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A light-switchable bidirectional expression system in filamentous fungus *Trichoderma reesei*

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ABSTRACT

The filamentous fungi *Trichoderma reesei* is widely used in the production of cellulolytic enzymes and recombinant proteins. However, only moderate success has been achieved in expressing heterologous proteins in *T. reesei*. Light-dependent control of DNA transcription, and protein expression have been demonstrated in bacteria, fungi, and mammalian cells. In this study, light inducible transactivators, a "light-on" bidirectional promoter and a "light-off" promoter were constructed successfully in *T. reesei* for the first time. Our light inducible transactivators can homodimerize and bind to the upstream region of artificial promoters to activate or repress genes transcription. Additionally, we upgraded the light-inducible system to on-off system that can simultaneously control the expression of multiple heterologous proteins in *T. reesei*. Moreover, a native cellulase-free background for the expression of heterologous proteins was achieved by knocking out the genes involved in transcriptional regulation and encoding of cellulases: *xyr1*, *cbh1*, and *cbh2*. Our light-switchable system showed a very little background protein expression and robust activation in the blue light with significantly improved heterologous protein expression. We demonstrate that our light-switchable system has a potential application as an on/off "switch" that can simultaneously regulate the expression of multiple genes in *T. reesei* under native cellulase-free background.

1. Introduction

The hypersecreting *Trichoderma reesei* (Teleomorph *Hypocrea jecorina*) is an important industrial workhorse for large-scale production of proteins, mainly cellulolytic enzymes (Merino and Cherry, 2007). Studies have demonstrated that secretion of native proteins in *T. reesei* could reach up to 100 g/L on optimal culture conditions, where the pool of secreted proteins is primarily dominated by cellulases (Kubicek et al., 2009; Schuster and Schmoll, 2010). Since the production of calf chymosin, expression of different heterologous proteins has been carried out in *T. reesei* for several decades, but with only moderate success (Harkki et al., 1989; Peterson and Nevalainen, 2012). Despite optimizations, the yield of heterologous proteins in *T. reesei* in controlled environment is considerably lower as compared to that of native proteins.

The excessive expression of native cellulases in *T. reesei* not only hampers the expression of heterologous proteins, but also makes purification and characterization of heterologous proteins extremely difficult from the native proteins (Singh et al., 2015). Deletion of the major transcriptional activator of cellulase, *xyr1* (xylanase regulator 1), is an alternative for achieving efficient heterologous protein expression by creating a relatively cellulase-free environment (Uzbas et al., 2012). The choice of promoter is a restrictive factor for heterologous protein expression. Some heterologous proteins have been successfully expressed in *T. reesei* by using cellulose-inducible promoters such as *cbh1*, *cbh2*, *xyn2*, and *egl2* (Geng et al., 2012; Meng et al., 2013; Miyauchi et al., 2013, 2014). However, irrelevant proteins are also induced with cellulose-inducible promoters, which contaminate the target proteins thereby increasing the difficulty in separation and characterization of heterologous protein. Constitutive promoters, that do not require inducers, effectively decrease the accumulation of irrelevant proteins (Li et al., 2012). Although the level of protein expression with constitutive promoters is often lower than that with a strong cellulose promoter, purification of target protein is often thought to be more attractive than working with a high irrelevant proteins background. However, one of the major challenges in utilizing constitutive promoters is that it cannot be selectively used to switch "on" or "off" the expression of the target gene.

Recently, light-dependent control of DNA transcription, and protein expression in bacteria, fungi, and mammalian cells have

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been demonstrated by using light-responsive transcription factors (Ohlendorf et al., 2012; Hughes et al., 2012; Wang et al., 2012, 2014a). In contrast to chemicals, light is an ideal inducer of gene expression as it is easy to obtain, highly tunable, easily delivered and, most importantly, can be instantly removed. Previously, we developed a light-mediated system based on synthetic blue light inducible transactivators (Wang et al., 2014a). Therefore, we attempted to construct a similar system in *T. reesei*. In this work, we upgraded the previously developed light-switchable system to an "on-off" system for regulating the simultaneous expression of multiple proteins in *T. reesei*. Here, we demonstrate that the light-switchable system has a potential application in controlling heterologous gene expression in filamentous fungus *T. reesei*, in a cellulase-free background.

2. Materials and methods

2.1. Strains and media

T. reesei RUT-C30 (ATCC 56765), a hypersecreting and catabolite-derepressed mutant (Peterson and Nevalainen, 2012), was used as host. *Escherichia coli* DH5α was used as the host strain for recombinant DNA manipulations. Luria-Bertani (LB) medium was used for *E. coli* and *Agrobacterium tumefaciens* culture. Basal fermentation medium (BFM) (10 to 40 g/L carbon source, 10 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L polypeptone, 0.5 g/L yeast extract, 1 g/L Tween 80, 10 mg/L $FeSO_4{\cdot}7H_2O$, 3.2 mg/L $MnSO_4{\cdot}4H_2O$, 2.8 mg/L $ZnSO_4{\cdot}7H_2O$ and 20 mg/L $CoCl_2{\cdot}6H_2O$) was used for fungal cultures. For analysis of the biomass in the presence of blue light, cultures were kept in constant light for 24 h. The mycelia were harvested using pre-weighed glass microfiber filters (Cat. No. 1822-055, Whatman, Kent, UK), washed with deionized water, dried at 80 °C for two days and then analyzed.

