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WASHINGTON UNIVERSITY IN ST. LOUIS
SCHOOL OF ENGINEERING AND APPLIED SCIENCE
DEPARTMENT OF ENERGY, ENVIRONMENTAL AND
CHEMICAL ENGINEERING

AN IMPROVED PLASMID VECTOR SYSTEM FOR
GENETIC ENGINEERING OF *SYNECHOCYSTIS* SP. PCC
6803, A MODEL CYANOBACTERIUM

By Arul Mozhy varman

Prepared under the direction of Professor Himadri Pakrasi

Thesis presented to the School of Engineering of Washington
University in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

March 2010

Saint Louis, Missouri

Abstract

Cyanobacteria are a group of photosynthetic organisms that consume carbon dioxide and produce oxygen. This group of bacteria inhabits varied ecological niches, an ability conferred by their diverse metabolic capabilities. Recent studies have shown that genetic engineering of cyanobacteria can be used to increase the efficiency of converting atmospheric CO₂ to products such as ethanol and butanol. Plasmid vectors are important tools to carry out such genetic manipulations. In this study, we constructed the set of vectors pSL2034 (6269 bp), pSL2035 (6113 bp), and pSL2036 (6113 bp) that can be used to transform the model cyanobacterium, *Synechocystis* sp. PCC 6803. We then verified the constructed vectors by conducting various restriction digestion experiments. Gene expression in this set of vectors is by the high light promoter of the *psbA2* gene, and these vectors are designed to target introduced DNA to the *psbA1* gene site in the *Synechocystis* 6803 genome, a location observed to be silent under most experimental conditions. Sequencing the vectors confirmed that the active promoter region of the *psbA2* gene is intact. To verify the functional performance of the *psbA2* promoter, the *isiA* ORF was cloned into pSL2035, and the *Synechocystis* 6803 transformants WT:2045 and PAL:2045 were generated. RT-PCR results from the transformants confirmed the constitutive expression of *isiA* mRNA, verifying the activity of the *psbA2* promoter. A 77 K fluorescence assay further showed the presence of the IsiA complex in the PAL transformants, PAL:2045. Henceforth, these vectors can be used to over express introduced genes in *Synechocystis* 6803.

Acknowledgements

I would like to express my sincere gratitude to Prof. Himadri Pakrasi for offering me a wonderful opportunity to work in his lab. I would also like to thank my committee members, Prof. Richard Axelbaum and Prof. Yinjie Tang for their help and discussions with my Master's thesis work. I would like to convey my sincere thanks to Dr. Michelle Liberton for her guidance throughout the project. She provided the kanamycin resistance cassette and the vector pSL2043. It was also a pleasure to have worked with Dr. Anindita Banerjee and Dr. Abhay Singh in my other projects. The experience in molecular biology experiments gained from these projects formed a basis for completing this project on time. I would also like to convey my thanks to Dr. Thanura Elvitigala, Dr. Haijun Liu and Jeff Cameron who have offered expert advice on different areas for my project. Jeff Cameron kindly provided me the primer JC44. I also wish to thank Lingxia Zhao for her support with various lab related work. The financial support was provided by National Science Foundation, FIBR.

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Chapter 1

Introduction

Cyanobacteria or blue-green algae represent one of the more ancient evolutionary lineages among bacteria. Morphologically, they range from unicellular to filamentous, possessing highly varying cell types. They occupy diverse habitats from fresh water to oceans, from soils to rocks and from hot springs to alkaline lakes. Characterized by their ability to carry out plant-like oxygen-evolving photosynthesis, they are an important primary producer in their ecology. They have also become a model organism for studying and understanding photosynthesis, and they are being used as a factory for the conversion of atmospheric CO₂ to useful products. The potential of cyanobacteria for the study of plant-related processes and for the production of bioproducts however can be highly enhanced by genetic engineering. In this study, an improved set of tools have been constructed to over-express foreign as well as host genes in a model cyanobacterium, *Synechocystis* sp. PCC 6803.

1.1 Transformation of cyanobacteria

The transformation of cyanobacteria is a first step in engineering them to produce bioproducts through genetic manipulation. *Transformation* refers to the uptake, incorporation, and expression of genetic materials from the environment. Transformation allows genetic manipulations and the ability to engineer an organism for the purpose of increasing the yield of a certain product. The

creation of a transgenic organism requires a protocol for transferring foreign DNA into the cell, and a system for stably maintaining and expressing it. A variety of approaches have been used to create transgenic organisms. In general, three methods are available for introducing foreign DNA into cyanobacteria: natural transformation, electroporation and conjugation.

