM) 0.5 μ L, homemade *Taq*¹⁴, 0.5 μ L, Primer A (100 μ M) 0.5 μ L, Primer B (100 μ M) 0.5 μ L, Template 0.5 μ L. Lower concentration primer stocks (10 μ M) can be used equally well.
- Run an agarose gel (e.g. 0.8%) of the PCR products. Deletion mutants will produce the Hyg band but not an ORF product. Ectopic integrants will show both bands. Wild type will produce only the ORF band.
NOTE: 15% glycerol is used to store our strains long-term at -80 $^{\circ}$ C.

Representative Results

The OSCAR method, in a single reaction, generates a plasmid containing the flanks of the target gene to be deleted surrounding the selectable marker cassette. The production of deletion constructs using OSCAR is very efficient. The system can, however, produce partial constructs containing some but not all three fragments (the two gene flanks and the selectable marker). Generally, the majority of *E. coli* transformants contain the correct OSCAR construct. For example, **Figure 2** depicts the OSCAR deletion construct for the *Verticillium dahliae* gene VDAG_06812. In this case the flanks of VDAG_06812 lack *Hind*III or *Kpn*I sites and therefore a plasmid insert of 4,247 bp should be released. **Figure 3** shows *Hind*III-*Kpn*I restriction enzyme double digestion confirmation of the correct plasmid structure except for lanes 5 and 11 representing anomalous constructs. **Figure 4** shows final confirmation of two different gene deletions by Southern blot. **Figure 5** shows a definitive PCR approach as an alternative to Southern blot.

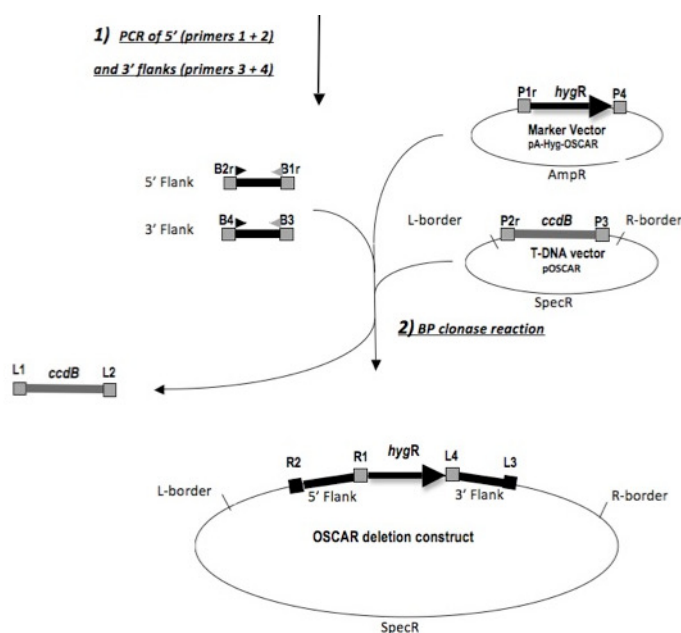


Figure 1: OSCAR deletion construction reaction. The OSCAR deletion construction process includes separate PCR amplification of the 5' and 3' gene flanks, followed by a BP clonase reaction with the combined purified flank products and the binary and selection marker plasmids. B1r, B2r, B3, B4, P1r, P2r, P3, P4, R1, R2, L3 and L4 represent lambda recombination sites. Re-print with permission from reference¹.

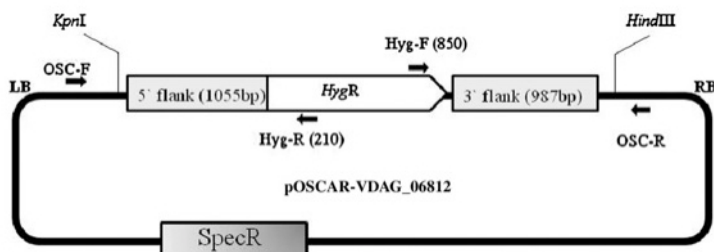


Figure 2: OSCAR deletion construct for *Verticillium dahliae* gene VDAG_06812. Positions of restriction enzyme recognition and primer annealing sites to verify correct deletion construct structure are shown. Re-print with permission from reference¹.

