Short Title: A Golden Gate-based toolkit for cyanobacteria

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Title: **CyanoGate: A modular cloning suite for engineering cyanobacteria based on the plant MoClo syntax**

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One-sentence summary: A Golden Gate-based assembly standard was developed for cloning and transformation in cyanobacteria that is compatible with, and builds on, the broadly established plant modular cloning syntax.

Author Contributions: RV, GARG, AAS and AM designed the study with input from BW; RV, GARG, AAS, AP, JM, MDG, KV performed experiments; VZ designed online software; BW contributed research reagents and materials. AM prepared the manuscript with input from experimentalists and BW, CJH and DL-S.

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ABSTRACT

Recent advances in synthetic biology research have been underpinned by an exponential increase in available genomic information and a proliferation of advanced DNA assembly tools. The adoption of plasmid vector assembly standards and parts libraries has greatly enhanced the reproducibility of research and the exchange of parts between different labs and biological systems. However, a standardised Modular Cloning (MoClo) system is not yet available for cyanobacteria, which lag behind other prokaryotes in synthetic biology despite their huge potential regarding biotechnological applications. By building on the assembly library and syntax of the Plant Golden Gate MoClo kit, we have developed a versatile system called CyanoGate that unites cyanobacteria with plant and algal systems. Here, we describe the generation of a suite of parts and acceptor vectors for making i) marked/unmarked knock-outs or integrations using an integrative acceptor vector, and ii) transient multigene expression and repression systems using known and previously undescribed replicative vectors. We tested and compared the CyanoGate system in the established model cyanobacterium *Synechocystis* sp. PCC 6803 and the more recently described fast-growing strain *Synechococcus elongatus* UTEX 2973. The UTEX 2973 fast-growth phenotype was only evident under specific growth conditions; however, UTEX 2973 accumulated high levels of proteins with strong native or synthetic promoters. The system is publicly available and can be readily expanded to accommodate other standardised MoClo parts to accelerate the development of reliable synthetic biology tools for the cyanobacterial community.

INTRODUCTION

Much work is focused on expanding synthetic biology approaches to engineer photosynthetic organisms, including cyanobacteria. Cyanobacteria are an evolutionarily ancient and diverse phylum of photosynthetic prokaryotic organisms that are ecologically important, and are thought to contribute *ca*. 25% to oceanic net primary productivity (Castenholz, 2001; Flombaum et al., 2013). The chloroplasts of all photosynthetic eukaryotes, including plants, resulted from the endosymbiotic uptake of a cyanobacterium by a eukaryotic ancestor (Keeling, 2004). Therefore, cyanobacteria have proved useful as model organisms for the study of photosynthesis, electron transport and associated biochemical pathways, many of which are conserved in eukaryotic algae and higher plants. Several unique aspects of cyanobacterial photosynthesis, such as the biophysical carbon concentrating mechanism, also show promise as a means for enhancing productivity in crop plants (Rae et al., 2017).

Furthermore, cyanobacteria are increasingly recognized as valuable platforms for industrial biotechnology to convert CO_2 and H_2O into valuable products using solar energy (Tan et al., 2011; Ducat et al., 2011; Ramey et al., 2015). They are metabolically diverse and encode many components (e.g. P450 cytochromes) necessary for generating high-value pharmaceutical products that can be challenging to produce in other systems (Nielsen et al., 2016; Wlodarczyk et al., 2016; Pye et al., 2017; Stensjö et al., 2017). Furthermore, cyanobacteria show significant promise in biophotovoltaic devices for generating electrical energy (McCormick et al., 2015; Saar et al., 2018).

Based on morphological complexity, cyanobacteria are classified into five sub-sections (I–V) (Castenholz, 2001). Several members of the five sub-sections have been reportedly transformed (Vioque, 2007; Stucken et al., 2012), suggesting that many cyanobacterial species are amenable to genetic manipulation. Exogenous DNA can be integrated into or removed from the genome through homologous recombination-based approaches using natural transformation, conjugation (tri-parental mating), or electroporation (Heidorn et al., 2011). Exogenous DNA can also be propagated by replicative vectors, although the latter are currently restricted to a single vector type based on the broad-host range RSF1010 origin (Mermet-Bouvier et al., 1993; Huang et al., 2010; Taton et al., 2014). Transformation tools have been developed for generating "unmarked" mutant strains (lacking an antibiotic resistance marker cassette) in several model species, such as *Synechocystis* sp. PCC 6803 (*Synechocystis* hereafter) (Lea-Smith et al., 2016). More recently, markerless genome editing using CRISPR-based approaches has been demonstrated to function in both unicellular and filamentous strains (Ungerer and Pakrasi, 2016; Wendt et al., 2016).

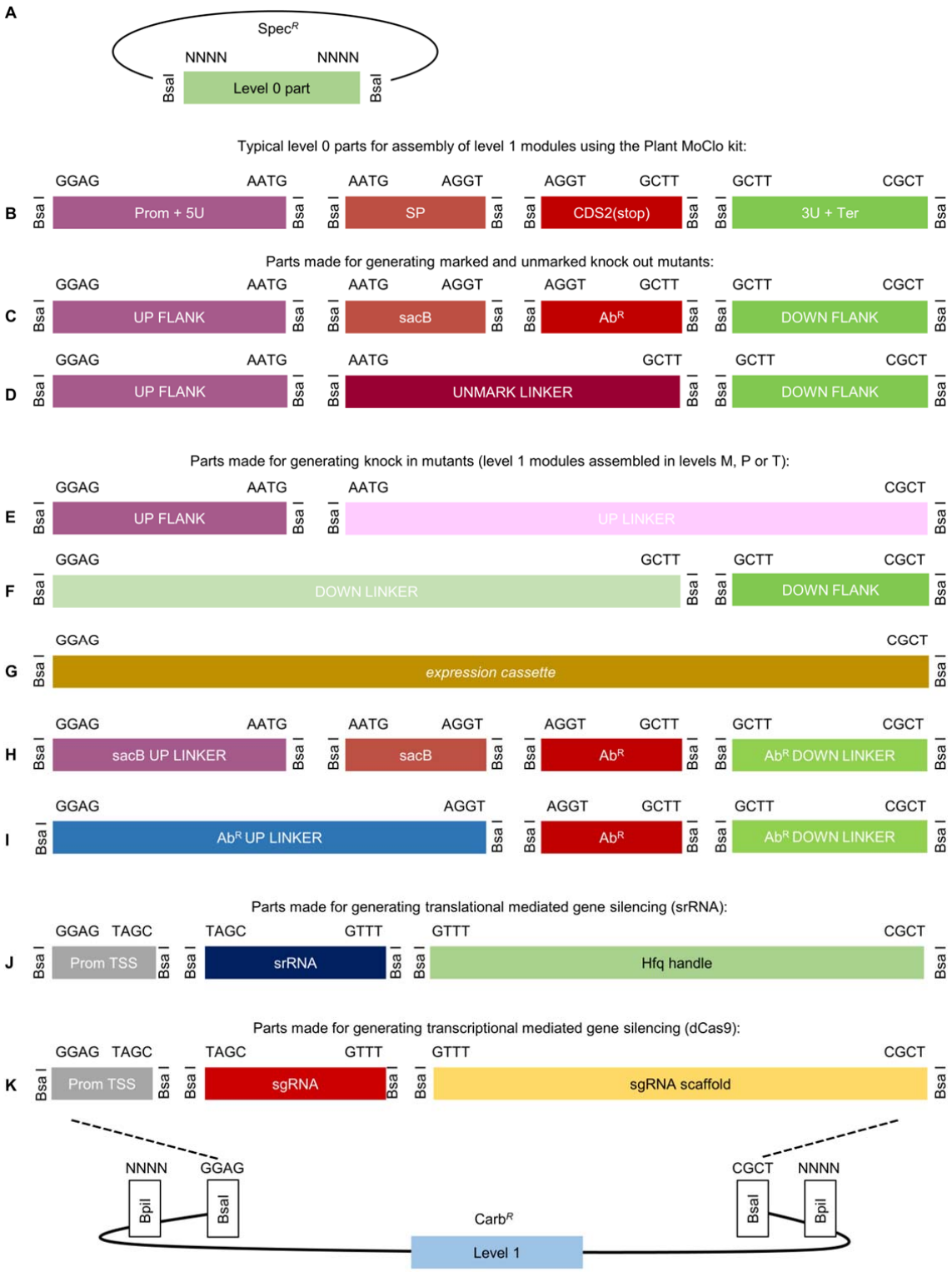
Although exciting progress is being made in developing effective transformation systems, cyanobacteria still lag behind in the field of synthetic biology compared to bacterial (heterotrophic), yeast and mammalian systems. Relatively few broad host-range genetic parts have been characterised, but many libraries of parts for constructing regulatory modules and circuits are starting to become available, albeit using different standards, which makes them difficult to combine (Huang and Lindblad, 2013; Camsund et al., 2014; Albers et al., 2015; Markley et al., 2015; Englund et al., 2016; Kim et al., 2017; Immethun et al., 2017; Taton et al., 2017; Ferreira et al., 2018; Li et al., 2018; Liu and Pakrasi, 2018; Wang et al., 2018). One key challenge is clear: parts that are widely used in *Escherichia coli* behave very differently in model cyanobacterial species, such as *Synechocystis* (Heidorn et al., 2011). Furthermore,

different cyanobacterial strains generally show a wide variation regarding functionality and performance of different genetic parts (e.g. promoters, reporter genes and antibiotic resistance markers) (Taton et al., 2014; Englund et al., 2016; Kim et al., 2017; Taton et al., 2017). This suggests that parts need to be validated, calibrated, and perhaps modified for individual strains, including model species and strains that may be more commercially relevant. Rapid cloning and assembly methods are essential for accelerating the 'design, build, test and learn' cycle, which is a central tenet of synthetic biology (Nielsen and Keasling, 2016).

