A synthetic transcription factor and core promoter system in *Picochlorum renovo* enables tunable gene expression

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Introduction

Microalgae are exceptional organisms due to their ability to convert CO_2 and sunlight into renewable biomass and bioproducts, significantly reducing carbon in our atmosphere and reducing the effects of global warming. *Picochlorum renovo* is a recently characterized microalga of industrial interest. Its rapid growth rate, and high temperature and salinity tolerances make *P. renovo* an attractive candidate for industrial scale cultivation and downstream production of sustainable fuels and chemicals. However, the current genetic toolbox for many non-model microalgae is limited and would greatly benefit from an orthogonal gene expression system to bypass host regulation as algal systems are robust and often interfere with integrated gene cassettes. Additionally, the engineering of complex metabolic pathways in eukaryotic organisms to optimize growth or biosynthesize high value products often requires tunable expression of each gene in a pathway.

Here we explore a tunable orthogonal gene expression system, originally designed for fungi, using a synthetic transcription factor (sTF) and universal core promoters (CPs) conferring expression of the fluorescent protein *mCherry* to quantify protein expression. The sTF paired with the relevant DNA binding site (BS) led to an ~5X increase in reporter gene expression compared to the native RuBisCo promoter, however had limited tunability with increasing BS number. A preliminary transcriptional analysis of our mRNA expression using qPCR detected a similar increase between zero and one BSs.

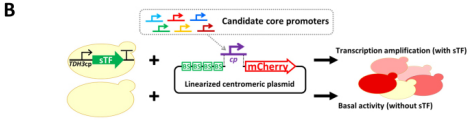
Additionally, quantification of *mCherry* expression under 34 different CPs paired with the sTF and BS showed an order of magnitude of expression tunability. With this sTF and CP system we aim to greatly improve growth rates and product titers in photosynthetic organisms, while also providing a potentially universal gene expression system for microalgae.

Fungal Synthetic Expression System

The synthetic expression system (SES) that we modeled our work after, was originally developed at the VTT Technical Research Centre of Finland, and designed and demonstrated in a variety of yeast and filamentous fungi. As previously stated, the SES system is composed of a sTF, CPs, and BSs. **(Below): (A)** Expression of an sTF can lead to tunable output via modulation of upstream binding site number. **(B)** Previously published gene cassette designed for universal gene expression in fungal systems to express the sTF and mCherry via universal core promoters (Rantasalo, et. al, 2018).

A

B



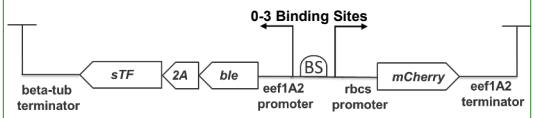
sTFs are composed of three parts: i) a modular DNA binding domain that can be obtained from diverse sources (e.g. bacterial transcription factors), ii) a VP-16 transcriptional activator domain from herpes simplex virus, and iii) a SV40 nuclear localization signal, from simian virus 40.

BSs are unique to their sTF and located just upstream of the CP. The sTF recognizes the BS which then amplifies transcription, with the addition of more BSs gene expression can be increased by recruitment of sTFs.

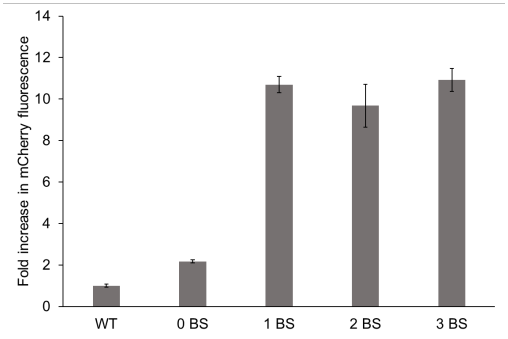
CPs were chosen based on transcriptomic data, targeting high expression genes in diverse fungi, and further restricted to promoters with a canonical TATA sequence that was a relatively short distance from the start codon (~80-180 bp), ultimately producing promoters with lengths of 140 to 200 bp.

