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Evaluation of synthetic promoters in *Physcomitrella patens*

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ABSTRACT

Securing a molecular toolbox including diverse promoters is essential for genome engineering. However, native promoters have limitations such as the available number or the length of the promoter. In this work, three short synthetic promoters were characterized by using the yellow fluorescent protein Venus. All of the tested promoters were active and showed higher mRNA expression than housekeeping gene *PpAct7*, and similar protein expression level to the *AtUBQ10* promoter. This study shows that few *cis*-regulatory elements are enough to establish a strong promoter for continuous expression of genes in plants. Along with this, the study enhance the number of available promotors to be used in *P. patens*. It also demonstrates the potential to construct multiple non-native promoters on demand, which would aid to resolve one bottleneck in multiple pathway expression in *P. patens* and other plants.

1. Introduction

Genes in eukaryotes consist of three major regions: the promoter, the coding strand, and the terminator. The promoter controls the gene expression by regulating the binding of transcription factors to recruit RNA polymerase [10]. Thus, the synthesis of mRNA is directly correlated with the promoter activity that may lead to the production of protein. The promoter is situated thousands of base pairs (bp) upstream from the transcription start site (TSS), to about 30 bp downstream from the TSS [18]. Promoters come in different type of switches, some are constitutive, some react to specific stimuli, and some are inducible and maintain a strict on/off style switch.

Plants are known to have larger promoter sequences than those found in fungi and prokaryotes. Plant promoters typically range from 500 bp to over 2000 bp [12]. As the possibilities in synthetic biology advances, it is often necessary to introduce multiple genes and promoters to achieve the desired traits. However, endogenous plant promoters are often of limited use in plant synthetic biology as multiple copies of the same promoter can trigger homology-dependent gene silencing [4]. Therefore, characterizing multiple promoters from heterologous species has become important for fine-tuning of multiple genes.

Physcomitrella patens is a plant model system that has been used extensively to study plant evolution, physiology, and development [25]. The full genome is sequenced [19] and development growth media and transformation methods are well described [1]. Its ability to perform efficient homologues recombination, that now can be explained with RecQ helicase function [27], is unique among plants and enable *in vivo* assembly of multiple DNA fragments followed by targeted genome integration by homologues recombination [9]. Collectively, such distinct features make *P. patens* attractive as an industrial production platform for small natural products, which requires integration of numerous genes [6,8,17,23,28].

Monocot housekeeping gene promoters and the 35S promoter from Cauliflower mosaic virus (CaMV) have shown a high-level of gene expression in *P. patens* [5,24]. However, the limited number of available promoters are a bottleneck in introducing complex pathways into *P. patens* and typically long plant promoters are difficult to handle in multiple gene integrations. Therefore, developing strong and short synthetic promoters has emerged as a major interest to provide a solution.

Synthetic promoters are relatively short (300–500 bp) and can

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be generated in countless number with similar strength or function, and could improve the genome stability [21]. *Cis*-regulatory elements (CREs) of native promoters are non-conserved among genes that are similarly expressed. Thus, the synthetic promoters can be reasonably constructed to give high gene expression with a smaller size [12]. The strength of synthetic promoters depends on the selection, spacing of CREs and the copy number. Using bioinformatic algorithms, novel CREs could be discovered, by comparing the upstream sequence of differentially regulated genes. These CREs could be put together rationally to design new synthetic promoters [21]. Furthermore, the strength of the synthetic promoter could be enhanced by proper spacing and increasing the CRE copy number [12].

Here, we have tested three synthetic promoters in *P. patens*, developed using an automated high-throughput screening method. The promoters were built using computational analysis of large transcriptomic functional data set to identify *cis*-elements, which form the building blocks of synthetic promoter libraries. All three synthetic promoters had higher mRNA expression than the housekeeping gene *PpAct7* and showed similar protein expression pattern to *AtUBQ*10 promoter.

2. Materials and methods

2.1. Promoter construction

The synthetic promoter library was constructed at Synpromics using random assembly techniques of *cis*-regulatory elements (CREs) through expression data analysis of *Zea mays*. Genes showing strong expression strength above *Ubiquitin1* transcripts were labeled constitutive. Using transcription factor binding site database TRANSFAC, CREs of the constitutively expressed genes were identified from the 1,500bp upstream and 500bp downstream of the transcription start site. Subsequently, Synpromics Ltd Syn-score algorithm was applied to the identified regions to rank the CREs [20]. Further, a synthetic promoter library was constructed by, random assembly of the chosen CREs (300 bp-800 bp), attached upstream to CaMV 35S minimal promoter (position −46 to +89).

