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Tools and Techniques

# Fluorescence Assisted Selection of Transformants (FAST): Using flow cytometry to select fungal transformants

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ABSTRACT

The availability of drug resistance markers for fungal transformation is often a limiting factor in both fungal genetics research and industrial applications. We describe a new technique using flow cytometry to select fungal transformants using well-known fluorescent proteins as markers for transformation. This new technique, Fluorescence-Assisted Selection of Transformants (FAST), was used for a transformation of *Fusarium oxysporum* with GFP as a marker targeted at a specific site on chromosome 14. The resulting strain was then transformed again with a gene replacement construct containing both RFP and a gene for drug resistance as markers. By directly comparing FAST with drug resistance selection we show that both methods yield comparable numbers of gene deletion mutants.



## 1. Introduction

The most common strategy for analyzing gene or protein function in fungi is by creating transgenic strains. Genes of interest can be either added by transformation with an expression cassette or deleted by introducing gene replacement constructs that exchange the gene of interest for a selection marker. Two of the most commonly used techniques for fungal transformation are electroporation of protoplasts or conidia (Jiang et al., 2013) and *Agrobacterium tumefaciens* mediated transformation (Michielse et al., 2005) to allow uptake of foreign DNA. The latter uses the bacterium *A. tumefaciens*, which normally infects and transforms plants and is a causal agent for crown gall disease, to introduce engineered transfer DNA into the fungus.

Both techniques rely on antifungal drug resistance genes to select for cells or hyphae that have successfully taken up foreign DNA. For each species of fungi only a few of such markers (generally not more than three) are available. This is one of the most notable restrictions in transforming filamentous fungi, especially when several rounds of transformation are required, for instance when investigating gene families or metabolic pathways. This problem has been highlighted by several researchers, each suggesting a method to circumvent this limitation (Hartl and Seiboth, 2005; Kopke et al., 2010; Watson and Wang, 2012; Zhang et al., 2013). The techniques proposed all rely on recombination to remove marker genes, either through the Flippase recombinase (FLP) and its Flippase recognition target FRT from the 2 μm plasmid from *Saccharomyces cerevisiae* (Kopke et al., 2010), Cre-Lox recombinases from bacteriophages (Zhang et al., 2013) or by spontaneous recombination after introducing repeats (Hartl and Seiboth, 2005; Zhang et al., 2013). By removing the marker after a transformation the same marker gene is available for consecutive manipulations. Several steps following the creation of transformants are required to cure the strains of the marker. This is achieved either by growing on medium inducing expression of the recombinase (Kopke et al., 2010), by anastomosis (Zhang et al., 2013) or by selecting for strains which spontaneously lost the marker due to recombination (Hartl and Seiboth, 2005; Zhang et al., 2013).

Here we demonstrate that flow cytometry can be used to screen spores for the insertion of DNA using fluorescent proteins such as GFP or RFP as a marker. The number of transformations using this method is limited to the number of different fluorescent proteins available that can be distinguished by flow cytometry. Since the method does not rely on antifungal resistance it could potentially be used in fungi where currently no markers for transformation are available. We call this new technique Fluorescence Assisted Selection of Transformants or FAST.

We demonstrate utility of this technique in the plant pathogenic fungus *Fusarium oxysporum*, which is in the top 10 of fungal pathogens in molecular plant pathology (Dean et al., 2012). This method could be adapted to many organisms across kingdoms. The only requirements are stable expression of transgenes and the production of transformed cells resilient to the stress of flow cytometry.

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## 2. Material and methods

### 2.1. Fungal strains

The fungal strain used to demonstrate FAST is *F. oxysporum f sp. lycopersici* (Fol) 4287 (Di Pietro and Roncero, 1996) with an insertion of a *HPH* hygromycin resistance cassette (Punt and van den Hondel, 1992) between the genes *FOXG_14191* and *FOXG_14192* on chromosome 14 (supercontig 22 397592; www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). This insertion was obtained by transformation of 4287 with plasmid p14H (described below). The strains used to determine which markers can be identified in FAST were 4287 transformed with pPK2-HPH-RFP (van der Does et al., 2008) and 4287 transformed with pPK2-HPH-GFP (Michielse et al., 2009). The *CFP* positive strain used for this purpose was the non-pathogenic strain Fo47 (Lemanceau and Alabouvette, 1991) transformed with pPK2-HPH-CFP (Ma et al., 2010).

### 2.2. Preparation of spores for FAST

*F. oxysporum* was grown in minimal liquid medium containing 3% sucrose, 0.17% yeast nitrogen base and 100 mM $KNO_3$ in a shaker (175 rpm) at 25 °C for 5 days. Spores were filtered through two layers of miracloth (Merck; pore size of 22–25 μm), counted and a spore suspension was made with a final density of $2 \times 10^6$ spores per milliliter. *A. tumefaciens* containing a binary vector for transformation to *F. oxysporum*, was grown to an OD of 0.45, then incubated for 6 h in Induction Medium (Mullins et al., 2001) supplemented with 200 mM acetosyringone (AS) and then mixed in a 1:1 ratio with the fungal spore suspension. 100 μl of this mix was then pipetted on a 0.45-μm pore, 45-mm diameter nitrocellulose filter (Whatman) on either Potato Dextro Agar or CM supplemented with AS and grown for 3–5 days. Filters were then transferred to PDA plates containing 200 μg $ml^{-1}$ cefotaxime. After another 3–5 days of incubation spores were scraped off of the filters using liquid minimal medium and transferred to liquid minimal medium. After two to three days of incubation (175 rpm, 25 °C) spores were collected for sorting by filtering through two layers of miracloth (Merck; pore size of 22–25 μm).