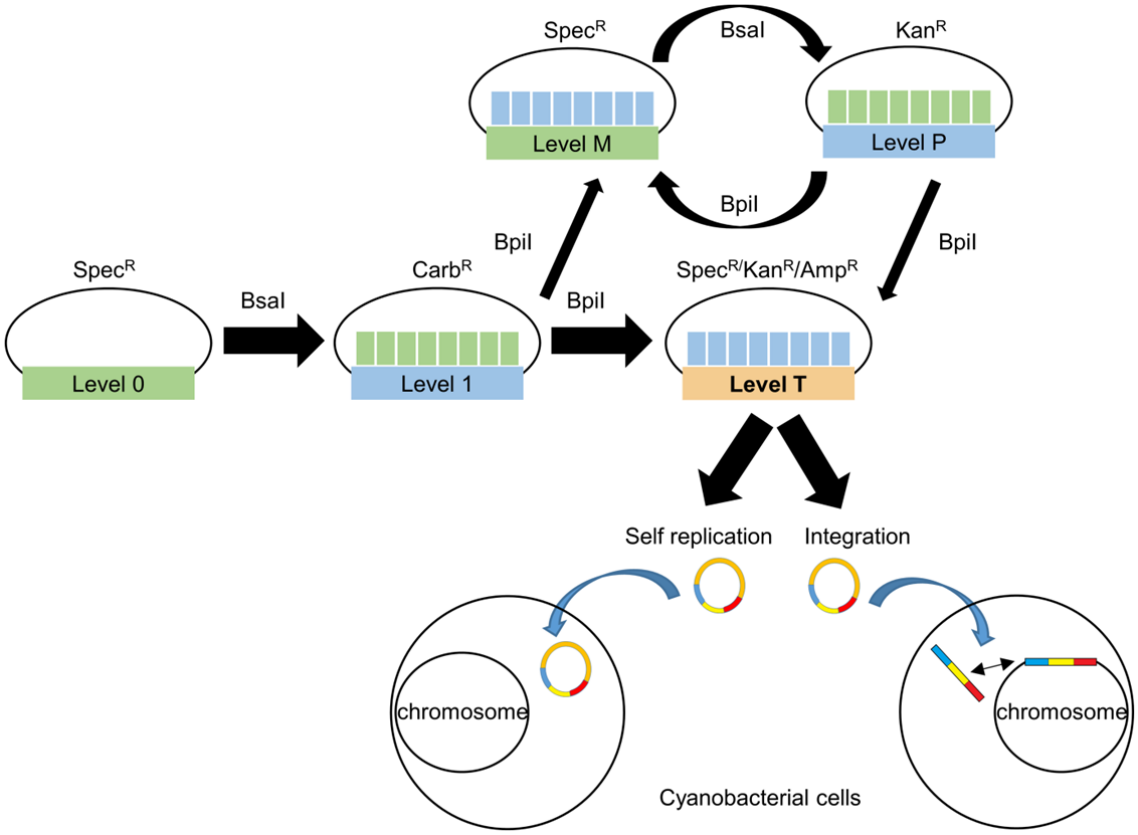
The adoption of new cloning and vector assembly methods (e.g. Isothermal (Gibson) Assembly and MoClo), assembly standards and part libraries has greatly enhanced the scalability of synthetic biology-based approaches in a range of biological systems (Moore et al., 2016; Vazquez-Vilar et al., 2018). Recent advances in synthetic biology have led to the development of standards for Type IIS restriction endonuclease-mediated assembly (commonly known as Golden Gate cloning) for several model systems, including plants (Sarrion-Perdigones et al., 2013; Engler et al., 2014; Andreou and Nakayama, 2018). Based on a common Golden Gate Modular Cloning (MoClo) syntax, large libraries are now available for fusion of different genetic parts to assemble complex vectors cheaply and easily without proprietary tools and reagents (Patron et al., 2015). High-throughput and automated assembly are projected to be widely available soon through DNA synthesis and construction facilities, such as the UK DNA Synthesis Foundries, where MoClo is seen as the most suitable assembly standard (Chambers et al., 2016).

Here, we describe the development of an easy-to-use system called CyanoGate that unites cyanobacteria with plant and algal systems. This system builds on the established Golden Gate MoClo syntax and assembly library for plants (Engler et al., 2014) that has been adopted by the OpenPlant consortium (www.openplant.org), iGEM competitions as "Phytobricks" and the MoClo kit for the microalga *Chlamydomonas reinhardtii* (Crozet et al., 2018). Firstly, we constructed and characterised a suite of known and new genetic parts (level 0) for use in cyanobacterial research, including promoters, terminators, antibiotic resistant markers, neutral sites and gene repression systems (Na et al., 2013; Yao et al., 2015; Sun et al., 2018). Secondly, we designed an additional level of acceptor vectors (level T) to facilitate integrative or replicative transformation. We characterised assembled level T vectors in *Synechocystis* and in *Synechococcus elongatus* UTEX 2973 (UTEX 2973 hereafter), which has a reported doubling time similar to that of *Saccharomyces cerevisiae* under specific

145 growth conditions (Yu et al., 2015; Ungerer et al., 2018a; 2018b). Lastly, we developed an
146 online tool for assembly of CyanoGate and Plant MoClo vectors to assist with the adoption of
147 the CyanoGate system.
148
149 **RESULTS AND DISCUSSION**
150 **Construction of the CyanoGate system**
151 The CyanoGate system integrates with the two-part Golden Gate MoClo Plant Tool Kit,
152 which can be acquired from Addgene [standardised parts (Kit #1000000047) and backbone
153 acceptor vectors (Kit # 1000000044), (www.addgene.org)] (Engler et al., 2014). A
154 comparison of the benefits of MoClo- and Gibson assembly-based cloning strategies is shown
155 in **Supplemental Information S1.** The syntax for level 0 parts was adapted for prokaryotic
156 cyanobacteria to address typical cloning requirements for cyanobacterial research (**Fig. 1**).
157 New level 0 parts were assembled from a variety of sources (**Supplemental Table S1**). Level
158 1, M and P acceptor vectors were adopted from the MoClo Plant Tool Kit, which facilitates
159 assembly of level 0 parts in a level 1 vector, and subsequently up to seven level 1 modules in
160 level M. Level M assemblies can be combined further into level P and cycled back into level
161 M to produce larger multi-module vectors if required (**Supplemental Information S2**).
162 Vectors >50 kb in size assembled by MoClo have been reported (Werner et al., 2012).
163 Modules from level 1 or level P can be assembled in new level T vectors designed for
164 cyanobacterial transformation (**Fig. 2**). We found that both UTEX 2973 and *Synechocystis*
165 produced recombinants following electroporation or conjugation methods with level T
166 vectors. For the majority of the work outlined below, we relied on the conjugation approach.
167
168 **Integration - generating marked and unmarked knock-out mutants**
169 A common method for engineering stable genomic knock-out and knock-in mutants in
170 several cyanobacteria relies on homologous recombination via integrative (suicide) vectors
171 using a two-step marked-unmarked strategy (Lea-Smith et al., 2016) (**Supplemental**
172 **Information S3**). Saar et al. (2018) recently used this approach to introduce up to five
173 genomic alterations into a single *Synechocystis* strain. Firstly, marked mutants are generated
174 with an integrative vector carrying two sequences (approximately 1 kb each) identical to the
175 regions of the cyanobacterial chromosome flanking the deletion/insertion site. Two gene
176 cassettes are inserted between these flanking sequences: a levansucrase expression cassette
177 (*sacB*) that confers sensitivity to transgenic colonies grown on sucrose and an antibiotic
178 resistance cassette (Ab^R) of choice. Secondly, unmarked mutants (carrying no selection

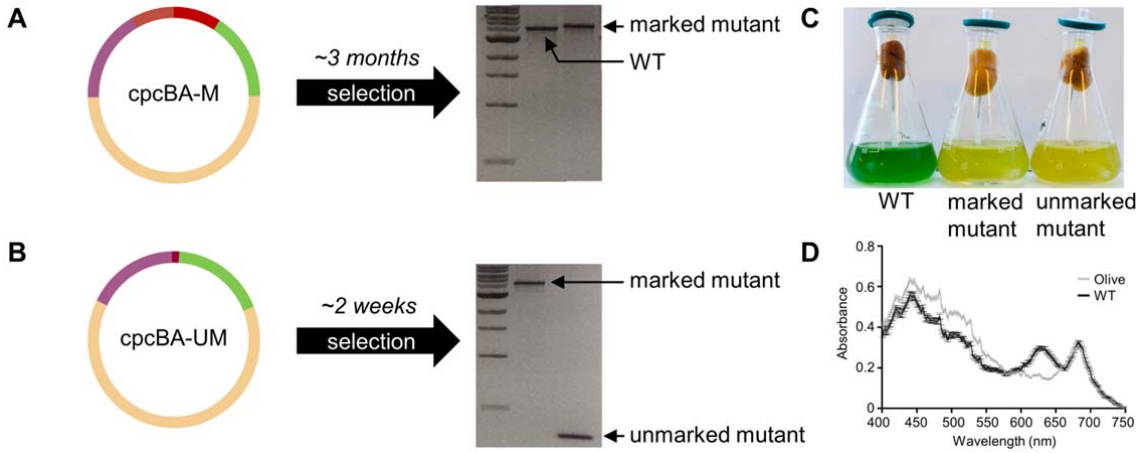


179 markers) are generated from fully segregated marked lines using a separate integrative vector
180 carrying only the flanking sequences and selection on plates containing sucrose.
181



We adapted this approach for the CyanoGate system (**Fig. 1**). To generate level 1 vectors for making knock-out mutants, sequences flanking the upstream (UP FLANK) and downstream (DOWN FLANK) site of recombination were ligated into the plant MoClo Prom+5U (with overhangs GGAG-AATG), and 3U+Ter (GCTT-CGCT) positions, respectively, to generate new level 0 parts (**Fig. 1B**). In addition, full expression cassettes were made for sucrose selection (*sacB*) and antibiotic resistance (Ab^{R}Spec, Ab^{R}Kan and Ab^{R}Ery) in level 0 that ligate into positions SP (AATG-AGGT) and CDS2 (stop) (AGGT-GCTT), respectively. Marked level 1 modules can be assembled using UP FLANK, DOWN FLANK, *sacB* and the required Ab^R level 0 part. For generating the corresponding unmarked level 1 module, a short 59-bp linker (UNMARK LINKER) can be ligated into the CDS1ns (AATG-GCTT) position for assembly with an UP FLANK and DOWN FLANK (**Fig. 1D**). Unmarked and marked level 1 modules can then be assembled into level T integrative vectors, with the potential capacity to include multiple knock-out modules in a single level T vector.

To validate our approach, we constructed the level 0 flanking vectors pC0.024 and pC0.025 and assembled two level T integrative vectors using pUC19-T (*cpcBA*-M and *cpcBA*-UM, with and without the *sacB* and Ab^R cassettes, respectively) to remove the *cpcBA* promoter and operon in *Synechocystis* and generate an "Olive" mutant unable to produce the



phycobiliprotein C-phycocyanin (Kirst et al., 2014; Lea-Smith et al., 2014) (**Fig. 3**; **Supplemental Table S1**). Following transformation with *cpcBA*-M, we successfully generated a marked Δ*cpcBA* mutant carrying the *sacB* and the Ab^RKan cassettes after selective segregation (*ca*. 3 months) (**Fig. 3A**). The unmarked Δ*cpcBA* mutant was then isolated following transformation of the marked Δ*cpcBA* mutant with *cpcBA*-UM and selection on sucrose (*ca*. 2 weeks) (**Fig. 3B**). Absence of C-phycocyanin in the Olive mutant resulted in a characteristic drop in absorbance at 625 nm (**Fig. 3D**) and a significant reduction in chlorophyll content compared to that in WT cells (28.4 ± 0.2 and 48.3 ± 0.2 amol chl $cell^{-1}$, respectively) (Kirst et al., 2014; Lea- Smith et al., 2014).

Generating knock-in mutants

Flexibility in designing level 1 insertion cassettes is needed when making knock-in mutants. Thus, for knock-in mutants the upstream and downstream sequences flanking the insertion site, and any required expression or marker cassettes, are first assembled into separate level 1 modules from UP FLANK and DOWN FLANK level 0 parts (**Fig. 1E, F**). Seven level 1 modules can be assembled directly into Level T (**Fig. 2**). Therefore, with a single pair of flanking sequences, up to five level 1 expression cassettes could be included in a Level T vector.

