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Letter

Construction of Fully Segregated Genomic Libraries in Polyploid Organisms Such as *Synechocystis* sp. PCC 6803

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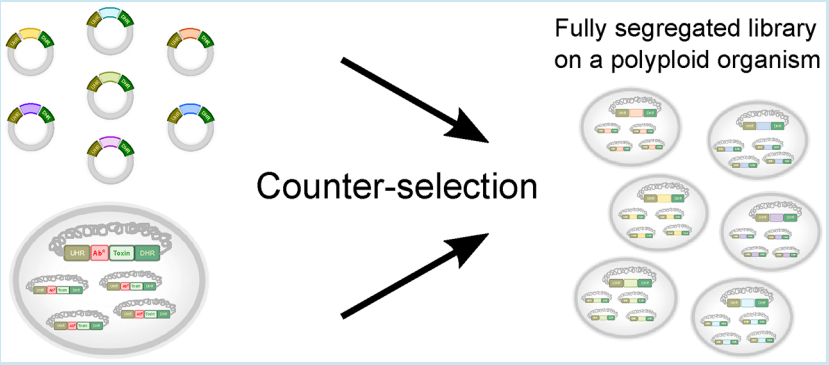

ABSTRACT: Several microbes are polyploid, meaning they contain several copies of their chromosome. Cyanobacteria, while holding great potential as photosynthetic cell factories of various products, are found among them. In these clades the diversity of genetic elements that serve within the basic molecular toolbox is often limiting. To assist mining for the latter, we present here a method for the generation of fully segregated genomic libraries, specifically designed for polyploids. We provide proof-of-principle for this method by generating a fully segregated genomic promoter library in the cyanobacterium *Synechocystis* sp. PCC 6803. This new tool was first analyzed through fluorescence activated cell sorting (FACS) and then a fraction was further characterized regarding promoter sequence. The location of libraries on the chromosome provides a better reflection of the behavior of its elements. Our work presents the first method for constructing fully segregated genomic libraries in polyploids, which may facilitate their usage in synthetic biology applications.

KEYWORDS: *polyploid microorganism, genomic library, fully segregated, counter-selection, cyanobacteria, promoters*

A genomic library is a collection of DNA fragments, ideally representing the entire DNA content of the genome from which the library was derived.[1] Genomic libraries are useful to screen for target DNA fragments contributing to desired complex phenotypes (*e.g.*, chemical tolerance), which are normally difficult to be rationally engineered.[2,3] Furthermore, genetic elements, such as promoters, that are indispensable in genetic engineering can also be identified. These include promoters not only with different strengths but also with different inducible properties.[4] Thus far, genomic libraries were mostly constructed either on a replicative plasmid or integrated in the chromosome of monoploid microorganisms (*i.e.*, organisms containing a single chromosome copy).[5] While for polyploid microorganisms, large genomic libraries have only been developed to explore native DNA fragments, such as promoters through a reporter (*e.g.*, fluorescence), on a replicative plasmid.[4] Because the copy number of the replicative plasmid may vary under the cultivation conditions tested,[6] this might compromise the correlation between the activity of the reporter and the promoter strength. For polyploid microorganisms as well, genomic libraries directly located on the chromosomes would have a relatively stable copy number in comparison to the ones located on replicative plasmids. But clearly, integrating such a library on all the chromosomes of a polyploid microorganism is a major challenge, without going through the regular time-consuming segregation steps for a limited number of representatives.[7] How can one ensure that the genomic library integrated on the chromosomes of polyploid microorganisms is fully segregated? In other words, how can one ensure that all existing copies of the chromosome contain the intended fragment?