Cyanobacterial transformation can be further divided into two groups based upon the type of plasmid vector used: a) Chromosomal or genomic transformation, in which the genome of the cyanobacterium is altered through recombination with the incoming DNA. (This type of transformation is useful to create mutations through *in vitro* manipulations in the genome). b) Transformation by shuttle vectors, in which the incoming DNA remains unaltered in the cyanobacteria and replicates on its own.

The transformation of cyanobacteria was first demonstrated for the species *Anacystis nidulans* 602 (Shestakov et. al., 1970), also known as *Synechococcus* PCC 7943 and was subsequently extended to other species as well. Several species of cyanobacteria are amenable to natural transformation, i.e., they can be transformed without any pretreatment. This includes *Synechococcus* sp. PCC 7942, *Synechococcus* sp. PCC 7002, *Synechocystis* sp. PCC 6803, and *Synechocystis* sp. PCC 6714 (Vermaas, 1996 & Grigorieva et al., 1982). The mechanism these cyanobacteria use to take up the foreign DNA is still poorly understood and it is not known why natural transformation is possible for only some strains. For *Synechocystis* 6803, it was demonstrated that pili are essential

for mobility and transformation (Yoshihara et. al., 2001). There are also reports on genetic exchange in mixed cultures of cyanobacteria (Porter, 1986).

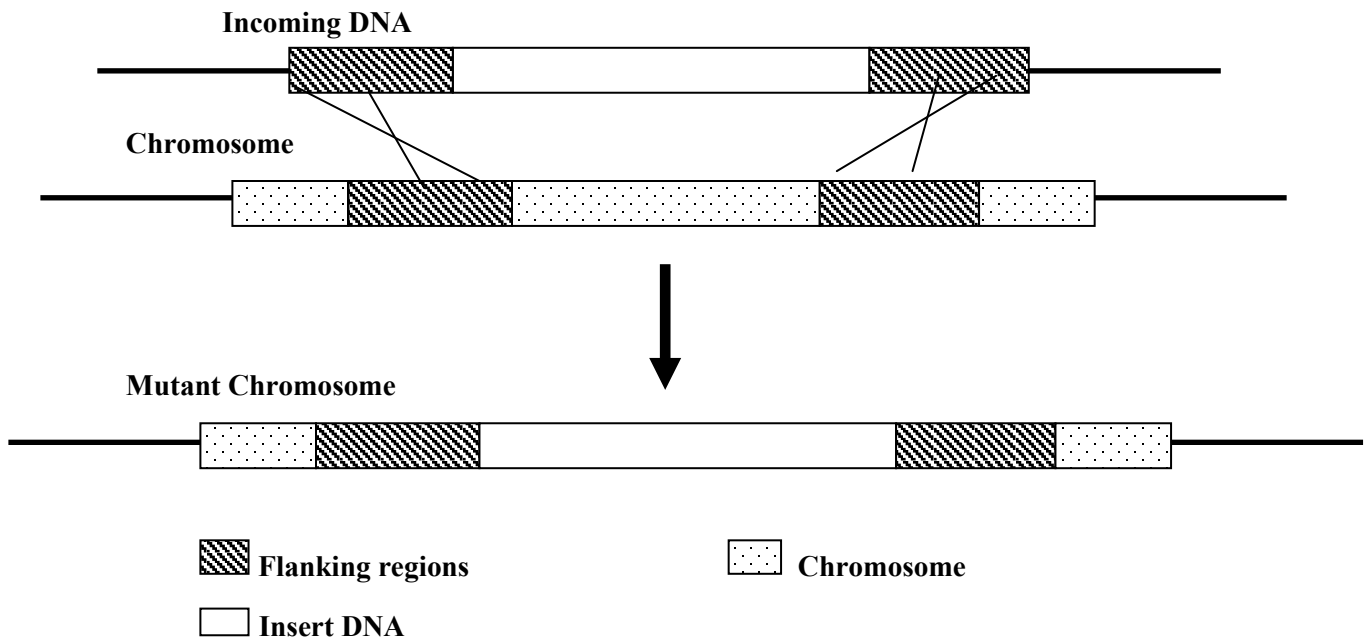


Figure 1. Double recombination between incoming DNA and chromosomal DNA

Homologous double recombination is essential for integrate the sequence of interest to the target site. Figure 1 shows a scheme of homologous double recombination for inserting DNA into the target site in the genome of cyanobacteria. Recombination will occur only between incoming DNA and genomic DNA with similar sequences. Regions of similarity are also called *flanking regions*, and they must be at least 200 bp for successful recombination. Higher transformation frequency and precision can be achieved by designing

longer flanking regions. This method is widely used to generate null mutants and site-directed mutants (Anderson et al., 1996). Along with the foreign DNA, a gene coding for antibiotic resistance is introduced, which allows selection for mutant strains. UV radiation has also been reported to stimulate illegitimate recombination in cyanobacteria, but lack of reproducibility has prevented its use in transformation (Labarre et al., 1989).