### 2.3. Construction of vectors and confirmation of insertion at the desired location

Plasmids p14H and p14HG were made by amplifying 1 kb segments of chromosome 14 from genomic DNA of strain 4287 using FP2900 and FP2901 and FP2902 and FP2903 (all primers used are listed in Supplementary Table S1). The segment amplified with FP2902 and FP2903 was cloned in pRW2h (Houterman et al., 2008) for p14H and pPK2-HPH-GFP (Michielse et al., 2009) using *Pac*I and *Kpn*I. These two vectors share the same backbone but differ in the markers present; pRW2h only contains the *HPH* resistance cassette, while *HPH* is fused to *GFP* in pPK2-HPH-GFP. The resulting plasmids and the fragment amplified with FP2900 and FP2901 were then digested with *Xba*I and *Hind*III and ligated to form p14H and p14HG, respectively. Insertion of either construct at the desired locus was determined using primer pair FP2906 and FP2907, which only gives a PCR product if the construct is not present at the desired locus. Strains negative for this PCR product were further analyzed with FP659 and FP3759 for proper insertion of the left flank and FP745 and FP3761 for the right flank.

Vector pGRBΔ*VIB* was made by amplifying the *Aspergillus nidulans gpd* promotor from pPK2-HPH-GFP using FP5547 and FP5548. This fragment and pHH01-RFP (Supplemental Fig. S2) were then digested using *Pac*I and *Bst*BI and ligated, yielding plasmid GPD-RFP-BLE (pGRB). pGRB and the right flank of *VIB1*, amplified from genomic DNA using FP5323 and FP5324, were digested with *Xba*I and ligated. The resulting plasmid and the left flank amplified using FP5325 and FP5326 were then digested using *Pac*I and ligated to form pGRBΔvib. Primers FP4208 and FP4209 were used to check for presence of the *VIB1* gene in transformants of *F. oxysporum*. In strains lacking the gene, FP659 and FP4206 were used to determine proper insertion of the left flank and FP745 and FP4207 for the right flank. Primers FP659 and FP750 were used to check for ectopic insertions of the left border and FP745 and FP746 were used to check for ectopic insertions of the right border (borders are retained only in case of ectopic insertions).

### 2.4. Flow cytometry

All flow cytometry experiments were performed using a BD Facsaria III.

Spore suspensions were prepared by filtering minimal medium grown cultures through 2 layers of Miracloth (Merck) directly before sorting (described above). Spore suspensions were diluted with MilliQ water and sample line pressure adapted resulting in 20,000 events per second (evt/s – the number of drops per second containing a particle which generates a signal) suitable for sorting with a 70 μm nozzle at a pressure of 70 psi. Front and side scatter area and width were used to exclude the largest cells and those with aberrant profiles to ensure a homogenous starting population.

GFP was excited with a 488 nm blue laser and detected using a 665 nm long pass and 530/30 nm band pass filter. RFP was excited with a 561 nm laser to excite and detected using a 630 nm long pass and 610/20 nm band pass filter. CFP was excited with a 407 nm laser and detected using a 595 nm long pass and 510/50 nm band pass filter. Populations constitutively expressing GFP or RFP were used to set PMT voltage to use the full range of detection and to be able to distinguish GFP and RFP positive populations. Application settings were created and used throughout the experiments to normalize population location on the plots.

Spores positive for the desired markers were gated and sorted onto plates containing PDA supplemented with 100 μg $ml^{-1}$ penicillin and 200 μg $ml^{-1}$ streptomycin in a 4 by 4 grid. Colonies were allowed to grow for 48 h, scraped off the plate and the presence of the fluorescent protein(s) confirmed using an Evos FL inverted microscope (AMG).

## 3. Results

### 3.1. Screening of F. oxysporum transformants using FAST

Transformants were created using an adaptation of the *A. tumefaciens*-mediated transformation (ATMT) protocol for *F. oxysporum* (Mullins et al., 2001). Fungi were coincubated with *A. tumefaciens* on filter pieces on solid medium for 2–3 days, and then transferred to solid medium containing antibiotics to inhibit growth of *A. tumefaciens*. The use of filter pieces limits the growth of *A. tumefaciens*, which was problematic during earlier attempts. Fungal spores were collected by scraping and grown in liquid medium to produce secondary spores for cell sorting, instead of being transferred to selective medium for selection as in the standard protocol. Spores from the liquid culture were obtained by filtering through miracloth and then analyzed using a BD Facsaria III cell sorter. Spores positive for the marker of interest were sorted onto plates after which they were allowed to grow into a colony and further analyzed using microscopy and PCR analysis to confirm the presence of fluorescent markers and determine the site of insertion (targeted or ectopic).