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Bioinformatic Prediction and High Throughput In Vivo Screening to Identify Cis-Regulatory Elements for the Development of Algal Synthetic Promoters

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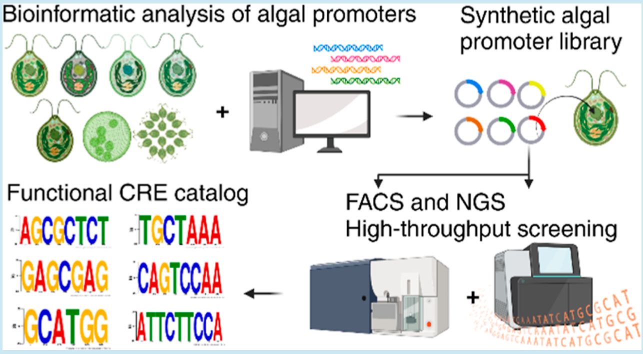
Bioinformatic analysis of algal promoters
Synthetic algal promoter library
Functional CRE catalog
AGCGCTCT
TGCTAAA
GAGCGAG
CAGTCCAA
GCATGG
ATTCTTCCA
FACS and NGS
High-throughput screening

ABSTRACT: Algae biotechnology holds immense promise for revolutionizing the bioeconomy through the sustainable and scalable production of various bioproducts. However, their development has been hindered by the lack of advanced genetic tools. This study introduces a synthetic biology approach to develop such tools, focusing on the construction and testing of synthetic promoters. By analyzing conserved DNA motifs within the promoter regions of highly expressed genes across six different algal species, we identified cis-regulatory elements (CREs) associated with high transcriptional activity. Combining the algorithms POWRS, STREME, and PhyloGibbs, we predicted 1511 CREs and inserted them into a minimal synthetic promoter sequence in 1, 2, or 3 copies, resulting in 4533 distinct synthetic promoters. These promoters were evaluated in vivo for their capacity to drive the expression of a transgene in a high-throughput manner through next-generation sequencing post antibiotic selection and fluorescence-activated cell sorting. To validate our approach, we sequenced hundreds of transgenic lines showing high levels of GFP expression. Further, we individually tested 14 identified promoters, revealing substantial increases in GFP expression—up to nine times higher than the baseline synthetic promoter, with five matching or even surpassing the performance of the native AR1 promoter. As a result of this study, we identified a catalog of CREs that can now be used to build superior synthetic algal promoters. More importantly, here we present a validated pipeline to generate building blocks for innovative synthetic genetic tools applicable to any algal species with a sequenced genome and transcriptome data set.

KEYWORDS: *synthetic biology, genetic engineering, algae, bioinformatics, cis-regulatory elements, promoters*

1. INTRODUCTION

Biotechnology, the use of biological organisms and processes to create products, has ancient roots dating back 10,000 years with the advent of agriculture and at least 6000 years ago in fermenting beverages and making cheese.[1] However, the modern era of biotechnology began in the 1970s with the development of molecular cloning technologies, leading to a new industry, which was first exemplified by companies like Genentech, Amgen, and Chiron. Today, biotechnology significantly impacts the world's economy and is predicted to reach almost 14% of the global economy by the year 2030.[2]

As the biotechnology industry has matured, it has become clear that more sustainable and efficient organisms need to be developed, ones that can meet the ever-growing demand for new biobased products, without overtaxing our already strained resources, like potable water and arable land.[3] Microalgae, microscopic aquatic organisms, are poised to become a cornerstone of future sustainable biotechnology, as these versatile organisms can be engineered for the efficient and environmentally favorable production of food and feed, therapeutics, biofuels, bioplastics, and many other products.[3] Numerous examples of current algae-based products exist, including omega-3 fatty acids and other nutritional supplements marketed for their health benefits, biopolymers, alternative food, and even biofuels.[4]

However, optimizing the production of these valuable products requires precise control over the genetics and metabolism of these organisms, which can be achieved today through targeted genetic modifications but at a much less

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sophisticated level than for other industrial biotechnology platforms. Fine-tuning metabolic pathways and expressing specific genes can significantly enhance the yield and quality of desired products, and to achieve this, the development of sophisticated genetic tools, including promoters capable of driving robust and tunable transgene expression. Currently available genetic tools for algae fall short of those available in other model organisms like bacteria and yeast.[5] While native promoters from the model green algae *Chlamydomonas reinhardtii*, such as the chimeric promoter AR1,[6] have been successfully utilized, their effectiveness pales compared to the diverse and powerful tools available in established industrial organisms.

Synthetic biology offers a promising avenue for overcoming these limitations and creating next-generation genetic tools for algae. This field of research focuses on designing and building biological systems with specific functions, and it has already yielded impressive results in other model organisms.[7,8] In the algae biotechnology field, synthetic biology is still in its initial stages. The first synthetic promoters in *C. reinhardtii* were created by analyzing the promoter regions of the 50 highest expressed genes in *C. reinhardtii* and identifying DNA motifs based on the presence of such elements in these genes' promoters, and on their positional relationship within the promoter region, using the POWRS algorithm.[9] Using this data, 25 synthetic algal promoters were made and the performance of which was compared to native hybrid promoter AR1 using in vivo expression assays, with seven of the synthetic promoters outperforming AR1, and one motif or CRE (CCCAT motif) was identified as a main driver of expression in one of the promoters.[10] In a different study, some of the motifs identified by Scranton et al. were used to design better synthetic promoters by inserting them into native chimeric promoters.[11] In another study, promoter regions of highly expressed genes were analyzed using software like WEEDER,[12] HOMER,[13] DREME,[14] and MEME,[15] followed by motif clustering and enrichment analysis. This process led to the identification of 13 putative DNA motifs that could function as transcriptional enhancers, which were synthesized and tested in vivo for their ability to drive the expression of a yellow fluorescent protein reporter gene. Out of these, five DNA motifs showed significantly higher reporter expression compared to AR1.[16] These studies have proven that it is possible to identify cis-regulatory motifs and utilize them to design functional synthetic promoters. However, the number of identified CREs remains limited, and the transcriptional activities of these elements have not been well characterized.

In this study, we sought to identify a large number of CREs by analyzing conserved DNA elements in the promoter regions of highly expressed genes across six different algal species. Using different bioinformatic algorithms, two pools of CREs were predicted: one containing 680 and the other 831 CREs. Consequently, two synthetic algal promoter (SAPS) libraries were built, with each CRE being tested by inserting 1, 2, or 3 tandem copies of a single CRE per synthetic promoter, thus generating 2040 promoters in SAPS1 and 2493 in SAPS2. We then transformed the promoter libraries into *C. reinhardtii* and assessed the impact of these CREs on gene expression using an in vivo reporter assay based on antibiotic selection and fluorescent protein gene expression, followed by Next-Generation Sequencing (NGS) to identify individual CREs. This enabled a high throughput comparison of relative promoter activity within a population containing thousands of transgenic lines to determine the relative ranking of these CREs. The sequencing data derived from the NGS analysis allowed us to create a catalog of more than 100 functional CREs in *C. reinhardtii* that can be used to generate functional synthetic algal promoter. Finally, we isolated 14 individual transformants containing synthetic promoters capable of driving green fluorescent protein (GFP) at high levels through fluorescence activated cell sorting (FACS), and quantitated GFP expression from these promoters in detail to demonstrate the utility of such synthetic promoters for driving recombinant gene expression.

2. RESULTS AND DISCUSSION

2.1. Bioinformatic Analysis of Conserved DNA Motifs in the Promoter Regions of the Highest Expressed Genes across Different Algal Species. In this study, we identified a comprehensive list of CREs from algal genomes that can promote transgene expression upon insertion into synthetic promoters. Our hypothesis was that promoters exhibiting high gene expression possess DNA binding sites for transcription factors that, upon binding, augment transcriptional activity. We anticipated these DNA binding sites to be over-represented in the promoter regions of genes with high expression levels. Furthermore, we expected these functional DNA sequences to be conserved across the promoter regions of orthologous genes in closely related species. While it is known that the same transcription factor can act as a repressor or as an activator depending on the genetic context,[17] our hypothesis partially infers that the ortholog genes of highly expressed genes in *C. reinhardtii* will also show a high transcriptional activity as a result of conserved CREs. A simple analysis showed that 80% of the 146 ortholog genes from *Chlamydomonas pacifica* could be found within the highest 200 expressed genes in that species, and 90% of the ortholog genes were found among the top 300 genes (data not shown).

Acknowledging the significant variability in motif prediction accuracy across data sets and species highlighted in prior studies, we elected a strategy that involved the application of three distinct motif prediction algorithms to identify CREs in *C. reinhardtii*.[18] Given these criteria, we employed the algorithms POWRS and STREME, in SAPS1, and PhyloGibbs, in SAPS2, to predict a total of 1471 potential CREs.

For SAPS1, the top 146 highly expressed genes in *C. reinhardtii* and their orthologues in *Chlamydomonas incerta* and *C. pacifica* were used as input sequences, anticipating an over-representation of transcriptionally activating CREs. Our constraints were kept lenient to minimize the number of false negatives, and we were confident in our high-throughput screening methods to manage even substantial quantities of false positives. To filter out ubiquitous DNA motifs in promoters unrelated to high transcriptional activity, control sequences were derived from the 146 least expressed genes in *C. reinhardtii* and *C. pacifica*. The input sequences are available in Supporting Information S1. The position-sensitive word set (POWRS) is a discriminative method that evaluates the significance of a motif through binomial distribution analysis of input and control sequences. Unlike other approaches, POWRS considers the motif's position in the sequences, which is often overlooked, without resorting to position weight matrices. Instead, it represents motifs using a single, nondegenerate consensus sequence along with some variants that differ by only one base pair. Simple, thorough, rapid, enriched motif elicitation (STREME), a widely popular tool designed for ungapped motif discovery, uses Fisher's Exact Test or the Binomial test to determine motif significance within the input sequences set