We decided to tackle this challenge by constructing a fully segregated chromosomal genomic library in polyploid microorganisms using a counter-selection approach (Figure 1). This

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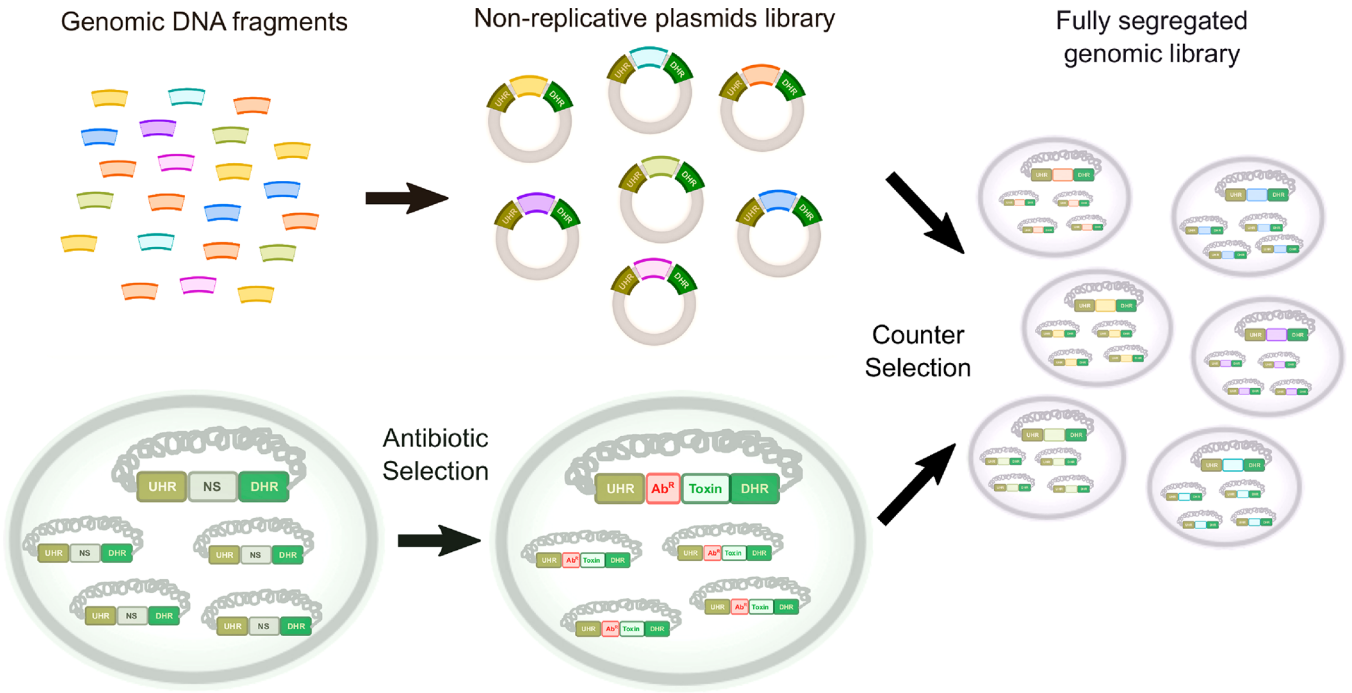

Figure 1. A schematic drawing of the construction of fully segregated genomic libraries in polyploid microorganisms. The full segregation of the genomic libraries is enabled through a counter-selection strategy, where a positive selection (*via* antibiotic resistance genes) and a counter (negative) selection (*via* conditional toxic genes) are implemented. Specifically, target genomic locus of the microorganism needs first to be fully integrated with both positive and counter selection genes *via* antibiotic selection and segregation (light green background). Then the prepared genomic DNA fragments are ligated to the nonreplicative vector flanking with upstream and downstream homologous regions of the target genomic locus, resulting in a plasmid library (light yellow background). After introducing the plasmid library into the microorganism, double crossover occurring at the target genomic locus will replace the selection genes with the DNA fragments. Cells with only fully replaced chromosomes can survive under counter-selection. Hence, the fully segregated genomic libraries are created in polyploid microorganisms (light purple background). NS, neutral site; UHR, upstream homologous region; DHR, downstream homologous region; Ab^R, antibiotic resistance gene; Toxin, conditional toxic gene. Five chromosomes inside the cells are depicted only to mimic the polyploidy.

