

Synechocystis was engineered to have a green-light-inducible gene

expression mechanism.

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Summary

Synechocystis sp. PCC6803's green-light detecting histidine kinase CcaS, the cognate response regulator CcaR, and the promoter of *cpcG2* were described in order to build a green-light-regulated gene expression system for cyanobacteria (*PcpcG2*). Gene expression from *PcpcG2* is activated by green-light illumination of CcaS and CcaR, which operate as a genetic controller. Native *PcpcG2*'s ability to respond to green light was studied utilising GFPuv as a reporter gene placed into a broad-host-range vector. The expression

Introduction

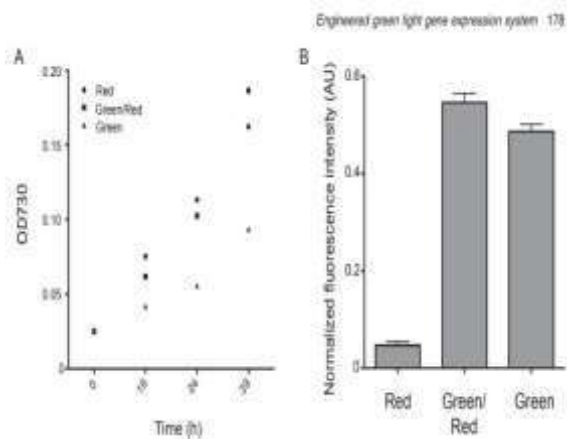
The capacity of cyanobacteria to directly transform carbon dioxide into the desired chemical, needing only sunshine, water, and a few inorganic substances, makes them a perfect host for the manufacture of biofuels or biomaterials. Because most cyanobacteria can be genetically modified, they can be used to produce biofuel and biomaterials more efficiently. There are a number of researchers who are trying to build biosynthetic pathways utilising components from *Escherichia coli* and other species (Atsumi et al., 2009; Liu and Curtiss, 2009; 2012; Oliver et al., 2013). well as the induction of specific genes (Briggs et al., 1990; Geerts et al., 1995; Lopez-Maury et al., 2002). The water recycling technique cannot use chemical inducers since they are difficult to remove from the growth media. The cyanobacterial bioprocess necessitates the development of a new gene expression system. In order to maintain an efficient photosynthesis or prevent photodamage from intense or short-wavelength light, cyanobacteria have a variety of light-sensing mechanisms. A variety of light-sensing systems regulate gene expression, enzymatic activity for the production of second messengers, or phototaxis response upon illumination with varying light, such as UV light; blue light (Yoshihara et al., 2004; Hirose and Yamamoto, 2008; 2010); green light (Terauchi et al., 2004; Hirose et al., of *PcpcG2* was

clearly induced by green-light illumination, however the amount of expression was much lower than that of *P_{trc}*, which has previously been described to be a constitutive promoter in cyanobacteria. Since the 5' untranslated region of the *cpcG2* gene lacks the ShineDalgarno-like motif, an insertion into this area resulted in enhanced CcaR expression. As a consequence, under green-light illumination, the modified green-light detecting system produced 40-fold greater levels of protein expression than the wild-type promoter did. An designed green-light gene expression system might be used to regulate gene expression in new cyanobacterial bioprocesses.

Though it has been hypothesised that some of the promoters are incompatible with cyanobacteria (Huang et al., 2010), it is unlikely that this is the case due to variations in RNA polymerase between bacteria and plants (Schneider and Hasekorn, 1988). Traditional bacterial gene expression systems use chemicals like isopropyl -D-1-thiogalactopyranoside (IPTG) and metal ions, which are impractical for large-scale cyanobacterial cultivation, as

2008; 2010) and red light (Narikawa, 2011; Song et al., 2011). (Yeh et al., 1997; Terauchi et al., 2004). A two-component regulation model underpins the vast majority of sensing systems.

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pKT230-PcpcG2-GFPuv-expressing *Synechocystis* sp. PCC6803 was able to control gene expression by exposing it to green light.

A. Growth curves in red, green, or both green and red light (green/red) are shown. The optical density at 730 nm was used to gauge growth.

B. After 39 h of incubation under each light irradiation, the fluorescence intensity of cells was normalised by their relative optical densities at 730 nm. A plate reader was used to assess the fluorescence intensity after each culture had been rinsed with phosphate buffered saline (ThermoFisher Scientific, Waltham, MA) comprised of a sensor and response regulator for histidine kinase. As a result of the sensor protein's kinase activity being activated by light absorption, the signal is sent to the cognate response regulator.

