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Efficient Multiplex Genome Editing of the Cyanobacterium *Synechocystis* sp. PCC6803 via CRISPR-Cas12a

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ABSTRACT

Cyanobacteria have been genetically modified to convert CO_2 into biochemical products, but efficient genetic engineering tools, including CRISPR-Cas systems, remain limited. This is primarily due to the polyploid nature of cyanobacteria, which hinders their effectiveness. Here, we address the latter by specifically (i) modifying the RSF1010-based replicative plasmid to simplify cloning efforts while maintaining high conjugation efficiency; (ii) improving the design of the guide RNA (gRNA) to facilitate chromosomal cleavage; (iii) introducing template DNA fragments as pure plasmids via natural transformation; and (iv) using *sacB* to facilitate replicative plasmid curing. With this system, the replicative plasmid containing both Cas12a and gRNA is introduced to *Synechocystis* sp. PCC6803 cells via conjugation to cleave the circular chromosomes. Template DNA plasmid that has meanwhile been assimilated will then repair it achieving the desired genetic modifications. This system was validated by successfully deleting various "neutral" chromosomal loci, both individually and collectively, as well as targeting an essential gene, *sll1797*. With the *sacB*-sucrose counter-selection, all deletions were simultaneously made markerless in <4 weeks. Moreover, we also integrate YFP with various protein degradation tags into the chromosome, allowing for their characterization at the chromosomal level. We foresee this system will greatly facilitate future genome engineering in cyanobacteria.

Photosynthetic cyanobacterial CO_2 conversion to biochemical compounds holds great potentials to achieve sustainability (Branco dos Santos, Du, and Hellingwerf 2014; Lips et al. 2018). Over the past two decades, we have witnessed numerous genetically modified cyanobacteria developed for the production of various chemicals. This is mainly achieved by knocking-out and/or -in gene(s) into the cyanobacteria to direct more metabolic flux toward the desired product. Traditional genetic engineering of cyanobacteria relies on an antibiotic resistance gene to replace the DNA target in the chromosome (Eisenhut et al. 2008). Hence, an antibiotic resistance marker stays in the chromosome for each genetic modification. Additionally, due to the polyploidy (i.e., multiple chromosomes) in many cyanobacterial species (Zerulla, Ludt, and Soppa 2016), a time-consuming segregation step is necessary to fully delete a gene in all copies of the chromosome. Generally, it takes at least 3 weeks to knock out a gene in *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis*) with this approach. The counter-selection approach could help to achieve a markerless modification, but then two rounds of transformation are needed that take at least 5–6 weeks (Cheah, Albers, and Peebles 2013). To fully explore the potentials of this so-called "cyanobacterial cell factories," an effective and efficient approach to aid genome engineering of cyanobacteria is urgently needed (Schmelling and Bross 2024).

As a robust and flexible tool for genome editing, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)

system has revolutionized genetic engineering among different model organisms (Pacesa, Pelea, and Jinek 2024). To successfully implement this system in cyanobacteria, three components are required: a Cas protein, a guide RNA (gRNA), and a DNA repair template. Cas protein will be directed by the gRNA to a specific genomic locus to cleave the circular chromosomes. To survive, cyanobacterial host needs to repair the broken chromosomes via an externally supplemented repairing DNA template. Depending on the template DNA provided, knock-out or -in can be easily achieved. CRISPR system is more advantageous over the traditional approaches for genome engineering as: (1) no antibiotic marker will be left (i.e., a markerless system); (2) potentially no segregation step is needed if the cleavage capability of the Cas protein is enhanced; and (3) multiplex genetic modifications (i.e., targeting two or more DNA locations within a chromosome) can be implemented. Given the advantages above, it is not surprising that both CRISPR-Cas9 and CRISPR-Cas12a (Cpf1), the two most popular CRISPR systems, have already been exploited in different cyanobacterial species (Baldanta, Guevara, and Navarro-Llorens 2022; Cengic et al. 2022; Racharaks, Arnold, and Peccia 2021; Ungerer and Pakrasi 2016). Despite these initial efforts (Table S1), there is still great room for improvement as the efficiency of genetic engineering in cyanobacteria is not as high as it could be (Ungerer and Pakrasi 2016), and the molecular cloning around it often requires more efforts than anticipated (Cengic et al. 2022). Therefore, we decided to carry out a few improvements to make the application of CRISPR systems in cyanobacteria simple and efficient.