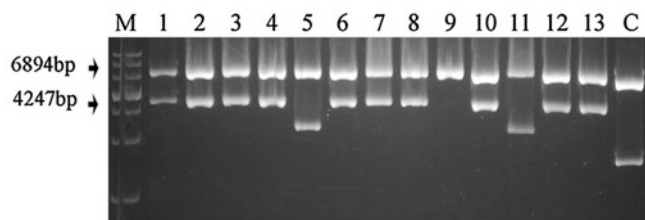


Figure 3: Restriction digestion confirmation of the deletion construct structure of VDAG_06812 with *KpnI* and *HindIII*. Plasmids were double digested and analyzed by gel electrophoresis on a 0.8% agarose gel. The correct construct generates two bands, of about 6.9 kb and 4.2 kb, representing the binary vector backbone and the HygR marker fused to the target gene flanks, respectively. Re-print with permission from reference¹.

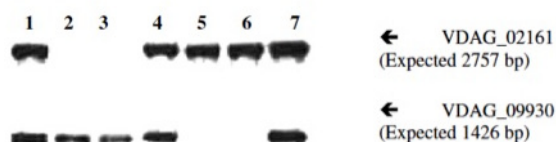


Figure 4: Southern blot hybridization confirming deletion of VDAG_02161 and VDAG_09930 in *V. dahliae* using OSCAR deletion constructs. Genomic DNAs were digested with *BclI*. They were then probed simultaneously with VDAG_02161 and VDAG_09930 ORF probes. Hybridization bands are 2,757 bp and 1,426 bp for the wild type alleles of VDAG_02161 and VDAG_09930, respectively. Samples are as follows: lane 1: wild type strain VdLs.17; lane 2: deletion mutant strain 02161.1; lane 3: deletion mutant strain 02161.3; lane 4: ectopic transformant strain Ect 02161.2; lane 5: deletion mutant strain 09930.3; lane 6: deletion mutant strain 09930.10; lane 7: ectopic transformant strain Ect 09930.4. Re-print with permission from reference¹.

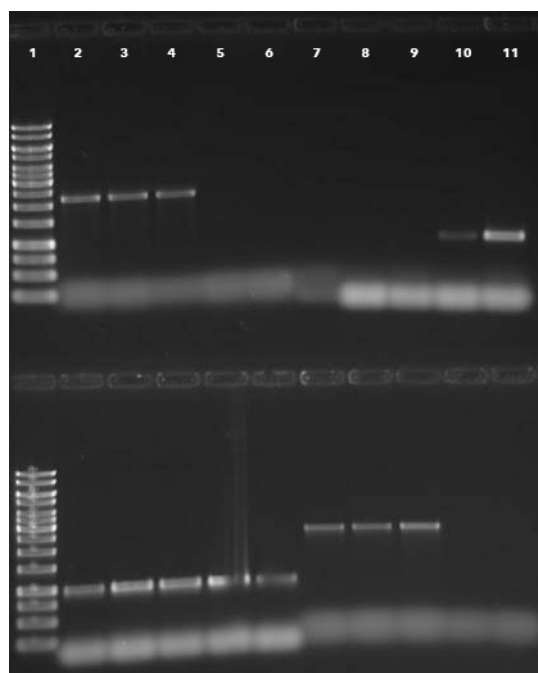


Figure 5: Deletion mutant confirmation by PCR. Analysis of deletion and ectopic *Fusarium verticillioides* transformants for gene FVEG_13253. Lanes 1 top and bottom, marker; lanes 2-6 top gel transformant PCR 5' out fragment amplification; lanes 7-11 top gel ORF amplification; lanes 2-6 bottom gel Hyg gene amplification; lanes 7-11 bottom gel 3' out fragment amplification. Samples are deletion strains 11/31 (lanes 2 and 7), 11/32 (lanes 3 and 8), 11/33 (lanes 4 and 9), and ectopic transformants 11/34 (lanes 5 and 10), and 11/35 (lanes 6 and 11).

