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Toolkit for Visualization of the Cellular Structure and Organelles in *Aspergillus niger*

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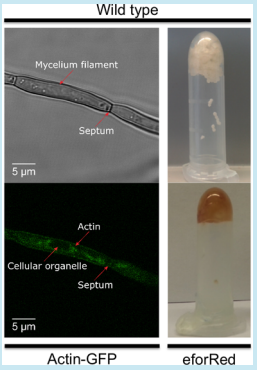
ABSTRACT: *Aspergillus niger* is a filamentous fungus that is extensively used in industrial fermentations for protein expression and the production of organic acids. Inherent biosynthetic capabilities, such as the capacity to secrete these biomolecules in high amounts, make *A. niger* an attractive production host. Although *A. niger* is renowned for this ability, the knowledge of the molecular components that underlie its production capacity, intercellular trafficking processes and secretion mechanisms is far from complete. Here, we introduce a standardized set of tools, consisting of an N-terminal GFP-actin fusion and codon optimized eforRed chromoprotein. Expression of the GFP-actin construct facilitates visualization of the actin filaments of the cytoskeleton, whereas expression of the chromoprotein construct results in a clearly distinguishable red phenotype. These experimentally validated constructs constitute the first set of standardized *A. niger* biomarkers, which can be used to study morphology, intercellular trafficking, and secretion phenomena.



Aspergillus niger is a well-established industrial host that is exploited for the production of organic acids and hydrolytic enzymes, of which citric acid and glucoamylase are well-known examples.[1] Its capacity to secrete these products in high amounts results in titers that exceed those of many contemporary production hosts. Nonetheless, the underlying secretion and intercellular trafficking mechanisms of *A. niger* are poorly understood and standardized biological tools and research techniques may contribute to the elucidation of the mechanisms of these basic biological processes.

In *A. niger* secretion occurs at the hyphal tips of the mycelium.[2] The Spitzenkörper is located here and acts as the organizing center, determining directionality of growth and serving as a supply center for exocytic vesicles. Intercellular transport between adjacent cells occurs via the septum, a dynamically regulated contractile ring that allows flow-through of nutrients and organelles. This process is called cytosolic streaming and occurs along actin filaments in the cytoskeleton of the cell.[3]

Methods for organelle visualization are essential to the study of these processes. To accommodate such studies, we introduce a GFP-actin fusion protein to expose actin filaments. However, the use of fluorescent proteins (FPs), although extensively used in cellular localization studies, is not suitable for simultaneous multiplex organelle visualization, since different excitation and emission wavelengths are required for different FPs. Moreover, the occurrence of spectral overlap renders the use of fluorescent proteins impractical when multiplexing visualization strategies.

In order to further expedite the investigation of secretion and organelle trafficking, we tailored a chromoprotein to suit the chassis of *A. niger*. Chromoproteins originate from corals and although they are related to the GFP family, they have evolved a unique chromophore.[4] Unlike fluorescent proteins, chromoproteins are visible to the naked eye. They undergo a conformational change that alters their color, thereby eliminating the occurrence of bleaching as well as spectral overlap, which can otherwise lead to signal crossover during microscopic imaging.[5]

The aim of this work was to establish a toolkit for the study of intercellular trafficking and secretion systems, consisting of a GFP-actin fusion and a mitochondrial targeted chromoprotein that allow for visualization of the cytoskeleton and organelles.

■ RESULTS AND DISCUSSION

The GFP-actin fusion protein was visible under the fluorescence microscope and was observed to encapsulate organelles (Figure 1A), which is in accordance with previous studies that have shown colocalization of organelles and actin.

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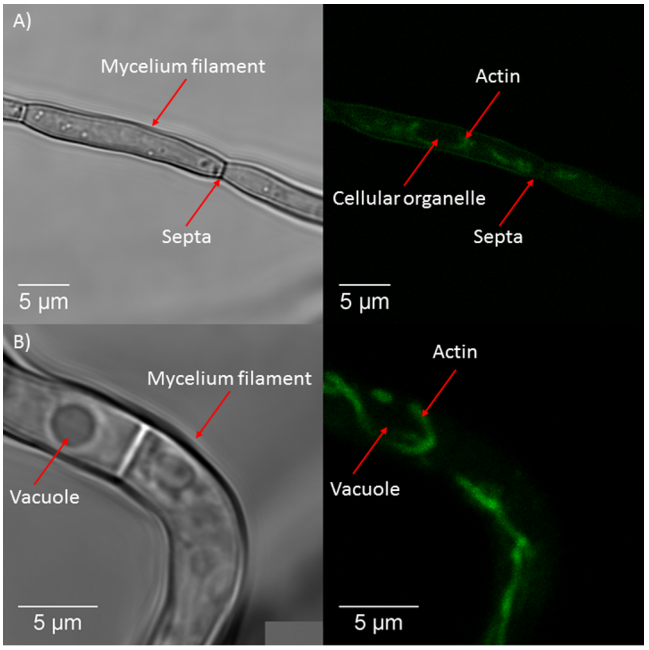

