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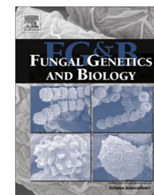

Tools and Techniques

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.

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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 2×10^6 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).

3.2. At least two different fluorescent markers can be used for FAST

The use of fluorescent proteins as markers for transformation requires good separation of the different fluorescent populations in cell sorter plots. We assessed separation of three fluorescent proteins for which an expression cassette was available in *F. oxysporum*. Spores from 4287 expressing *GFP* or *RFP* and spores from Fo47 expressing *CFP* were analyzed both individually and as a mixture of spores with different fluorescent proteins. RFP-positive populations were expected to be easily distinguishable from CFP- and GFP-positive populations as they are excited with a different wavelength laser and their emission spectra do not overlap. When comparing the signal in the RFP detection channel and forward scatter – a measure for size and complexity of cells – the RFP-positive cells are clearly differentiated from the green and cyan cells (Fig. 1A, RFP-positive spores in red). Thus, RFP-positive cells can be selected from a pool of GFP- and CFP-marked spores. GFP-marked spores were also easily distinguishable from CFP- and RFP-populations, with no overlap between GFP-positive and -negative populations (Fig. 1B, GFP-positive spores in green). CFP-positive and -negative populations overlapped (Fig. 1C). When plotting CFP against GFP detection we saw that the intensity of GFP-positive spores in the CFP channel rises with GFP intensity, meaning the brighter GFP-positive cells produce the same signal as CFP-positive cells in the CFP detector (Fig. 1D). This is due to a portion of the signal from the GFP cells being detected in the CFP channel. By measuring which fraction of the GFP signal from a GFP-positive sample is detected in the CFP channel this portion can be calculated and used for fluorescence compensation. After fluorescence compensation, where the fraction of the GFP signal detected in the CFP signal is subtracted from the CFP signal, this linear relationship between the GFP and CFP signal for the GFP positive cells is lost, resulting in better separation of the populations (Fig. 1E). Three populations of cells, each positive for one of the fluorescent proteins are shown in Fig. 1F. Although it is clear that the CFP-positive cells are indeed most strongly fluorescent in the CFP channel there is considerable overlap with the other strains. Spore populations from wild-type strains lacking any fluorescent protein also overlap with the CFP positive population, limiting usefulness of CFP for FAST with our laser and filter setup, which requires the selection of very small populations from a much larger pool of negatives. Taken together, these results suggest that GFP and RFP can be used in the same strain for consecutive manipulations.

3.2.1. GFP as a marker for a targeted transformation

To investigate the feasibility of FAST in *F. oxysporum* a strain already carrying an insertion of a hygromycin resistance cassette was transformed with vector p14HPH-GFP, which carries the same resistance gene fused to *GFP* and flanked by two sequences corresponding to a region on chromosome 14, such that homologous recombination could occur at that site. After transformation cultures were grown for two or three days, spore suspensions were obtained by filtering and a million cells analyzed using flow cytometry.

As expected, the vast majority of cells were negative for GFP as shown in blue in Fig. 2A. A small and distinct population that was positive for GFP was also present (Fig. 2A, green). This population averaged 16 ± 9 per million spores. Individual spores from the GFP-positive population were sorted onto plate to determine fluorescence and the site of insertion of the T-DNA. Twenty-nine individual cultures from 29 individual plates were screened for