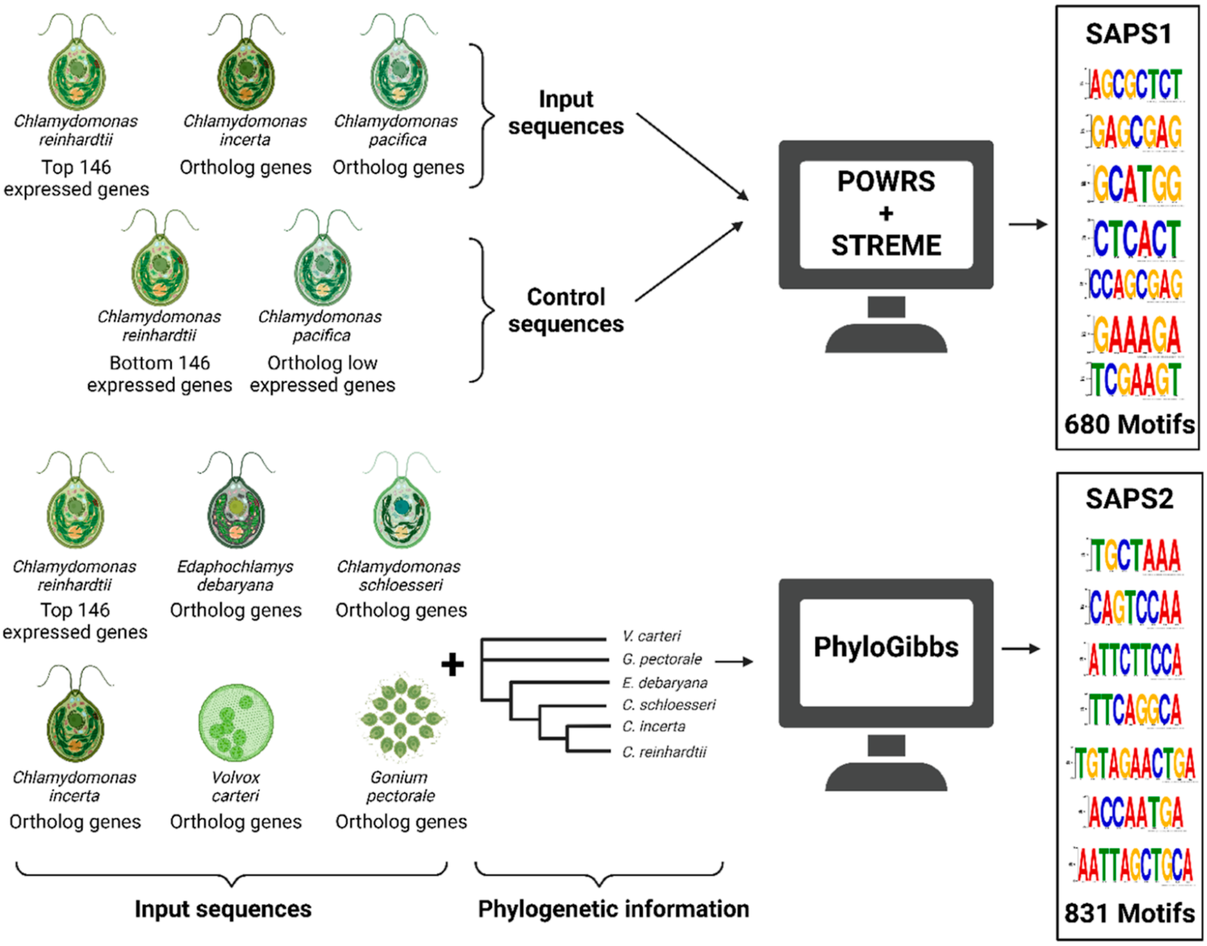

Figure 1. Identification and utilization of CREs in SAPS1 and SAPS2 libraries. Utilizing the top 146 highly expressed genes from *C. reinhardtii* and their orthologs in *C. incerta* and *C. pacifica*, alongside the least expressed 146 genes from *C. reinhardtii* and their orthologs in *C. pacifica* as controls, 680 CREs were predicted via POWRS and STREME for SAPS1 promoters. For SAPS2, the same approach applied to *C. reinhardtii*'s highly expressed genes and orthologs in *E. debaryana*, *C. schloesseri*, *C. incerta*, *V. carteri*, and *G. pectoral*, incorporating phylogenetic distances, resulted in 831 CREs through PhyloGibbs.

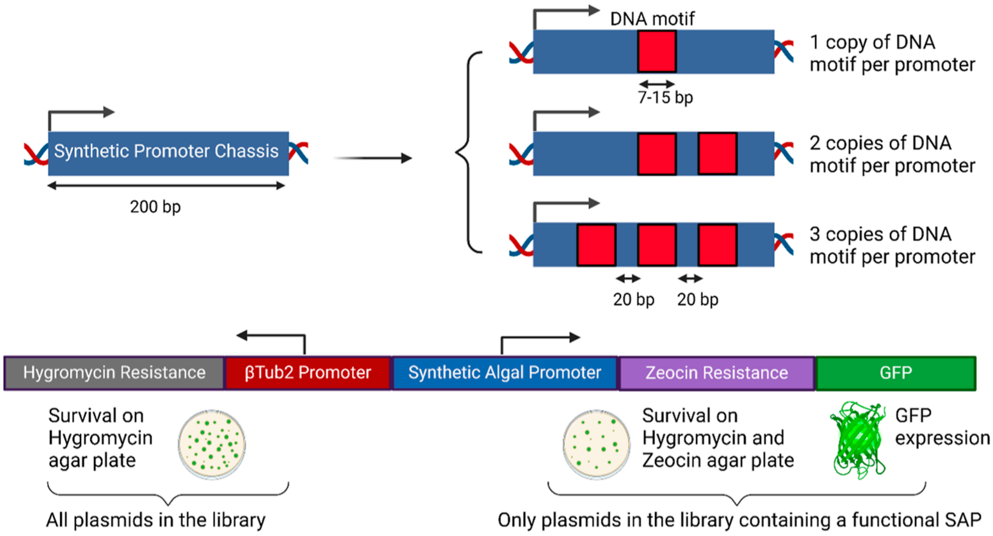
Synthetic Promoter Chassis
200 bp
DNA motif
7-15 bp
1 copy of DNA motif per promoter
2 copies of DNA motif per promoter
3 copies of DNA motif per promoter
20 bp
20 bp
Hygromycin Resistance
βTub2 Promoter
Synthetic Algal Promoter
Zeocin Resistance
GFP
Survival on Hygromycin agar plate
Survival on Hygromycin and Zeocin agar plate
GFP expression
All plasmids in the library
Only plasmids in the library containing a functional SAP

Figure 2. Design of synthetic promoter and expression vector. This figure illustrates the construction of synthetic promoters using a generated 200 bp DNA sequence with specific constraints. Predicted CREs were integrated in 1, 2, or 3 tandem copies at positions −104, −76, and −132 from the start site. The spacing between the motifs was standardized to 20 bp. These synthetic promoters were positioned upstream of a GFP-fused zeocin resistance gene for expression analysis. For comparison, an β-tubulin-2 promoter was utilized to regulate hygromycin resistance, serving as a control.

relative to the control sequences. Only the motifs found in at least two of the three species were selected. This resulted in the prediction of 640 different CREs (Figure 1 and Supporting Information S1).

For SAPS2, the same 146 promoter sequences from *C. reinhardtii* were used as input sequences, but the orthologue sequences used were those of *Edaphochlamys debaryana*, *Chlamydomonas schloesseri*, *C. incerta*, *Volvox carteri* and *Gonium pectorale*. These species were selected to cover a spectrum of evolutionary distances, ranging from the closely related *C. incerta* and *C. reinhardtii*, to more distant species like *V. carteri*, with a divergence comparable to that between humans and chickens, estimated at 310 million years ago (Supporting Information S1). PhyloGibbs predicts DNA motifs by combining overrepresentation analysis with evolutionary conservation across multiple sequence alignments of orthologous sequences. It uses a Bayesian model to discern functional binding sites, factoring in phylogenetic relationships, and applies simulated annealing alongside Monte Carlo Markov chain sampling to rigorously evaluate and assign probabilities to these sites. This resulted in predicting 831 different CREs (Figure 1 and Supporting Information S1).

2.2. Insertion of Putative Cis-Regulatory DNA Elements into a Synthetic Promoter Driving Zeocin Resistance and GFP Expression. The synthetic promoter chassis (SPC) was designed as a random 200 bp sequence with the following two constraints: the high GC content should resemble that of promoters in *C. reinhardtii* as described in Scranton et al.,[10] and the sequence should not contain any of the putative CREs to be tested in SAPS1 and SAPS2.

We engineered the synthetic promoters by inserting the predicted CREs in tandem arrays of 1, 2, or 3 copies. The insertion sites for these CREs were standardized across all of the promoters. Specifically, promoters with a single CRE had this element positioned at −104 relative to the transcriptional start site (TSS), with a variation for this position of ±2 bp. For promoters containing two CREs, the elements were positioned at −104 and −76 ± 2 bp. Lastly, promoters with three CRE copies had their CREs located at −132 ± 4, −104 ± 2, and −76 ± 2 bp (Figure 2). This positioning was designed to keep approximately 20 bp of spacing between CREs to reduce steric hindrances between transcription factors binding to the promoter. This resulted in 2040 synthetic promoters in SAPS1 and 2493 synthetic promoters in SAPS2. These were subsequently cloned into a vector named pLibrary, culminating in the formation of two libraries: Synthetic Algal Promoters 1 (SAPS1) and 2 (SAPS2).

The synthetic promoters were made to drive the antibiotic resistance gene fused to the reporter gene GFP. This configuration allowed the selection of functional synthetic promoters through Zeocin resistance. Additionally, the GFP signal provides a measurable indication of the transcriptional activity of each promoter.

A high-throughput strategy for comparing the transcriptional activities of synthetic promoters within the same library was developed, combining differential antibiotic selections and NGS. This approach is predicated on the assumption that synthetic promoters demonstrating high transcriptional activity will sufficiently drive the expression of the *ble* gene to enable a significant proportion of transformants to surpass the zeocin survival threshold. Consequently, synthetic promoters with high transcriptional activity will have a higher relative abundance within the algal population post-transformation and zeocin selection. To accurately measure the relative abundance of each synthetic promoter within the algal libraries, we incorporated a secondary selection gene (APHVII) that imparts Hygromycin resistance. This gene was controlled by the well-established endogenous β-tubulin 2 promoter,[19] conferring equal hygromycin-resistant capacity to all plasmids within both SAPS libraries. We strategically positioned this secondary selection gene upstream of the synthetic promoter with transcription heading away from the synthetic promoter. This placement was essential to mitigate any promoter trapping effects that might influence the transcriptional activity of a synthetic promoter, especially if it were to integrate immediately downstream of a strong endogenous promoter during transformation of the synthetic promoter construct into the genome. Furthermore, to prevent transcriptional readthrough from the Hygromycin resistance cassette into the synthetic promoter, we encoded the cassette on the opposite DNA strand relative to the synthetic promoter (Figure 2).

Under hygromycin selection, each synthetic promoter's relative abundance should simply reflect its initial abundance within the physical DNA library utilized for cell transformation. The relative abundance under hygromycin and zeocin selection should provide insights into the transcriptional activity of the synthetic promoter driving the *ble* gene, as only strong synthetic promoters should allow sufficient transcription of the *ble* gene to enable ble resistance. Nonetheless, this measure is influenced by the inherent abundance of each promoter in the physical DNA library as well. To account for this, the data are normalized by dividing the relative abundance under hygromycin and zeocin selection by the relative abundance under hygromycin alone, deriving what we termed the "HygZeo ratio". This ratio indicates the extent to which a synthetic promoter is over- or underrepresented in the hygromycin and zeocin condition compared to hygromycin alone, which can be used as a relative measurement of the transcriptional activity of each synthetic promoter compared to the rest of the promoters within the library.