Gene expression or flagella movement are activated when phosphorylated response regulator (PRR) attaches to a promoter's upstream region. This gene, PcpG2, has been shown to be chromatically controlled by the sensor Histidine Kinase CcaS and the cognate response regulator CcaR in *Synechocystis* PCC6803, a marine bacterium (Hirose et al., 2008). In the presence of green light, CcaS performs autophosphorylation, which is followed by phosphotransfer to CcaR and dephosphorylation of CcaR. A gene expression system was designed to control cyanobacteria's gene expression in response to green light, which is not a primary source of photosynthesis. Using the *Synechocystis* sp. PCC6803 green-light sensing system, we created a

cyanobacteria-specific green-light-regulated gene expression system that included the green-light sensing histidine kinase CcaS, the cognate response regulator CcaR, and PcpG2. Gene expression from PcpG2 is activated by green-light illumination of CcaS and CcaR, which operate as a genetic controller.

Discussion and conclusions

GFPuv reporter protein gene was cloned from *Synechocystis* PCC6803 and placed upstream of the PcpG2 promoter on the broad-host-range vector pKT230 as stated in Supplementary material (Bagdasarian et al., 1981) (Table S1). Under red-light illumination, plasmid was precultured and converted into *Synechocystis* sp. PCC6803. The fluorescence intensity of the cultures was then assessed after the cultures were incubated under continuous irradiation with green light, red light, or both. A rise in the fluorescence intensity of GFPuv-derived fluorescence was seen under green, red, and even red-green light illumination. When the culture's optical density (OD) at 730 nm was normalised, these fluorescence intensities were practically equal (Fig. 1). CcaS phosphatase activity has been shown to dephosphorylate CcaR as a consequence of red-light illumination repressing CcaS. (Hirose et al., 2008). We found that green light may activate CcaS, resulting in transcription via PcpG2, even when red light is also being illuminated simultaneously, which is required for effective cell development. However, the GFPuv fluorescence intensity of expression.

In comparison to gene expression driven by the Ptrc promoter, the intensity was rather low (Abe et al., 2013). We initially focused on the gene dosage impact of CcaS on the GFPuv expression level in an effort to enhance gene expression. The phosphorylation of CcaR by CcaS may not occur in all *Synechocystis* sp., PCC6803 cells that are continually exposed with green and red light. Increasing CcaS in *Synechocystis* sp. PCC6803 may result in an increase in CcaR phosphorylation, which may lead to an increase in GFPuv expression in response to green-light illumination. Plasmids containing PcpG2-GFPuv and the ccaS gene under the native promoter of *Synechocystis* sp. PCC6803 were produced to boost CcaS activity by raising its expression level (Fig. 2A) (Table S1). Expression of both endogenous and extracellular CcaS demonstrated approximately comparable GFPuv-derived fluorescence intensities (Fig. 2B).

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The kinase activity of CcaS toward CcaR is increased by green light, but the dephosphorylation activity of CcaS toward CcaR is suppressed by red light. Because the ratio of kinase to dephosphorylation activity did not change as a consequence of increased CcaS expression, the degree of CcaR phosphorylation remained steady. The impact of CcaR's gene dosage on GFPuv expression was then examined.

Exogenously inserted PcpG2, encoded in the broad-host-range vector pKT230, with a copy number more than 10, may not be completely activated by the greenlight gene expression system since endogenous CcaR was used (Barth and Grinter, 1974). PcpG2GFPuv and the *Synechocystis* sp. PCC6803 ccaR gene were thus co-transfected into a plasmid (Table S1), and the GFPuv expression level was compared under green and red light illumination (Fig. 2A).

Because of this, GFPuv-derived fluorescence intensity was roughly three times greater under green and red light illumination compared to cells simply expressing CcaR. (Fig. 2B). In this study, it was shown that boosting the CcaR expression level increased the green-light gene expression system's ability to induce gene expression. Exogenously overexpressed CcaR is phosphorylated by certain endogenously present histidine kinases, which may be causing an increase in fluorescence intensity when the cells are illuminated with red light rather than green. The binding and activation of a similar promoter by several response regulators has also been described in unphosphorylated conditions (Schar et al., 2005; Kato et al., 2008).