approach includes a positive selection (*via* antibiotic resistance genes) and a counter (negative) selection (*via* conditional expression of toxic genes). Specifically, both selection cassettes need first to be fully integrated into the target genomic locus of the targeted polyploid microorganism. This is achieved by the positive selection with increasing dosage of the antibiotic through possibly several rounds of segregation. During the positive selection process, the toxic gene for counter-selection is regulated as nonfunctional (*e.g.*, noninduced or no substrate available). This polyploid microorganism, with fully integrated chromosomal selection cassette, is served as the background strain ready for transformation. Then, the genomic DNA fragments are prepared either through partial enzymatic digestion or mechanical forces to a target size range. Those fragments will later be inserted into a nonreplicative vector, flanking with upstream and downstream homologous regions of the target genomic locus of the background strain, resulting in a plasmid library. After introducing the plasmid library into the background strain, the selection cassette will be replaced with a random DNA fragment though double crossover at the target genomic locus, by homologous recombination. With controlled functional expression of the toxic gene (*e.g.*, adding an inducer or substrate) for counter-selection, only the cells with fully replaced chromosomes can survive. This would lead to fully segregated chromosomal genomic libraries created in polyploid microorganisms.

To test this approach, polyploid cyanobacteria—promising photosynthetic microbial hosts that can be employed to directly convert atmospheric CO_2 to biochemical compounds[8]—were chosen. As one of the model cyanobacterial species, *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*), which has multiple chromosome copies, was selected as a case study. The chromosome copy number in *Synechocystis* is variable and can be affected by growth phase and by physical and chemical factors.[9−11] Nevertheless, despite the variation, the copy number of chromosomes in polyploid cyanobacteria is reported to be much smaller and less variable than the copy number of exogenous plasmids. This has been observed in the expression levels obtained through plasmid expression in comparison with chromosomal expression in *Synechocystis* sp. PCC 6803,[12−14] *Synechococcus* sp. PCC 7000,[15] and *Synechococcus elongatus* PCC 7942.[16]

Despite the efforts to identify native promoters in cyanobacteria, the number of well characterized constitutive and inducible native promoters is still considered to be somewhat small.[17−20] Because a large number of the promoters that have been applied in the cyanobacterial studies come directly from other model organisms such as *Escherichia coli*,[19] we deemed that a fully segregated chromosomal genomic library would be useful in order to mine the native cyanobacterial promoters.

Overall, in this work we successfully constructed a fully segregated genomic library on the chromosomes of *Synechocystis* (Figure 2A). As a conceptual framework, we screened and characterized in total 72 native promoters with their sequences analyzed. Our work marks the first report of