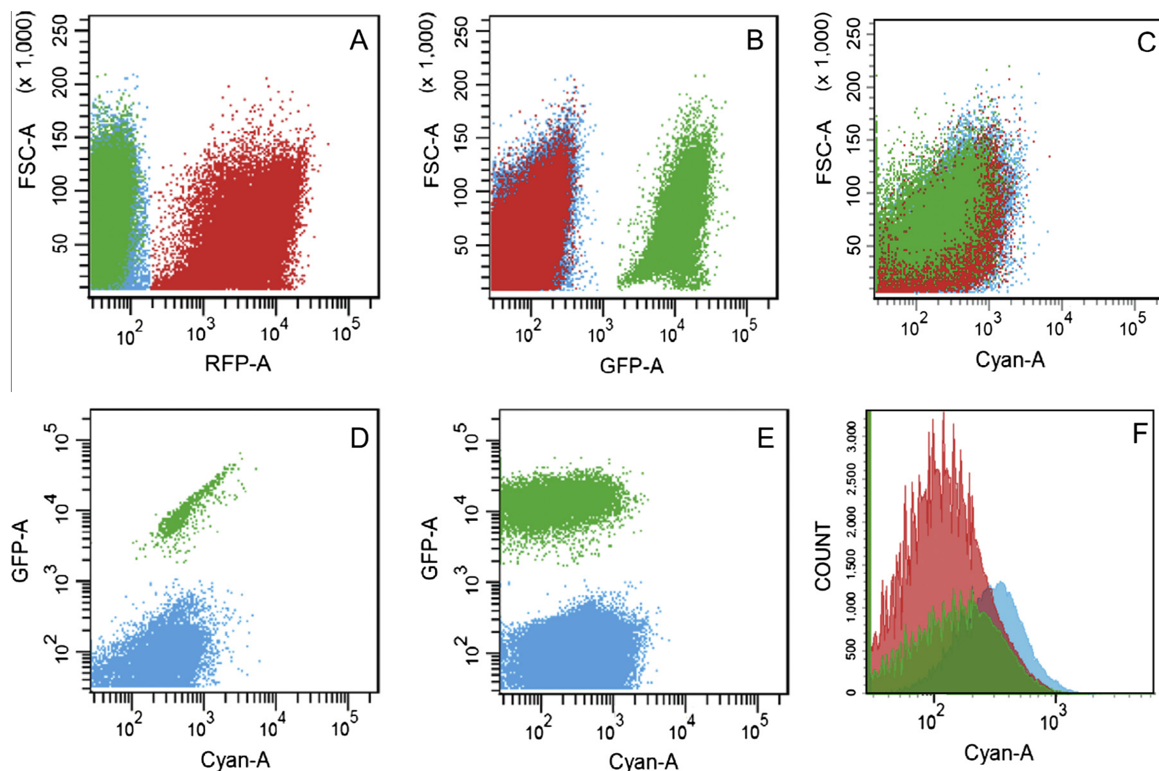

Fig. 1. Separation of populations of fluorescent spores labeled with RFP, GFP or CFP. Colors correspond to the fluorescent proteins detected. Axis labels show detection channel. Units on the axis represent arbitrary units for the area under the curve of the signal detected. This is specific for each instrument and experimental setup as the sensitivity of the detectors can be adjusted so that it matches the range of signal from a particular biological sample. Panels A, B and C show separation when measured in one channel. Panels D and E show separation of GFP- and CFP-positive populations before and after calculating fluorescence compensation. Panel F shows histograms for different fluorescent populations as detected in the Cyan channel.