Previously, all the CRISPR-Cas12a components were delivered to the cyanobacteria via conjugation through the RSF1010-based pSL2680 plasmid (Ungerer and Pakrasi 2016). Straightforward as it is, this low-copy replicative plasmid is already relatively big (~7 kb). After inserting the *cas* gene and one or more repair template DNAs (each with ~1000 bp homologous regions), the final construct exceeds 15 kb, which poses additional challenges for molecular cloning. In addition, single-strand DNA (ssDNA) will generate during the plasmid miniprep. This is due to the MobA protein that reversibly nicks a DNA strand at oriT sequence, forming a covalent complex with the 5′-end of the cleaved strand to initialize conjugation. During the alkaline lysis step of the plasmid preparation, nicked DNA denatures to generate single-stranded DNA, rendering it unsuitable for cloning, disrupting restriction digests and enzymatic reactions (Bishé, Taton, and Golden 2019; Taton et al. 2014). To optimize the latter, we began by analyzing the key components of the pSL2680 plasmid to determine if any elements could be relocated. This would allow us to reduce the size of the plasmid backbone.

We assume the Mob elements on the pSL2680 plasmid are only needed during conjugation to facilitate the transfer of this plasmid from *Escherichia coli* to *Synechocystis* (Elhai and Wolk 1988). Hence, we removed the Mob elements (~2.6 kb) from the pSL2680 replicative plasmid and expressed them in another plasmid ("Mob plasmid") but let both plasmids stay in the same *E. coli* cell (Figure 1A). This allows the Mob elements expressed from the Mob plasmid to be used by the replicative plasmid for conjugation. Additionally, the removal of Mob elements prevents the generation of the ssDNA in the replicative plasmid, which will further simplify and facilitate the molecular cloning process. We compared the conjugation efficiency between the original RSF1010-based replicative plasmid and the two plasmid systems (replicative plasmid and Mob plasmid in one cell). No measurable differences in terms of positive colonies could be observed (Figure S1). To further reduce the size of the replicative plasmid, we propose that the repair template DNA (~1 kb per target gene) can be supplied as a separate plasmid, which *Synechocystis* can directly assimilate through natural transformation (Figure 1A). Thus, during conjugation, when different *E. coli* strains are mixed with

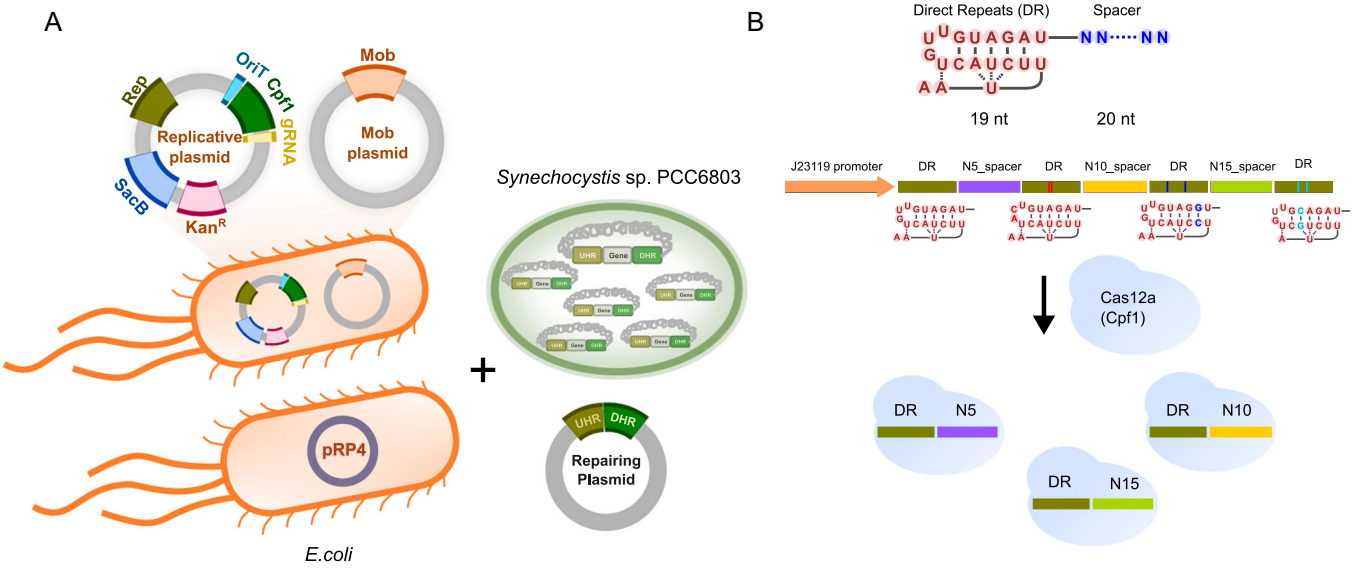

FIGURE 1 | Schematic illustration of the optimized CRISPR-Cas12a system in *Synechocystis*. (A) Through conjugation, a replicative plasmid encoding Cas12a and gRNA is introduced into *Synechocystis*, where it targets and cleaves the circular chromosomes. Meanwhile, an externally supplied repair plasmid is taken up by *Synechocystis* to facilitate the repair of the cleaved chromosomes. (B) Structure of the gRNA and CRISPR array, illustrating how this system enables multiplex genome editing. Colors in the DR represent the nucleotides that were altered to make all the DRs nonidentical.