Under red-light illumination, unphosphorylated CcaR may contribute to the background expression. A combination of promoter activity, ribosome binding site strength (RBS), mRNA structure around the RBS and start codon, and certain trans elements, such as antisense RNA, govern protein production levels primarily. Upstream of the start codon, the PcpG2 promoter lacks a characteristic ShineDalgarno (SD) sequence. At reality, just 26% of cyanobacterial genes include an SD-like motif in the ideal place, making them devoid of SD-like sequences (Ma et al., 2002). S1 ribosomal protein detects AU-rich sequences in the 5'-untranslated region (UTR) upstream of the initiation codon in some of these genes that lack an SD-like sequence (Tzareva et al., 1994). PcpG2 expression may possibly be controlled

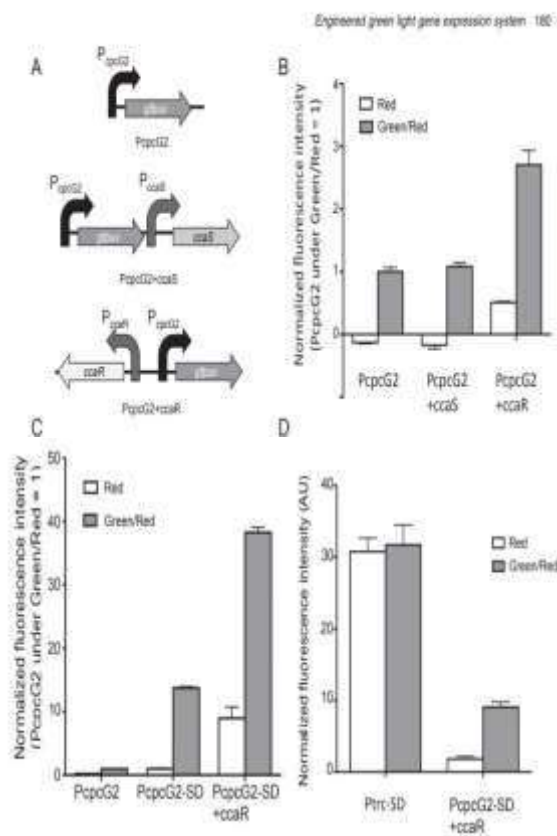
by the S1 ribosomal protein since the upstream region of PcpG2 contains multiple consecutive AU bases (5'-UUAAG UUUAAUUACUAACUUUAUCU-3').

Mfold (Zuker, 2003) was unable to anticipate a stable secondary structure in the area, as indicated by the programme. It was decided to test the impact of inserting an SD-like region on GFPuv gene expression under PcpG2 in light of the low levels of expression seen under PcpG2.

The 5'-UTRs of cyanobacterial genes were screened for SD-like sequence possibilities. Cyanobacteria produce a number of photosynthesis-related proteins, including the psbA-encoded D1 protein and the cpcB encoded c-phycoerythrin, both of which include SD-like sequences in their 5'-UTRs. *Synechocystis* sp. PCC6803's 16S rRNA has an SD-like sequence that is entirely complementary to cpcB's SD-like sequence and is highly conserved throughout other cyanobacteria. That's why it seemed to us that the SD-like sequence of the CPC gene in *Synechococcus* sp.

PCC6803 would have a major effect on the organism. The 5'-UUAAGUAGGAGAUAAAAC-3' SD-like sequence obtained from the cpcB gene of *Synechococcus* sp. PCC7002 was inserted before the GFPuv start codon under the PcpG2 promoter (Table S1). When GFPuv-derived fluorescence was lit with both red and green light, the SD-like sequence added to PcpG2 increased its intensity by around 15 times (PcpG2-SD in Fig. 2C).

Reverse transcription polymerase chain reaction study indicated that the GFPuv mRNA in PcpG2-SD culture was twice as much as in PcpG2 (data not shown), possibly due to improved binding of the ribosome to the mRNA. Because translation efficiency has increased, rather than transcription, GFPuv fluorescence has risen. The expression level in *Escherichia coli* was boosted by the AU-rich stretch upstream of the SD sequence (Komarova et al., 2002). As a result, we believe that the addition of



In *Synechocystis* PCC6803, a green-light detecting system was tested under red or green/red light, as shown in Fig. 2.

- A. In this image, you can see a schematic depiction of each construct injected into pKT230, including PcpG2 (GFPuv) and PcpG2-GFPuv-PCcas (ccaS) and PcpG2-GFPuv-PCcas (ccaR).
- B. PcpG2, PcpG2+ccaS, or PcpG2+ccaR in *Synechocystis* PCC6803 under green/red or red/green light, respectively. PcpG2 normalised under green light is used as a reference point for all other values (set as 1).
- C. PcpG2, pKT230-PcpG2-SD-GFPuv (PcpG2-SD), and PcpG2-SD-GFPuv+ccaR (PcpG2-SD+ccaR) relative fluorescence intensity under red and green/red light for *Synechocystis* sp. PCC6803. PcpG2 normalised under green light is used as a reference point for all other values (set as 1).
- D. In *Synechocystis* sp. PCC6803 the SD-like sequence of cpcB and PcpG2-SD+ccaR was

substituted with the SD-like sequence of pKT230PtrcSD-GFPuv (Ptrc-SD), resulting in a relative normalised fluorescence intensity of 1.5. Using the cell's optical density, each value is normalised. A gene expression method that produces green light