Figure 1. *A. niger* mycelium in phase contrast microscopy (left panel) showing a mycelium filament. On the right-hand side labeled GFP-actin is visualized by fluorescence microscopy. The actin filaments are detectable by the presence of the GFP labeled actin. Red arrows indicate the visible structures. In A the upper part of the image the actin seems to encapsulate an organelle. In B the GFP labeled actin can be seen encapsulating a vacuole. In both parts of the image the scale bar represents 5 μm.

It was also visible at some of the septa (Figure 1B), although with low intensity and therefore continuous actin filaments that facilitate transport of nutrients and organelles via cytosolic streaming and the Spitzenkörper could not be visualized. This may be explained by the fact that the native actin gene was still present; only a fraction of the actin was GFP-labeled. A recommendation for future work is lowering the expression of the native actin gene or replacing it with the N-terminal GFP-actin construct under control of a tunable promoter. A previous attempt to replace the native actin gene with a C-terminal actin-GFP fusion construct in *Aspergillus nidulans* was unsuccessful.[6] Other interesting studies would include tagging of actin-binding proteins, such as myosin, which has been hypothesized to direct actin polymerization and drive endocytosis at the tip.[6]

The original eforRed chromoprotein was obtained from the 2012 team IGEM2012_Uppsala_University (BBa_K592012).[7] Transformation of *A. niger* with this construct downstream of the native *pkiA* promoter did not result in an observable red phenotype, which might be explained by the fact that the chromoprotein was codon optimized for *E. coli* (ECO). Therefore, we designed an *A. niger* codon optimized eforRed chromoprotein construct (ACO). As our main interest was organelle visualization, we included a mitochondrial localization sequence (MLS-ACO). After plating the transformants, initially no change in mycelial color was observed compared to the wild type strain. However, when grown in liquid medium a clear red color could be observed in comparison to the wild type (see Figure 2). The fact that red colored mycelium was only observed in liquid medium might be related to morphological differences arising specific culture conditions. More replicate experiments with different chromoproteins may shed light on this discrepancy. Follow-up microscopic imaging experiments have to be done to determine whether the proteins are in fact effectively targeted to the mitochondria.

The availability of simple and standardized biomarkers, such as GFP fusions and chromoproteins, facilitates experimental set-ups for a variety of different research topics, including