Synechocystis, the repair template DNA can be added directly as a separate plasmid. This approach allows for further reduction in the size of the replicative construct (~1 kb per target gene). Overall, the plasmid size was reduced by ~3.6 kb for a single gene knockout and by about 5.6 kb for a triple knockout. These improvements to the RSF1010-based replicative vector will greatly simplify molecular cloning.

Building on the success of reducing the replicative plasmid size, we next explored strategies to enhance the cleavage efficiency of Cas12a. This approach aims to eliminate the segregation step, as an enhanced Cas12a cleavage efficiency would theoretically cut all wild-type chromosomes until a fully segregated mutant is obtained. First, we shortened the length of the direct repeats (DR) from 36 nt to 19 nt (Figure 1B), a modification previously reported to enhance Cas12a cleavage efficiency in yeast (Świat et al. 2017). Their study demonstrated that short DR (19 nt) exhibited more than twice the editing efficiency compared to long DR (36 nt)(Świat et al. 2017). Enhanced editing efficiency with short DR has also been demonstrated in a previous study conducted in mammalian cells (Zetsche et al. 2017). Additionally, we altered the DR sequences to make them nonidentical (Figures 1B and S2), which could improve the genetic stability of the gRNA array by preventing spacer excision through DNA self-recombination. Spacer loss through recombination between DR has been identified as a strategy cells use to evade genome editing (Csörgő et al. 2020). A previous study showed that modifying the DR sequences, while preserving the stem-loop structure, can prevent spacer excision and thereby improve the editing efficiency (Csörgő et al. 2020).

With these adjustments, the optimized CRISPR-Cas12a system functions as follows (Figure 1A): the replicative plasmid carries both the Cas12a (Cpf1) gene and gRNA, while the Mob plasmid, containing the mob elements, remains within the same *E. coli* cell. A second *E. coli* strain, harboring the conjugal plasmid pRP4, is used to facilitate conjugation. During conjugation, these two types of *E. coli* are mixed with *Synechocystis* and a pure DNA repair template. Following conjugation, the replicative plasmid is introduced into *Synechocystis*, where Cas12a guided by gRNA cleaves the target genomic locus. The repair template DNA, assimilated by *Synechocystis* through natural transformation, repairs the broken chromosome, thereby achieving the desired genetic modification. A control group is always included, where the repair template DNA is not supplemented. In this case, *Synechocystis* chromosomes are cleaved but cannot be repaired, and if cleavage is effective, only a few colonies will form. In contrast, when the repair template DNA is provided, dozens of colonies emerge. Our next step is to experimentally validate this optimized CRISPR-Cas12a system in *Synechocystis*.

To validate this optimized system, we first performed single-, double-, and triple-knockouts of "neutral" genes in the *Synechocystis* chromosome (Pinto et al. 2015). The neutral genes chosen are *sll1476* (N5), *slr1396* (N10), *slr0271* (N15). We began by conducting single knockouts for each gene, using replicative plasmids each containing a specific gRNA. For each knockout, we supplemented with 2 μg of repair template DNA plasmid, which included ~500 bp of each homologous region inserted into a high-copy plasmid. We obtained 38 colonies for N5, 40 colonies for N10, and 82 colonies for N15, while the negative control (lacking only the repair template DNA plasmid) yielded only 1 colony. Since the negative control group contains Cas12a and gRNA but lacks the repair template DNA, *Synechocystis* chromosomes are cleaved but cannot be repaired, leading to cell death unless few cells manage to escape editing. This negative control is essential for assessing the effectiveness of Cas12a-mediated chromosomal cutting. By comparing the number of colonies between the experimental and negative control groups, we can estimate the proportion of successfully edited cells. In the single knockout experiment, only one colony was observed in the negative control, while multiple colonies appeared in the experimental group. This suggested that nearly all colonies in the experimental group were likely positive. Therefore, for each gene, only three colonies were randomly selected, re-streaked, and validated by PCR using gene-specific primers. At least one primer was designed outside the homologous regions to prevent amplification of any residual template DNA plasmid (Figure 2A). All colonies displayed complete gene deletion immediately without repatching of the colonies as confirmed by the results (Figure 2B).