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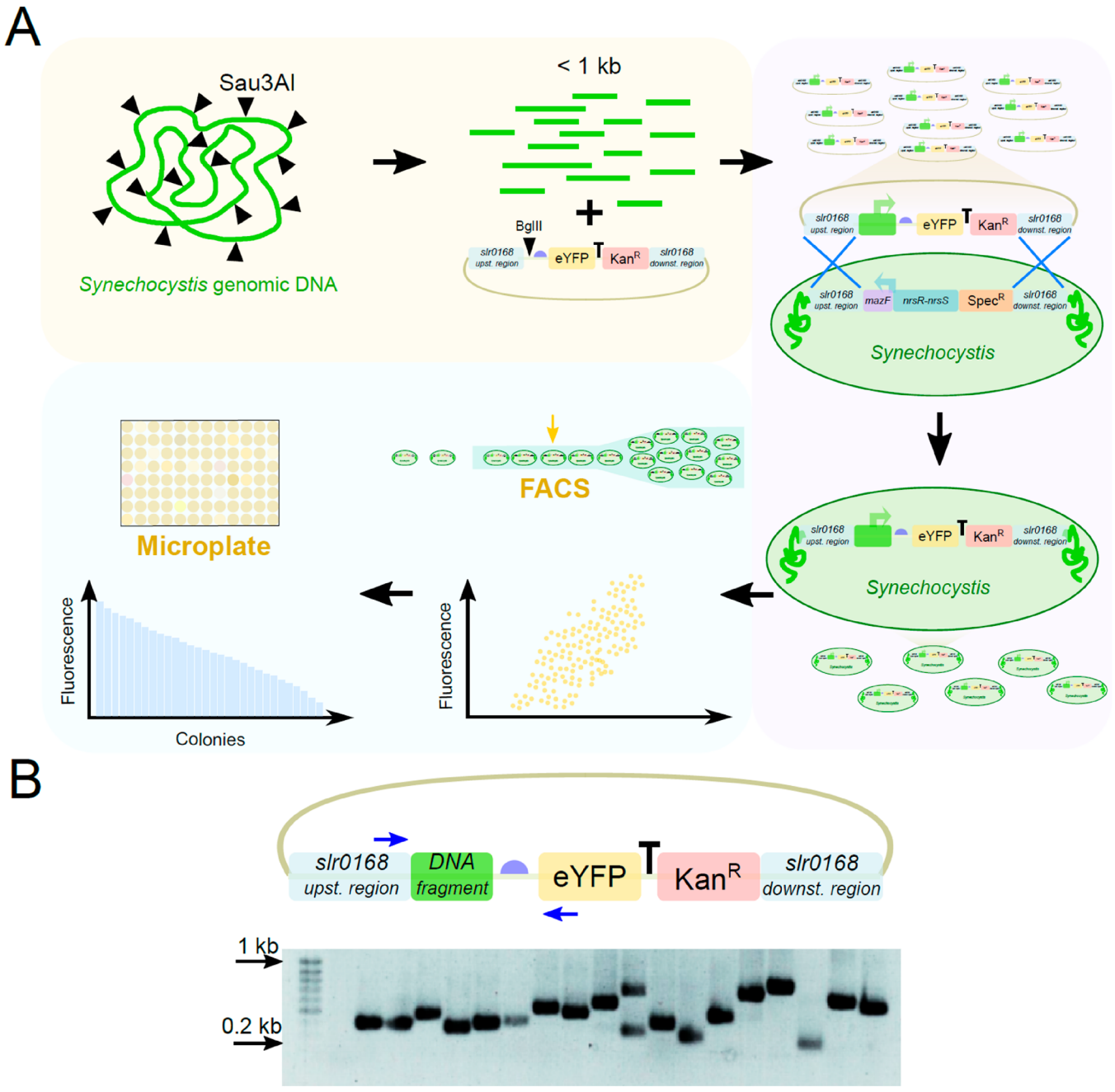

Figure 2. Construction of a fully segregated genomic promoter library in *Synechocystis*. (A) The whole process can be divided as three main modules (as indicated with different color backgrounds): (1) inserting the enzymatic digested genomic DNA fragments to the integrative vector targeting the *slr0168* genomic locus of *Synechocystis*; (2) transforming *Synechocystis* with the plasmids containing DNA fragments to obtain the fully segregated promoter library on the *Synechocystis* chromosome; (3) characterizing the promoter library in *Synechocystis via* the fluorescence intensity. (B) PCR verification of a few representative *E. coli* colonies harboring the genomic library. The gel picture shows that the size of genomic DNA fragments generated to create the promoter library are ranging roughly from 200 to 1000 bp. Blue arrows indicate the position of the primers.

construction of a fully segregated chromosomal genomic library in polyploid microorganisms, without going through the time-consuming segregation step of only a small number of representatives.[7] This library could in principle be exploited either for fundamental research (*e.g.*, detailing complex phenotypes) or synthetic biology applications (*e.g.*, mining genetic elements).