Linker parts (20 bp) UP FLANK LINKER and DOWN FLANK LINKER were generated to allow assembly of level 0 UP FLANK and DOWN FLANK parts into separate level 1 acceptor vectors. Similarly, level 0 linker parts were generated for *sacB* and Ab^R (**Fig. 1H, I**). Level 1 vectors at different positions can then be assembled in level T (or M) containing one or more expression cassettes, an Ab^R of choice, or both *sacB* and Ab^R (**Fig. 2**).

Using this approach, CyanoGate can facilitate the generation of knock-in mutants using a variety of strategies. For example, if retention of a resistance marker is not an experimental requirement (e.g. Liberton et al., 2017), only a single antibiotic resistance cassette needs to be included in level T. Alternatively, a two-step marked-unmarked strategy could be followed, as for generating knock-out mutants.

Whereas knock-out strategies can target particular loci, knock-in approaches often rely on recombination at designated 'neutral sites' within the genome of interest that can be disrupted with no or minimal impact on the growth phenotype (Ng et al., 2015; Pinto et al., 2015). Based on loci reported in the literature, we have assembled a suite of flanking regions to target neutral sites in *Synechocystis* (designated 6803 NS1-4) (Pinto et al., 2015), *Synechococcus* sp. PCC 7002 (PCC 7002 hereafter) (designated 7002 NS1 and NS2) (Ruffing et al., 2016; Vogel et al., 2017), and neutral sites common to UTEX 2973, PCC 7942 and *Synechococcus elongatus* PCC 6301 (designated 7942 NS1-3) (Bustos and Golden, 1992; Kulkarni and Golden, 1997; Andersson et al., 2000; Niederholtmeyer et al., 2010) (**Supplemental Table S1**). Pinto et al. (2015) have qualitatively compared the impact of the four *Synechocystis* neutral sites assembled here under several different growth conditions, and observed that insertions at 6803 NS3 and NS4 had no significant effect on growth compared to that of WT cultures, whereas insertions at NS2 and NS1 had small but significant effects depending on the growth conditions. Several studies have used 6803 NS3, for example, to engineer a *Synechocystis* strain for the bioremediation of microcystins (Dexter et al., 2018) and the development of T7 polymerase-based synthetic promoter systems (Ferreira et al., 2018). For the two PCC 7002 neutral sites, growth rates with insertions at 7002 NS1 were slightly reduced (Vogel et al., 2017), but not significantly affected with insertions at 7002 NS2 (Ruffing et al., 2016). Insertions at the three 7942 neutral sites reportedly have no phenotypic effect on morphology or growth rate (Clerico et al., 2007; Niederholtmeyer et al., 2010) and have been used to study mRNA stability and translation (Kulkarni and Golden, 1997), circadian rhythms (Anderson et al. 2000), chromosome duplication (Watanabe et al., 2017) and to engineer PCC 7942 for synthesising heterologous products (Niederholtmeyer et al., 2010; Gao et al., 2016). When using the neutral sites supplied with CyanoGate (or others), we would still recommend a thorough growth analysis under the specific culturing conditions being tested to identify any potential impact of the inserted DNA on growth phenotype.

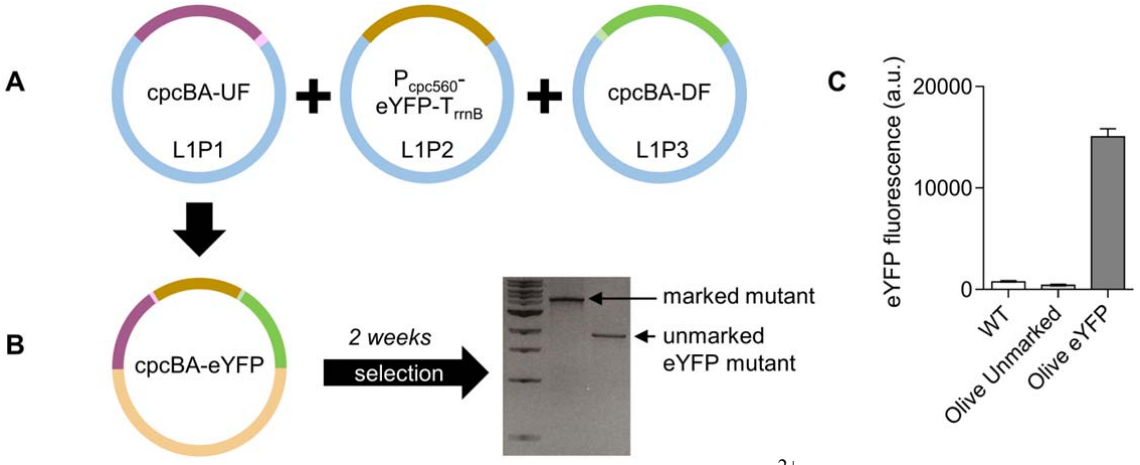
To validate our system, we generated a level T vector carrying the flanking regions for the *cpcBA* operon and an eYFP expression cassette (*cpcBA*-eYFP) (**Fig. 4A, B; Supplemental Table S1**). We successfully transformed this vector into our marked "Olive" *Synechocystis* mutant, and generated a stable olive mutant with constitutive expression of eYFP (Olive-eYFP) (**Fig. 4C**).

Expression comparison for promoter parts in *Synechocystis* and UTEX 2973

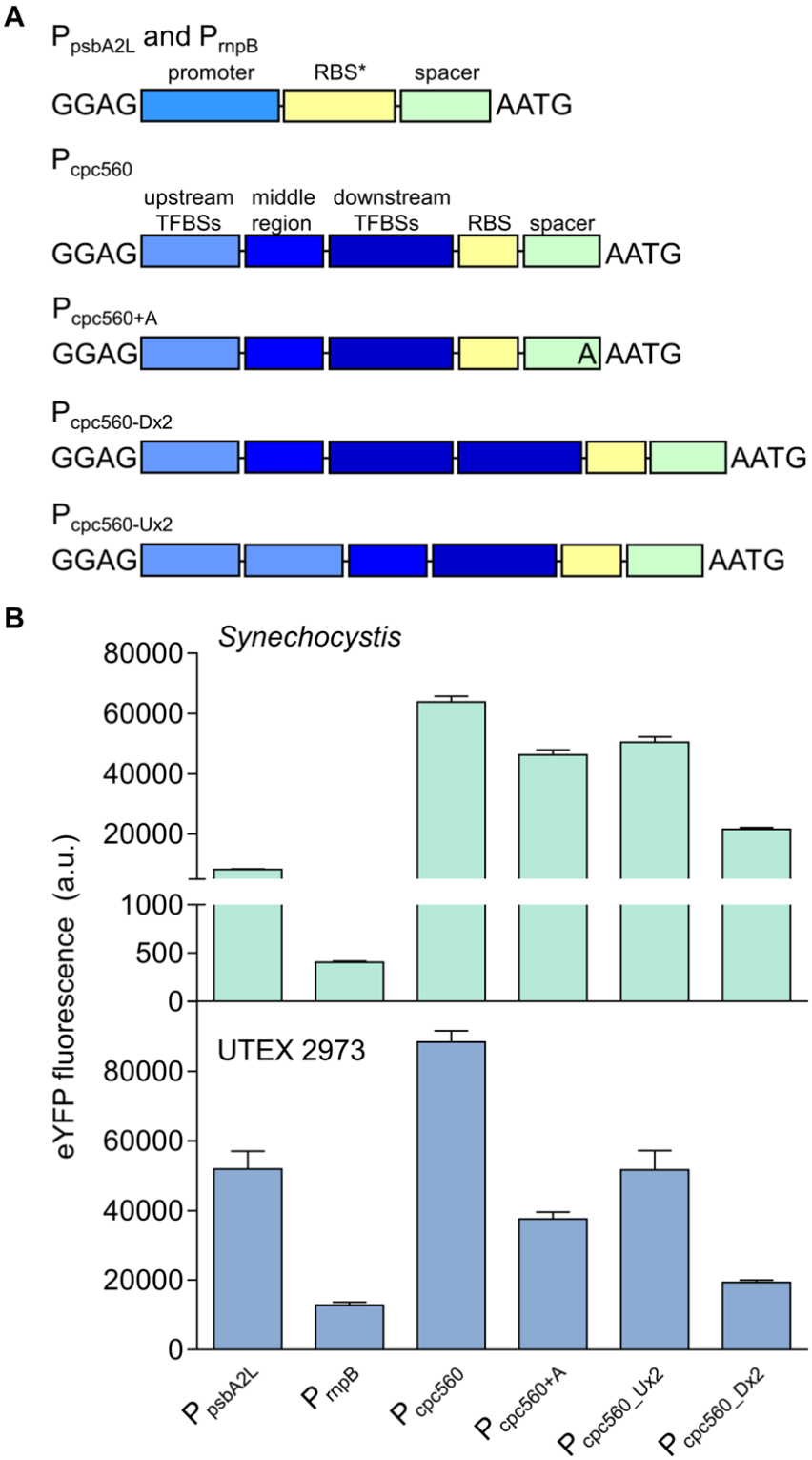
We constructed level 0 parts for a wide selection of synthetic promoters and promoters native to *Synechocystis*. Promoters were assembled as expression cassettes driving eYFP in replicative level T vector pPMQAK1-T to test for differences in expression when conjugated into *Synechocystis* or UTEX 2973. We first compared the growth rates of *Synechocystis*, UTEX and PCC 7942 [a close relative of UTEX 2973 (Yu et al., 2015)] under a variety of different culturing conditions (**Supplemental Figure S1**). We found that growth rates were comparable between *Synechocystis* and PCC 7942 at temperatures below 40°C regardless of light levels and supplementation with CO_2. In contrast, UTEX 2973 grew poorly under those conditions. UTEX 2973 only showed an enhanced growth rate at 45°C under the highest light tested (500 µmol photons m^{-2} s^{-1}) with CO_2, whereas all three strains failed to grow at 50°C. These results confirm that the enhanced growth phenotype reported for UTEX 2973 requires specific conditions as reported by Ungerer et al. (2018a; 2018b). Furthermore, they are consistent with recent reports that this phenotype is linked to an increased stress tolerance, which has been attributed to a small number of nucleotide polymorphisms (Lou et al., 2018; Ungerer et al., 2018b). We proceeded with CyanoGate part characterisations and comparisons under the best conditions achievable for *Synechocystis* and UTEX 2973 (see Materials and Methods) (**Supplemental Figure S2A**).

Promoters native to Synechocystis

We assembled several previously reported promotors from *Synechocystis* in the CyanoGate kit. These include six inducible/repressible promoters (P_{nrsB}, $P_{coaT,}$ P_{nirA}, P_{petE}, P_{isiAB}, and P_{arsB}), which were placed in front of the strong, synthetic *Synechocystis* ribosomal binding site (RBS*) (Heidorn et al., 2011) as used in Englund et al. (2016) (**Supplemental Table S1; Supplemental Information S4**). P_{nrsB} and P_{coaT} drive the expression of nickel and cobalt ion efflux pumps and are induced by Ni^{2+}, and Co^{2+} or Zn^{2+} , respectively (Peca et al., 2008; Blasi et al., 2012; Guerrero et al., 2012; Englund et al., 2016). P_{nirA}, from the nitrate assimilation operon, is induced by the presence of NO_3^- and/or NO_2^- (Kikuchi et al., 1996; Qi et al., 2005).