2.2. RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

Approximately 100 mg of *T. reesei* mycelium was harvested. Total RNA was extracted using a FastRNA Pro Red Kit (MPbio, Irvine, CA, USA), according to the manufacturer's instructions. Reverse transcription was performed with 1000 ng of total RNA, using TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen, Beijing, China), according to the manufacturer's instructions. For RT-qPCR, the TransStart TipTop Green qPCR SuperMix (TransGen) was used with 200 nM of forward and reverse primers (Table S1 in Supplementary materials) and 1 μL of 10-fold diluted cDNA in a final volume of 20 μL. Gene transcription was analyzed using SYBR green assays. As previously described *sar1* and *rpl6e* genes served as reference (Steiger et al., 2009; Tisch et al., 2011). Thermocycling was performed in an ABI StepOne Plus thermocycler (Applied Biosystems, Foster City, CA, USA).

2.3. DNA cloning

The LML3.0 s cassette (Zhang et al., 2016) was used as a tool for gene replacement in *T. reesei*. The light-inducible transactivator G-linker-V sequence, encoding the Gal4 (1–65), Vivid (37–186), linker, and VP16 activation domain, was synthetized by GENEray (Shanghai, China). To generate chimeric fusion protein G1A, VP16 activation domain of G1 V were replaced with sequences encoding Ace2 (59–341) activation domain, isolated from *T. reesei* cDNA, by NotI and MauBI (Fig. 1B).

The amino acid sequences of the designed linkers are described in Fig. S1A. The transactivating sequences that replaced the native *xyr*1 regulator were expressed under the control of the native *xyr*1 promoter or a constitutive promoter *pdc* (Li et al., 2012) from *T. reesei* (Fig. S1B). The light-inducible transactivators include Pxyr1-G1A, Pxyr1-G1 V, Pxyr1-G2 V, Pxyr1-G3 V, Pxyr1-G4 V, Pxyr1-GPAV, and Ppdc-GPAV (Fig. 1B).

The "light-on" reporter was generated by overlapping 5 × UASG sequence (a *cis*-acting regulatory sequence to bind the Gal4 protein), 132p, which targets *cbh*1 locus (Wang et al., 2014a) (Fig. S1C). The reporter structures, including 5U-132-RFP, 5U-132-GFP, 5U-132-NI and 5U-132-V, were generated by ligating genes encoding red fluorescent protein (RFP), green fluorescent protein (GFP), NlsLacI, LacI with a nuclear localization sequence (GenBank accession no. EHK22773.1) (Labow et al., 1990), and EGV, an alkaline cellulase from *Humicola insolens* (Schülein, 1997), into *Pml*I and *Mau*BI sites (Fig. 1C).

"Light-on" bidirectional promoter was generated by overlapping reversed 132p and 5U-132 element, which targets *cbh*1 locus (Fig. 1C). The reporter structures including 5U–2 × 132-(V + V), 5U–2 × 132-(RFP + V) and 5U–2 × 132-(GFP + NI) were generated.

The "light-off" promoter was generated by inserting *lacO* sequences within the *pdc* promoter region (Labow et al., 1990). The insertion was located between the transcription start site and TATA box region (Fig. 1D). Multiple tandem operators, as well as single operators, were also used in this construction. The reporter structures, including pdc-V, pdc-O1-V, pdc-O2-V, pdc-O3-V and pdc-O2-RFP, which targets *cbh*2 locus, were generated by ligating genes encoding EGV or RFP into *Pml*I and *Mau*BI sites, respectively (Fig. 1D).

Deletion cassettes consisting of 1 kb fragments of *xyr1*, *cbh*1 or *cbh*2 specific flanking regions interrupted by transactivator, "light-on" or "light-off" reporter structures with LML 3.0 s cassette were assembled by pEASY-Uni Seamless Cloning and Assembly Kit (TransGen, Beijing, China). Individual flanking regions were amplified from *T. reesei* genomic DNA using KOD Polymerase (Toyobo, Osaka, Japan). Corresponding sequences are described in Supplementary materials.

2.4. Characterization of the transformants

The disruption vectors were introduced into *T. reesei* RUT-C30 by *Agrobacterium*-mediated transformation (Lv et al., 2012). In all the purified transformants, type of integration for the target gene deletion construct was determined by diagnostic PCR (Zhang et al., 2016). Single copy integration for selected strains was verified by qPCR. Ectopic integration of the deletion cassettes led to an additional gene copy in *T. reesei* genome.

2.5. Light induction and heterologous protein production

To induce transcription and protein expression, *T. reesei* strains cultured in the 12-well plate (culturing procedure described in Supplementary materials) were exposed to blue light (460 nm peak, 11, 21, 32, 53, 78, 137, 314 and 550 W/m^2) using an automated timer (Fig. S2A). To induce alkaline cellulase expression, transformants cultured in 500 mL flasks (culturing procedure described in supplementary methods) were exposed to a 1-min pulse of blue light (190 W/m^2) every 5 min using an automated timer (Fig. S2B). Further, to determine alkaline cellulase expression, transformants cultured in 7-L jar fermentor (culturing procedure described in supplementary methods) were exposed to a 1-min pulse of blue light (944 W/m^2) every 10 min using an automated timer (Fig. S2C). The system is sensitive to ambient light. In this study, other experimental procedures were carried out under dark or red LED light.

2.6. Detection of fluorescence

Expression of fluorescent proteins in the cultures was detected using an S Plan Fluor ELWD 40 ×, 0.45 numerical aperture (NA)