Once the barrier of the cell envelope is passed, another obstacle for stable integration is the presence of native restriction endonucleases. The presence of such endonucleases varies among different strains. Methylation of incoming DNA can overcome this problem by providing protection from restriction digestion in many cases. Conjugation is employed as a general procedure to transform cyanobacteria using a helper plasmid encoding methylases, that protect the incoming DNA from endogenous restriction enzymes.

Cyanobacterial strains contain more than one genome copy per cell, depending on growth rate and growth stage (Vermaas, 1996). Hence, a mutant strain of interest would be one in which the target sequence in all genomic copies are replaced with the introduced sequence. The segregation of wild type genomes into mutant copies is achieved by continuously streaking the colonies onto plates that contain high level of antibiotics to which the transformant is resistant. In some cases, complete segregation is not possible if the deletion confers significant disadvantages to the organisms under the culture conditions. A complete segregation of colonies is verified by a colony PCR or, in some cases, by a genomic PCR.

1.2 Model organism

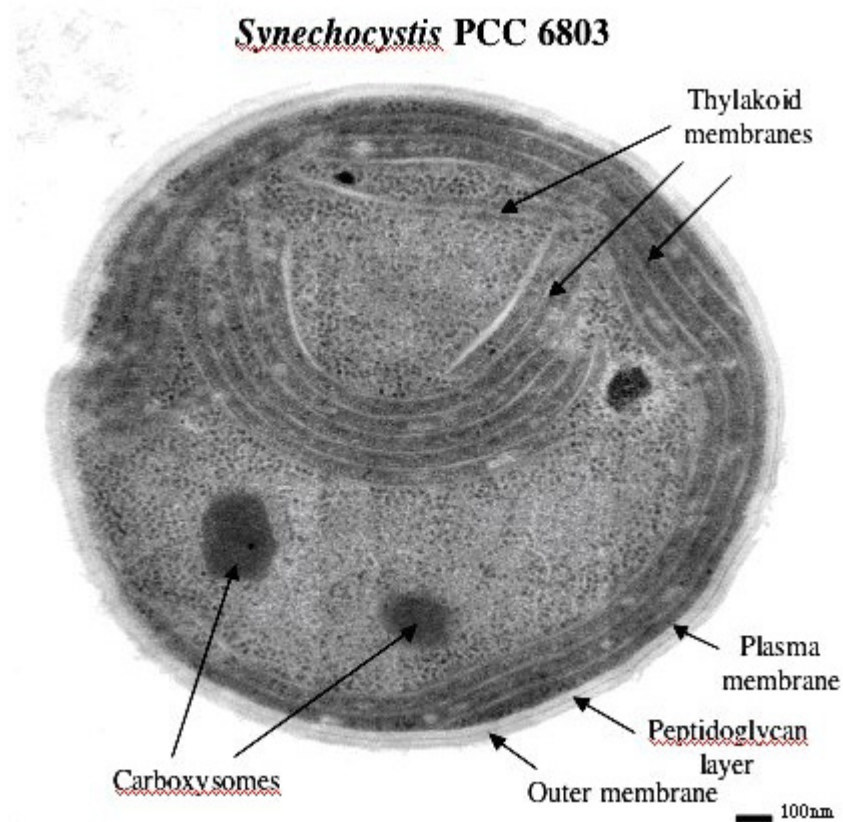


Figure 2. Thin section electron micrograph of a *Synechocystis* 6803 cell (Liberton et al., 2006)

Synechocystis sp. PCC 6803 is a unicellular, non-nitrogen fixing cyanobacterium and a ubiquitous inhabitant of fresh water. It is spherical and divides by binary fission. *Synechocystis* 6803 was the first photoautotrophic organism to be fully sequenced (Kaneko et al., 1995). The circular genome is 3,573,470 bp, and sequence information is accessible through CyanoBase at www.kazusa.or.jp/cyano/. The genomic sequence of *Synechocystis* 6803 revealed

the presence of 3167 genes and relative map positions of each gene. Similarity searches have helped identify the function of more than half of the genes (Ikeuchi, 2001). *Synechocystis* 6803 undergoes natural transformation and can be grown heterotrophically in the presence of glucose. All these factors, along with the establishment of databases for *Synechocystis* 6803, make it an extremely versatile model organism.