293 P_{petE} drives the expression of plastocyanin and is induced by Cu^{2+}, which has previously been
294 used for the expression of heterologous genes (Guerrero et al., 2012; Camsund et al., 2014).
295 The promoter of the *isiAB* operon (P_{isiAB}) is repressed by Fe^{3+} and activated when the cell is
296 under iron stress (Kunert et al., 2003). P_{arsB} drives the expression of a putative arsenite and
297 antimonite carrier and is activated by AsO_2^- (Blasi et al., 2012).
298
299 We also cloned the *rnpB* promoter, P_{rnpB}, from the Ribonuclease P gene (Huang et al., 2010),
300 a long version of the *psbA2* promoter, P_{psbA2L}, from the Photosystem II protein D1 gene
301 (Lindberg et al., 2010; Englund et al., 2016) and the promoter of the C-phycocyanin operon,
302 P_{cpc560} (also known as P_{cpcB} and P_{cpcBA}) (Zhou et al., 2014). P_{rnpB} and P_{psbA2L} were placed in
303 front of RBS* (Heidorn et al., 2011) (**Fig. 5A**). To build on a previous functional
304 characterisation of P_{cpc560} (Zhou et al., 2010), we assembled four variants of this strong
305 promoter. Firstly, $P_{cpc560+A}$ consisted of the promoter and the 4-bp MoClo overhang AATG.
306 Secondly, P_{cpc560} was truncated by one bp (A), so that that the start codon was aligned with
307 the native P_{cpc560} RBS spacer region length. Zhou et al. (2014) identified 14 predicted
308 transcription factor binding sites (TFBSs) in the upstream region of P_{cpc560} (-556 to -381 bp)
309 and removal of this region resulted in a significant loss of promoter activity. However,
310 alignment of the reported TFBSs showed their locations are in the downstream region of the
311 promoter (-180 to -5 bp). We identified 11 additional predicted TFBSs using Virtual
312 Footprint (Munch et al., 2005) in the upstream region and hypothesised that the promoter
313 activity may be modified by duplicating either of these regions. So thirdly, we generated
314 P_{cpc560_Dx2} containing a duplicated downstream TFBS region. For P_{cpc560_Dx2}, only the region
315 between -31 to -180 bp was duplicated to avoid repeating the Shine-Dalgarno (SD) sequence.
316 Fourthly, we duplicated the upstream region to generate P_{cpc560_Ux2}. We then assembled P_{rnpB},
317 P_{psbA2L} and the four P_{cpc560} variants with eYFP and the *rrnB* terminator (T_{rrnB}) into a Level 1



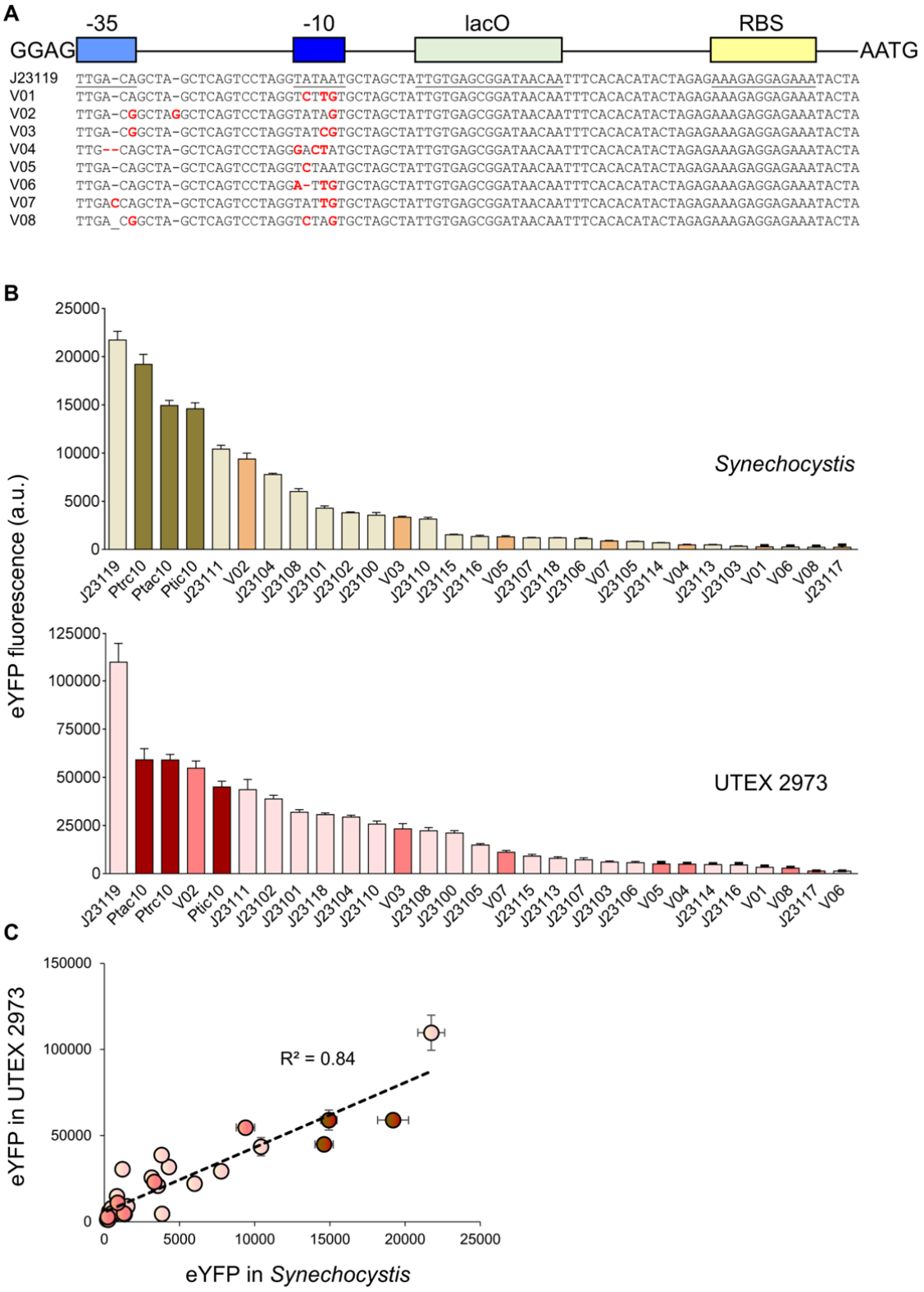
318 expression cassette, and subsequently into a level T replicative vector (pPMQAK1-T) for
319 expression analysis (**Supplemental Table S2**).
320

In *Synechocystis* the highest expressing promoter was P_{cpc560} (**Fig. 5B**), which indicated that maintaining the native RBS spacer region for P_{cpc560} is important for maximising expression. Neither P_{cpc560_Dx2} nor P_{cpc560_Ux2} resulted in higher expression levels compared to that of P_{cpc560}. P_{cpc560_Dx2}–driven expression was strongly decreased compared to that of P_{cpc560}, suggesting that promoter function is sensitive to modification of the downstream region and this region could be a useful target for modulating P_{cpc560} efficacy. Previous work in *Synechocystis* has suggested that modification of the middle region of P_{cpc560} (-380 to -181 bp) may also affect function (Lea-Smith et al., 2014). P_{psbA2L} produced lower expression levels than any variant of P_{cpc560} in *Synechocystis*, whereas P_{rnpB} produced the lowest expression levels. The observed differences in expression levels are consistent with those in other studies with *Synechocystis* (Camsund et al., 2014; Englund et al., 2016; Liu and Pakrasi, 2018).

In UTEX 2973, the trend in expression patterns was similar to that in *Synechocystis* (**Fig. 5B**). However, the overall expression levels of eYFP measured in UTEX 2973 were significantly higher than in *Synechocystis*. P_{cpc560} was increased by 30%, whereas P_{rnpB} showed a 20-fold increase in expression relative to *Synechocystis*. The relative expression strength of P_{psbA2L} was also higher than in *Synechocystis*, and second only to P_{cpc560} in UTEX 2973. As promoters derived from P_{psbA} are responsive to increasing light levels (Englund et al., 2016), the increased levels of expression for P_{psbA2L} may be associated with the higher light intensities used for growing UTEX 2973 compared to that used for *Synechocystis*. Background fluorescence levels were similar between UTEX 2973 and *Synechocystis* conjugated with an empty pPMQAK1-T vector (i.e. lacking an eYFP expression cassette), which suggested that the higher fluorescence values in UTEX 2973 were a direct result of increased levels of eYFP protein.

Heterologous and synthetic promoters

A suite of twenty constitutive synthetic promoters was assembled in level 0 based on the modified BioBricks BBa_J23119 library of promoters (Markley et al., 2015), and the synthetic P_{trc10}, P_{tic10} and P_{tac10} promoters (Huang et al., 2010; Albers et al., 2015) (**Supplemental Table S1; Supplemental Information S4**). We retained the broad-range BBa_B0034 RBS (AAAGAGGAGAAA) and *lac* operator (*lacO*) from Huang et al. (2010), for future *lacI*-based repression experiments (*lacI* and the P_{lacIQ} promoter are included in the CyanoGate kit) (Bhal et al., 1977). We cloned eight new variants (J23119MH_V01-8) with



355 mutations in the canonical BBa_J23119 promoter sequence (**Fig. 6A**). Additionally, we
356 included the L-arabinose-inducible promoter from *E. coli* (P_{BAD}) (Abe et al., 2014).
357

We encountered an unexpected challenge with random internal deletions in the -35 and -10 regions of some promoters of the BBa_J23119 library and *trc* promoter variants when cloning them into level 0 acceptors. Similar issues were reported previously for the *E. coli* EcoFlex kit (Moore et al., 2016) that may relate to the functionality of the promoters and the host vector copy number in *E. coli*, which consequently resulted in cell toxicity and selection for mutated promoter variants. To resolve this issue, we generated a low copy level 0 promoter acceptor vector compatible with CyanoGate (pSB4K5 acceptor) for cloning recalcitrant promoters (**Supplemental Table S1; Supplemental Information S4**). Subsequent assemblies in level 1 and T showed no indication of further mutation.