We then proceeded with multiple gene knockouts, starting with the simultaneous deletion of N5 and N10. To achieve this, we inserted a CRISPR array capable of producing gRNAs for both N5 and N10 into the replicative plasmid. The repair DNA template was designed with upstream and downstream homologous regions for both N5 and N10, arranged sequentially in a high-copy plasmid. We added 10 μg of this pure template plasmid to ensure adequate repair of the broken chromosomes at both genomic loci. Out of 40 colonies screened, 28 showed successful (and immediate) gene deletion for both N5 and N10 (Figure 2C).

Next, we selected N5, N10, and N15 to test the feasibility of our optimized system for triple knockout (Figure 2D). We anticipated more repair template DNA was needed due to the simultaneous cutting and repairing of three genomic loci. A CRISPR array containing gRNAs for N5, N10, and N15 was inserted into the replicative plasmid, and homologous regions for all three genes were sequentially constructed in a high-copy plasmid. We added 12 or 18 μg of the repair DNA plasmid for different experimental conditions. Since DNA cleavage will occur at three distinct genomic loci in this scenario, we anticipated that the cells would require an even greater amount of repairing plasmid DNA to effectively mend the broken chromosomes. With 12 μg of the repair plasmid, only four colonies appeared, and no gene deletions were observed. In contrast, with 18 μg of the repair plasmid, 14 colonies emerged, and 5 of these, exhibited complete deletion of all three genes. This demonstrates that a greater amount of repair template DNA is indeed required to achieve fully multiplex genome editing, as 12 μg of the repair plasmid appears insufficient for the cells to effectively repair their broken chromosomes. Hence only with 18 μg of the repair plasmid, more colonies, particularly positive colonies, were observed. Despite the limited number of positive colonies (5 out of 14, i.e., 36%), the system can still achieve simultaneous triple gene knockout without additional segregation.

We observed varying genomic editing efficiencies among different "neutral" gene targets and across the number of multiplex knockouts. For instance, the N15 knockout yielded twice as many positive