2.3. Assessment of Synthetic Promoter Activity and CRE Copy Effect in *C. reinhardtii* Using Zeocin Selection and Next-Generation Sequencing. The SAPS1 and SAPS2 libraries were transformed to *C. reinhardtii* using electroporation. Post-transformation, each sample was divided into two parts: 10% of the volume was plated on TAP agar plates with 30 μg/mL Hygromycin (Hyg), and the remaining 90% on plates with 30 μg/mL Hygromycin and 10 μg/mL Zeocin (Hyg/Zeo). To ensure full representation of our promoter libraries in the high throughput in vivo testing, we established the goal of obtaining over 200,000 total transgenic lines (colonies) per library. This target assumes that only 1% of the obtained colonies are likely to exhibit significant transgene expression, due to factors such as random integration of transgenes into the nuclear genome leading to positional effects, RNA gene silencing, exonuclease activity causing truncated or damaged transgenes, and other epigenetic effects causing gene silencing.[20]

After performing over 60 transformations per library, around 280,000 SAPS1 colonies and 180,000 SAPS2 colonies were obtained on Hyg plates. The numbers on Hyg/Zeo plates were 12,000 colonies for SAPS1 and 14,000 colonies for SAPS2. Analyzing these figures and factoring in the 1:9 split in each transformation, it was calculated that only 0.47% of SAPS1 clones and 0.86% of SAPS2 colonies that survived in Hyg could also endure in Hyg/Zeo conditions. This means that even though only 12,000 and 14,000 colonies were obtained on the

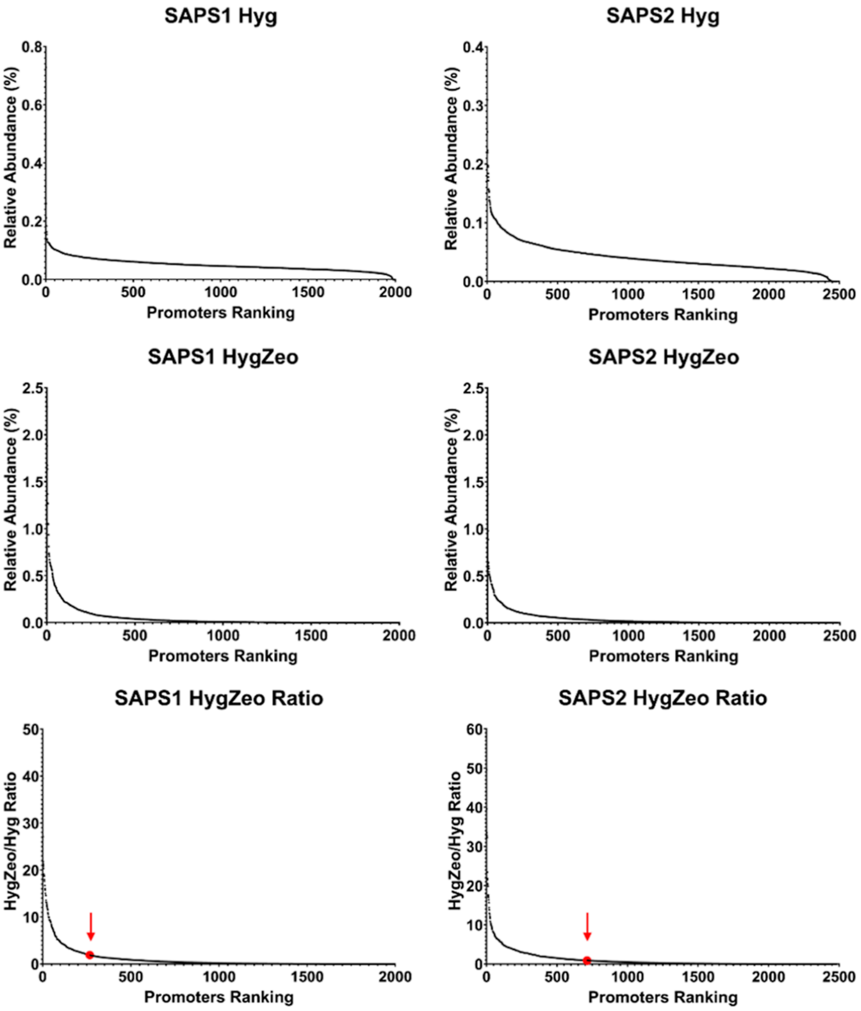

Figure 3. High throughput evaluation of the synthetic promoter libraries through relative abundance measurements. The transformants resulting from the transformation of SAPS1 and SAPS2, selected on either hygromycin (Hyg) or hygromycin and zeocin (HygZeo), were pooled in liquid cultures. Through PCR, their promoter sequences were amplified and analyzed through NGS. The relative abundances, calculated as the percentage of reads per promoter compared to the total amount of reads, are shown. Promoters are ranked based on their relative abundance, with the promoter showing the highest abundance in each graph receiving the ranking of 1. The relative abundance of each promoter under HygZeo selection is divided by the relative abundance of the same promoter under Hyg selection, obtaining the HygZeo ratio. The HygZeo ratio of the SPC is highlighted in red, with a red arrow indicating its position.

Hyg/Zeo plates, the actual number of CFUs that were screened for transgene expression was much higher: 25,520,000 for SAPS1 and 1,620,000 for SAPS2. These numbers are in accordance with what has been previously reported in the literature[20] for this type of selection. Additionally, this large number of transformants gives us confidence in our ability to thoroughly investigate these promoter libraries.

The algal colonies were separately pooled for each condition: SAPS1 in Hyg, SAPS1 in Hyg/Zeo, SAPS2 in Hyg, and SAPS2 in Hyg/Zeo, and these were then cultured in TAP liquid cultures. Subsequently, we took aliquots from each pool for genomic extraction and purification. We then amplified the synthetic promoter region of the integrated plasmid using PCR, as depicted in the Supporting Information S3, Figure S1. The NGS process yielded approximately 150 and 120 million reads for SAPS1 and SAPS2, respectively. We processed and analyzed this data using the Galaxy platform,[21] obtaining a detailed examination of the promoter distribution within each sample.

Each processed read was matched with its corresponding promoter sequence. We then calculated the relative abundance of each promoter within SAPS1 and SAPS2 under the conditions Hyg and Hyg/Zeo, expressing the results as a percentage (Figure 3). Promoters show an average relative abundance of 0.05% in the initial Hyg library, aligning with expectations for 2000 evenly distributed promoters (Figure 3A). As previously explained, for each library, we divided the relative abundance of each promoter in the Hyg/Zeo condition (Figure 3B) by their relative abundance in the Hyg condition, obtaining the normalized score “HygZeo ratio” (Figure 3C). Using this metric, we ranked the promoters within the SAPS1 and SAPS2 libraries, from highest to lowest, the number 1 promoter being the one with the highest score and thus the one with the highest transcriptional activity (Supporting Information S2).

In Figure 3, each promoter is depicted according to its relative abundance, with the most abundant ones positioned nearest to the graph’s origin. This arrangement, which places promoters in descending order of abundance across the graph, effectively illustrates the distribution patterns of the entire promoter library tested. In the Hyg condition for both libraries (Figure 3A), the distributions display a striking similarity between the mean and

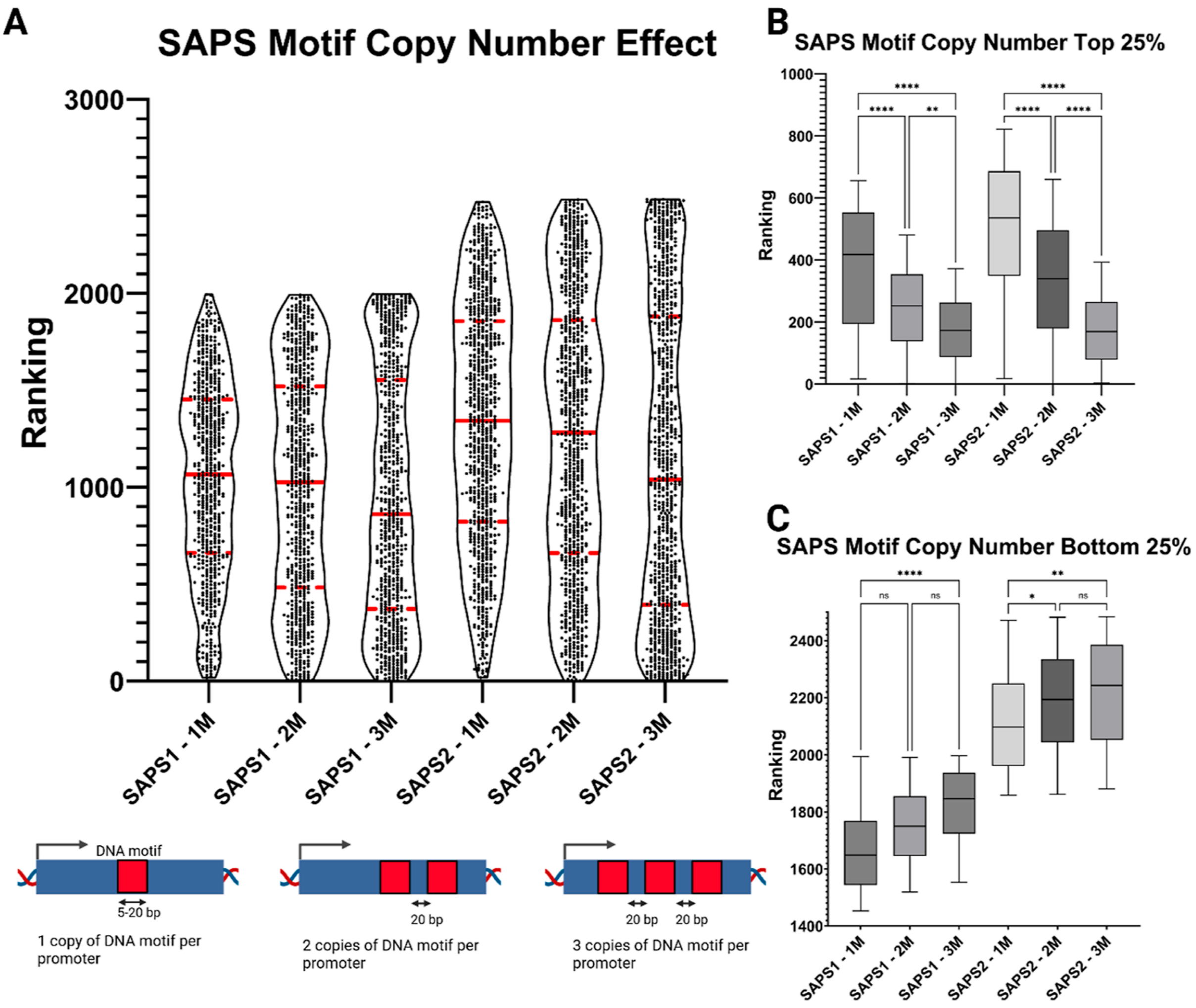

Figure 4. Assessing the impact of the motif copy number on synthetic promoter efficiency. (A) This figure presents a truncated violin plot illustrating synthetic promoters' performance, categorized by motif copy number and library, as measured by their HygZeo ratio rankings. The rankings are based on the HygZeo ratio, with rank 1 indicating the highest ratio. A continuous red line marks the median value, while dashed red lines denote the quartile divisions. (B) Additional insets display the top 25% (upper quartile) and, (C) bottom 25% (lower quartile) of data points in a box-and-whiskers format, employing Tukey's method for whisker calculation to identify outliers. The statistical significance of differences in median values across groups was determined using the Kruskal–Wallis test, followed by posthoc analysis with Dunn's multiple comparisons correction. Significance levels are indicated as follows: * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