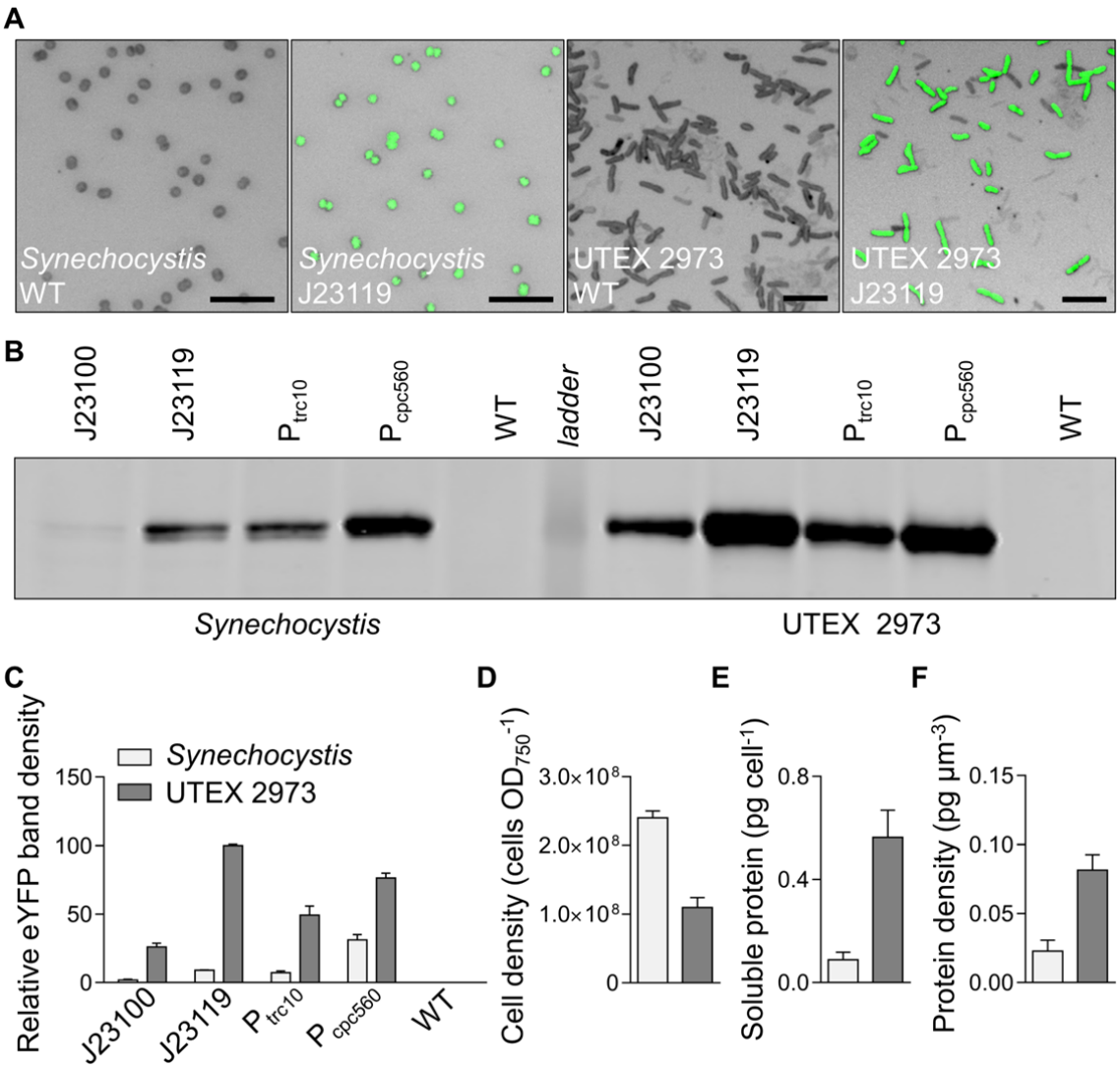
We then tested the expression levels of eYFP driven by the synthetic promoters in *Synechocystis* and UTEX 2973 following assembly in pPMQAK1-T (**Fig. 6B; Supplemental Table S2**). The synthetic promoters showed a 120-fold dynamic range in both cyanobacterial strains. Furthermore, a similar trend in promoter expression strength was observed (R^2 = 0.84) (**Fig. 6C)**. However, eYFP expression levels were on average eight-fold higher in UTEX 2973 compared to that in *Synechocystis*. In *Synechocystis*, the highest expression levels were observed for J23119 and P_{trc10}, but these were still approximately 50% lower than values for the native P_{cpc560} promoters (**Fig. 5B**). The expression trends for the BBa_J23119 library were consistent with the subset reported by Camsund et al. (2014) in *Synechocystis*, whereas the observed differences between P_{trc10} and P_{cpc560} were similar to those reported by Liu and Pakrasi (2018).

In contrast, the expression levels in UTEX 2973 for J23119 were approximately 50% higher than P_{cpc560}. Several synthetic promoters showed expression levels in a similar range to those for the native P_{cpc560} promoter variants, including P_{trc10}, J23111 and the J23119 variant V02. V02 is identical to J23111 except for an additional 'G' between the -35 and -10 motifs, suggesting that small changes in the length of this spacer region may not be critical for promoter strength (similar expression levels were also observed for these two promoters in *Synechocystis*). In contrast, a single bp difference between J23111 and J23106 in the -35 motif resulted in an eight- and ten-fold reduction in expression in *Synechocystis* and UTEX 2973, respectively. The results for UTEX 2973 were unexpected, and to our knowledge no studies to date have directly compared these promoters in this strain. Recent work has examined the expression of β-galactosidase using promoters such as P_{cpc560} and P_{trc} in UTEX 2973 (Li et al., 2018). Li et al. (2018) highlighted that different growth environments (e.g.

light levels) can have significant effects on protein expression. Changes in culture density can also affect promoter activity, such that protein expression levels can change during the exponential and stationary growth stages depending on the promoter and expression vector used (Ng et al., 2015; Madsen et al., 2018). Here we tracked eYFP expression levels over time for three days during early and late exponential growth phase for *Synechocystis* and UTEX 2973. Although expression levels for each promoter fluctuated over time, with peak expression levels at 24 hr and 48 hr in UTEX 2973 and *Synechocystis*, respectively, the overall expression trends were generally consistent for the two strains (**Supplemental Figure S2B**).

Protein expression levels in *Synechocystis* and UTEX 2973

To investigate further the increased levels of eYFP expression observed in UTEX 2973 compared to that in *Synechocystis*, we examined cell morphology, protein content and eYFP protein abundances in expression lines for each strain. Confocal image analysis confirmed the coccoid and rod shapes of *Synechocystis* and UTEX 2973, respectively, and the differences in cell size (van de Meene et al. 2006; Yu et al., 2015) (**Fig. 7A**). Immunoblot analyses of eYFP from protein extracts of four eYFP expressing strains correlated well with previous flow cytometry measurements (**Fig 5; 6**). eYFP driven by the J23119 promoter in UTEX 2973 produced the highest levels of eYFP protein (**Fig. 7B, C**). Although the density of cells in culture was two-fold higher in *Synechocystis* compared to that in UTEX 2973 (**Fig. 7D)**, the protein content per cell was six-fold lower (**Fig. 7E**). We then estimated the average cell volumes for *Synechocystis* and UTEX 2973 at 3.91 ± 0.106 μm^3 and 6.83 ± 0.166 μm^3 (n = 50 each), respectively, based on measurements from confocal microscopy images (**Supplemental Figure S3**). Based on those estimates, we calculated that the density of soluble protein per cell was four-fold higher in UTEX 2973 compared to that in *Synechocystis* (**Fig. 7F**). Thus, we hypothesised that the enhanced levels of eYFP observed in UTEX 2973 were a result of the expression system harnessing a larger available amino acid pool. Mueller et al. (2017) have reported that UTEX 2973 has an increased investment in amino acid content compared to that in PCC 7942, which may be linked to higher rates of translation in UTEX 2973. Therefore, UTEX 2973 continues to show promise as a bioplatform for generating heterologous protein products, although future work should study production rates under conditions optimal for faster growth (Lou et al., 2018; Ungerer et al., 2018a). Recent characterisation of the UTEX 2973 transcriptome will also assist with native promoter characterisations (Tan et al., 2017).

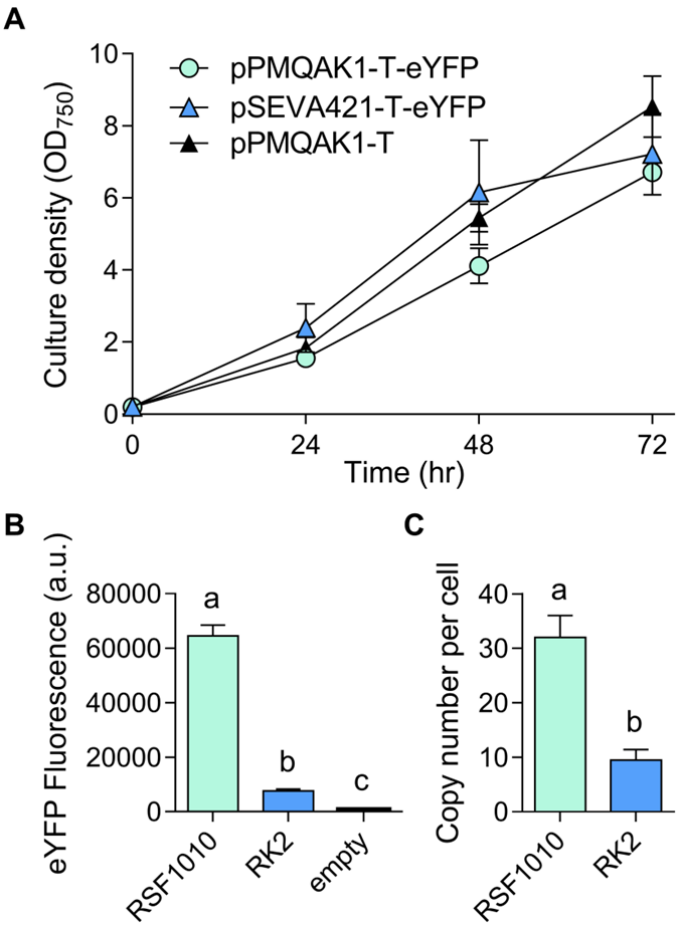


426

427 **The RK2 origin of replication is functional in *Synechocystis***

428 Synthetic biology tools (e.g. gene expression circuits, CRISPR/Cas-based systems) are often
429 distributed between multiple plasmid vectors at different copy numbers in order to synthesise
430 each component at the required concentration (Bradley et al., 2016). The large RSF1010
431 vector is able to replicate in a broad range of microbes including gram-negative bacteria such
432 as *E. coli* and several cyanobacterial species. However, for 25 years it has remained the only
433 non-native vector reported to be able to self-replicate in cyanobacteria (Mermet-Bouvier et
434 al., 1993). Recently, two small plasmids native to *Synechocystis*, pCA2.4 and pCB2.4, have
435 been engineered for gene expression (Armshaw et al., 2015; Ng et al., 2015; Liu and Pakrasi,
436 2018). The pANS plasmid (native to PCC 7942) has also been adapted as a replicative vector,
437 but so far it has been only shown to function in PCC 7942 and *Anabaena* PCC 7120 (Chen et
438 al., 2016). Similarly, the high copy number plasmid pAQ1 (native to PCC 7002) has been

