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Standardization of Functional Reporter and Antibiotic Resistance Cassettes to Facilitate the Genetic Engineering of Filamentous Fungi

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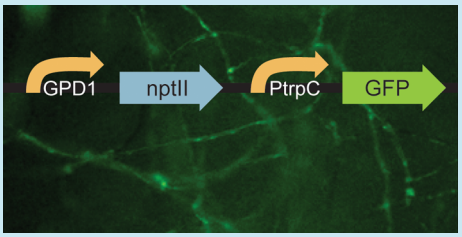
ABSTRACT: The unique physiological properties of fungi are useful for a myriad of applications, which could greatly benefit from increased control of native pathways and introduction of recombinant genes. However, fungal genetic engineering is still limited in scope and accessibility, largely due to lack of standardization. To help standardize the genetic engineering of filamentous fungi, we created BioBricks of commonly used antibiotic resistance genes, neomycin phosphotransferase (*nptII*) and hygromycin phosphotransferase (*hph*), which confer resistance to G418 (Geneticin) and hygromycin B, respectively. Additionally, we created a BioBrick of the constitutive *trpC* promoter, from the tryptophan biosynthesis pathway of *Aspergillus nidulans*, and used it to create a composite part including the *GFP* gene. The functionality of these parts was demonstrated in the model fungal organism *Cochliobolus heterostrophus*, and as these tools are in modular BioBrick format, they can be easily used to facilitate genetic engineering of other fungal species.



Filamentous fungi have been used traditionally for the production of various cheeses and other fermented foods.[1] In recent years, more researchers and corporations have recognized these fungi for their hyphal structure and useful secondary metabolites. In particular, higher order fungi have shown potential for sustainable biomaterials development.[2] An advantage of using fungi as biomaterials is that the structure of the hyphal cell wall, primarily composed of glucose polymers and the rigid polymer chitin, forms light yet durable materials.[2] Using synthetic biology, researchers are able to utilize the diverse properties of fungi and manipulate them for a variety of scientific and industrial purposes. The first step toward accomplishing such goals is to standardize basic molecular genetic tools, such as effective antibiotic resistance constructs and functional reporters.

One aim of this study was to standardize constructs of commonly used resistance genes, neomycin phosphotransferase (*nptII*) and hygromycin B phosphotransferase (*hph*) from *Escherichia coli*, which provide resistance to the antibiotics G418 and hygromycin B respectively.[3,4] The efficacy of these resistance genes was demonstrated with the model ascomycete *C. heterostrophus*.

Identification of gene regulatory mechanisms that are effective across evolutionarily distant fungi is critical for the standardization of fungal genetic engineering. Herein, we describe the standardization of the *trpC* promoter, from the tryptophan biosynthesis pathway of the ascomycete *A. nidulans*, which has been shown to effectively regulate transcription in a wide array of filamentous fungi.[4,5] A standard genetic construct was created for use in determining the efficacy of the *trpC* promoter within any fungal chassis, which can help streamline research on novel fungal organisms.

The entirety of this project was conceptualized and conducted by Cornell University undergraduates participating in the 2013 International Genetically Engineered Machine (iGEM) competition. The genetic constructs created in this study are all available in the Registry of Standard Biological Parts (http://parts.igem.org).

RESULTS AND DISCUSSION

The coding sequences for *nptII* and *hph* were isolated from pNG and pUCATPH, respectively, and mutagenized using PCR to remove internal restriction sites and comply with the RFC[10] assembly standard.[6] Silent mutations were introduced to remove a *Pst*I site from *nptII* and *Eco*RI and *Pst*I sites from *hph*. Each coding sequence was then cloned into the modular BioBrick vector, pSB1C3, and submitted to the Registry of Standard Biological Parts (pSB1C3−K1021001, pSB1C3−K1021002). These parts, being modular, can be easily combined with other BioBricks and used in research on different fungal organisms. Both pHPVEL1 and pNG were incorporated into *C. heterostrophus* and shown to confer the respective antibiotic resistances (Figure 1).