To initialize, *Synechocystis* genomic DNA was isolated and partially digested by Sau3AI to a size of approximately 200 bp to 1 kb (Figure 2B). Those DNA fragments were inserted on a suicide plasmid (at the *Bgl*II restriction site) that integrates at the *slr0168* genomic locus (neutral site) of *Synechocystis*. Between the homologous regions, and downstream of the DNA fragments inserted, a ribosome binding site, a fluorescence reporter (eYFP), and a terminator were attached (Figure 2A). This enables the screening and quantitation of the promoter strength using the fluorescence intensity as a proxy. Additionally, a kanamycin resistance gene is included in this vector for propagation purposes. To introduce this plasmid library containing random DNA fragments into *Synechocystis*, and ensure their complete segregation, we adopted a counter-selection strategy that has been developed for markerless

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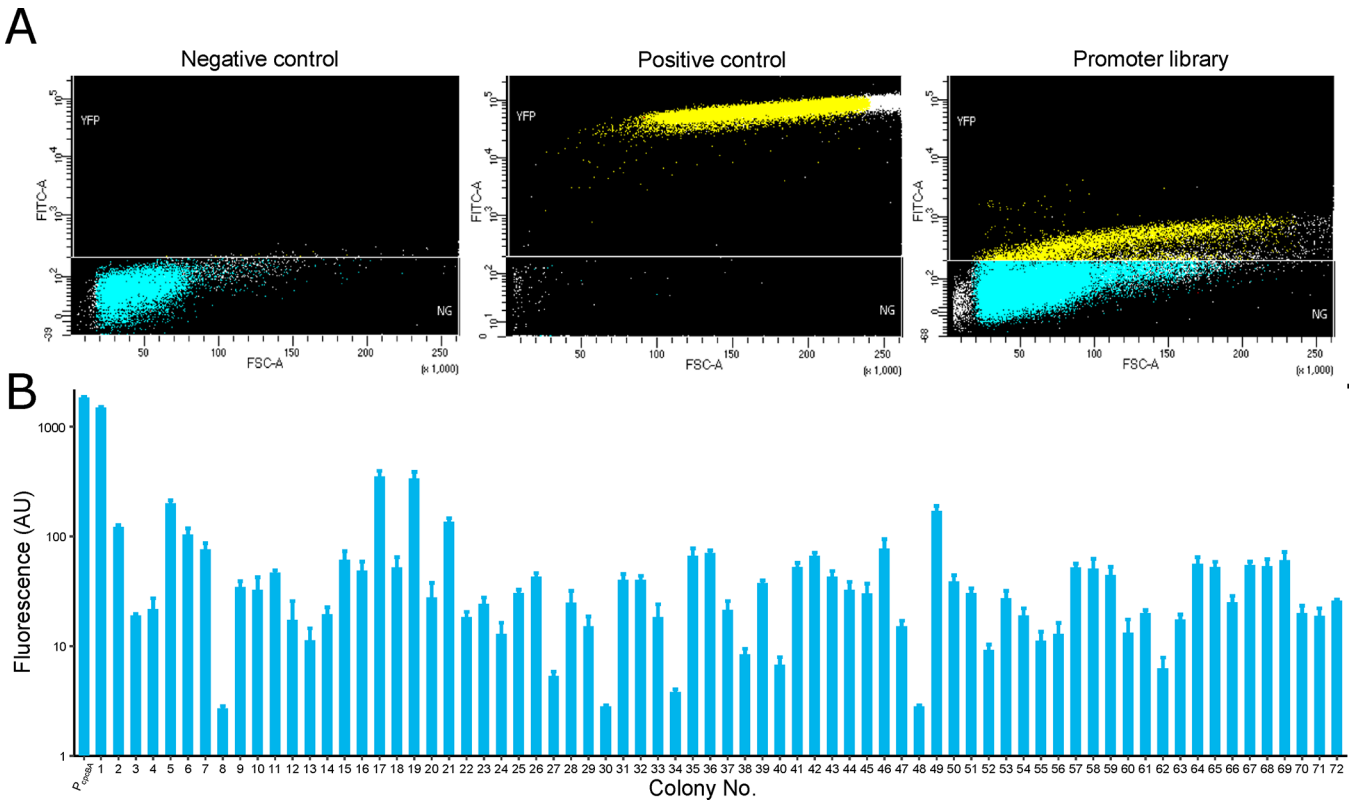