SD-like region had a synergistic impact on protein translation with the AU-rich motif. A pKT230derived vector containing PcpG2-SD-GFPuv and ccaR under the control of its native promoter was then created (Table S1). When we added an SD-like region to the CcaR gene, we hoped to see a synergistic impact of both accelerated transcription and enhanced translation. Cells producing GFPuv without the SDlike sequence or the exogenous CcaR plasmid had fluorescence roughly 40 times less than cells expressing GFPuv when illuminated in red and green (Fig. 2C). The nearly threefold rise from the exogenous CcaR (Fig. 2B) and the almost 15-fold increase from the insertion of the SD-like sequence (Fig. 2A) are virtually directly cumulative (Fig. 2C). The GFPuv expression level under the Ptrc promoter, which also contains an SD-like region, was three times lower than under the built vector (Fig. 2D). Due to a low ON/OFF ratio in *Synechocystis* sp. PCC6803 using

IPTG as inducer (Huang et al., 2010), the PcpG2SD+ccaR system is an appealing option for an inducible gene expression system for *Synechocystis* sp. PC6803. The use of induced promoters, a kind of genetic tool, is critical in the study of gene function and the regulation of protein synthesis. However, nothing is known about cyanobacteria's inducible promoters. Traditional *E. coli* inducible promoters like lactose promoter or derivatives fail in *Synechocystis* sp. PCC6803 (Huang et al., 2010) due to structural changes in RNA polymerase (Schneider and Hasekorn, 1988). In *Synechocystis* sp. PCC 6803, Guerrero and colleagues (2012) found that IPTG induction of the PA1lacO1 promoter was superior to that of the Ptrc promoter. However, synthetic chemical inducers, such as IPTG, which is frequently employed in *E. coli*, are not cost-effective.

Because heavy metal ions are hazardous, nrsB, a nickel-inducible promoter in cyanobacteria that has a high ON/OFF ratio, cannot be used for large-scale

production of biofuel or biomaterials. When using the light-inducible promoter psbA1 and psbA2 to regulate protein expression in cyanobacteria, dark culture is required before induction can occur (Agrawal et al., 2001). In order to regulate gene expression, we developed a green-light sensing system with a high protein expression level and a high ON/OFF ratio in this work. We can culture cyanobacteria with optimum development before gene activation since green light is not needed for photosynthesis. Because CcaS, which possesses a phycocyanobilin chromophore, is known to be activated by green light and inhibited by red light, we assumed that the simultaneous illumination with red and green light would cancel each other out, resulting in a very modest activation of target gene expression (Hirose et al., 2008). Even with red-light illumination, we discovered that green-light illumination had a substantial effect on the expression of target genes. Gene function analysis or biofuel or biomaterial manufacturing might both benefit from this green-light sensor device.

We used an SD-like motif to boost gene expression in this investigation. The use of several RBSs of varying strengths in biofuel production applications might be beneficial in optimising the expression levels of the enzymes in the biosynthetic pathway to minimise unwanted byproducts. With the use of a programme created by Salis and colleagues (2009), a succession of SD-like sequences varying in strength may be easily generated. Gene expression for biofuel and/or biomaterials that need numerous genes to be synthesised will be the final aim. As of right now, we've been able to use green light to control the expression of only one gene product. Single gene expression, the transcription factor and/or gene product regulating cell survival, may alter biofuel and/or biomaterial production levels, even if just one gene expression is controlled. Finally, in *Synechocystis* sp. PCC6803, we discovered a greenlight detecting system composed of the genes ccaS, ccaR, and the target promoter PcpG2. By illuminating PcpG2 with green light and red light, this method stimulated the target gene expression from PcpG2. While maintaining suitable growth conditions with red light, green light may influence gene expression in cyanobacteria. Further engineering of the green-light sensor resulted in a 40-fold increase in target gene expression by boosting CcaR expression and introducing an SD like region. Ptrc, a powerful promoter employed in cyanobacteria recombinant

DNA research, yielded levels of expression equivalent to those found here. An designed green-light gene expression system might be used to regulate gene expression in new cyanobacterial bioprocesses.

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