2.2. Growth media

P. patens (Gransden ecotype, International Moss Stock Center #40001) was grown on solid and liquid PhyB media [1] under sterile conditions, with continuous 20–50 W/m^2 light intensity at 23 °C.

2.3. DNA preparation and transformation protocol

DNA fragments for transformation were prepared in blocks as described below. The first block is a 2.7 kb region with 108 5′ neutral locus, G418 selection marker with CaMV 35S promoter/CaMV poly(A) signal was amplified from the pRH004 plasmid. The second block is the synthetic promoter sequences developed by Synpromics, and the *Arabidopsis Ubiquitin10* (*AtUBQ10*) promoter was amplified with 20–22 nt overhangs homologous to block one and three. Block 3 is with the Venus fluorescent protein, OCS terminator and the 108 locus homologous recombination flanking region was amplified from pRH004 plasmid (Fig. 1A). Purified 1.5 pmol of each DNA block (Fig. 1B) was transfected into the isolated moss protoplasts during transformation process and selected for positive colonies according to previously published methods [8,9].

2.4. Arabidopsis Ubiqutin10 promoter

Arabidopsis UBQ10 promoter with a length of 634 bp of [3] was cloned from *Arabidopsis* genomic DNA using primer set; UBQ10 F 5′-GTCGACGAGTCAGTAATAAACGG-3′ and UBQ10 R 5′-CTGTTAATCAGAAAAACTCAGATTAATC-3'. For moss transformation, 22-nt overhangs that are identical to the next fragments (block one and three) were attached to both ends by second PCR using overhang primers.

2.5. Detection of Venus fluorescence

Venus fluorescence was detected on protonemal cells grown for seven days in PhyB liquid media. *P. patens* protonema cells were visualized and photographed using a confocal laser-scanning microscope. Z-stacks were performed on each line using the 488 nm laser line and YFP emission filter. Z-stacks were put together using the Zeiss software built-in maximum projection function. Fluorescent level of each promoter lines was calculated from digital Images using the software ImageJ (https://imagej.nih.gov/ij/). A previously published method on fluorescent cell analysis was used to calculate the corrected total cell fluorescence (CTCF) levels [2,13].

For each cell, measurements were taken for the cell area, integrated density, and mean grey value. Final corrected total cell fluorescence (CTCF) was calculated using the following formula.

CTCF = Integrated Density - (Area of selected cell X*x* Mean fluorescence of background reading)

2.6. RNA extraction and qPCR

Total RNA was extracted from the appropriate lines (7 days after blending), using Spectrum™ Plant Total RNA Kit (Sigma, STRN250). To synthesize cDNA, 1 μg of extracted total RNA was reverse transcribed by iScript cDNA synthesis kit (Bio-rad, 1708891), followed by PCR amplification of the following transcripts, *PpAct7* and *Venus*. PCR reactions were carried out using a Qiagen kit with denaturation at 95 °C 5 min, 40 cycles with 95 °C for 10 s and 60 °C for 10 s, and with a melting curve analysis to check the specificity. Relative *Venus* gene expression from each promoter line was analyzed by $Exp_{Venus} = 2^{\Delta Ct[promoter]}$, $\Delta C_t[promoter] = C_t[Actin]\text{-}C_t[Venus]$.

3. Results and discussion

3.1. Assembly of cis-regulatory elements to construct constitutive plant promoters

To generate synthetic promoters, functional CREs should be collected since these will form the building blocks of the synthetic promoters. We used an automated high-throughput screening method. In this method, computational analysis of large transcriptomic functional data sets of *Zea mays* was used to identify CREs from constitutively expressed genes. We ranked collected CREs by applying Syn-score algorithm and randomly assembled selected CREs to generate promoter library [20]. This technique has the advantaged that elements are selected based on the requirements for the synthetic promoters (e.g., inducible, constitutive and tissue- or developmental stage-specific), which is a more focused approach than using completely random elements. The promoter candidates consist of randomly assembled CREs of varying lengths up to 30 bp. Therefore, the promoter length and the position of the CREs vary (Fig. 1C). It has been shown that the

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