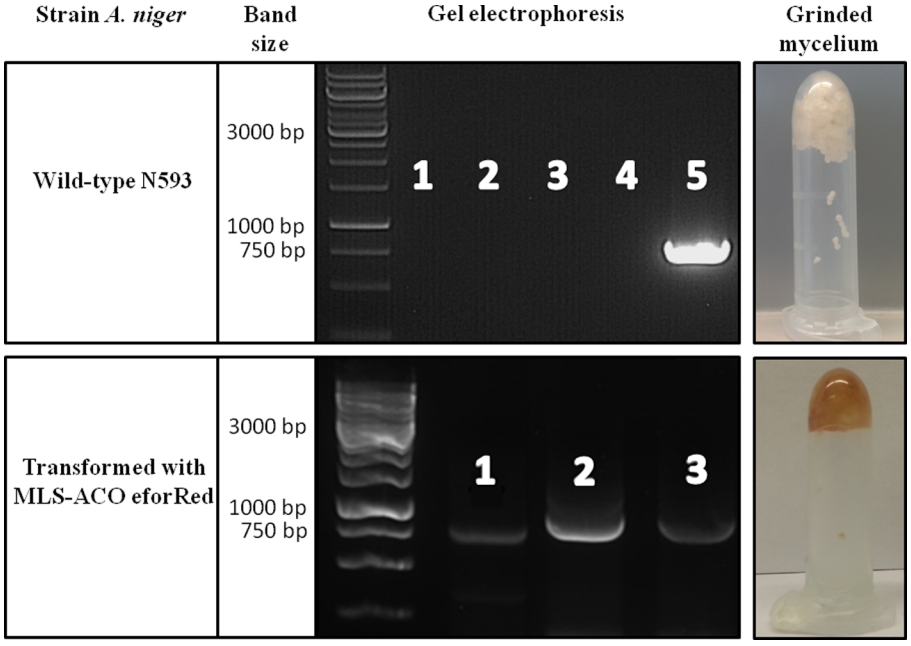

Figure 2. Mycelium, when grown in liquid medium, of *A. niger* transformed with the MLS-ACO eforRed construct displays a clearly discernible red color compared to the mycelium of WT strain. Presence of the MLS-ACO eforRed construct (expected size 732 bp) in transformed *A. niger* was confirmed via PCR (lane 1−3; bottom gel picture). In contrast, no presence of the MLS-ACO eforRed construct was confirmed in wild type *A. niger* (lane 1−3; top gel picture). Positive control (lane 5 top; lane 3 bottom) was the PCR product of a plasmid containing the MLS-ACO eforRed construct (size 732 bp).

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promoter identification and validation, neutrally marked strains for competition and fitness experiments, multiplex organelle visualization, without the risk of spectral overlap, for insight in organelle distribution and dynamics, qualitative testing of gene expression, and most specifically, the analysis of processes underlying intercellular trafficking and secretion.

In conclusion, our research shows that the GFP-actin fusion protein and the eforRed chromoprotein are expressed in *A. niger*. A *pkiA* promoter and *xlnD* terminator have been shown to be functional in combination with the chromoprotein coding genes and complement the toolkit. Documentation on all four aforementioned parts can be found in the Registry of Standard Biological Parts (Bba_K1023001 (*pkiA*), Bba_K1023002 (*xlnD*), Bba_K1023006 (GFP-actin), and Bba_K1023017 (MLS-ACO eforRed).

MATERIALS AND METHODS

Parts Acquisition. *GFP-Actin.* Genomic DNA from *A. niger* N593, a *pyrA* mutant of *A.niger* CBS102.49 was isolated to serve as a template. Primers (LS_NsiI_actin_for: (5′-ATGCATATGGAAGGTAAGGGCTTCCTGC-3′), LS_NotI_actin_rev: (5′-GCGGCCGCTTAGAAGCACTTGCGGTGGACG-3′) were used to amplify the actin gene (An15g00560) by PCR with Phusion High-Fidelity DNA polymerase (New England Biolabs, Leiden, The Netherlands). Optimization of the PCR yielded an optimal annealing temperature of 65 °C and 0.4 mM Mg^{2+} was added as a cofactor for the DNA-polymerase. The primers introduced 5′ overhang extensions that included the *NotI* and *NsiI* restriction sites, for cloning into the Funbrick vector containing a *xlnD* promoter and terminator[9] (L. van der Straat and L.H. de Graaff, personal communication) and the standard BioBrick restriction sites. The amplified product was gel purified using the QIAquick Gel Extraction Kit (QIAgen, Venlo, The Netherlands) and ligated into pJET (CloneJET PCR Cloning Kit, Thermo Scientific, Landsmeer, The Netherlands) with the use of T4 DNA ligase (New England Biolabs, Leiden, The Netherlands). Subsequently *Escherichia coli* DH5α was transformed with the recombinant plasmid via electroporation and grown in lysogeny broth (LB, 10 g/L NaCl, 10 g/L Bacto tryptone, 5 g/L yeast extract) medium with ampicillin (100 μg/mL). Successful cloning was confirmed by plasmid isolation using the GeneJet Plasmid Miniprep Kit (Thermo Scientific, Landsmeer, The Netherlands) and restriction analysis (Figure 2).