Figure 3. Characterization of the *Synechocystis* genomic promoter library *via* fluorescence intensity measurement either by FACS (A) or microplate (B). For FACS, the promoterless strain that was unable to express eYFP was used as negative control and also to determine the fluorescence threshold (indicated by the white line). The strain expressing eYFP driven by the strong constitutive PcpcBA promoter was used as positive control. Individual colonies that display fluorescence below the threshold were assigned the color blue, while the ones above the threshold were shown in yellow color. The *Synechocystis* cells transformed with the genomic promoter library showed heterogeneity in the fluorescence intensity, with low or mild expression in most of the population. In the microplate, the fluorescence intensity represents the transcriptional strength of the 72 promoters sequenced, in the order of Table S1, compared with the strength of PcpcBA. Fluorescence was normalized by OD_{730} of the cultures. The error bars indicate the standard deviation of three replicates.

genetic modification.[21] To implement this strategy, we first build a *Synechocystis* strain, that has a fully segregated *mazF* (toxic) cassette, expressed under the strict control of a nickel-inducible promoter, at the *slr0168* genomic locus. This was done by selection using spectinomycin. Cultures of this *Synechocystis* strain were then incubated with the plasmid library in a shake flask without antibiotics for 24 h. Then, kanamycin was added to ensure the integration of the promoter library targeting the *slr0168* genomic locus (replacing the *mazF* cassette) and pushing the cells toward the direction of complete segregation. After a few days, nickel was added in the shake flask to select the cells with fully segregated inserts. In order to have a representative promoter library, it is critical to determine the timing of adding nickel. This should not be too soon to avoid killing the cells that were not yet fully segregated, but should also not be too late, which would favor fast growers to take over the population, reducing library representativeness. We tested the effect of adding nickel at different days after adding kanamycin, and more varied colonies with fully segregated chromosomes (verified by PCR in Figure S1) were obtained when nickel was added after 4 days (data not shown here).

The strength of each randomly inserted DNA fragment with promoter activity was then checked by fluorescent activated cell sorting (FACS) (Figure 3A). The *Synechocystis* strain without any DNA fragment insertion serves as the negative control (important to set the lower threshold of fluorescence). While the strain carrying a strong constitutive promoter (PcpcBA) was used as the positive control.[22] Our results indicated that a small proportion (~12.2%) of the total cells analyzed (50 000) sparked fluorescence at different intensities. For the rest of the cells the fluorescence was below the threshold, indicating the DNA fragments inserted did not drive eYFP expression. For the cells displaying significant fluorescence, it is of course interesting to characterize the underlying sequence responsible for its intensity. Therefore, cells that displayed fluorescence above the set threshold were collected by FACS and spread on BG11 plates to isolate single colonies. To first have an overview of the colony heterogeneity, we performed a colony PCR to specifically amplify the DNA fragment inserted. Our results (Figure S2) showed that for a sample of 18 colonies tested, the size of the DNA fragment varies, but always falls into the expected range (200 bp to 1 kb). This again corroborates the idea that our approach is feasible and can lead to the successful construction of a genomic promoter library directly on the chromosomes.

As a proof-of-principle validation of our approach in terms of mining native promoters, we picked in total 72 colonies from the plate for further characterization. To measure the fluorescence of each colony, we applied a 96-well plate method.[23] The results showed a wide distribution of the fluorescence from all the colonies, which reflects the diverse promoter strength of the respective DNA fragments (Figure 3B). We next sequenced the inserted DNA fragment acting as

