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Rapid evolution of regulatory element libraries for tunable transcriptional and translational control of gene expression

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ABSTRACT

Engineering cell factories for producing biofuels and pharmaceuticals has spurred great interests to develop rapid and efficient synthetic biology tools customized for modular pathway engineering. Along the way, combinatorial gene expression control through modification of regulatory element offered tremendous opportunity for fine-tuning gene expression and generating digital-like genetic circuits. In this report, we present an efficient evolutionary approach to build a range of regulatory control elements. The reported method allows for rapid construction of promoter, 5′UTR, terminator and *trans*-activating RNA libraries. Synthetic overlapping oligos with high portion of degenerate nucleotides flanking the regulatory element could be efficiently assembled to a vector expressing fluorescence reporter. This approach combines high mutation rate of the synthetic DNA with the high assembly efficiency of Gibson Mix. Our constructed library demonstrates broad range of transcriptional or translational gene expression dynamics. Specifically, both the promoter library and 5′UTR library exhibits gene expression dynamics spanning across three order of magnitude. The terminator library and *trans*-activating RNA library displays relatively narrowed gene expression pattern. The reported study provides a versatile toolbox for rapidly constructing a large family of prokaryotic regulatory elements. These libraries also facilitate the implementation of combinatorial pathway engineering principles and the engineering of more efficient microbial cell factory for various biomanufacturing applications.

1. Introduction

The plummeting cost of gene synthesis and facile gene assembly platforms allow metabolic engineers to rapidly reprogram a cell's genetic blueprint at a speed and scale never seen before [1]. Metabolic engineering has become an enabling technology to construct efficient microbial cell factories and a major driver for next-generation bio-economy [2]. By engineering heterologous pathways or endogenous metabolism, metabolic engineers have now been able to produce a large portfolio of commodity chemicals [3,4], novel materials [5], sustainable fuels [6,7] and pharmaceuticals [8,9] from renewable feedstocks. This is often achieved through sophisticated metabolic engineering strategies including overexpression of rate-limiting steps [10], deletion of competing pathways [11], managing ATP [12,13] and recycling NADPH and other cofactors [13]. While these approaches have been shown to effectively improve cellular productivity and yield, the attempt to engineer a specific pathway often requires proper balancing of precursor pathway and fine-tuning of gene expression.

Engineering microbial overproduction phenotypes remains a daunting task as it usually involves the manipulation of a handful of precursor or rate-rating pathways that are subject to tight cellular regulation. For example, precursor flux improvement by overexpression of heterologous pathways may not be accommodated by downstream pathways; intermediate accumulation or depletion may compromise cell viability and pathway productivity [14]; and overexpressed heterologous protein may penalize the cell with additional energy cost and elicit cellular stress response [15]. As an attempt to address these issues, the practice of metabolic balancing has shifted towards redistributing metabolic flux through synthetic biology approaches, including modification of plasmid copy

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number [16], promoter strength [17], gene codon usage [18] and RBS strength [15,19].

Along this line, combinatorial transcriptional engineering coupled with efficient gene assembly tools has offered tremendous opportunities for customized optimization of multi-gene pathways. Excellent examples include construction of yeast xylose pathways by promoter shuffling [20], heterologous production of anti-cancer taxol precursors in *E. coli* [21] and rapid assembly and screening multi-gene mutant pathway libraries in *E. coli* and yeast [22,23], and combinatorial optimization of fatty acids pathway to produce advanced biofuels [24,25], multiplexed regulatory RNA [26] and global transcriptional machinery engineering [27] to produce L-tyrosine. Design of experiment (DoE) statistical procedures have been recently applied to rapidly locate the overproduction genotype out of a large number of pathway candidates. Without testing all the possible gene expression combinations, factorial design and empirical equations allow metabolic engineers to correlate gene expression level with metabolites titer; and facilitate the extrapolation of the optimal gene expression patterns leading to the desired overproduction phenotype [28,29].

As an emerging discipline, synthetic biology is becoming increasingly important to design, construct and optimize metabolic pathways leading to desired overproduction phenotype in genetically tractable organisms. One of the major challenges for heterologous expression of multi-gene pathways is to balance the expression level of each of the enzymes among the selected pathways and achieve optimal catalytic efficiency [30]. Thus, delicately designed molecular control elements has been integrated into cell chassis to enable the host strain to precisely respond to environmental stimuli or cellular intermediates and drive carbon flux toward a target pathway. For example, engineering promoter architecture has achieved tunable gene expression in both *E. coli* [31] and yeast [32] at transcriptional levels; engineered metabolite-responsive riboswitches [33] and synthetic ribosome binding sites [19] can be used to precisely control protein expression at the translational level; metabolic flux channeling by spatial recruitment of desired metabolic enzymes at stoichiometric ratio on a synthetic protein scaffold can efficiently prevent the loss of intermediates due to diffusion [34]. In a word, advances in synthetic biology have accelerated our ability to design and construct cell factories for metabolic engineering applications.