Primer	Use	Sequence
Primer O1-(attB2r)	Amplification of 5' flank, primer forward	5'-GGGGACAGCTTTCTTGTACAAAGTGGAA
Primer O2-(attB1r)	Amplification of 5' flank, primer reverse	5'-GGGGACTGCTTTTTTGTACAAACTTGT
Primer O3-(attB4)	Amplification of 3' flank, primer forward	5'-GGGGACAACCTTTGTATAGAAAAGTTGTT
Primer O4-(attB3)	Amplification of 3' flank, primer reverse	5'-GGGGACAACCTTTGTATAATAAAGTTGT

Table 1: Specific OSCAR primer 5' extensions added to gene specific primer sequences.

Primer	Use	Sequence
OSC-F	OSCAR forward sequencing primer, sequences sense strand of 5' flank	5'-CTAGAGGCGCGCCGATATCCT
OSC-R	OSCAR reverse sequencing primer, sequences anti-sense strand of 5' flank	5'-CGCCAATATATCCTGTCAAACACT
HygR (210)	Reverse sequencing primer, sequences anti-sense strand of 5' flank	5'-GCCGATGCAAAGTGCCGATAAACA
HygF (850)	Forward sequencing primer, sequences sense strand of 3' flank	5'-AGAGCTTGGTTGACGGCAATTTCCG
New Hyg Marker Forward	To amplify the hygromycin resistance gene as a control (together with New Hyg Marker Reverse primer below)	5'-GACAGGAACGAGGACATTATTA
New Hyg Marker Reverse	To amplify the hygromycin resistance gene as a control (together with New Hyg Marker Forward primer above)	5'-GCTCTGATAGAGTTGGTCAAG

Table 2: Primers for analysis of OSCAR deletion constructs.

Discussion

One Step Construction of *Agrobacterium*-Recombination-ready-plasmids (OSCAR) has been successfully employed with an ever-increasing number of Ascomycota fungi. The method should also easily be applicable to the Basidiomycota and species from other fungal phyla (with appropriate promoters driving selectable marker genes), assuming *Agrobacterium* mediated transformation and homologous recombination are possible. Additional marker vectors have been generated to diversify choices of anti-fungal compound as well as allow the production of double and higher order mutants. These include resistance to G418, nourseothricin, and recently the herbicides glufosinate ammonium and chlorimuron ethyl⁴.

The method was worked out for use with *Verticillium dahliae* from which the images presented here are primarily derived¹. More recently OSCAR has been used extensively for deletion of genes in the mycotoxigenic fungus *Fusarium verticillioides*. Over 60 deletion constructs have been made and deletions mutants for more than 30 genes generated (unpublished).

In its current iteration the process requires about 4-6 weeks to have a set of confirmed deletion mutants in hand. The following is a brief list of the major OSCAR steps and approximate time needed to accomplish them: 1) Design and procurement of OSCAR primers based on genome data (5 days), 2) flank amplification and cloning, (2 days), 3) clone confirmation by miniprep and sequencing (1 week), 4) introduction of plasmid into *Agrobacterium* for ATMT (4 days), 5) production of transformants by ATMT in *Fusarium verticillioides* and grow out (2 weeks, note that this is dependent on growth rate of the fungus under study), thermolysis and PCR determination of transformant genotypes (2 days), 6) single sporing, genotype confirmation and storage (1-2 weeks).

The adaptation of OSCAR to true high throughput has not been attempted to our knowledge but should be practical. The reactions are sufficiently robust to allow for successful application in multi-well format. The use of robotics would certainly advance this approach. The OSCAR method has the time saving advantage of requiring a single recombination reaction containing the amplified flanks and two plasmids pOSCAR and pA-Hyg OSCAR. Similar approaches require generation of an entry clone that has to be confirmed and then used in a second reaction to generate the construct of interest. Plasmids pOSCAR and pA-Hyg OSCAR mentioned are available via Addgene or the Fungal Genetics Stock Center¹⁵. Other OSCAR marker plasmids such as pA-Bar-OSCAR and pA-Sur-OSCAR may be available from authors⁵.

Disclosures

The authors have nothing to disclose.

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