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promoters in those 72 colonies (Table S1). The results indicated that all the fragments with promoter activity had unique DNA sequences, though some colonies have overlapping sequences that cover the same region of the chromosomal locus (specifically, colonies 11, 13, 22, 35, and 39 in Table S1). Some sequences showed the typical promoter location (5′ UTR region), other sequences were part of a gene, and others overlap two neighbor genes. The ones located as part of a gene suggest that some regions of the annotated genes could also be regulatory elements of other flaking coding sequences, or the promoters driving the expression of sRNAs.[24,25] Some of the promoters found belong to regions of the genes coding for proteins involved in replication and transcription such as ligase, DNA gyrase, and transcriptional regulators. Others were part of genes coding for important metabolic enzymes such as ATP synthase, NADH dehydrogenase, phosphorylases, transferases, synthases, and dehydrogenases. The promoters that were related with photosynthesis were part of genes coding for phycocyanin, plastocyanin, and thioredoxin. Furthermore, some sequences found are part of the genes encoding for hypothetical proteins. This can be an indication of the regulatory role of the regions that are not coding for known proteins, or that are still not well characterized. Overall, these findings validate the potential of constructing a promoter library on the chromosome to identify native promoters that can modulate different levels of target gene expression for genetic engineering purposes.

Genomic libraries serve as an important tool that can be utilized to map important DNA fragments/genes for both fundamental and applied research.[26] In terms of exploring native promoters in polyploid microorganisms, a genomic library integrated on the chromosome is more preferred (although challenging) than on a replicative plasmid. This is because the copy number of the chromosome is relatively stable, while the replicative plasmid copy number may vary more. This may be due mostly to the independent replication system of the replicative plasmid such that its replication is loosely controlled by the cells. Furthermore, it is also difficult for cells to equally distribute the replicative plasmids to the daughter cells *via* the plasmid partition mechanism during cell division.[27,28] Therefore, when cells have different copy numbers of the replicative plasmid, the reporter gene dosage is expected to be different. Under this situation, it would be problematic to directly estimate the promoter strength based on the activity of the reporter (*e.g.*, fluorescence). In this work, we tackled this challenge using a counter-selection approach and successfully constructed a fully segregated genomic library on the chromosomes of the cyanobacterium *Synechocystis* as a case study. As a conceptual validation, we screened in total 72 native promoters that showed a wide distribution of promoter strength. Because cyanobacteria hold great promise to be developed as photosynthetic cell factories, the native promoters mined here through a genomic library may greatly facilitate various synthetic biology implementations. This approach to construct a fully segregated genomic library is also applicable to other polyploid microorganisms, where desired DNA fragments contributing to target phenotypes can be reliably mapped for both fundamental and applied research.

METHODS

General Cultivation Conditions. *E. coli* strain DH5α was used as the host for plasmid molecular cloning. It was grown at 37 °C in Lysogeny Broth medium (LB) in an incubator with a shaking speed of 200 rpm or on LB plates containing 1.5% (w/v) agar. The concentration of antibiotics used were 50 μg mL^{-1} for kanamycin and 50 μg mL^{-1} for spectinomycin. *Synechocystis* sp. PCC6803 (glucose tolerant, obtained from D. Bhaya, Stanford University, USA) was cultivated at 30 °C in liquid BG-11 medium supplemented with 10 mM TES-KOH (pH = 8), in a shaking incubator at 120 rpm (Innova 44, New Brunswick Scientific) under constant moderate red light illumination (~30 μmol photons m^{-2}s^{-1}), or on BG-11 agar plates supplemented with 10 mM TES-KOH and 0.3% (w/v) sodium thiosulfate, in an incubator with white light (~30 μmol photons m^{-2} s^{-1}) and 1% CO_2. For *Synechocystis* mutant construction, kanamycin, spectinomycin, or nickel sulfate were added to the medium with a final concentration of 50 μg mL^{-1}, 20 μg mL^{-1}, and 15 μM, respectively. Biomass concentration in the cultures was measured by optical density at 730 nm (OD_{730}) in a spectrophotometer (Lightwave II, Biochrom).