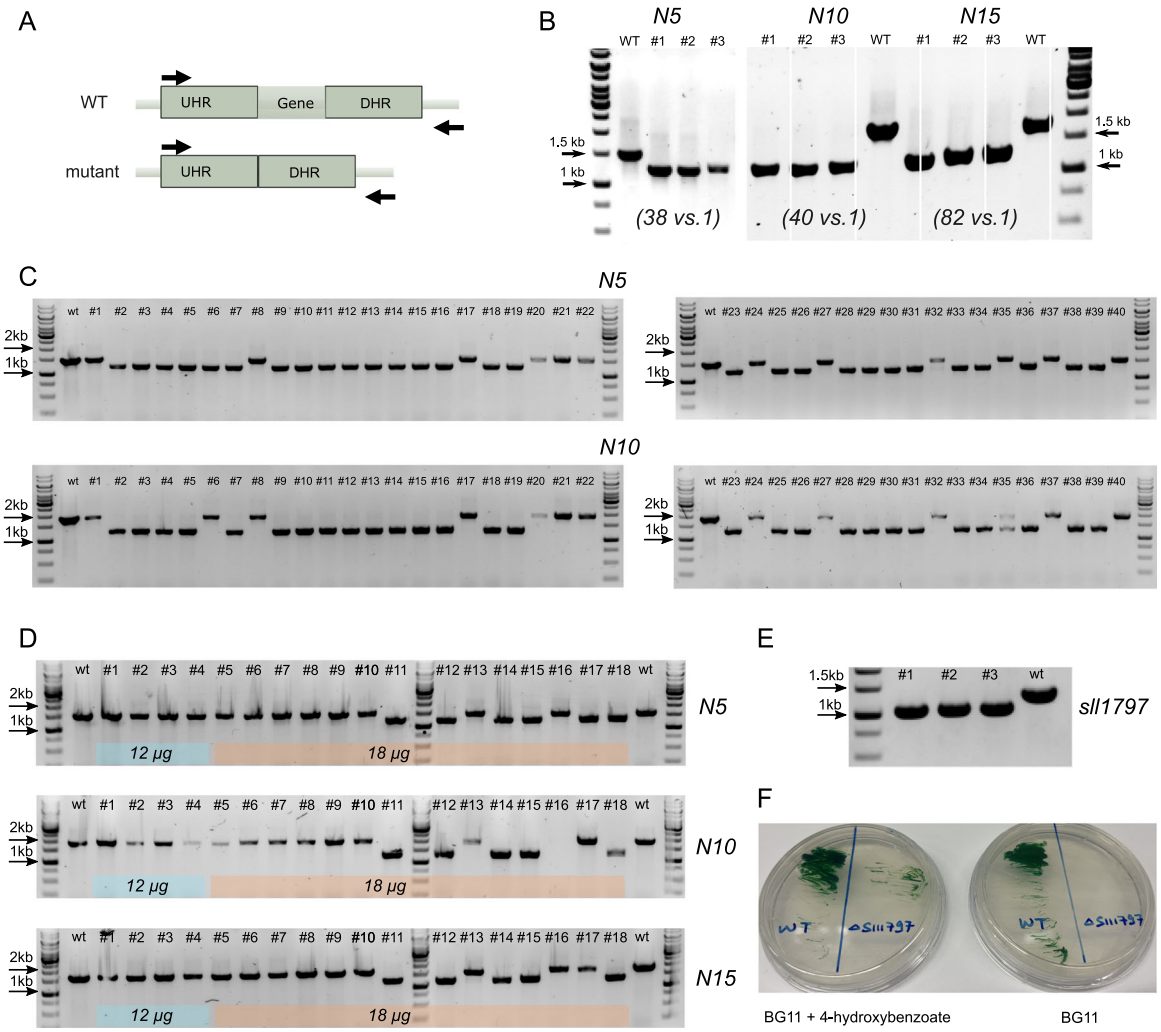

FIGURE 2 | Gene knockouts in *Synechocystis* with the optimized CRISPR-Cas12a system. (A) Schematic representation of the primers designed for PCR validation of gene knockouts. (B) Single-gene knockouts of N5, N10, and N15, with 2 μg of repair plasmid supplemented. The numbers in parentheses indicate the number of colonies obtained for the experimental group versus the negative control. (C) Simultaneous double knockout of N5 and N10, with 10 μg of repair plasmid supplemented. (D) Simultaneous triple knockout of N5, N10, and N15, with varying amounts of repair plasmid supplemented. (E) PCR validation of the knockout of *sll1797*. (F) Growth of the wild type and Δ*sll1797* strain on the BG11 plate with/without 0.1 mM 4-hydroxybenzoate supplementation.

colonies compared to the single knockouts of N5 and N10. Given that equal amounts of repair template DNA with ~500 bp homologous regions were provided for each target on the same plasmid vector, it is unlikely that these differences are due to variations in template DNA availability. Additionally, the negative controls for the single knockouts consistently produced only one colony, indicating effective chromosomal DNA cleavage. This suggests that the differences in editing efficiency are not primarily due to DNA cleavage, which is largely determined by gRNA design. However, the chromosomal repair process may play a role, as not all genomic loci might be equally accessible for repair due to the supercoiled structure of the chromosome (Martis et al. 2019). A decrease in editing efficiency with an increasing number of knockouts has been reported previously (Cengic et al. 2022), but the exact reasons remain unclear in this study. Potential factors include uneven expression of gRNAs in the CRISPR array when multiple gRNAs are assembled (Liao et al. 2019), or spacer excision caused by sequence similarity between DR (Csörgő et al. 2020).

To evaluate whether our optimized CRISPR-Cas12a system can effectively target and knock out essential genes, we selected *sll1797* as a test case. The *sll1797* gene encodes chorismate pyruvate-lyase, an enzyme that catalyzes the first and critical step in plastoquinone biosynthesis. Previous studies reported that the Δ*sll1797* mutant, generated by replacing the gene with an antibiotic resistance cassette, could only grow when supplemented with 100 μM 4-hydroxybenzoate (Pfaff et al. 2014). Using our optimized system, we targeted *sll1797* and obtained 22 colonies in the experimental group, while no colonies were observed in the control group. PCR validation of three randomly selected colonies confirmed complete segregation in all samples (Figure 2D). When re-streaked on BG11 plates, the mutants demonstrated growth only when supplemented with 100 μM 4-hydroxybenzoate, consistent with previous findings (Figure 2E). These results highlight the robustness of our optimized CRISPR-Cas12a system for knocking out essential genes, further expanding its applicability.