median values (SAPS1 mean: 0.049, median: 0.046, std: 0.026; SAPS2 mean: 0.040, median: 0.034, std: 0.028), indicative of a predominantly symmetrical distribution with a minor skewness toward higher values, likely attributable to a limited number of promoters exhibiting notably elevated relative abundances within the physical DNA library. Under the HygZeo condition (Figure 3B), both libraries exhibit a marked departure from their previous symmetrical distribution, with a pronounced shift toward skewness (SAPS1 mean: 0.049, median: 0.004, std: 0.159; SAPS2 mean: 0.040, median: 0.006, std: 0.114). This is characterized by a drastic reduction in the relative abundance of most promoters, while a select few display a substantial increase in their relative abundance. When the relative abundances of each synthetic promoter under the HygZeo condition are normalized by dividing them by their corresponding abundances under the Hyg condition, we obtain the HygZeo ratio (Figure 3C).

Analysis reveals that in both the SAPS1 and SAPS2 libraries, around 80 and 75% of the promoters, respectively, have a HygZeo ratio below 1 which indicates a reduction in their relative abundance when exposed to Zeocin. Moreover, the SPC without added CREs was also present in both libraries, and it ranked 266th out of 2040 in SAPS1 and 712th out of 2493 in SAPS2. This can be observed in Figure 3C, in which the position of the SPC is indicated with a large red dot and a red arrow pointing at it. From these data, it can be assumed that the promoters with a significantly higher HygZeo ratio than that of the SPC contain a functional CRE. Promoters with the highest HygZeo ratio scores were selected that were at least three times the population's standard deviation above the SPC HygZeo ratio. As a result, 37 promoters for SAPS1 and 40 for SAPS2 were identified, each exhibiting a relative abundance increase exceeding 9-fold, over the empty promoter construct (Supporting Information S2). The best performing promoters present a relative abundance increase of 58-fold for SAPS1 and 49-fold for SAPS2, highlighting the dramatic effect that some CREs had on transcriptional activity, and hence Zeocin resistance, on the synthetic promoter that contained them.

The use of scoring of the promoters based on their relative abundance in HygZeo selection compared to Hyg alone has

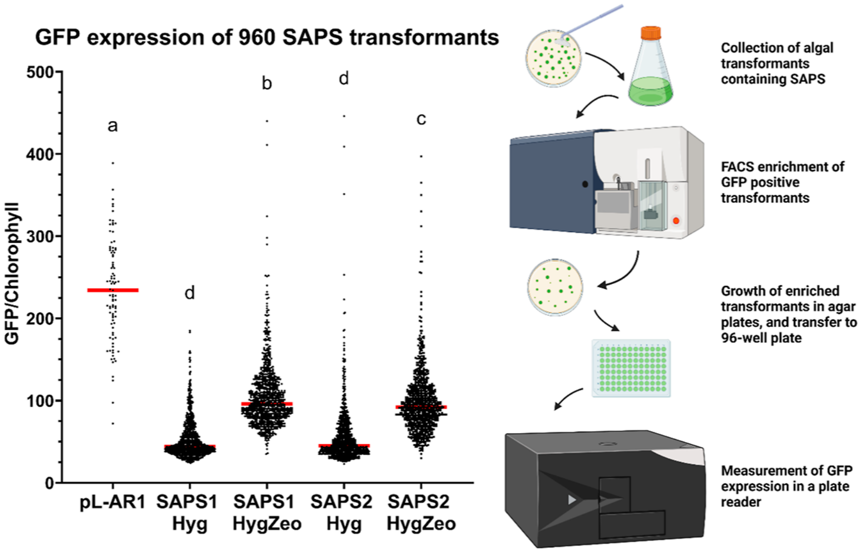

Figure 5. Analysis of the GFP expression of 960 SAPS transformants. The collected transformants for each library and condition were enriched for high GFP expression by sorting the top 5% of GFP events. The cells were plated and inoculated into 96-well plates, allowed to grow for a couple of days and measured in a plate reader. GFP fluorescence, normalized against chlorophyll fluorescence, was measured in a plate reader (excitation: 505/6 nm, emission: 536/20 nm for GFP; excitation: 440/9 nm, emission: 680/20 nm for chlorophyll). Each sample has 960 data points, except for the positive control that contains 96 data points belonging to a clonal strain expressing GFP using the pL-AR1 vector. The statistical significance of differences in median values across groups was determined using the Kruskal−Wallis test, followed by posthoc analysis with Dunn's multiple comparisons correction. Samples annotated with different letters are statistically different from each other, with an adjusted *p*-value below 0.05.

advantages and disadvantages. The advantages are that this analysis allows for simple and fast high throughput screening of hundreds of thousands of individual transformation events and thousands of functional promoters. The disadvantage is that they are susceptible to noise. The first source of noise is the fact that transgenes are randomly integrated into the nuclear genome of *C. reinhardtii*, which allows for the possibility of a synthetic promoter landing in a highly permissible place and overestimating its transcriptional strength, and vice versa. To reduce this noise, we cloned the Hyg resistance cassette upstream from the synthetic promoter and in the opposite DNA chain to avoid readthrough. Another possible source of noise is the fact that a percentage of transgenes integrated into the genome may integrate as truncated vectors. In this case, it would be possible for a promoter to have a damaged *ble* gene but still be counted in the Hyg sequencing sample, which would underestimate its true increase in relative abundance in the HygZeo sample. Additionally, research suggests that a significant percentage of *C. reinhardtii* transformants may contain multiple insertions.[22] This would lead to inaccurate scoring as both promoters present in the transgenic cell would be measured and thus assumed promoter activity could be a combination of their individual transcriptional activities.

We inserted CREs into synthetic promoters in 1, 2, or 3 tandem copies so that we could identify both basic transcriptional activity of a CRE, as well as explore any potential dose-dependent effect. This concept suggests that a greater number of CRE copies could lead to enhanced transcriptional activity, a hypothesis supported by previous research.[23] Analyzing the ranked synthetic promoters from each SAPS library, differentiated by the number of CRE copies, revealed a notable trend (Figure 4A). Promoters containing a single CRE tended to cluster around the distribution median, indicating a moderate transcriptional activity. In contrast, promoters with two CRE copies demonstrated a broader distribution, with transcriptional activity deviating from the median, both upward and downward. This divergence was even more pronounced in promoters with three CRE copies (Figure 4A). When the top 25% of promoters from each library were isolated and separated by copy numbers, a significant difference in the means of their distributions was observed (Figure 4B). As the copy number of CREs increased, a reduction in the mean of these populations was noted, indicating that promoters with a higher number of CRE copies generally exhibit enhanced transcriptional activity. Conversely, analysis of the bottom 25% ranked promoters revealed an increase in the mean of this population with rising copy numbers, suggesting that certain CREs may serve as repressor binding sites, where an increase in their copy number results in diminished promoter transcriptional activity (Figure 4C).

These findings suggest that our initial hypothesis is likely correct in that increases in copy number of individual CREs either enhance (transcriptional activation) or diminish (transcriptional repression) the transcriptional activity of the synthetic promoter in which they are inserted. The proximity of the CRE to the TSS is recognized as critically important in native promoters.[23] It is conceivable that the optimal activity of the CRE is due to the positioning of the third motif copy at −132 base pairs rather than the presence of additional CRE copies. The extensive range of CREs evaluated is anticipated to counterbalance the impact of this confounding variable. Although the positional effect likely influences some promoters, it is improbable that optimal performance across all CREs would occur at the −132 bp site. The insertion sites for all CREs were kept constant to limit experimental variables despite the acknowledged significance of their positions. Accounting for variable positions would have increased the number of synthetic promoters to test to an unmanageable number.

2.4. Selection of Algal Transformants Containing Synthetic Promoters Showing High GFP Expression as Measured via FACS.

To validate the findings obtained from our high throughput promoter scoring system, we decided to evaluate the effectiveness of the synthetic promoters based on their ability to drive the expression of GFP. We identified several hundred transformants from each library harboring a synthetic promoter able to drive high levels of GFP. We then sequenced those individual transgenic lines to identify the sequence of the promoters and compared those results to the ones obtained through the HygZeo ratio.

The algal transformants that harbored either the SAPS1 or SAPS2 libraries and were selected on Hyg or Hyg/Zeo, were subsequently combined in liquid cultures. From each pool of transformants, we isolated the top 5% of GFP expressors using FACS, and then plated those cells on Hyg or Hyg/Zeo plates. After the cells grew to form colonies, we picked 960 colonies, from each library and condition, into ten 96-well plates containing TAP media and allowed them to grow for several days. Then, we measured their GFP signal in a plate reader, which we normalized by dividing it over their chlorophyll fluorescence as a measure of general cell growth.

In Figure 5, we show the normalized GFP signals compared to 96 clones from a strain producing GFP driven by the strong native AR1 promoter. From the figure, we can observe that upon selection of SAPS solely on Hyg, significant GFP expression is observed in some cases, while most transformants exhibit minimal, if any, GFP expression. Upon selection of the libraries on Hyg/Zeo, both libraries showed a significant shift toward higher GFP expression with certain transformants exhibiting GFP expression levels that surpassed those of the native AR1 promoter. This observation aligns with our expectations and corroborates the findings presented in Figure 3, which indicated that while most transformants harboring a SAPS promoter lack a highly functional promoter, a small subset possesses promoters with substantially enhanced transcriptional activity.