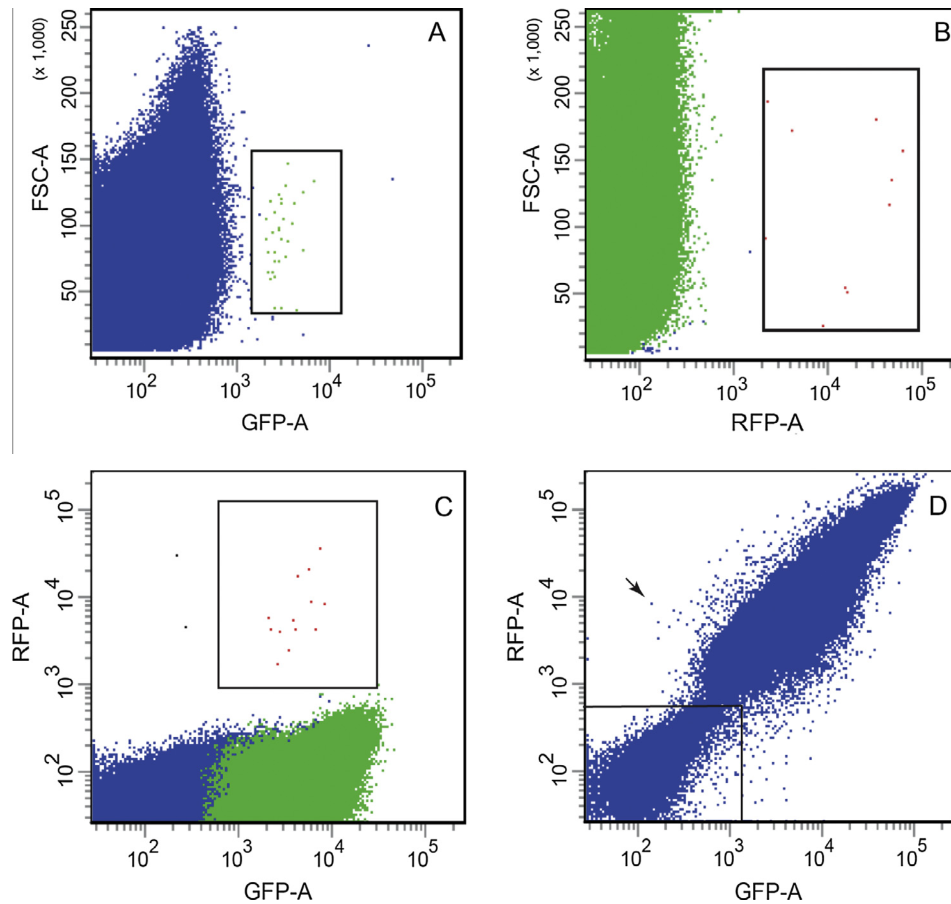

Fig. 2. (A) Non-fluorescent spores transformed with a construct carrying GFP. Cells in the gate (indicated by a square) were selected for further analysis. (B) and (C) A GFP-positive strain, obtained from the transformation shown in panel A, transformed with a construct carrying RFP. Cells positive for both markers were selected for further analysis. (D) A strain obtained from the second transformation, with both GFP and RFP, grown in liquid culture, showing double (GFP- and RFP-) positive cells in blue and debris in the lower left corner (in the square; <1% of total). Arrow indicates spores that have spontaneously lost the marker.

fluorescent spores. From each of the 29 cultures 16 individual spores were transferred to plates using the cell sorter and allowed to grow into a colony for 2–3 days. For each plate three individual colonies were transferred to minimal medium in a 96 wells plate, allowed to grow for one day and then checked for fluorescence using an EVOS inverted microscope. 88% of the colonies were confirmed to be positive for GFP. DNA was isolated from each of the positive colonies and the site of insertion was checked by PCR. Only one of these colonies had the insertion at the target site on chromosome 14 – this strain was called 4287-14HG. A transformation of wild-type *Fol4287* using hygromicin resistance as a selectable marker performed using the same construct did not yield any insertions at the target site, out of around 90 transformants tested (data not shown). A very low frequency of homologous recombination has often been observed when the target site is present on chromosome 14 of *F. oxysporum* strain 4287 (our unpublished observations). These experiments demonstrate that FAST can be used to screen for transformants of *F. oxysporum*.

3.2.2. Screening for insertion of a second fluorescent marker

To investigate the potential of FAST for consecutive transformations, 4287-14HG was transformed again. This strain, carrying p14HPH-GFP, was transformed with pGRBΔ*VIB*. These two constructs share the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promotor and *trpC* terminator from *A. nidulans* but the latter carries *RFP* and a phleomycin resistance gene instead of the *HPH-GFP* fusion gene. In pGRBΔ*VIB*, *RFP* and *BLE* are flanked by two 1 kb fragments corresponding to the flanks of the *F. oxysporum* homolog of the *Neurospora crassa VIB-1* gene, which is involved in vegetative incompatibility (Xiang and Glass, 2004). Homologous recombination in these flanks should result in the replacement of *VIB1* with *BLE* and *RFP*, leading to loss of this gene. FAST experiments were performed in the same way as described above. Cells positive for RFP were sorted onto a plate, checked for fluorescence using fluorescence microscopy and further analyzed using PCR to confirm gene replacement. The same construct was used to do normal ATMT of the same strain using zeocin resistance (conferred by *BLE*) to select for transformants. Co-cultivations of *F. oxysporum* and *A. tumefaciens* for both FAST and ATMT were performed on the same day with the same cultures to directly compare both methods. Forty zeocin resistant colonies were picked from ATMT and 40 plates were used for FAST.