The *trpC* promoter was amplified from pUCATPH and inserted into pSB1C3 to make pSB1C3−K1021007. The composite part BBa_K1021023 was then constructed by placing the *GFP* reporter, BBa_K082003, under the control of the *trpC* promoter, BBa_K1021007. pNG-K1021023 was made by inserting the composite part BBa_K1021023 into

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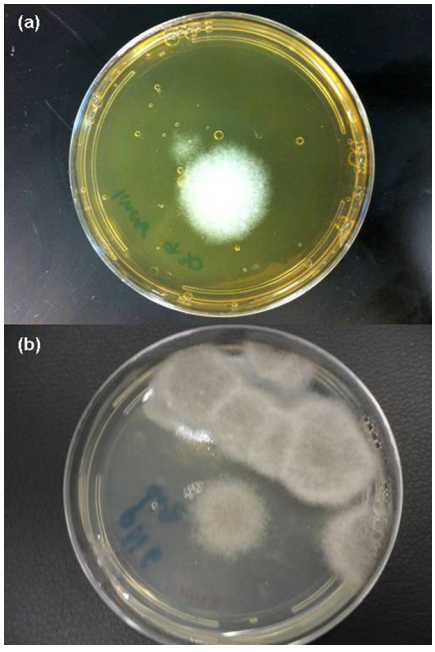

Figure 1. (a) Transformants carrying pHPVEL1 plated on hygromycin B. (b) Transformants carrying pNG plated on G418. Negative control strain of *C. heterostrophus* showed no growth in the presence of either G418 or hygromycin B.

pNG, adjacent to the G418 resistance cassette, containing the *C. heterostrophus GPD1* promoter (Figure 2). The native promoter driving the resistance gene facilitates transformation via homologous recombination into the *C. heterostrophus* genome.

C. heterostrophus was transformed with both pNG and pNG-K1021023. After 1 week of growth, *C. heterostrophus* cells transformed with pNG-K1021023 were resistant to G418 and displayed visible GFP fluorescence whereas the control strain transformed with only the pNG vector was resistant to the drug, but cells did not fluoresce (Figure 3). This suggests that

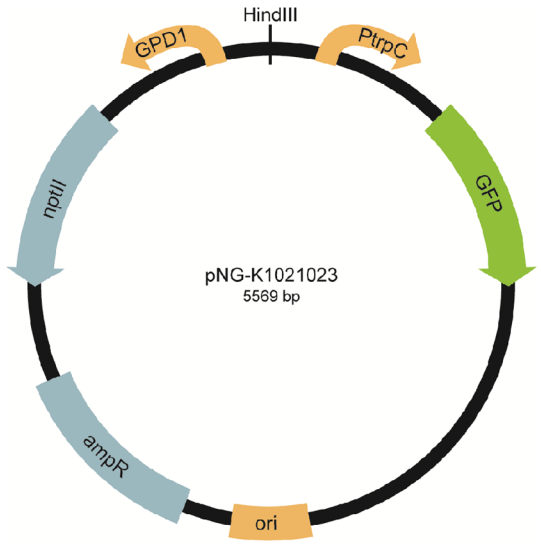

Figure 2. *GFP* driven by the constitutive *trpC* promoter from *A. nidulans* (BBa_K1020123) was inserted in the pNG vector adjacent to the *C. heterostrophus GPD1* promoter in the G418 resistance cassette to make pNG-K1021023.