960 individual transformants were measured on a plate reader for GFP expression, and several of the best GFP expressing transformants of SAPS1 and SAPS2 from HygZeo selection were individually sequenced to identify the synthetic promoter responsible for the high GFP expression. This resulted in a total of 115 SAPS1 and 254 SAPS2 transformants being sequenced, with promoters' sequences being overrepresented in those with elevated HygZeo scores (Supporting Information S2). Out of those, 20 of the transformants sequenced for SAPS1 and 32 for SAPS2 belong to promoters with a HygZeo ratio of 3 standard deviations above the mean. The frequency at which promoters with such a HygZeo ratio appear in high GFP sequenced individuals is over 9-fold and over 8-fold higher than would be expected from a random selection of promoters. These findings validate the HygZeo scoring shown in Figure 3 and provide us with a list of synthetic promoters containing predicted CREs shown to be able to drive the gene ble and GFP to high levels. In Table 1, we present a list of such synthetic promoters, described by the CRE they contain and the number of copies.

We must note that the Sanger sequencing of the synthetic promoters within individual algal clones allowed us to identify the rate of multiple insertions, a risk factor that could greatly impact the conclusions of our synthetic promoter screening. Each synthetic promoter consists of both common and unique segments, where the different CREs are located. In single promoter insertions, the chromatogram shows distinct and clear peaks for each base pair throughout the entirety of the sequence. Meanwhile, in multiple promoter insertions, the chromatogram shows clear and distinct peaks in the common sequences and overlapping peaks at the motif locations. Analysis of the sequenced promoters indicated that approximately 3.7% had multiple insertions, which we did not expect to have a major impact on our results.

Table 1. Validated Synthetic Promoters Containing Predicted CREs[a]

SAPS ranking	CRE copy number	DNA sequence	SAPS HygZeo ratio
		SAPS1 Promoters	
3	**3**	**ACGCAAAG**	**21.81**
4	**3**	**TCCAAATA**	**21.29**
7	**2**	**GAAAGCAGGG**	**19.10**
8	2	AGCTGCAC	18.82
11	**2**	**CCAGCAAA**	**17.63**
12	3	AAGCACAA	16.71
14	3	TCGCATGG	15.73
16	**3**	**AAGCCAAA**	**15.06**
20	3	AGAGCAGC	13.12
23	2	TGCATGGG	12.67
28	3	GCGAGCAGG	11.59
31	**3**	**CGCACAAG**	**10.57**
36	3	GCGAGCAG	9.91
		SAPS2 Promoters	
2	**2**	**GCGAAGGCGA**	**39.60**
5	**2**	**CCTAGCAG**	**32.30**
6	**3**	**TCAAACACA**	**23.45**
8	2	AGCGATCG	21.58
11	**3**	**AGCGATCG**	**19.86**
12	3	AACGTTCTTGAT	17.64
14	3	AAGAGGAGGTG	17.03
16	**3**	**AGCTTCATTCTC**	**16.37**
19	3	CTTTAAGACGTT	14.38
22	3	CATACGCGAAAGC	12.94
25	3	ATCGCGGA	11.97
28	3	ACGAGCCAACG	10.87
37	3	AATTAGCTGCAT	9.62
38	3	CGAAGCTCTCAT	9.35

[a] This table shows synthetic promoters that showed a HygZeo ratio higher than three standard deviations above the SPC and were identified in individually sequenced high GFP expressors. Highlighted in bold are promoters that were individually tested, with the results shown in Figure 7.

We observed significant differences in the performance of the synthetic promoters from SAPS1 and SAPS2. At first, it appears that the synthetic promoters from SAPS2 were generally more effective than those from SAPS1. This can be observed in the percentage of colonies obtained under Hyg selection compared to those under HygZeo selection. For SAPS1, only 0.47% of the colonies survived in HygZeo compared to those that survived in Hyg alone, and that number increased to 0.86% in the case of SAPS2. This difference in performance can also be seen in Figure 2, where 266 out of 2040 SAPS1 show a higher HygZeo ratio than the SPC compared to the 712 out of 2483 SAPS2. Moreover, in Figure 4 it can be observed that the effect exerted on the transcriptional activity of the promoters by the number of CREs inserted is steeper in SAPS2. All of these indicate that the SAP2 library has more functional CREs than the SAP1 library. However, when we measured the GFP expression of the libraries, SAPS1 promoters appeared to perform slightly better. This can be seen in Figure 5, where the overall GFP expression of the transformants tested for SAPS1 HygZeo was slightly superior to that of SAPS2 HygZeo. Thus, it seems the SAPS2 library has more functional CREs than the SAPS1 library but that the SAPS1 library has CREs that outperforms SAPS2 CREs when GFP expression is measured.

Selected transformants, one from SAPS1 and two from SAPS2, were subjected to imaging using a fluorescence microscope and compared with a GFP expressing clone containing the positive control promoter AR1. As shown in Figure 6, the cells exhibit red autofluorescence attributed to chlorophyll, while GFP, which is localized to the nucleus as a GFP/Ble fusion protein, can be visualized as a small green dot in the middle of the cells. The observed phenotypes in a selected subset of clones containing SAPS indicate that while AR1 promotes GFP expression in a robust and uniform manner, these specific SAPS clones drive GFP expression at higher levels but with notable heterogeneity and induce a stressed phenotype, evidenced by cell enlargement in the affected cells. This stress may be linked to the significant accumulation of recombinant GFP within the nucleus, although such observations are not necessarily representative of all SAPS-containing transformants.

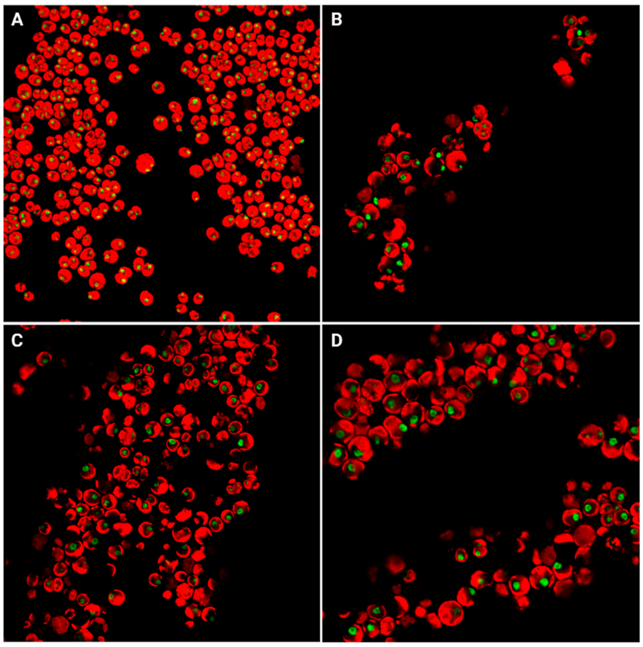

Figure 6. Differential GFP expression in synthetic promoter transformants via fluorescence microscopy. This image displays GFP levels in clonal cells, each with distinct promoters: (A) pL-AR1, (B) SAPS1 example, (C,D) SAPS2 examples. Captured on a Leica DMi8 Inverted confocal microscope at 400× optical magnification with 0.75× digital zoom. Red indicates chlorophyll autofluorescence (excitation of 555 nm, emission of 617 nm); green signifies GFP (excitation of 484 nm, emission of 525 nm).

2.5. Comparison of Transcriptional Activity of Individual Synthetic Promoters as Measured by GFP Expression.

To further validate our findings and accurately quantify the transcriptional activity of novel synthetic promoters, individual transformants with synthetic algal promoters were subjected to further analysis. A selection of 14 synthetic promoters was made based on a combined assessment of those exhibiting a Hyg/Zeo ratio significantly above that of the SPC and the data from individually sequenced diverse synthetic promoters, highlighting a strategic integration of both data sets to identify promoters with varied anticipated transcriptional strengths. Those promoter constructs were individually transformed into *C. reinhardtii*, and their transcriptional activity was assessed by their GFP expression quantified through FACS of a population of transgenic lines from the single promoter. We also measured their transformation efficiency as a metric for transcriptional activity given that others have used this metric to evaluate the utility of a promoter in vivo[11] and the fact that our HygZeo ratio can be thought of as a relative transformation efficiency score.

In Figure 7A, we can see the transformation efficiencies of all 14 SAPS compared to the two control promoters: a vector with the same design as those carrying synthetic promoters but containing only the SPC without motifs inserted (pL0), and an identically designed vector carrying the native AR1 promoter (pL-AR1). As shown, pL0 shows minimal transcriptional activity but is still able to generate colonies under Zeocin selection, with a transformation efficiency of approximately 57 colony forming units (cfu) per μg of DNA. In contrast, well-characterized and robust promoter AR1 showed a transformation efficiency of approximately 3000 cfu/μg of DNA under the same conditions. Comparatively, the SAPS chosen for this experiment show transformation efficiencies ranging from approximately 500 to 3000 cfu/μg of DNA. This data suggests that in terms of transformation efficiencies, some of the SAPS are as good as AR1, a strong chimeric endogenous promoter. As a direct comparison with pL0, we see that its transformation efficiency is improved by 53-fold with the addition of 3 tandem copies of the motif AGCTTCATTCTC. Other notable examples include the addition of 3 tandem copies of motifs TCCAAATA, ACGCAAAG and AGCGATCG, where the transformation efficiency was increased 41, 35 and 30-fold, respectively. All of the SAPS tested yielded superior transformation efficiencies compared to pL0, except for pL1_70_1, which contained a single copy of the motif GGTACGGC.

In Figure 7B, the transcriptional activity of individual promoters, as measured by GFP expression in the top 1000 registered events during FACS analysis, is depicted. It is observed that pL0 exhibits minimal GFP activity, with a median GFP expression marginally above the background, as evidenced by a comparison with the wild-type sample. All SAPS displayed a median GFP expression exceeding that of pL0, with improvements ranging from 1.4-fold to 9.2-fold (after background subtraction). pL-AR1 was observed to have a median GFP expression 8.2-fold higher than that of pL0. Among the SAPS evaluated, five exhibited GFP expression levels comparable to or in one case surpassing those of AR1. The SAPS, along with the CREs they contain, listed in descending order of GFP expression, are as follows: pL1_7_2 with 2 copies of GAAAGCAGGG, pL2_5_2 with 2 copies of CCTAGCAG, pL1_3_3 with 3 copies of ACGCAAAG, pL2_6_3 with 3 copies of TCAAACACA, and pL1_67_1 with 1 copy of GGCGAGCA. It was observed that the top two SAPS tested harbored an identical DNA sequence within their CREs, specifically the motif AGCAG, with both containing two copies. Additionally, pL1_67_1 possesses the motif AGCA that becomes an AGCAG due to the occurrence of a G immediately downstream from its insertion site. This notable occurrence suggests that the AGCAG motif may function as a transcription factor binding site.