For FAST the population of spores positive for both GFP and RFP as determined by FACS (Fig. 2A and C) was 21.5 ± 24 per million. Two thirds of colonies emerging from spores positive for both markers on the FACS were positive for both markers when checked microscopically. From each plate two colonies were tested for gene replacement of *VIB1* with *BLE* by PCR. From the 31 plates we tested, in thirty-four colonies from 25 different plates gene replacement was successful. This is a relatively high frequency of homologous recombination, but not unusual for *F. oxysporum*. Re-culturing of these strains showed the bulk of the population positive for both markers and, as always observed, some debris (not fluorescent in either channel), indicating stable expression of both markers (Fig. 2D). In some cases colonies were positive for RFP but negative for GFP. For six out of seven tested, we confirmed replacement of

the *GFP* gene by the *RFP* gene by PCR. Reculturing these strains and analyzing them by flow cytometry confirmed stable expression of both markers (Fig. 2F). There was a small population that had apparently lost the GFP protein – PCR analysis of several colonies emerging from these spores showed that *GFP* was indeed missing but not *SIX1* and *SIX6*, both of which are genes for small secreted proteins located on the same chromosome (Ma et al., 2010). This indicates that spontaneous loss of GFP occurs, albeit at very low frequency.

Out of the 31 transformants generated in the same experiments using phleomycin resistance to select transformants there were 24 successful gene replacements for *VIB1*.

4. Discussion

Here we demonstrate that it is possible to screen for transformants of the fungus *F. oxysporum* based on the introduction of genes for fluorescent proteins, without the need to introduce genes conferring drug resistance. We show this can be achieved both ectopically and targeted at specific sites. We also show that FAST can be used to select transformants of a strain that already contains a fluorescent marker.

FAST expands the repertoire of available markers for transforming filamentous fungi, and potentially, organisms as diverse as bacteria, plant cells and human cell lines, provided separate transformed cells can be produced and sorted. The extra transformation markers provided by FAST can be used as a marker for creating gene deletion mutants or for adding genes which is particularly useful when researching gene families, biosynthetic pathways or duplications where redundancy often means single deletion mutations have no effect. Using flow cytometry it is also possible to select against the presence of a fluorescent marker so each marker is also a negative marker. It is possible to distinguish RFP and GFP that are widely used. In our set-up Cyan fluorescence was also detectable but not sufficiently strong and distinct to be practical for selection of transformants. There are no special requirements for the vectors used beyond the gene for a fluorescent protein and efficient uptake by the target organism.

Screening transformants directly for GFP has been performed in plants such as sugarcane and wheat (Elliott et al., 1998; Jordan, 2000). Screening in these cases was performed by microscopic examination of candidates, made possible by a very high frequency of 1–5% of embryos that were transformed and the macroscopic nature of plants. The observed frequency for *F. oxysporum* was around 0.003% of spores co-incubated with *Agrobacterium*. This low frequency precludes visual inspection as a viable option for screening. Using flow cytometry it is possible to screen up to 20,000 cells per second, allowing quick selection of transformants even with very low transformation efficiency. Potentially multicellular spores or compartments could also be used as long as they do not exceed the maximum particle size for the cell sorter used.

Alternative techniques currently available to enhance the number of repetitive transformations of a single strain all rely on recombination, which is often laborious and time consuming. They also require vectors carrying specified recombinases and recombination sites, which entail limits on possible cloning strategies. Still, leaving no markers at all by excising a marker through recombination is generally more desirable, especially for commercial or environmental applications (Watson and Wang, 2012). On the other hand, leaving *GFP* in a strain is certainly more desirable than drug resistance genes since unwanted growth can still be controlled using anti-fungal drugs. Since flow cytometry can also be used to select against markers it is also possible to select cells that have spontaneously lost the marker gene. Our results also show that FAST can be used to swap cassettes at a target site.

FAST does have certain limitations. Due to the high costs of maintaining and operating a FACS machine and the expertise required these are not accessible to everyone. Further, a limited number of fluorescent markers is available which could be used for this technique. Other than the three already discussed here a Far Red fluorescent protein like mPLUM could be suitable (Wang et al., 2004). A brighter blue fluorescent protein such as eBFP2 (Ai et al., 2007) would likely be more easily distinguishable than CFP used in this paper. Adding even more markers is not feasible at the moment due to overlap in excitation and emission spectra for the different fluorescent proteins with the lasers and filters available for cell sorting. Despite these restrictions, we propose that FAST is a worthwhile addition to the technology available to transform filamentous fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.02.003.

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