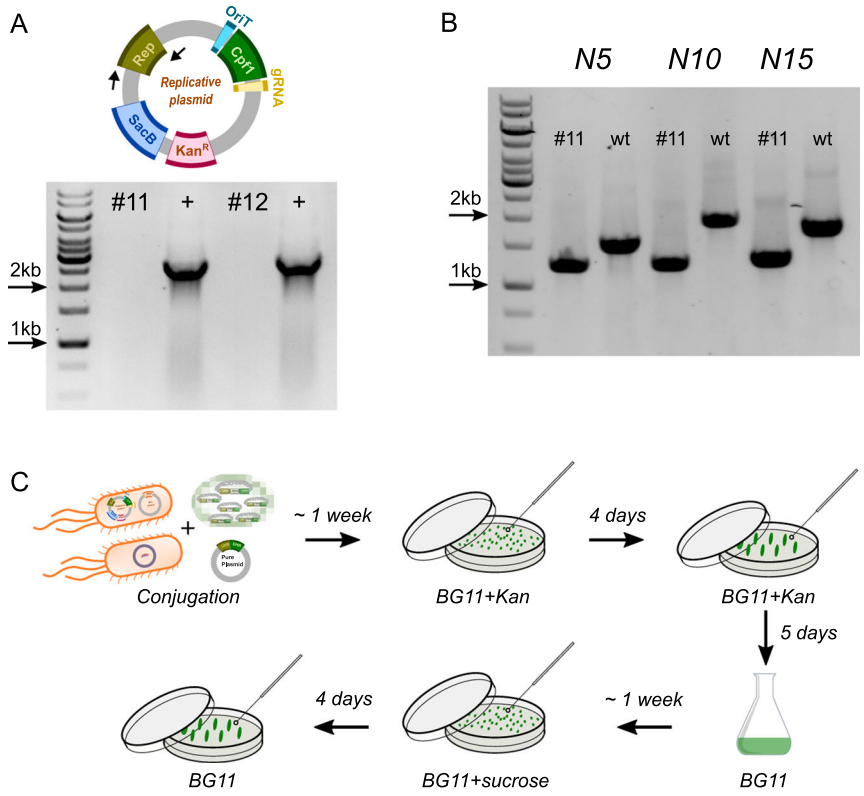

FIGURE 3 | Further steps of gene knockout in *Synechocystis*. (A) PCR validation of the plasmid curing via the *sacB*-sucrose counter selection. Arrows inside the plasmid drawing indicate the primers designed to check the presence of the replicative plasmid. Two colonies (#11 and #12) from the previous triple knockout experiment were selected. The positive control used pure replicative plasmid as the PCR template. (B) PCR validation with the pure genomic DNA extracted from colony #11. (C) Schematic overview of the entire process, including the estimated time required for each step.

In previous genetic engineering methods, at least three serial cultivations and multiple colony picks were required to ensure the spontaneous curing of the replicative plasmid (Ungerer and Pakrasi 2016). We see this step can be shortened via the *sacB*-sucrose counter-selection, as demonstrated in other studies (Niu et al. 2019). We implemented this approach by inserting the *sacB* gene into the replicative plasmid. Following the deletion of target gene(s) and PCR validation, we cultured the cells in liquid BG11 media without antibiotic supplementation. The cultures were then spread on BG11 plates containing 5% sucrose. Colonies that appeared after about 1 week were tested by PCR using two primers specific to the plasmid backbone. If the replicative plasmid was successfully cured, no PCR product would be amplified. We selected two colonies from the triple gene knockout and verified plasmid curing using this *sacB*-sucrose system. PCR results confirmed that the replicative plasmid was cured in both colonies (Figure 3A). To complete the process, we performed an additional PCR validation on the genomic DNA from the triple-gene knockout mutant. The results (Figure 3B) confirmed that indeed all three genes were fully removed. Time-wise, to give an overview of the entire process (Figure 3C), it takes ~2 weeks to determine whether the target gene(s) have been deleted, followed by an additional 2 weeks for plasmid curing and final confirmation.

To assess whether this optimized CRISPR-Cas12a system can facilitate gene integration into the chromosome, we targeted the insertion of a YFP expression cassette at the *slr0168* neutral site. This YFP gene was tagged with various protein degradation tags (Landry, Stöckel, and Pakrasi 2013), allowing the characterization of those tags at the chromosome level, as opposed to the plasmid level (Landry, Stöckel, and Pakrasi 2013). We argue that chromosomal level may be preferred if we would like to use those tags to knock down native genes already in the chromosome. We selected three protein degradation tags—*E. coli* native, *Synechocystis* #, and *Synechocystis* *—based on their differing degradation efficiencies. Additionally, we used two promoters: the strong J23119 and the medium-to-weak J23110. After confirming through PCR that all constructs were correctly inserted (immediately fully segregated using our new method) at the *slr0168* site (Figure 4A), we measured YFP fluorescence and normalized it against a promoterless control (RBS only). Our results (Figure 4B) demonstrated that the J23119 promoter is more than ten times stronger than the J23110 promoter. With the J23110 promoter, the *E. coli* native degradation tag reduced YFP levels to near-background, but some YFP remained with the strong J23119 promoter. Conversely, the *Synechocystis* * tag resulted in background YFP levels even with the J23119 promoter. Interestingly, the RBS-only construct exhibited higher fluorescence than several mutants with protein degradation tags. This is likely due to the native promoter in the chromosome still driving YFP expression in the RBS-only mutant, while the tags effectively reduced YFP levels. Our findings clearly demonstrate that this optimized CRISPR-Cas12a system is also effective for integrating small DNA fragments (~1 kb) into the chromosome.