2.6. Cis-Regulatory Element Placement within Highly Expressed Genes in *C. reinhardtii*.

After identifying functional CREs that enhance gene expression when inserted into a synthetic promoter (Table 1), we analyzed their placement in promoters of highly and low expressed (control) genes in the *C. reinhardtii* genome, excluding those with introns

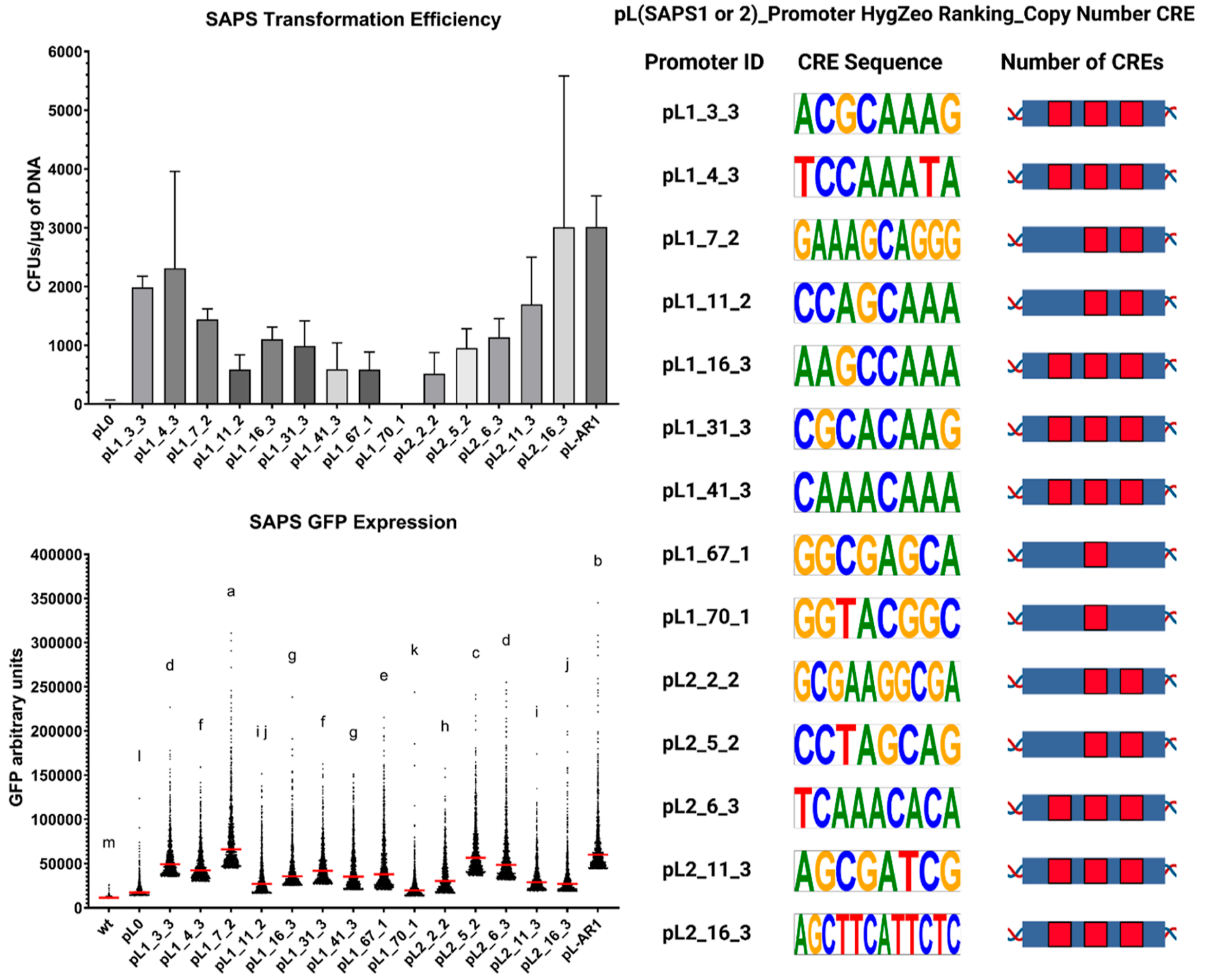

Figure 7. Evaluation of individual synthetic promoters. Top panel: transformation efficiency of synthetic promoters, measured in CFUs using 1 μg of DNA for each, performed in triplicates, with pL0 and pL-AR1 as reference promoters. Bottom panel: GFP expression analyzed via FACS, pooling colonies from three transformations, showcasing the top 1000 GFP events per promoter, including wild-type (wt) cells for comparison. Statistical analysis was conducted with the Kruskal−Wallis test and Dunn's posthoc correction; promoters with differing letters signify statistically significant differences (adjusted p-value <0.05). The promoter IDs structure is specified on the top right part of the figure: the first indicates the SAPS origin (1 or 2), the second number represents the HygZeo ratio ranking within their library, and the third number specifies the CRE copy number for that promoter.

in their 5′ UTRs. Results showed that highly expressed promoters had an average of 0.84 CRE occurrences per gene, compared to 0.35 in lowly expressed promoters and 0.23 in random sequences. This indicates a 3.7-fold and 1.6-fold higher CRE representation in highly and control expressed genes, respectively. Allowing one mismatch in the CRE increased the occurrence of these CREs in promoters but reduced over-representation in the highly expressed genes. Visualization of CRE distribution relative to the TSS revealed no significant differences beyond noted overrepresentation (Supporting Information S3, Figures S3−S6). Two-thirds of the CRE occurrences were duplicated within the same promoter. Some transcription factors are known to bind as homodimers. Applying this constraint, we found 0.42 potential homodimer binding sites per gene in highly expressed genes, 0.29 in lowly expressed genes, and 0.07 in random sequences. This suggests a significant role for CRE homodimers in gene transcription in *C. reinhardtii*.

No distinct patterns in CRE positioning relative to the TSS were observed without allowing for mismatches, except for the element TGCATGGG, which clustered between −40 and −80 of the TSS, corroborating findings by Scranton et al.[10] (Supporting Information S3, Figures S7 and S8). With one mismatch allowed, motifs ATCGCGGA, TCGCATGG, and AGCTGCAC all showed some clustering in the −40 to −80 region, and motifs TGCATGGG, AAGCCAAA, and TCGCATGG clustered in the +40 to +80 region. These patterns suggest specific binding locations for these CREs, yet the evidence presented here is not conclusive. Further research will be needed to elucidate the mechanisms behind CRE-mediated gene regulation in algae, considering consensus sequences, positioning relative to the TSS, transcription factor interactions, chromatin state, and environmental factors, before we can fully understand the complexities of transcription regulation in *C. reinhardtii*.

3. CONCLUSIONS

We now have a functional list of CREs that are available to build synthetic promoters for any green algae species. The natural next

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step is to combine different CREs in different combinations to explore potential synergistic effects and determine how far transgene expression can be pushed using these synthetic promoters. As an additional next step, we will test these CREs to identify those that increase gene expression under constant light and constant dark for *C. reinhardtii*, but this same procedure could be used to test any number of different growth conditions. In this way, CREs that respond to different environmental cues could be engineered into a single promoter to finely tune gene regulation in *C. reinhardtii* and in any other algae species in which this strategy is deployed.

Advances in synthetic biology tools can be used to engineer cells to more efficiently produce a desired bioproduct, but this information can also be used to obtain a complete understanding of how the molecular machinery of the cell interacts with genetic information to yield different phenotypes. As the physicist Richard Feynman said, "What I cannot create, I do not understand", we strive to grasp how genetic regulation works in microalgae by creating new genetic tools. In this work we strove to generate a comprehensive list of putative CREs that could then be used to create synthetic promoters of multiple functions, and we believe the list here is a very good start to building tools necessary to take algae biotechnology to a new level.

4. METHODS

4.1. Bioinformatic Analysis and Synthetic Promoter Chassis Design. All genome and annotation data were sourced from the NCBI.[24] The data to determine the highest expressed genes can be found at the NCBI Gene Expression Omnibus repository under accession number GSE71469 (GSE71469_ChlamydomonasSynchronousDiurnalExpressionRPKM.txt.gz).[25] To ensure the binding sites were still functional in short vectors and to include all core promoter elements, which are known to exist 200 bp upstream of the TSS. The promoter length was approximated to be 500 bp upstream of the translational start site. Furthermore, given its more reliable annotation, the translational start site was chosen instead of the TSS as the reference location. Using the aforementioned transcriptomic data set, we determined the top 200 highest expressed nuclear genes in *C. reinhardtii* based on the average gene expression over a diurnal cycle. Then we searched for orthologues in the highest 500 expressed genes in *C. pacifica*, using transcriptomic data not yet published from our lab, and in all the genes in *C. incerta* due to lack of transcriptomic data. Genes that did not have an ortholog in either data set were discarded, yielding 146 genes. These are named the top 146 highest expressed genes in *C. reinhardtii* for ease of notation but are a subset of the top 200 highest expressed genes.

4.1.1. SAPS1 CRE Prediction. Two algorithms were used to predict the CREs in SAPS1: POWRS and STREME.[26] As input sequences, the promoter sequences of the top 146 highest expressed genes in *C. reinhardtii* were selected, based on its diurnal transcriptome. The lowest 146 expressed genes were used as control sequences (Supporting Information S1). We used Reciprocal Best Hit and BLASTN (*e*-value $<10^{-5}$) algorithms[27] to identify orthologues of the selected genes in *C. incerta* and *C. pacifica* (Supporting Information S1). The latest species is a novel extremophile green alga from the genus Chlamydomonas that was isolated at the University of California San Diego campus. In total, the number of orthologs for the 146 highly expressed genes from *C. reinhardtii* were: 146 genes in *C. pacifica* and 158 genes in *C. incerta*. For the lowest expressed genes, 116 ortholog genes were found in *C. pacifica*. The algorithm POWRS was run with parameters of "min_genes" 120, "window_width" 8, "cluster_limit" 800, and "seed_size" 8. STREME was executed using the parameters "minw" 6 and "maxw" 15, and only motifs that were 8 base pairs or more identical between at least two of the following species: *C. reinhardtii*, *C. incerta*, or *C. pacifica*, were chosen. In both cases, only the motifs that showed a *p*-value smaller than 1×10^{-2} were selected. This resulted in the prediction of 680 CREs (Supporting Information S1).