Synthetic Transcription in *P. renovo*

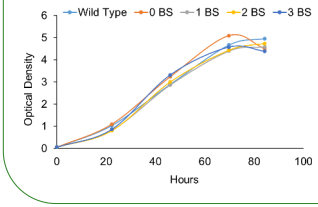
Synthetic transcription factors (sTF) have been established in bacterial and yeast systems to allow for tunable expression of genes of interest, an important ability when fine tuning multiple genes needed for certain metabolic engineering approaches. **(Below):** A synthetic transcription factor containing a DNA binding domain tethered to a transcriptional activation domain is expressed, concurrently a gene of interest is expressed with DNA binding sites matching the DNA binding domain of the sTF, by altering the number of DNA binding sites tunability of gene expression can be achieved.



(Below): A synthetic transcription factor was expressed in *P. renovo*, enhancing expression of the reporter gene *mCherry*. Transformants with no DNA binding sites (BS) showed an ~2-fold increase in fluorescence over wild type background, due to the expression level of the native promoter used, inclusion of 1-3 binding sites increased *mCherry* fluorescence by ~5 fold compared to 0 binding sites (~10 fold compared to wildtype). No tunability in gene expression was observed by increasing the number of DNA binding sites, however additional DNA binding domains are under evaluation that have showed greater tunability in model heterotrophic systems.



We have hypothesized that the plateau in *mCherry* fluorescence between 1, 2, and 3 BSs could be due to three possible bottle necks in the production process. Either there is no difference in transcription due to a lack of free RNA polymerases, or lack of binding space for additional sTFs. Additionally, there could be differences in translation due to a lack of free ribosomes, while transcription rates still increase with the number of BSs. To assess this question, we have begun preliminary qPCR on the four strains. The data as of now, shows an approximate 4.3-fold *mCherry* transcript increase from 0 to 1 binding site, in line with the observed increase from the protein level fluorescent data. Additional analyses are underway to differentiate transcription rates between 1, 2, and 3 BSs. Additionally, a series of controls have been generated, including no sTF and no promoter.

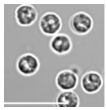


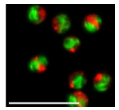
(Left): No impact on growth was observed due to expression of the synthetic transcription factor, or due to the increased expression of transgenic *mCherry*.

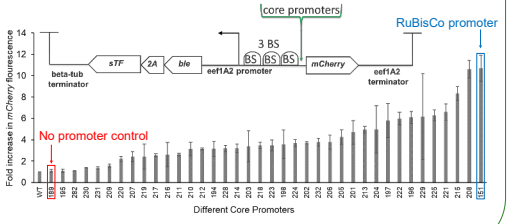
Core Promoters in *P. renovo*

To compare *mCherry* expression between all CP strains, WT, a native RuBisCo promoter high expression control (**151**), and a no promoter control (**189**), each strain was measured concurrently in black 96-well plates. Four unique strains containing each CP were grown up together in normal conditions to approximately the same OD_{750} and all measurements were taken with a WT control as well as chlorophyll measurements to indicate any substantial differences in cell number or health. **(Below):** The results showed a wide range of expression levels from background to very high. This gives us gene expression tunability through the addition of different CPs, which are a relatively low bp addition to any transformational DNA cassette. In addition, there was relatively low variance between unique strains containing the same CP, indicating functional orthogonality of the system.

(Right): Bright field and chlorophyll (green) + *mCherry* (red) fluorescence images of *P. renovo*, 10 µm scale bar







Conclusions and Future Work

The use of different CPs in our integration cassette appears to have substantial tunability with consistent resulting expression despite random integration. This infers that we are successfully bypassing host regulation and have a wide range of choices for desired expression levels.

Our current SES model does not appear to have tunability via BSs past the first BS. However, the ability of BSs to increase the expression of one or more transgenes could prove valuable for a variety of genetic engineering pursuits. Additionally, the sTF utilized herein also showed limited tunability in fungal systems, however a variety of sTFs are available with varying DNA binding domains, which showed greater tunability in fungal systems. As such, future research on sTFs in *P. renovo* will target these other sTFs and altering the linker region length between numerous BSs.

Future Work:

- Integrate different known sTFs
- Continue to add BSs and assess tunability of expression
- Space out BSs to allow for better binding of multiple sTFs
- Random integration of BSs into sTF expressing strains to produce an overexpression library for *P. renovo*

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