439 engineered for heterologous expression, but up to now it has only been used in PCC 7002
440 (Xu et al., 2011). To expand the replication origins available for cyanobacterial research
441 further we tested the capacity for vectors from the SEVA library to replicate in *Synechocystis*
442 (Silva-Rocha et al., 2013).
443
444 We acquired three vectors driven by three different replication origins [pSEVA421 (RK2),
445 pSEVA431 (pBBR1) and pSEVA442 (pRO1600/ColE1)] and carrying a spectinomycin
446 antibiotic resistance marker. These vectors were domesticated and modified as level T
447 acceptor vectors, assembled and then transformed into *Synechocystis* by electroporation or
448 conjugation. Only *Synechocystis* strains conjugated with vectors carrying RK2 (pSEVA421-
449 T) grew on spectinomycin-containing plates (**Supplemental Table S1; Supplemental**
450 **Information S4**). To confirm that RSF1010 and RK2 replication origins can replicate
451 autonomously in *Synechocystis*, we recovered the pPMQAK1-T or pSEVA421-T vector from
452 lysates of axenic *Synechocystis* strains previously conjugated with each vector by
453 transformation into *E. coli*. The identity and integrity of pPMQAK1-T and pSEVA421-T
454 extracted from transformed *E. coli* colonies were confirmed by restriction digest and Sanger
455 sequencing.
456
457 We then assembled two level T vectors with an eYFP expression cassette (P_{cpc560}-eYFP-
458 T_{rrnB}) to produce pPMQAK1-T-eYFP and pSEVA421-T-eYFP, which were conjugated into
459 *Synechocystis* (**Fig. 8; Supplemental Table S2**). Both pPMQAK1-T-eYFP and pSEVA421-
460 T-eYFP transconjugates grew at similar rates in 50 μg ml^{-1} kanamycin and 5 μg ml^{-1}
461 spectinomycin, respectively (**Fig. 8A**). However, eYFP levels were 8-fold lower in
462 pSEVA421-T-eYFP, suggesting that RK2 has a reduced copy number relative to RSF1010 in
463 *Synechocystis* (**Fig. 8B**). We measured the heterologous plasmid vector copy number in
464 strains expressing pSEVA421-T or pPMQAK1-T and estimated an average copy number per
465 cell of 9 ± 2 and 31 ± 5, respectively (**Fig. 8C**). The copy number for pPMQAK1-T was
466 similar to values reported previously for RSF1010-derived vectors in *Synechocystis* (*ca*. 30)
467 (Ng et al., 2000). Our results are also consistent with the lower copy numbers in *E. coli* for
468 vectors with RK2 (4–7 copies) compared to those with RSF1010 (10–12 copies) replication
469 origins (Frey et al., 1992; Blasina et al., 1996). Furthermore, we compared the genome copies
470 per cell between transformants and wild-type strains and found no significant differences -
471 the average value was 11 ± 2, which is consistent with the typical range of genome copy
472 numbers observed in *Synechocystis* cells (Zerulla et al., 2016).



Gene repression systems

CRISPR (clustered regularly interspaced short palindromic) interference (CRISPRi) is a relatively new but well characterised tool for modulating genes expression at the transcription stage in a sequence-specific manner (Qi et al., 2013; Behler et al., 2018). CRISPRi typically uses a nuclease deficient Cas9 from *Streptococcus pyogenes* (dCas9) and has been demonstrated to work in several cyanobacterial species, including *Synechocystis* (Yao et al., 2015), PCC 7002 (Gordon et al., 2016); PCC 7942 (Huang et al., 2016) and *Anabaena* sp. PCC 7120 (Higo et al., 2018). A second approach for gene repression uses rationally designed small regulatory RNAs (srRNAs) to regulate gene expression at the translation stage (Na et al., 2013; Higo et al., 2016). The synthetic srRNA is attached to a scaffold to recruit the Hfq protein, an RNA chaperone that is conserved in a wide-range of bacteria and cyanobacteria, which facilitates the hybridization of srRNA and target mRNA, and directs mRNA for degradation. The role of cyanobacterial Hfq in interacting with synthetic srRNAs is still unclear (Zess et al., 2016). However, regulatory ability can be improved by introducing Hfq from *E. coli* into *Synechocystis* (Sakai et al., 2015). Both CRISPRi- and

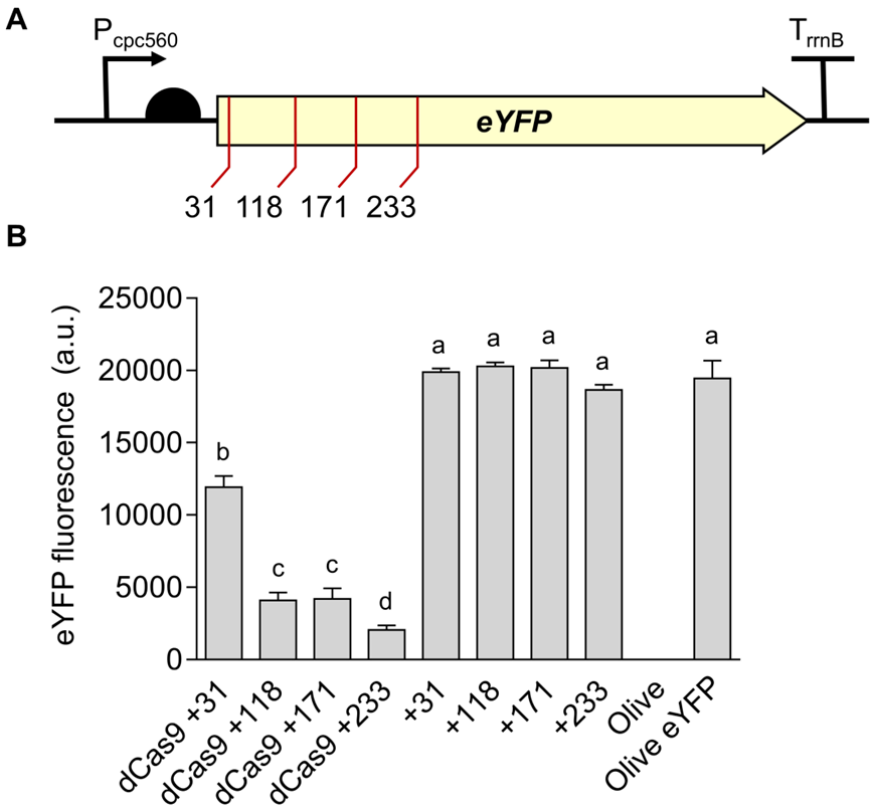
srRNA-based systems have potential advantages as they can be used to repress multiple genes simultaneously.

To validate the CRISPRi system, we assembled an expression cassette for dCas9 (P_{cpc560}-dCas9-T_{rrnB}) on the Level 1 position 1 vector pICH47732, and four different sgRNA expression cassettes (P_{trc10_TSS}-sgRNA-sgRNA scaffold) targeting eYFP on the Level 1 position 2 vector pICH47742 (Engler et al., 2014) (**Supplemental Table S2**). For assembly of CRISPRi sgRNA expression cassettes in level 1, we targeted four 18–22-bp regions of the eYFP non-template strand with an adjacent 3' protospacer adjacent motif (PAM) of 5′-NGG-3′, as required by *S. pyogenes* dCas9 (**Fig. 9A**). The sgRNA sequences contained no off-target sites in the *Synechocystis* genome (confirmed by CasOT; Xiao et al., 2014). The sgRNAs were made by PCR using two complementary primers carrying the required overhangs and *Bsa*I sites, and were assembled with P_{trc10}_TSS promoter (pC0.203) and the sgRNA scaffold (pC0.122) (**Fig. 1K**). Level T vectors were assembled carrying dCas9 and a single sgRNA, or just the sgRNA alone. We subsequently conjugated the Olive-eYFP mutant and tracked eYFP expression.

Transconjugates carrying only the sgRNA showed no reduction in eYFP level compared to that in non-transconjugated Olive-eYFP (**Fig. 9B**). However, all strains carrying dCas9 and a sgRNA showed a decrease in eYFP that ranged from 40–90% depending on the sgRNA used. These reductions are similar to those observed previously in PCC 7002 and in *Synechocystis* (Yao et al., 2016; Gordon et al., 2016) and demonstrated that CRISPRi system is functional in the CyanoGate kit.

CONCLUSION

The CyanoGate kit was designed to increase the availability of well characterised libraries and standardised modular parts in cyanobacteria (Sun et al., 2018). We aimed to simplify and accelerate modular cloning methods in cyanobacterial research and allow integration with the growing number of labs that rely on the established common plant and algal syntax for multi-part DNA assembly (Patron et al., 2015; Crozet et al., 2018). Here, we have demonstrated the functionality of CyanoGate in sufficient detail to show that it is straightforward to adopt and functionally robust across two different cyanobacterial species. CyanoGate includes parts for usage in other cyanobacterial species and could likely be utilised also in non-cyanobacterial microbes amenable to transformation (e.g. *Rhodopseudomonas* spp.) and adapted for use in



subcellular eukaryotic compartments of prokaryote origin (e.g. chloroplasts) (Economou et al., 2014; Doud et al., 2017; Leonard et al., 2018). In addition to the parts discussed, we have also assembled a suite of 21 terminators (**Supplemental Table S1**). To increase the accessibility and usability of the CyanoGate, we have included the vector maps for all parts and new acceptors (**Supplemental Information S4**), implemented support for Cyanogate assemblies in the online DNA "Design and Build" portal of the Edinburgh Genome Foundry (dab.genomefoundry.org) (**Supplemental Information S5**), and submitted all vectors as a toolkit for order from Addgene (Addgene Kit #1000000146; www.addgene.org/kits/mccormick-cyanogate).

Standardisation will help to accelerate the development of reliable synthetic biology tools for biotechnological applications and promote sharing and evaluation of genetic parts in different species and under different culturing conditions (Patron et al., 2015). Going forward, it will be important to test the performance of different parts with different components (e.g. gene expression cassettes) and in different assembly combinations. Several groups using plant MoClo assembly have reported differences in cassette expression and functionality depending on position and orientations (e.g. Ordon et al., 2017), which highlights a key synthetic

biology crux - the performance of a system is not simply the sum of its components (Mutalik et al., 2013; Heyduk et al., 2018).

The increasing availability of genome-scale metabolic models for different cyanobacterial species and their utilisation for guiding engineering strategies for producing heterologous high-value biochemicals has helped to re-invigorate interest in the industrial potential of cyanobacteria (Knoop et al., 2013; Hendry et al., 2016; Mohammadi et al., 2016; Shirai et al., 2016). Future efforts should focus on combining genome-scale metabolic models with synthetic biology approaches, which may help to overcome the production yield limitations observed for cyanobacterial cell factories (Nielsen et al., 2016), and will accelerate the development of more complex and precise gene control circuit systems that can better integrate with host metabolism and generate more robust strains (Bradley and Wang 2015; Jusiak et al., 2016; Luan and Lu, 2018). The future development of truly 'programmable' photosynthetic cells could provide significant advancements in addressing fundamental biological questions and tackling global challenges, including health and food security (Dobrin et al., 2016; Medford and Prasad, 2016; Smanksi et al., 2016).

MATERIALS AND METHODS

Cyanobacterial culture conditions

Cyanobacterial strains of *Synechocystis*, UTEX 2973 and *Synechococcus elongatus* PCC 7942 (PCC 7942 hereafter) were maintained on 1.5% (w/v) agar plates containing BG11 medium. Liquid cultures were grown in Erlenmeyer flasks (100 ml) containing BG11 medium (Rippka et al., 1979) supplemented with 10 mM $NaHCO_3$, shaken at 100 rpm and aerated with filter-sterilised water-saturated atmospheric air. *Synechocystis* and PCC 7942 strains were grown at 30°C with continuous light (100 µmol photons m^{-2} s^{-1}) and UTEX 2793 strains were grown at 40°C with 300 µmol photons m^{-2} s^{-1} in an Infors Multitron-Pro supplied with warm white LED lighting (Infors HT).

Growth analysis

Growth of *Synechocystis*, UTEX 2973 and PCC 7942 was measured in a Photon Systems Instrument Multicultivator MC 1000-OD (MC). Starter cultures were grown in a Photon Systems Instrument AlgaeTron AG 230 at 30°C under continuous warm-white light (100 µmol photons m^{-2} s^{-1}) with air bubbling and shaken at 160 rpm unless otherwise indicated.