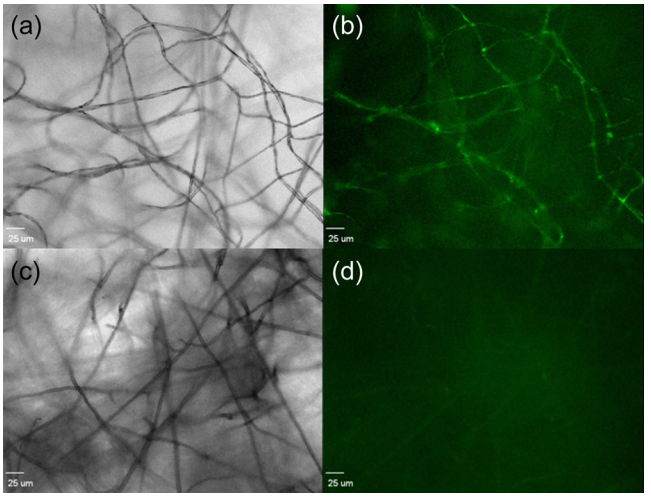

Figure 3. *C. heterostrophus* was transformed independently with pNG-K1021023 and with pNG. Only the former resulted in fluorescence under 395 nm excitation wavelength. Images were taken using the Olympus BX61 microscope and Slidebook 5.0 software. (a) Bright field image of pNG-K1021023 transformation. (b) Fluorescence image of pNG-K1021023 transformation. (c) Bright field image of pNG transformation. (d) Fluorescence image of pNG transformation.

pNG-K1021023 is functional in *C. heterostrophus* and that BBa_K1021023 is effective as a fungal reporter. This BioBrick, when assembled with a resistance cassette, can be used as a test sequence for future researchers to determine the activity of the promising *trpC* promoter within their respective fungal species. Some potential obstacles for researchers may include isolating viable fungal protoplasts from other fungal species and optimizing transformation efficiency. Even with our relatively well-characterized strain of *C. heterostrophus*, developing a precise and reliable methodology for fungal transformation proved to be a time-consuming and tedious process.

METHODS

Strains and Plasmids. *C. heterostrophus* strain C5 was cultured in complete medium, as described in Turgeon et al. (2010).[9] *E. coli* DH5α was used for standard cloning procedures and cultured in LB medium.[7] Engineered strains were maintained on solid LB agar.

The plasmids pNG, pHPVEL1, and pUCATPH containing the genes *nptII*, *hph*, and the *trpC* promoter, respectively, were used as starting materials for new constructions.[5,8] The high copy number vector pSB1C3 and the BioBrick plasmid pSB1C3-K082003 were obtained from the Registry of Standard Biological Parts. The parts pSB1C3-K1021001, pSB1C3-K1021002, pSB1C3-K1021007, and pSB1C3-K1021023 created in this study were submitted to the Registry of Standard Biological Parts and are compatible with the RFC[10] assembly standard.[6]

Protoplasting and Transformation of *Cochliobolus heterostrophus*: Protoplasts were created and transformed as described in Turgeon et al. (2010). Fungal cell walls were enzymatically digested using Driselase (Sigma-Aldrich D9515) and Glucanex (provided by B. G. Turgeon) to create protoplasts.[9] Prior to transformation, the constructs were linearized using a *Hin*dIII restriction site external to the genes of interest to increase the rate of recombination.[9] Protoplasts were then incubated with recombinant DNA and polyethylene glycol (PEG), allowing the DNA to enter the cells and integrate

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Table 1. Plasmids Used in This Study and Their Respective Origins[a]

plasmid name	gene of interest	source
pNG	*nptII*	B. G. Turgeon[4]
pHPVEL1	*hph*	B. G. Turgeon[4]
pUCATPH	*PtrpC, hph*	B. G. Turgeon[8]
pSB1C3	high copy vector	iGEM Registry
pSB1C3-K082003	*GFP*	iGEM Registry
pSB1C3-K1021001	*nptII* BioBrick	this study
pSB1C3-K1021002	*hph* BioBrick	this study
pSB1C3-K1021007	*PtrpC* BioBrick	this study
pSB1C3-K1021023	*PtrpC+GFP* BioBrick	this study
pNG-K1021023	*PtrpC+GFP* with G418 resistance cassette	this study

[a]The right side of the hyphenated plasmid name (e.g., K1021001) corresponds to the respective BioBrick name in the Registry of Standard Biological Parts (http://parts.igem.org).

into the genome via homologous recombination.[10] Candidate transformed cells were grown under the appropriate selective pressure at 30 °C for approximately a week until distinct colonies of successful transformants were visible (Figure 1).

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Author Contributions

S.S. conceived and developed the project idea with input from G.T., D.W., A.C., E.H., O.S., and N.B. S.S., A.C., E.H., O.S., and N.B. designed and performed experiments. S.S., A.C., and E.H. wrote the manuscript and D.W. and G.T. supervised all fungal experimentation.

Notes

The authors declare no competing financial interest.

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