Regardless of this development, it is necessary to further expand synthetic biology toolbox and diversify the number of regulatory elements that could be used for pathway fine-tuning. In this report, we present an evolutionary approach to efficiently construct regulatory element libraries that cover a broad range of gene expression dynamics. Our library construction approach has been successfully applied to construct the T7 promoter library, lactose repressor binding site (lacO) library, prokaryotic ribosome binding site library, T7 terminator library and the *trans*-activating RNA library. The constructed regulatory element libraries will find wide applications in transcriptional and translational fine-tuning, as well as sampling the multidimensional gene expression space to rapidly locate the desired phenotype. We envision this evolutionary approach will provide an efficient solution to balance multiple gene pathway and accelerate strain engineering for cost-competitive manufacturing of bioproducts.

2. Results and discussion

2.1. An efficient evolutionary approach to construct regulatory element library

Traditional regulatory element library construction are primarily relied on error-prone PCR [35], which typically suffers from biased mutational spectrum with transition mutation occurring more frequent than transversion mutation. Here we adopted a novel library construction approach [36] that capitalizes on partially overlapped 60-mer synthetic oligos from DNA vendors like IDT and Sigma. Customized oligos with point mutations or degenerate bases flanking the regulatory element region can be easily fused to a plasmid backbone via highly efficient Gibson assembly. The vector backbone contains the replication origin, antibiotic maker and a reporter gene, allowing for easy transformation, plasmid propagation and reporter activity screening.

Gibson assembly mix contains polymerase, ligase and T5 exonuclease activity. T5 exonuclease chews back the 5′ overhang and introduces a 3′ overhang which complements the primers. Then the polymerase extends the single strand DNA and fills up the gaps. Taq ligase joins the 5′-phosphate and 3′-hyroxyl group to form a phosphodiester bond. Because of the activity of the T5 exonuclease, a special consideration for designing the synthetic overlapping oligos is to leave the overhang at the 3′ end to avoid the digestion by T5 exonuclease (Fig. 1). Another important aspect is to keep the completeness of the library. One cannot pick a single colony to retrieve the library. Instead, a bacteria “lawn” from the agar plates were directly retrieved for plasmid mini-preparation. The plasmid library was later retransformed to the proper chassis strains (in our case, BL21(DE3)star) for proper protein expression and reporter activity quantification (Fig. 1). This approach combines the high mutation frequency of the degenerate synthetic oligos with the highly efficient gene assembly platform (specifically Gibson Assembly), offers us a rapid evolutionary strategy to construct regulatory element libraries.

2.2. A hybrid T7 promoter and lactose repressor-binding site library

Promoter is the DNA sequence that caries transcriptional start signal. Promoters consists of specific response elements that provide the binding site for RNA polymerase and for transcription factors to regulate transcription. These transcription factors could be activators or repressors that are responsible for turning on or shutting down gene expression. Prokaryotic promoter generally contains two conserved sequence elements: the -10 (TATAAT box) and -35 (TTGACA sequence) region. Recruited by sigma factors, RNA polymerase will specifically interact with the -10/-35 region and form a RNAP-promoter complex to initiate transcription.

We first test if we can modify the bacteriophage T7 promoter as it is widely used in various metabolic engineering applications. The strength and efficiency of T7 RNA polymerase (RNAP) are determined by two factors: the recruitment of T7 RNAP and sigma factors on the promoter core region (-35 and -10) and the proceeding of the RNAP across the repressor binding region (lacO) [37]. To generate the T7 promoter library, we designed the synthetic overlapping oligos in such a way that we can mutate both the sigma factor binding region (-35 and -10) and the lacI repressor binding region (lacO) (Fig. 2A). IPTG induces the dissociation of lacI repressor from the lacO site and thus allows T7 RNAP to read across the DNA template and give rise to fluorescence signals. With green fluorescence protein as readout, we quantified the T7 promoter transcriptional activity of the constructed promoter candidates. Reporter output results indicate that the constructed library covers a broad range of transcriptional dynamics spanning across three orders of magnitude (Fig. 2B). For instance, the highest transcriptional activity obtained (21,340 FU/OD with promoter No. 2) was about 7-fold higher than the original T7 promoter (3942 FU/OD with promoter No. 48); the lowest transcriptional activity (22.3 FU/OD with promoter No. 46) achieved was about three order of magnitude lower than the highest promoter (No. 2).

We then sequenced 18 promoters and the sequencing results

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