4.1.2. SAPS2 CRE Prediction. The algorithm PhyloGibbs[28] was used to predict the CREs in SAPS2. The top 146 highest expressed genes in *C. reinhardtii* were used as input sequences, as well as the orthologue genes for the highest 146 from *C. reinhardtii* found in the species *Edaphoclamys debaryana* (90 genes), *C. schloesseri* (132 genes), *C. incerta* (158 genes), *V. carteri* (4 genes) and *Gonium pectorale* (92 genes) (Supporting Information S1). For each gene in *C. reinhardtii*, the orthologue sequences from all species were passed to the PhyloGibbs algorithm after performing multiple sequence alignments. Notably, each gene had at least one orthologue in another species. DiAlign[29] was utilized, following the parameter guidelines recommended by Siddharthan et al.,[28] for deducing the multiple sequence alignments of the promoter regions across the top 146 genes in *C. reinhardtii* and their orthologs. The phylogenetic relationships among these species were elucidated using Benchmarking Universal Single-Copy Orthologs (BUSCO) genes.[30] For constructing the phylogenetic tree, multiple sequence alignments were conducted with MUSCLE,[31] gene trees were inferred via CLUSTALW,[32] and the species tree was constructed using ASTRAL III.[33] Since species information is already incorporated in the algorithm, we only filtered motifs based on the *p*-value, with a cutoff of 10^{-4}. This methodology resulted in the identification of 831 CREs.

4.1.3. Synthetic Promoter Chassis. The synthetic promoter chassis (SPC), a 200 bp sequence on which CREs are embedded, was designed in-silico using a random sequence generator based on probabilities derived from Scranton et al.[10] The first 60 bp have probabilities for ACGT of [0.2, 0.25, 0.35, 0.2], while the next 140 bp have probabilities of [0.28, 0.24, 0.2, 0.28]. After the sequence was generated, if any of the predicted CREs in SAPS1 and SAPS2 were present, the SPC sequence was regenerated to exclude the CRE. This process ensures SPC closely resembles the native core promoter of *C. reinhardtii* while avoiding interference from CREs during testing.

4.2. Expression Vector Design. The expression vector is designed to have two open reading frames in opposite directions. One ORF contains a Hygromycin resistance cassette composed of: the *C. reinhardtii* *β*2-tubulin promoter from −1 to −199 (both positions included), the 113 bp of the 5′ UTR of the same gene, the APH7 gene which confers *C. reinhardtii* with hygromycin resistance, and 455 bp of the 3′ UTR of the *β*2-Tubulin gene. The other ORF contains a zeocin resistance gene fused to the fluorescent protein GFP mclover. A promoter being tested, described below, is placed upstream of the 23 bp 5′ UTR of the *C. reinhardtii* *rbcs2* gene followed by the coding region, and ending with the 234 bp of the 3′ UTR of the *C. reinhardtii* *rbcs2* gene. The coding region, from the start to stop codon, contains 1563 bp and is composed of several parts. There are two introns, rbcs2 intron 1 (positions 169...313) and 2 (positions 927...1255). The first protein is the CDS of the *ble* gene, codon optimized for the nuclear genome of *C. reinhardtii*, which confers resistance against the antibiotic zeocin. The second protein is the GFP variant mclover, which was also codon optimized. Finally, there is the 8 DYKDDDDK amino acid tag at the C-terminus.

The promoters used to drive the *ble*-GFP fusion were the AR1 promoter[34] in the case of pLibrary-AR1 (pL-AR1), or the SPC previously described in the case of pLibrary-0 (pL0). Any tested SAPS were used as promoters replacing the SPC. Sequences fully annotated for the vectors pL0 and pL-AR1 can be found in Supporting Information S1. The sequence of all the SAPS1 and SAPS2 assembled are also included in the same file. The strain and annotated plasmid sequence for pL0, were deposited in the public instance of the Agile BioFoundry Inventory of Composable Elements (ICE)[35] Registry (https://public-registry.agilebiofoundry.org/folders/40) and are physically available upon reasonable request.

4.3. Promoter Library Synthesis and Assembly into the Vector.

4.3.1. Promoter Library Synthesis and Assembly into Vector. Promoter libraries SAPS1 and SAPS2 were synthesized by Twist Bioscience (South San Francisco, CA), containing 2040 and 2493 variants, respectively. The sequences of all the SAPS designed can be found in Supporting Information S1. In brief, assembly of the promoter libraries into the pL0 vector was achieved by using Gibson Assembly, reactions with PCR-amplified promoter libraries and pL0 backbone containing Gibson overhangs designed by j5[36] (https://j5.jbei.org/). Following assembly, each library was transformed into *E. coli* via electroporation and subsequently extracted for downstream steps. Details of these steps are described below. Unless otherwise specified, all primers were synthesized by Integrative DNA Technologies (Coralville, IA), and all PCR reactions were set up with Q5Master Mix (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol.

Promoter libraries SAPS1 and SAPS2 were amplified with j5-designed primers "barcode_pool_F" (5′-TGCTGGAAGTGTCATAGCGCAAGAAAGNNNNNNNNNGTCCTTCCCGGGCCAGGC-3′) and "barcode_pool_R" (5′-TCTCTTGTAAAAAAGTAGTTGAGGATCCCCCACTTATTGCG-3′), and the following thermal cycler conditions: 98 °C for 3 min, 10 cycles of 98 °C for 20 s, 63 °C for 30 s, 72 °C for 40 s, and a final extension of 72 °C for 3 min. SAPS1 and SAPS2 were amplified with 10 PCR cycles to limit the amplification bias. The pL0 backbone was amplified using j5-designed primers "backbone_F" (5′-TGGGGGATCCTCAACTACTTTTTTACAAGAGAAGTCACTCAACATC-3′) and "backbone_R" (5′-TTTCTTGCGCTATGACACTTCCAGC-3′), using thermal cycler conditions consisting of 98 °C for 3 min, 25 cycles of 98 °C for 15 s, 63.5 °C for 30 s, 72 °C for 3.5 min, and a final extension of 5 min at 72 °C. Following amplification, the backbone PCR reaction was digested overnight with DpnI (New England Biolabs, Ipswich, MA) at 37 °C. All amplicons were then verified by gel electrophoresis and purified using a Wizard Gel Purification kit (Promega, Madison, WI).

Gibson assemblies of each promoter library were executed using NEBuilder Hifi Assembly Master Mix (New England Biolabs, Ipswich, MA) following the manufacturer's protocol. Assemblies were then transformed into MegaX DH10β T1R Electrocomp Cells (Thermo Fisher Scientific, Waltham, MA), in 1 mm sterile cuvettes (VWR, Radnor, PA) with a Gene Pulser Xcell (Bio-Rad Laboratories Inc., Hercules, CA) at 2.0 kV, 200 Ω, and 25 μF. For each promoter library, four electroporation reactions were executed in parallel to increase the diversity of promoters transformed. The four reactions were pooled following recovery and plated on Bioassay Qtrays (Molecular Devices, San Jose, CA) containing 250 mL of 100 μg/mL Carbenicillin LB agar. After 18 h of growth at 37 °C, each promoter library's Qtray was scrapped into 35 mL of 100 μg/mL Carbenicillin LB media using cell scrapers (Sarstedt, Newton, NC), and extracted using a Midiprep kit (Qiagen, Hilden, Germany).

4.3.2. Library Preparation and QC. To verify the assemblies and assess the distribution of promoter variants, promoter regions of each promoter plasmid library were amplified and ligated to sequencing adapters and then purified and sequenced using Next Generation Sequencing (NGS). This process is described below. Promoter regions of each plasmid library were amplified with primers "screen_F" (5′-GGAAGTGTCATAGCGCAAGAA-3′) and "screen_R" (5′-AGATGTTGAGTGACTTCTCTTGTAA-3′), and thermal cycler conditions consisting of 98 °C for 30 s, 7 cycles of 98 °C for 10 s, 54.8 °C for 30 s, 72 °C for 40 s, and a final extension of 72 °C for 1 min. The promoter region was amplified with 7 PCR cycles to limit amplification bias. Following verification with gel electrophoresis, amplicons were purified using a Wizard Gel Purification kit. Amplicons were then A-Tailed and ligated to xGen-UDI-UMI Adapters (Integrative DNA Technologies, Coralville, IA) using a KAPA HyperPrep PCR-Free kit (Roche, Basel Switzerland) according to the manufacturer's protocol. The resulting libraries were purified twice at 0.9× using Ampure XP Beads (Beckman Coulter, Indianapolis IN), and quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and Qubit dsDNA High Sensitivity kit (Thermo Fisher Scientific, Waltham, MA). One nanogram of each library was then run on a Bioanalyzer 2100 (Agilent, Santa Clara, CA) with a High Sensitivity DNA Kit (Agilent, Santa Clara, CA) to assess quality and adapter ligation.

Each library was then sequenced on a Miseq (Illumina, San Diego, CA) using a MiSeq Reagent Kit v2 (500 cycles) with paired-end sequencing. Each library's R1 and R2 reads were then interleaved into one FASTQ using BBTools reformat.[37] Lastly, sequencing data was analyzed by synbioqc-libqc[38] to verify promoter pool assembly and quantify promoter variant diversity per promoter pool. Histograms visualizing the distribution of promoter variant counts per library are shown in Supporting Information S3 and Figure S2.

4.4. Algal Strain Used and Culture Conditions.

The algal strain used to perform this study was *C. reinhardtii* cc-1690. The algae was cultured in TAP media,[39] grown under continuous light with a photosynthetically active radiation of 125 μE/m^2/s, on a shaker table rotating at 125 rpm, and a constant temperature of 25 °C.

4.5. Algal Transformation and Selection.

The plasmid DNA was subjected to double digestion using *Kpn*I and XbaI enzymes (New England Biolabs, Ipswich, MA, USA), followed by purification with the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI, USA), without fragment separation. DNA concentration was quantified using the Qubit dsDNA High Sensitivity Kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA amount for each transformation was adjusted to ensure 1 μg of payload DNA, based on the payload DNA/backbone DNA ratio. Transformation is performed through electroporation as described in this study.[40]

Transformant selection occurred on two types of plates. For library transformations, resuspensions of 1 mL in TAP were divided: 100 μL onto TAP agar with 30 μg/mL Hygromycin (Hyg plates) and 900 μL onto TAP agar with 30 μg/mL Hygromycin plus 15 μg/mL Zeocin (HygZeo plates). Individual promoter transformations involved plating the entire 1 mL resuspension on HygZeo plates.