MLS-ACO eforRed. Plasmids containing *Escherichia coli* codon-optimized (ECO) chromoprotein coding genes were obtained from the iGEM Uppsala 2012 team (Bba_K592009, Bba_E1010) and the iGEM Braunschweig 2013 team (Bba_K1073020 - Bba_K1073027). An improved *A. niger* codon-optimized eforRed chromoprotein coding construct (Bba_K1023017) was designed that had a 5′ end mitochondrial localization sequence. The construct has been codon-optimized according to a codon usage table obtained from IDT (Coralville, Iowa, U.S.A.) taking into account the standard 10 restriction sites. It was then synthesized by IDT, resulting in two 500 bp double-stranded DNA sequence blocks. Using the Gibson Assembly Cloning Kit (New England Biolabs), we obtained a pJET plasmid that had the synthetic eforRed coding part inserted. PCR was performed to amplify the ECO chromoprotein coding parts and the synthetic eforRed coding part using the Phusion High-Fidelity DNA polymerase (New England Biolabs). Primers were designed that provided the parts with five prime overhang extensions. These overhangs include specific restriction sites that had to be cleaved for further assembly of the parts into our *A. niger* vector (*NotI* and *NsiI*) and into the standard BioBrick vector according to the RFC 10 BioBrick assembly method. Our *A. niger* vector contained a *pkiA* promoter[9] (part Bba_K1023001) that induces expression of downstream genes in *A. niger* and an *xlnD* terminator (part Bba_K1023002). An ampicillin resistance gene allowed for selection of *E. coli* transformants. An orotidine-5′-phosphate-decarboxylase (*pyrA*) gene,[8] which selects for uridine prototrophy, was used as a selection marker for uridine auxotrophic *A. niger* N593 strain transformants. Backbone vectors and part amplicons were restriction digested; resulting fragments were separated via gel electrophoresis and extracted using the QIAquick Gel Extraction Kit (QIAgen). Subsequent ligation, using T4 DNA ligase (New England Biolabs) then resulted in assembly of the recombinant plasmids.

Transformation and Expression in *A. niger*. *GFP-Actin.* *A. niger* N593 protoplasts were transformed by the Funbrick vector containing the gene encoding the GFP-actin fusion, plated on selective minimal medium salts (MMS) plates (6.0 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4$.7 H_2O, 1 mL/L Vishniac, 325.2 g/L sucrose, 1.2% (w/v) agar, pH 6.0) and incubated for 4 days at 30 °C. Colonies were streaked on selective plates containing minimal medium (6.0 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4$.7 H_2O, 1 mL/L Vishniac, 50 mM sucrose, 0.2% casamino acids, 0.1% yeast extract, 15 g/L agar, pH 6.0) to obtain single *A. niger* colonies from which spores were collected. These were grown on plates with complete medium (6.0 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4$.7 H_2O, 2 g/L meat peptone, 1 g/L yeast extract, 1 g/L casamino acids, 0.3 g/L yeast ribonucleic acids, 1 mL/L Vishniac, 2 mL/L vitamins solution, 50 mM sucrose, 15 g/L agar, pH 6.0) for 4 days at 30 °C after which spores were collected. Samples of *A. niger* mycelia used for fluorescence microscopy were made by inoculating 350 spores per chamber in 200 μL minimal medium containing 30 mM sorbitol using chambered glass slides. The fungal strains were grown overnight at 30 °C. To induce the *xlnD* promoter,[10] 10 mM xylose was added and incubated for 1 h at 30 °C. Images were acquired with a Leica TCS SP5X confocal microscope using a Leica HCX PL Apo 63×/1.2 water objective. Data were analyzed using ImageJ software.

MLS-ACO eforRed. *E. coli* DH5α electrocompetent cells were transformed using our plasmids in electroporation. Lysogeny broth (LB) agar plates, with either kanamycin, chloramphenicol, and ampicillin depending on the plasmid, were then inoculated with these cells and were incubated overnight at 37 °C. Colony-PCR with DreamTaq DNA polymerase (Thermo Scientific) was performed to identify colonies harboring recombinant plasmid. The transformant colonies were inoculated in liquid LB medium, and plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit (Thermo Scientific). *A. niger* N593 protoplasts were transformed with plasmids containing the synthetic eforRed construct and were plated on MMS plates. Transformants were selected for by their uridine independence and spores were harvested. Complete medium was inoculated with these spores and subsequently grown for 3 days at 30 °C on MMS plates again. Fungal genomic DNA was extracted and transformants were validated by PCR with primers that annealed to the *pkiA* 5′ end and to the *xlnD* 5′ end. All

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constructs were sequence−sequence verified (GATC Biotech, Constance, Germany).

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Author Contributions

⊥E.tB., M.K., and M.L. contributed equally. E.tB., J.H., and M.L. conceived the study and collected and interpreted data. E.tB. and M.K. participated in the preparation of the manuscript. S.C., Y.D., J.M.V., V.W., T.J.B.G., R.G.A.vH., D.I.O., T.S., L.vdS., L.H.dG., and M.W.J.vP. helped interpret data and helped preparing the manuscript. T.J.B.G., R.G.A.vH., D.I.O., T.S., L.vdS., L.H.dG., and M.W.J.vP. supervised the research.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GFP, green fluorescent protein; CDS, coding sequence; WT, wild type; ECO, *Escherichia coli* codon optimized; ACO, *Aspergillus niger* codon optimized; MLS, mitochondrial localization sequence; MMS, minimal medium salts; FP, fluorescent protein

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