These were grown to an optical density at 750 nm (OD_{750}) of approximately 1.0, and used to seed 80-ml cultures for growth in the MC at a starting OD_{720} of ~0.2 (the MC measures culture growth at OD_{720}). Cultures were then grown under continuous warm-white light at 30°C (300 µmol photons m^{-2} s^{-1}) with air bubbling or 30°C (under 300 or 500 µmol photons m^{-2} s^{-1}) or 41, 45 and 50°C (500 µmol photons m^{-2} s^{-1}) with 5% CO_2 bubbling until the fastest grown strain was at OD_{720} = ~0.9, the maximum accurate OD that can be measured with this device. A total of 5–6 replicate experiments were performed over two separate runs (16 in total), each inoculated from a different starter culture.

Vector construction

Level 0 vectors

Native cyanobacterial genetic parts were amplified from genomic DNA using NEB Q5 High-Fidelity DNA Polymerase (New England Biolabs) (**Fig. 1**; **Supplemental Table S1**). Where necessary, native genetic parts were domesticated (i.e. *Bsa*I and *Bpi*I sites were removed) using specific primers. Alternatively, parts were synthesised as Gblocks® DNA fragments (Integrated DNA Technology) and cloned directly into an appropriate level 0 acceptor (see **Supplemental Information S4** for vector maps) (Engler et al. 2014).

Golden Gate assembly reactions were performed with restriction enzymes *Bsa*I (New England Biolabs) or *Bpi*I (Thermofisher), and T4 DNA ligase (Thermofisher) (see **Supplemental Information S2, S3 and S6** for detailed protocols). Vectors were transformed into One Shot TOP10 chemically competent *Escherichia coli* (Thermofisher) as per the manufacturer's instructions. Transformed cultures were grown at 37°C on [1.5% (w/v)] LB agar plates or in liquid LB medium shaking at 260 rpm, with appropriate antibiotic selection for level 0, 1, M and P vectors as outlined in Engler et al. (2014).

Level T acceptor vectors and new level 0 acceptors

A new level T vector system was designed that provides MoClo-compatible replicative vectors or integrative vectors for genomic modifications in cyanobacteria (Heidorn et al., 2011) (**Fig. 2; Supplemental Table S1; Supplemental Information S4**). For replicative vectors, we modified the pPMQAK1 carrying an RSF1010 replicative origin (Huang et al., 2010) to make pPMQAK1-T, and vector pSEVA421 from the Standard European Vector Architecture (SEVA) 2.0 database (seva.cnb.csic.es) carrying the RK2 replicative origin to make pSEVA421-T (Silva-Rocha et al., 2013). Replicative vector backbones were

607 domesticated to remove native *Bsa*I and *Bpi*I sites where appropriate. The region between the
608 BioBrick's prefix and suffix was then replaced by a *lacZ* expression cassette flanked by two
609 *Bpi*I sites that produce overhangs TGCC and GGGA, which are compatible with the plant
610 Golden Gate MoClo assembly syntax for level 2 acceptors (e.g. pAGM4673) (Engler et al.,
611 2014). For integrative vectors, we domesticated a pUC19 vector backbone and introduced
612 two *Bpi*I sites compatible with a level 2 acceptor (as above) to make pUC19A-T and
613 pUC19S-T. In addition, we made a new low copy level 0 acceptor (pSC101 origin of
614 replication) for promoter parts based on the BioBrick standard vector pSB4K5 (Liu et al.,
615 2018). DNA was amplified using NEB Q5 High-Fidelity DNA Polymerase (New England
616 Biolabs). All vectors were sequenced following assembly to confirm domestication and the
617 integrity of the MoClo cloning site.
618
619 *Level 0 parts for CRISPRi and srRNA*
620 A nuclease deficient Cas9 gene sequence sourced from Addgene (www.addgene.org/44249/)
621 was domesticated and assembled as a level 0 CDS part (**Supplemental Table S1; S2**) (Qi et
622 al., 2013). Five promoters of different strengths were truncated to the transcriptional start site
623 (TSS) and cloned into a new level 0 acceptor vector with the unique overhangs GGAG and
624 TAGC (**Fig. 1**). Two new level 0 parts with the unique overhangs GTTT and CGCT were
625 generated for the sgRNA scaffold and srRNA HFQ handle (based on MicC) (Na et al., 2013),
626 respectively. Assembly of level 1 expression cassettes proceeded by combining appropriate
627 level 0 parts with a PCR product for either a srRNA or sgRNA (**Fig. 1**).
628
629 **Cyanobacterial transformation and conjugation**
630 Transformation with integrative level T vectors was performed as in Lea-Smith et al. (2016).
631 For transformation by electroporation, cultures were harvested during the 'exponential'
632 growth phase (OD_{750} of ~0.6) by centrifugation at 4,000 *g* for 10 min. The cell pellet was
633 washed 3 times with 2 ml of sterile 1 mM HEPES buffer (pH 7.5), re-suspended in water
634 with 3–5 µg of level T vector DNA and transferred into a 0.1-cm electroporation cuvette
635 (Scientific Laboratory Suppliers). Re-suspended cells were electroporated using an
636 Eppendorf 2510 electroporator (Eppendorf) set to 1200 V. Sterile BG-11 (1 ml) was
637 immediately added to the electroporated cells. Following a 1-hr incubation at RT, the cells
638 were plated on 1.5% (w/v) agar plates containing BG-11 with antibiotics at standard working
639 concentrations to select for transformed colonies. The plates were sealed with parafilm and
640 placed under 15 µmol photons m^{-2} s^{-1} light at 30°C for 1 day. The plates were then moved to

30 µmol photons m^{-2} s^{-1} light until colonies appeared. After 15–20 days, putative transformants were recovered and streaked onto new plates with appropriate antibiotics for further study.

Genetic modification by conjugation in *Synechocystis* was facilitated by an *E. coli* strain (HB101) carrying both mobilizer and helper vectors pRK2013 (ATCC® 37159™) and pRL528 (www.addgene.org/58495/), respectively (Tsinoremas et al., 1994). For UTEX 2973, conjugation was facilitated by a MC1061 strain carrying mobilizer and helper vectors pRK24 (www.addgene.org/51950/) and pRL528, respectively. Cultures of HB101 and OneShot TOP10 *E. coli* strains carrying level T cargo vectors were grown for approximately 15 hr with appropriate antibiotics. Cyanobacterial strains were grown to an OD_{750} of ~1. All bacterial cultures were washed three times with either fresh LB medium for *E. coli* or BG11 for cyanobacteria prior to use. *Synechocystis* cultures (100 µl, OD_{750} of 0.5–0.8) were conjugated by combining appropriate HB101 and the cargo strains (100 µl each) and plating onto HATF 0.45-µm transfer membranes (Merck Millipore) placed on LB: BG11 (1: 19) agar plates. For UTEX 2973 conjugations, appropriate MC1061 and the cargo strains (100 µl each) were initially combined and incubated at 30°C for 30 min, then mixed with UTEX 2973 cultures (100 µl, OD_{750} of 0.5–0.8) and incubated at 30°C for 2 hr, and then plated onto transfer membranes as above. *Synechocystis* and UTEX 2973 transconjugates were grown under culturing conditions outlined above. Following growth on non-selective media for 24 hr, the membranes were transferred to BG11 agar plates supplemented with appropriate antibiotics. Colonies were observed within a week for both strains. Chlorophyll content of wild-type (WT) and mutant strains was calculated as in Lea-Smith et al. (2013).

Fluorescence assays

Transgenic strains maintained on agar plates containing appropriate antibiotics were used to inoculate 10-ml seed cultures that were grown to an optical density at 750 nm (OD_{750}) of approximately 1.0, as measured with a WPA Biowave II spectrometer (Biochrom). Seed cultures were diluted to an OD_{750} of 0.2, and 2-ml starting cultures were transferred to 24-well plates (Costar® Corning Incorporated) for experiments. *Synechocystis* and UTEX 2973 strains were grown in an Infors Multitron-Pro in the same culturing conditions described above. OD_{750} was measured using a FLUOstar OMEGA microplate reader (BMG Labtech). Fluorescence of eYFP for individual cells (10,000 cells per culture) was measured by flow cytometry using an Attune NxT Flow Cytometer (Thermofisher). Cells were gated using

675 forward and side scatter, and median eYFP fluorescence was calculated from
676 excitation/emission wavelengths 488 nm/515–545 nm (Kelly et al., 2018) and reported at 48
677 hr unless otherwise stated.
678
679 **Cell counts, soluble protein and eYFP quantification**
680 *Synechocystis* and UTEX 2973 strains were cultured for 48 hr as described above, counted
681 using a haemocytometer and then harvested for soluble protein extraction. Cells were pelleted
682 by centrifugation at 4,000 *g* for 15 min, re-suspended in lysis buffer [0.1 M potassium
683 phosphate buffer (pH 7.0), 2 mM DTT and one Roche cOmplete EDTA-free protease
684 inhibitor tablet per 10 ml (Roche Diagnostics)] and lysed with 0.5 mm glass beads (Thistle
685 Scientific) in a TissueLyser II (Qiagen). The cell lysate was centrifuged at 18,000 *g* for 30
686 min and the supernatant assayed for soluble protein content using Pierce 660nm Protein
687 Assay Reagent against BSA standards (Thermo Fisher Scientific). Extracts were subjected to
688 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a 4–12% (w/v)
689 polyacrylamide gel (Bolt® Bis-Tris Plus Gel; Thermo Fisher Scientific) alongside a SeeBlue
690 Plus2 Prestained protein ladder (Thermo), transferred to polyvinylidene fluoride (PVDF)
691 membrane then probed with monoclonal anti-GFP serum (AbCAM) at 1: 1,000 dilution,
692 followed by LI-COR IRDye ®800CW goat anti-rabbit IgG (LI-COR Inc.) at 1: 10,000
693 dilution, then viewed on an LI-COR Odyssey CLx Imager. eYFP protein content was
694 estimated by immunoblotting using densitometry using LI-COR Lite Studio software v5.2 .
695 Relative eYFP protein abundance was estimated by densitometry using LI-COR Lite Studio
696 software v5.2.
697
698 **Plasmid vector and genome copy number determination**
699 The genome copy number and copy number of heterologous self-replicating plasmid vectors
700 in *Synechocystis* was estimated using a quantitative real-time PCR (qPCR) approach adapted
701 from Zerulla et al. (2016). Cytoplasmic extracts containing total cellular DNA were harvested
702 from *Synechocystis* cultures after 48 hr growth (OD_{750} = *ca*. 5) according to Zerulla et al.
703 (2016). Cells in 10 ml of culture were pelleted by centrifugation at 4,000 *g* for 15 min,
704 disrupted by shaking at 30 Hz for 10 min in a TissueLyser II with a mixture of 0.2-mm and
705 0.5-mm acid washed glass beads (0.35 g each), and then resuspended in dH_2O. The culture
706 cell count was determined prior to harvest using a haemocytometer and checked again after
707 cell disruption to calculate the efficiency of cell disruption. A standard curve based on a
708 dilution series of vector DNA was generated and used for qPCR analysis in parallel with

extracts carrying the same vector. Two DNA fragments (*ca*. 1 kb) targeting two separate loci (*petB* and *secA*) were amplified from isolated genomic DNA from *Synechocystis* using standard PCR (Pinto et al., 2012). DNA mass concentrations were determined photometrically and the concentrations of DNA molecules were calculated from the known molecular mass. As above, a standard curve based on a dilution series of the two fragments was generated to estimate genome copy number in the extracts (Zerulla et al., 2016). The Ct of the extracts were then plotted against the linear portion of the standard curves to estimate plasmid vector copy number and genome copy number per cell. Oligonucleotides used are summarised in **Supplemental Table S3**.