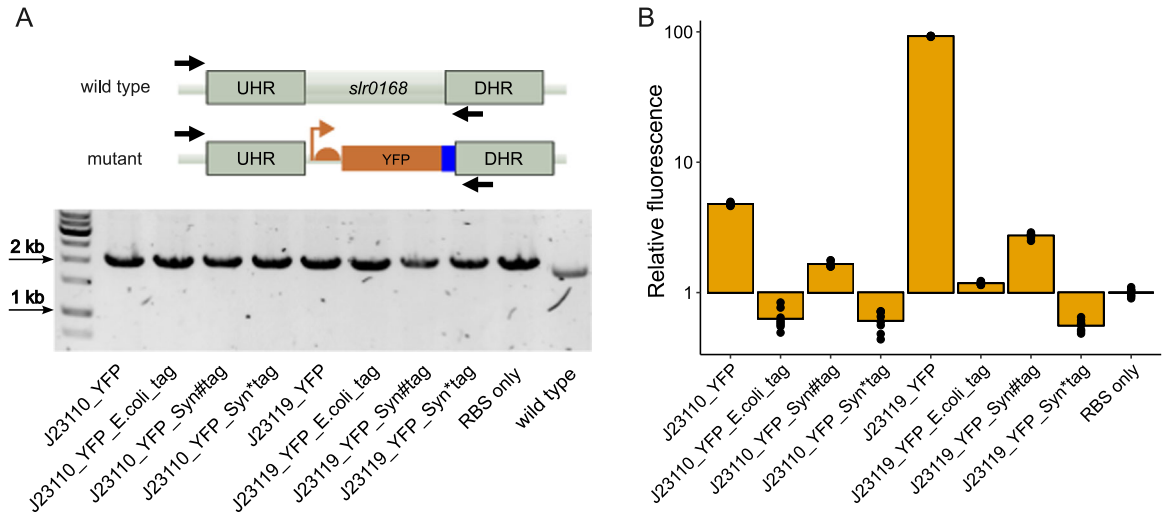

FIGURE 4 | Application of CRISPR-Cas12a for YFP knock-in with various protein degradation tags at the *slr0168* genomic locus. (A) Schematic representation of the genetic constructs, with primers indicated by black arrows. Blue box is the representation of the protein degradation tag. The gel image shows the correct sizes for each construct. All mutants were generated without the need for segregation. (B) Fluorescence measurements for each construct, normalized to the "RBS only" control. Blue box is the representation of the protein degradation tag. Each dot represents a biological replicate.

Genetically engineered cyanobacteria capable of photosynthetic CO_2 conversion into valuable chemicals represent a promising approach to sustainability. However, the development of effective genetic engineering tools for polyploid cyanobacteria has been relatively limited. While the CRISPR-Cas system has been employed in cyanobacteria to advance genetic engineering, there remained significant room for improvement in efficiency. In this study, we have built upon previous research to enhance the efficiency of genetic manipulation in *Synechocystis*. We anticipate that these advancements will significantly aid in the development of *Synechocystis* cell factories and other genetic engineering studies. Additionally, this optimized CRISPR-Cas12a system could be adapted for use in other cyanobacterial model species.

1 | Materials and Methods

1.1 | Strains and Growth Conditions

Molecular cloning was carried out in *E. coli* XL-1 blue grown at 37°C in LB medium, either liquid in flask with a shaking speed of 200 rpm, or solidified in plates (by adding agar 1.5% w/v). In order to propagate a specific plasmid, the appropriate antibiotics were added to the culture medium, with a final concentration of 100 mg L^{-1} for ampicillin and 50 mg L^{-1} for kanamycin.

A glucose-tolerant wild type (WT) strain of *Synechocystis* sp. PCC 6803, obtained from D. Bhaya, Stanford University, Stanford, CA, was used in this study. All the strains of *Synechocystis* (i.e., WT and constructed mutants) have been grown in BG-11 medium (van Alphen et al. 2018) supplemented with 10 mM TES-NaOH (pH 8.0) as a buffer, at 30°C in a shaking incubator (Innova 43, New Brunswick Scientific) at 120 rpm, under constant moderate white-light illumination (~40 mol photons m^{-2} s^{-1}, measured with a LI-250A light meter, LI-COR).

1.2 | Plasmid and Strain Construction

Cas12a (from *Francisella novicida*) and the DNA fragment used for gRNA insertion were PCR-amplified using the pSL2680 plasmid as the template. The *sacB* gene (from *Bacillus subtilis*) was PCR-amplified using the pPSBA2KS plasmid as the template. All PCR reactions were performed using Phusion High-Fidelity PCR Master Mix (Thermo Scientific) for enhanced accuracy. Following enzymatic digestion and ligation, these fragments were assembled into the pSEVA251 plasmid with *oriT* sequences replaced by the *oriT* sequences from pSL2680 plasmid. This eventually results to the replicative plasmid pWD301.