4.6. Next-Generation-Sequencing of the Algal Libraries.

4.6.1. Genome Extraction. Genomic DNA was extracted from algal cells using SDS DNA extraction buffer (1% SDS, 20 mM Tris−HCl pH 8.0, 2 mM Sodium EDTA pH 8.0, and 200 mM NaCl). Approximately 50 μL of packed cells from late-log phase culture were lysed in 1625 μL of this buffer at 65 °C. The lysate was then subjected to extraction with an equal volume of phenol/chloroform/isoamyl Alcohol pH 8.0 and centrifuged at 5000 rcf for 4 min. The aqueous phase was washed twice with 1875 μL of ethanol-stabilized chloroform and once with an equal volume of chloroform. DNA was precipitated from the final aqueous phase by adding an equal volume (750 μL) of isopropanol, followed by centrifugation at 16,000 rcf for 30 min at 4 °C. The DNA pellet was washed thrice with 70% ethanol, air-dried, and resuspended in 150 μL of 1× TE pH 8.0. Quantification was performed by using a Qubit HS DNA kit, yielding concentrations suitable for use in Q5 PCR reactions.

4.6.2. Library Amplification. The libraries were prepared for sequencing by PCR using the extracted genomic DNA as a template. The PCR protocol involved two amplification steps using New England Biolabs (NEB, Ipswich, MA, USA) reagents. In the first PCR, 500 ng of DNA genomic DNA was amplified over 13 cycles using a master mix containing Q5 Phusion Buffer, GC Enhancer, primers, dNTPs, and Q5 Polymerase from NEB, followed by gel purification. The primers used for this round were forward primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGgctggaagtgtcatagcgcaag and reverse primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGcccacttattgcgagactgggc.

The second PCR, with 20 cycles, utilized 5 ng of the purified amplicon from the first round of PCR per reaction with a similar master mix composition. This round included the addition of barcodes for posterior sequencing deconvolution. The primers used were:

SAPS1 Hyg forward: AATGATACGGCGACCACCGAGATCTACACTCGTGGAGCGTCGTCGGCAGCGTC.

SAPS1 Hyg reverse: CAAGCAGAAGACGGCATACGAGATCGCTCAGTTCGTCTCGTGGGCTCGG.

SAPS1 HygZeo forward: AATGATACGGCGACCACCGAGATCTACACTATAGTAGCTTCGTCGGCAGCGTC.

SAPS1 HygZeo reverse: CAAGCAGAAGACGGCATACGAGATATATGAGACGGTCTCGTGGGCTCGG.

SAPS2 Hyg forward: AATGATACGGCGACCACCGAGATCTACACCTACAAGATATCGTCGGCAGCGTC.

SAPS2 Hyg reverse: CAAGCAGAAGACGGCATACGAGATTATCTGACCTGTCTCGTGGGCTCGG.

SAPS2 HygZeo forward: AATGATACGGCGACCACCGAGATCTACACTGCCTGGTGGTCGTCGGCAGCGTC.

SAPS2 HygZeo reverse: CAAGCAGAAGACGGCATACGAGATCTTATGGAATGTCTCGTGGGCTCGG.

Postamplification, the products were cleaned up and diluted to a uniform concentration of 80 ng/μL, and 25 μL of each were pooled, ensuring equal molar ratios for subsequent steps.

4.6.3. Next-Generation Sequencing. For the sequencing analysis, we utilized the services of Azenta (Chelmsford, Massachusetts, USA). Our sample was composed of equimolar parts of our four libraries as previously described. The libraries were designed to be compatible with Illumina's sequencing platform, including standard Illumina adapter sequences, primer binding sites for both Read 1 and Read 2, as well as dual index sequences with a length of 10 base pairs each for sample deconvolution. We employed a sequencing configuration of 2 × 150 bp with 150 million reads, which fully covered our 222 bp amplicon and allowed 78 bp of overlapping sequences in the middle, and the read depth was more than enough to guarantee full coverage of our library.

4.6.4. Sequence Data Processing. For NGS data analysis we employed a sophisticated Galaxy[21] workflow for processing and analyzing sequencing data. The workflow began with the processing of paired-end raw reads, followed by adapter trimming and quality control measures. Next, the reads were merged based on overlap criteria. This step was crucial for reconstructing the complete sequence of our amplicons. Subsequently, specific genomic regions, namely, promoters, were extracted from these merged reads. The extracted sequences were then aligned to a promoter database, facilitating the identification of promoter regions in our sample. Postalignment, the data was converted into a suitable format for further analysis. The final steps of the workflow focused on extracting and sorting relevant read and sequence identifiers, culminating in a count of distinct reads and barcodes. This workflow enabled a comprehensive sequencing data analysis, ensuring accurate identification and quantification of genomic elements of interest. The workflow can be visited at the following link: https://usegalaxy.eu/u/yasintorres/w/synthetic-algal-promoter-library---count-analysis.

4.6.5. CRE Abundance Analysis. Each synthetic promoter was correlated to its corresponding read count. The total reads for each library were summed to determine the library's total reads. Relative abundance of each promoter (SAPS1 Hyg, SAPS1 HygZeo, SAPS2 Hyg, and SAPS2 HygZeo) in the library was calculated by dividing the individual promoter reads by the total library reads. Finally, to obtain the HygZeo ratio, the relative abundance of each promoter under the HygZeo condition was divided by its abundance under the Hyg condition, for both SAPS1 and SAPS2. Spreadsheets with the calculations can be found in the Supporting Information S2. The results obtained were graphically represented by using GraphPad Prism (GraphPad Software, San Diego, California, USA).

4.7. In Vivo CRE Activity Based on Fluorescence Activated Cell Sorting.

In this study, we utilized Fluorescence-Activated Cell Sorting to analyze GFP expression in transformed *C. reinhardtii* cells in a similar manner as described in Sproles et al.[20] The analysis was performed using a Sony MA900 Cell Sorter with 405, 488, 638, and 561 nm lasers. Our gating strategy focused on cell size and chlorophyll autofluorescence, an excitation laser at 488 nm, and an emission filter at 695/50 nm to isolate single cells. We sorted and analyzed the top 5% of cells with the highest GFP expression, excitation laser, 488 nm, and emission filter, 525/50 nm. Data for 500,000 events per sample were collected in FCS files and then analyzed using FlowJo (FlowJo LLC, Ashland, Oregon, USA). The top 1000 GFP data points per sample were extracted, and graphs were generated using GraphPad Prism (GraphPad Software, San Diego, California, USA).

The cells were collected on 15 mL conical tubes containing 1 mL of TAP media. After sorting, the cells were plated onto HygZeo plates, and colonies were visible 3−5 days after.

4.8. Fluorescent Confocal Microscopy.

High-resolution confocal imaging was performed at the UCSD Microscopy Core using a Leica DMi8 Inverted Confocal Microscope (Leica Microsystems, Wetzlar, Germany). Samples were illuminated with a tunable White Light Laser, with excitation and emission wavelengths selected for chlorophyll (excitation: 555 nm, emission: 617 nm) and GFP (excitation: 484 nm, emission: 525 nm). The oil immersion 40× objective lens with a numerical aperture of 1.3 and a digital zoom of 0.75× was utilized. Laser power, gain, and pixel dwell time were adjusted to optimize the image clarity and minimize photobleaching. Images were acquired with a resolution of 2048 × 2048 pixels, using Leica LAS X software (Leica Microsystems, Wetzlar, Germany). Spectral deconvolution was conducted within the software to mitigate fluorophore signal overlap, and channels were merged to assess fluorophore colocalization, ensuring precise and artifact-free data for subsequent analysis.

4.9. Sequencing of Individual Transformants. Individual transformants grown on HygZeo plates were patched onto similarly treated plates with drawn grids for clone tracking. Subsequently, these transformants were picked into 96-well plates, each well containing 200 μL of TAP medium supplemented with 10 μg/mL Zeocin. After a growth period of 3 days, measurements were conducted by using a Tecan Infinite m200 Pro plate reader (Tecan Group Ltd., Männedorf, Zurich, Switzerland). Two key parameters were assessed: GFP fluorescence, indicative of promoter transcriptional activity and chlorophyll concentration for cell number normalization. GFP was measured at an excitation wavelength of 505/9 nm and an emission wavelength of 536/20 nm, with the plate reader set to a gain of 245. Chlorophyll measurements were taken at an excitation wavelength of 440/9 nm and an emission wavelength of 680/20 nm, with a gain of 160. Transformants exhibiting the highest GFP-to-chlorophyll ratio were selected for further analysis.

For DNA extraction, the chosen transformants from the original HygZeo plates were resuspended in 20 μL of 10× Tris–EDTA (TE) buffer and subjected to a boiling step at 95 °C for 10 min. Following this, 2 μL of the resultant lysate was used as a template for the PCR amplification. The PCR employed the primers: forward GATCACAAGCTCGAGTGGCC and reverse GACCGCGCTGATGAACAGGG. The amplified PCR products were then purified using a standard cleanup kit. Finally, these purified samples were forwarded to Eton Bioscience Inc. for Sanger sequencing analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.4c00199.

Zip file containing the input DNA sequences used for all three algorithms: POWRS, STREME, and PhyloGibbs, and it also contains the DNA sequences for vectors pLibrary-0 and pLibrary-AR1 (ZIP)

Excel sheet containing data used in the generation of figures and results: SAPS1 Hyg—Contains data regarding the counts for each SAPS1 under Hyg; SAPS2 Hyg—Contains data regarding the counts for each SAPS2 under Hyg; SAPS1 HygZeo—Contains data regarding the counts for each SAPS1 under HygZeo; SAPS2 HygZeo—Contains data regarding the counts for each SAPS2 under HygZeo; SAPS1—Contains the compiled data for all SAPS1 counts and the calculated HygZeo ratios; SAPS1—Contains the compiled data for all SAPS1 counts and the calculated HygZeo ratios; All sequenced promoters—Contains the corresponding promoter ID for each individually sequenced transformant; Unique sequenced promoters—Contains the corresponding promoter ID for each unique sequenced promoter (XLSX)

NGS Reads Annotated (Figure S1); distribution of promoter variants in SAPS1 and SAPS2 (Figure S2); distribution of CREs in highly expressed promoters (Figure S3); distribution of CREs in control promoters (Figure S4); distribution of CREs with 1 mismatch in highly expressed promoters (Figure S5); distribution of CREs with 1 mismatch in control promoters (Figure S6); position distributions of CREs relative to the TSS (Figures S7 and S8) (PDF)

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Notes

The authors declare the following competing financial interest(s): S.P.M. was a founder of and holds an equity stake in Algenesis Inc., a company that could potentially benefit from this research. N.J.H. declares financial interests in TeselaGen Biotechnologies and Ansa Biotechnologies.

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■ ABBREVIATIONS

SAPS, synthetic algal promoters; SPC, synthetic promoter chassis; CRE, cis-regulatory elements; FACS, fluorescence activated cell sorting; NGS, next-generation sequencing; GFP, green fluorescent protein; Hyg, hygromycin; Zeo, zeocin; CFU, colony forming unit; pL0, pLibrary-0; pL-AR1, pLibrary-AR1; WT, wild-type; POWRS, position-sensitive word set; STREME, simple, thorough, rapid, enriched motif elicitation; TSS, transcriptional start site

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