Plasmid and Strain Construction. All plasmids, strains, and primers are listed in the Table S2. To construct the required plasmids, both homologous regions of *slr0168* were amplified from the genomic DNA of *Synechocystis*, using Herculase II polymerase (Agilent) and primers designed to introduce restriction sites for *Kpn*I in the 5′ of the upstream region, *Hin*dIII in the 3′ of the downstream region, and XbaI in between the two regions. The fragments were fused together and completely amplified using *Pfu* DNA Polymerase (Thermo Scientific). After gel extraction and purification (Thermo Scientific), the fused fragment was inserted in the vector (prepared by digestion of the plasmid pFL-XN with *Kpn*I and *Hin*dIII). This resulted in the new plasmid pFLXN, serving as the background plasmid for the following constructs. To construct the plasmid pFLXN-MAZF, kanamycin resistant fragment from the selection cassette of the pWD42 plasmid was first replaced with the spectinomycin resistant fragment, resulting in the plasmid pWD007. Then, the new selection cassette was cut from pWD007 by double digestion with SpeI and AvrII, and inserted into the pFLXN plasmid digested by XbaI.

To generate the CPC-YFP cassette, the region containing the PcpcBA promoter (including the RBS), eYFP, terminator BB0014 and kanamycin resistant cassette (Kan^R) was first PCR amplified from pHKH-cpcBA-YFP. Then, this fragment was further prepared by XbaI and NheI digestion and inserted into the pFLXN plasmid digested by XbaI, resulting in the plasmid pFLXN-CPC-YFP. To generate the RBS-YFP cassette, only the region containing the ribosome binding site from the PcpcBA promoter (excluding the promoter region), eYFP, terminator BB0014, and Kan^R was PCR amplified from pHKH-cpcBA-YFP and then inserted into the pFLXN plasmid following the same above approach. This resulted in the plasmid pFLXN-RBS-YFP. For the construction of the pFLXN-LIB-YFP plasmid, a *Bgl*II site was additionally added before the RBS during the PCR amplification of the RBS-YFP cassette, to allow later introduction of the genomic DNA fragments of the library. All the fragments amplified in this study were confirmed by Sanger sequencing at Macrogen Europe (The Netherlands).

Synechocystis mutants Syn-MAZF, Syn-RBS-YFP, and Syn-CPC-YFP were created by natural transformation of the corresponding plasmid to *Synechocystis* wild type as described previously.[29] Fully segregation of each mutant was verified by PCR using the genomic DNA as the template for 35 cycles. To obtain a fully segregated genomic library integrated in the

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Synechocystis chromosomes, we adopted a liquid transformation approach to introduce the promoter library to Syn-MAZF. First, fresh Syn-MAZF cells were collected from 20 mL liquid culture ($OD_{730} \approx 1$). After being washed twice with fresh BG11 medium through centrifugation (3900 rpm, 10 min), cells were further concentrated to a total volume of 200 μL. The pFLXN-LIB-YFP plasmids were mixed with these cells to reach a final plasmid concentration of 30 $\mu g\ mL^{-1}$, and then incubated at 30 °C with moderate light intensity (white light, 30 μmol photons $m^{-2}\ s^{-1}$) for 5 h. Next, the mixture was inoculated in 20 mL of BG11 (without antibiotic) and incubated at 30 °C in a shaking incubator at 120 rpm (Innova 44, New Brunswick Scientific) under constant moderate red-light illumination (~30 μmol photons $m^{-2}\ s^{-1}$). After further incubation for about 24 h, kanamycin was added to the cultures at a final concentration of 50 $\mu g\ mL^{-1}$. After another 4 days of incubation, nickel sulfate was added to the cultures at a final concentration of 15 μM. When the cultures reached $OD_{730} \approx 1-1.5$, a sample was collected to perform FACS analysis and the rest was concentrated to make glycerol stocks.

Genomic DNA Promoter Library Construction, Fluorescence Analysis. See Supporting Information for details.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00353.

Methods for genomic DNA promoter library construction and fluorescence analysis; List of sequences from the selected promoter library in Table S1; Plasmids, strains and primers used in this study in Table S2; Colony PCR verification of 10 representative colonies showing that the constructed genomic DNA library in *Synechocystis* was fully segregated in Figure S1; PCR verification gel picture of a few representative *Synechocystis* colonies from the constructed fully segregated genomic DNA library in Figure S2 (PDF)

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Author Contributions

PCB, WD, and FBS designed the experiments; PCB performed most of the experiments with technical assistance from TS and HPH; PCB, WD, and FBS wrote the manuscript. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

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