Confocal laser scanning microscopy

Cultures were imaged using Leica TCS SP8 confocal microscopy (Leica Microsystems) with a water immersion objective lens (HCX APO L 20x/0.50 W). Excitation/emission wavelengths were 514 nm/527–546 nm for eYFP and 514 nm/605–710 nm for chlorophyll autofluorescence.

SUPPLEMENTAL DATA

Supplemental Figure S1. Comparison of growth for *Synechocystis*, PCC 7942 and UTEX 2973 under different culturing conditions.

Supplemental Figure S2. Growth and expression levels of heterologous and synthetic promoters in *Synechocystis* and UTEX 2973.

Supplemental Figure S3. Cell volume calculations for *Synechocystis* and UTEX 2973 from confocal microscopy images.

Supplemental Table S1. Table of all parts from CyanoGate kit generated in this work.

Supplemental Table S2. List of level T vectors used in this study.

Supplemental Table S3. Sequences of synthetic oligonucleotides used to determine copy number.

Supplemental Information S1. Comparison of Gibson Assembly and Golden Gate Assembly.

Supplemental Information S2. Detailed assembly strategies using the CyanoGate kit.

Supplemental Information S3. Integrative engineering strategies using the CyanoGate kit.

Supplemental Information S4. Sequence maps (.gb files) of the components of the CyanoGate kit.

Supplemental Information S5. Protocol and online interface for building CyanoGate vector assemblies.

Supplemental Information S6. Protocols for MoClo assembly in level -1 through to level T.

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762

763 **FIGURE LEGENDS**

764 **Figure 1. Adaptation of the Plant Golden Gate MoClo level 0 syntax for generating level**
765 **1 assemblies for transfer to Level T.** A, The format for a level 0 MoClo acceptor vector
766 with the part bordered by two BsaI sites. B, Typical level 0 parts from the Plant MoClo kit
767 (38), where parts of the same type are bordered by the same pair of fusion sites (for each
768 fusion site, only the sequence of the top strand is shown). Note that the parts are not drawn to
769 scale. C and D, The syntax of the Plant MoClo kit was adapted to generate level 0 parts for
770 engineering marked and unmarked cyanobacterial mutant strains (20). E to I, To generate
771 knock-in mutants, short linker parts (30 bp) were constructed to allow assembly of individual
772 flanking sequences, or marker cassettes (Ab^R or *sacB*) in level 1 vectors for subsequent
773 assembly in level T. J and K, Parts required for generating synthetic srRNA or CRISPRi level
774 1 constructs. See **Supplemental Information S2 and S3** for workflows. Abbreviations:
775 3U+Ter, 3'UTR and terminator; Ab^R, antibiotic resistance cassette; Ab^R DOWN LINKER,
776 short sequence (~30 bp) to provide CGCT overhang; Ab^R UP LINKER, short sequence (~30
777 bp) to provide GAGG overhang; CDS2(stop), coding sequence with a stop codon; DOWN
778 FLANK, flanking sequence downstream of target site; DOWN FLANK LINKER, short
779 sequence (~30 bp) to provide GGAG overhang; Prom+5U, promoter and 5' UTR; Prom TSS,
780 promoter transcription start site; *sacB*, levansucrase expression cassette; *sacB* UP LINKER,
781 short sequence (~30 bp) to provide GAGG overhang; sgRNA, single guide RNA; SP, signal
782 peptide; srRNA, small regulatory RNA; UP FLANK, flanking sequence upstream of target
783 site; UP FLANK LINKER, short sequence (~30 bp) to provide CGCT overhang; UNMARK
784 LINKER, short sequence to bridge UP FLANK and DOWN FLANK.

785

786 **Figure 2. Extension of the Plant Golden Gate MoClo Assembly Standard for**
787 **cyanobacterial transformation.** Assembly relies one of two Type IIS restriction
788 endonuclease enzymes (*Bsa*I or *Bpi*I). Domesticated level 0 parts are assembled into level 1
789 vectors. Up to seven level 1 modules can be assembled directly into a level T cyanobacterial
790 transformation vector, which consists of two sub-types (either a replicative or an integrative
791 vector). Alternatively, larger vectors with more modules can be built by assembling level 1
792 modules into level M, and then cycling assembly between level M and level P, and finally
793 transferring from Level P to level T. Antibiotic selection markers are shown for each level.
794 Level T vectors are supplied with internal antibiotic selection markers (shown), but additional
795 selection markers could be included from level 1 modules as required. See **Supplemental**

796 **Table S1** and **Supplemental Information S4** for the full list and maps of level T acceptor
797 vectors.
798
799 **Figure 3. Generating knock-out mutants in cyanobacteria.** A, Assembled level T vector
800 *cpcBA*-M (see Fig. 1C) targeting the *cpcBA* promoter and operon (3,563 bp) to generate a
801 marked Δ*cpcBA* "Olive" mutant in *Synechocystis* sp. PCC 6803. Following transformation
802 and segregation on kanamycin (*ca*. 3 months), a segregated marked mutant was isolated (WT
803 band is 3,925 bp, marked mutant band is 5,503 bp, 1-kb DNA ladder (NEB) is shown). B,
804 Assembled level T vector *cpcBA*-UM (see Fig. 1D) for generating an unmarked Δ*cpcBA*
805 mutant. Following transformation and segregation on sucrose (*ca*. 2 weeks), an unmarked
806 mutant was isolated (unmarked band is 425 bp). C, Liquid cultures of WT, marked and
807 unmarked Olive mutants. D, Spectrum showing the absorbance of the unmarked Olive mutant
808 and WT cultures after 72 hr of growth. Values are the average of four biological replicates ±
809 SE and are standardised to 750 nm.
810
811 **Figure 4. Generating knock-in mutants in cyanobacteria.** A, Assembly of level 1 modules
812 *cpcBA*-UF (see Fig. 1E) in the level 1, position 1 acceptor (L1P1), P_{cpc560}-eYFP-T_{rrnB} (see
813 Fig. 1G) in L1P2 and *cpcBA*-DF (see Fig. 1F) in L1P3. B, Transfer of level 1 assemblies to
814 level T vector *cpcBA*-eYFP for generating an unmarked Δ*cpcBA* mutant carrying an eYFP
815 expression cassette. Following transformation and segregation on sucrose (ca. 3 weeks), an
816 unmarked eYFP mutant was isolated (1,771 bp). C, Fluorescence values are the means ± SE
817 of four biological replicates, where each replicate represents the median measurements of
818 10,000 cells.
819
820 **Figure 5. Expression levels of cyanobacterial promoters in *Synechocystis* and UTEX**
821 **2973.** A, Structure of the cyanobacterial promoters adapted for the CyanoGate kit. Regions of
822 P_{cpc560} shown are the upstream transcription factor binding sites (TFBSs) (-556 to -381 bp),
823 middle region (-380 to -181 bp), and the downstream TFBSs, ribosome binding site (RBS)
824 and spacer (-180 to -5 bp). B, Expression levels of eYFP driven by promoters in
825 *Synechocystis* and UTEX 2973 calculated from measurements taken from 10,000 individual
826 cells. Values are the means ± SE from at least four biological replicates after 48 hr of growth
827 (average OD_{750} values for *Synechocystis* and UTEX 2973 cultures were 3.5 ± 0.2 and 3.6 ±
828 0.2, respectively). See **Supplemental Figure S2** for more info.
829

Figure 6. Expression levels of heterologous and synthetic promoters in *Synechocystis* and UTEX 2973. A, Structure and alignment of eight new synthetic promoters derived from the BioBricks BBa_J23119 library and P_{trc10} promoter design (18). B, Expression levels of eYFP driven by promoters in *Synechocystis* and UTEX 2973 calculated from measurements taken from 10,000 individual cells. C, Correlation analysis of expression levels of synthetic promoters tested in *Synechocystis* and UTEX 2973. The coefficient of determination (R^2) is shown for the J23119 library (red), new synthetic promoters (pink) and *trc* variants (dark red). Values are the means ± SE from at least four biological replicates after 48 hr of growth (average OD_{750} values for *Synechocystis* and UTEX 2973 cultures were 3.5 ± 0.2 and 3.6 ± 0.2, respectively). See **Supplemental Figure S2** for more info.

Figure 7. Protein expression levels in *Synechocystis* and UTEX 2973 cells. A, Confocal images of WT strains and mutants expressing eYFP (eYFP fluorescence shown in green on bright field images) driven by the J23119 promoter (bars = 10 μm). B, Representative immunoblot of protein extracts (3 μg protein) from mutants with different promoter expression cassettes (as in **Fig. 6**) probed with an antibody against eYFP. The protein ladder band corresponds to 30 kDa. C, Relative eYFP protein abundance relative to that in UTEX 2973 mutants carrying the J23119 expression cassette. D to F, Cell density (D), protein content per cell (E) and protein density per estimated cell volume (F) for *Synechocystis* and UTEX 2973. Asterisks (*) indicate significant difference ($P < 0.05$) as determined by Student's *t*-test. Values are the means ± SE of four biological replicates.

Figure 8. Cell growth and expression levels of eYFP with the RK2 replicative origin in *Synechocystis*. A, Growth of strains carrying RK2 (vector pSEVA421-T-eYFP), RSF1010 (pPMQAK1-T-eYFP) or empty pPMQAK1-T, with cultures containing appropriate antibiotic selection. Growth was measured as OD_{750} under a constant illumination of 100 μmol photons $m^{-2}s^{-1}$ at 30°C. B, Expression levels of eYFP after 48 hr of growth calculated from measurements taken from 10,000 individual cells. C, Plasmid copy numbers per cell after 48 hr of growth. Letters indicating significant difference ($P < 0.05$) are shown, as determined by ANOVA followed by Tukey's HSD tests. Values are the means ± SE of four biological replicates.

Figure 9. Gene regulation system using CRISPRi in *Synechocystis*. A, Four target regions were chosen as sgRNA protospacers to repress eYFP expression in Olive-eYFP (**Fig. 4**):

‘CCAGGATGGGCACCACCC’ (+31), ‘ACTTCAGGGTCAGCTTGCCGT’ (+118), ‘AGGTGGTCACGAGGGTGGGCCA’ (+171) and ‘AGAAGTCGTGCTGCTTCATG’ (+233). B, eYFP fluorescence of Olive-eYFP expressing constructs carrying sgRNAs with and without dCas9 (representative of 10,000 individual cells). Untransformed Olive-eYFP and the Olive mutant were used as controls. Letters indicating significant difference ($P < 0.05$) are shown, as determined by ANOVA followed by Tukey’s HSD tests. Values are the means ± SE of four biological replicates.

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