For gRNA insertion, annealed oligos, pWD301, BsaI (New England Biolabs), T4 ligase, and buffer (New England Biolabs) were combined in a PCR tube and subjected to 30 cycles of two-step incubation (37°C for 5 min, 16°C for 5 min). After transforming into competent *E. coli* XL-1 Blue cells (Mix & Go! *E. coli* Transformation Kit, Zymo), white colonies were selected for Sanger sequencing.

To construct the repair DNA plasmid, homologous regions (~500 bp each) were amplified using Phusion High-Fidelity PCR. The upstream and downstream homologous regions were assembled via overlapping PCR and inserted into a BioBrick T-vector (Zhu et al. 2015). For creating a CRISPR array with multiple gRNAs, primers were designed with overlapping nucleotides (Figure S3). All oligos were phosphorylated at the 5′-OH group (T4 Polynucleotide Kinase, Thermo Scientific) and annealed using a PCR program (37°C for 30 min, 95°C for 3 min, followed by a gradual decrease to 4°C at 0.1°C/s). The mixture was used directly for ligation into the replicative plasmid.

The mob elements (*mobA, mobB*, and *mobC* genes) were PCR amplified and inserted into the pFL-SN backbone, generating

the "Mob plasmid." Constructs for protein degradation tags were generated by introducing different promoters and protein degradation tags via specific primers (Table S3).

For the construction of *Synechocystis* mutants via conjugation, overnight cultures *of E. coli* (~16 h at 33°C) were prepared. Fresh *Synechocystis* cultures were grown to an OD of ~1. A 0.5 mL aliquot of each *E. coli* culture was collected, washed three times with LB medium by centrifugation (5000 rpm, 5 min), and concentrated to a final volume of 100 μL. The two *E. coli* cultures were then combined, resulting in a total volume of 200 μL. *Synechocystis* cells at an OD of 1 equivalent (1 mL of OD = 1 culture) were harvested by centrifugation (5000 rpm, 5 min) and, if no antibiotics were used during cultivation, could be directly used for conjugation. If antibiotics were previously used, the cells were then washed three times with BG11 medium, similar to the *E. coli* preparation, and concentrated to 100 μL. The *Synechocystis* suspension was mixed with the 200 μL of *E. coli* culture, creating a final volume of 300 μL. This mixture was split into two equal portions (150 μL each). Pure repair plasmids were added to one tube, while an equal volume of water was added to the other as a control. The mixtures were spread onto a commercial membrane (0.45 μm, Pall Corporation) placed on a BG11 agar plate (without antibiotic) and incubated under illumination for 16–24 h. After incubation, the membrane was transferred to a BG11 agar plate containing kanamycin. Colonies typically emerged after about 1 week.

For replicative plasmid curing, *Synechocystis* cultures were inoculated into liquid BG11 medium immediately after PCR validation. Once the OD reached ~1, 5 mL of the cultures were collected by centrifugation at 5000 rpm for 5 min. The cell pellets were then resuspended in 150 μL of BG11 medium and spread onto BG11 plates containing 5% sucrose. Colonies typically appeared after about 1 week.

Two Python scripts (gRNA_design.py and Primer_design_cr_array.py) were developed to design primers for gRNA and the CRISPR array, respectively (https://gitlab.com/mmp-uva/crispr-cas12a-synechocystis). The list of plasmids used in this study is provided in Table S2, while the primers are detailed in Table S3. The plasmid sequence maps for the replicative plasmid (pWD301) and Mob plasmid (pWD133) were uploaded (https://gitlab.com/mmp-uva/crispr-cas12a-synechocystis). All constructs were validated by Sanger sequencing (Eurofins).

1.3 | YFP Fluorescence Measurement

All *Synechocystis* protein-degradation-tag mutants were inoculated into liquid BG11 medium. Once the OD reached 1, 150 μL of the cultures were transferred to a black, flat-bottom 96-well microplate (Greiner). YFP fluorescence was measured using a fluorescence plate reader (SYNERGY Mx, BioTeK) with an excitation wavelength of 500 ± 9 nm and emission at 532 ± 9 nm.

Author Contributions

Wei Du conceived and designed this study under the supervision of Filipe Branco dos Santos; Luna L. Meister optimized the conjugation method; Tobias van Grinsven constructed majority of the protein-degradation-tag mutants. Wei Du and Filipe Branco dos Santos wrote the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article and from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.