DNA uptake in *Synechocystis* 6803 is particularly high for cells at exponential growth phase ($1-3 \times 10^8$ cells/ml). The transformation efficiency of *Synechocystis* 6803 is in the range of 10^{-3} to 10^{-5} % (Vermaas, 1996). Transformation efficiency depends on factors such as age of cells, length of sequence similarity between the flanking regions and time of incubation. To have a successful transformation, the minimum concentration of plasmid should be 0.02 $\mu\text{g/ml}$, and the size of flanking regions should be at least 0.2 kb (Zang, 2007). Normal doubling time of *Synechocystis* 6803 typically ranges from 10 to 15 hours depending upon aeration and light intensity. Their growth is normal at a light intensity between 40 and 70 $\mu\text{E m}^{-2} \text{s}^{-1}$, but photo-inactivation can occur at higher intensities. Most researchers believe genetic recombination in *Synechocystis* sp. PCC 6803 happens exclusively by double recombination (Vermaas, 1996; Ikeuchi et al., 2001), although some contradictory reports exist (Labarre, 1989).

1.3 Cloning and plasmid vectors

Cloning in E coli refers to inserting a DNA fragment into a plasmid vector and generating large quantities of the clone through replication in a competent bacterial strain (Casali et al., 2003). This allows us to further investigate the DNA of interest by conducting various molecular analyses and expression studies. The vector and foreign DNA are generally digested with restriction enzymes that generate compatible ends for cloning to proceed. In some cases, T4 DNA polymerase is used to modify the ends. The cleaved fragments are mixed in the presence of T4 DNA ligase, resulting in the generation of vectors containing the inserted DNA. The cloning systems initially could clone only very small DNA fragments. Recent developments, however, let us clone very large fragments.

A cloning vector must contain three common features (Ausubel, 2002):

- a) Replication origin: the site at which replication begins
- b) Selection marker: necessary for the maintenance of a plasmid vector in cells and for the screening of recombinants. Genes encoding resistance to antibiotics are the most common selection markers used.
- c) Multi-cloning sites: unique restriction sites in a vector arranged close to each other.

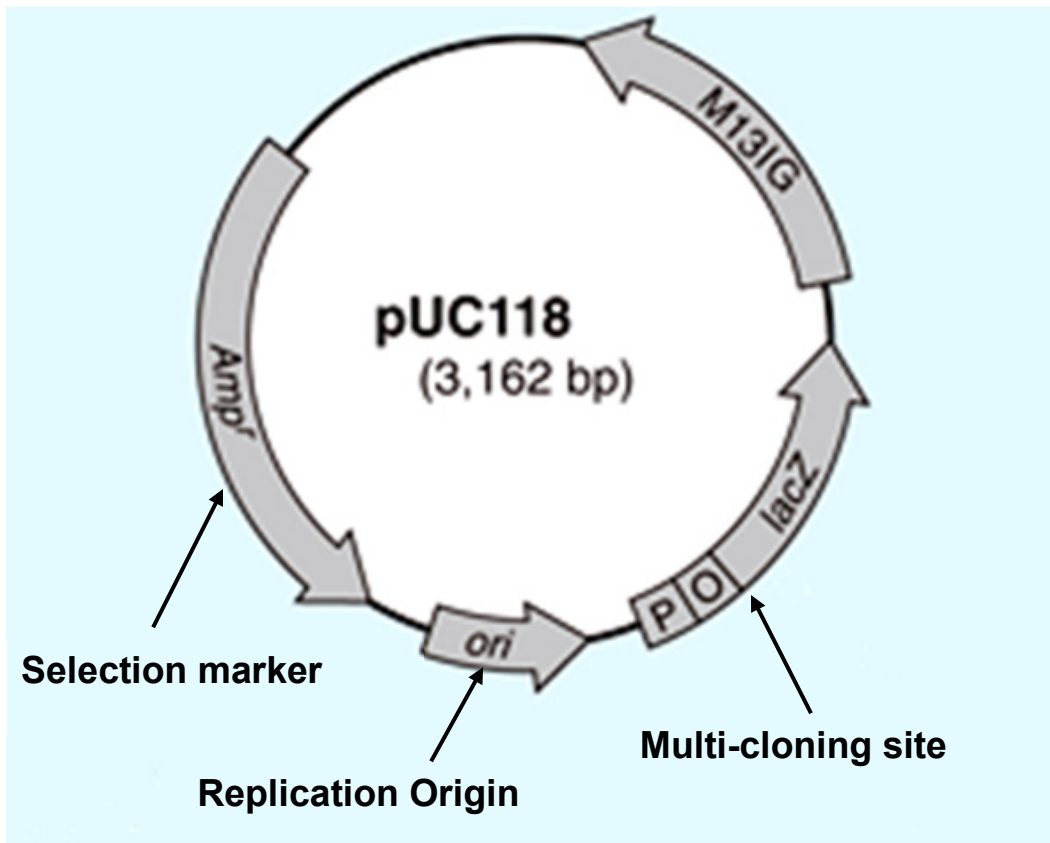


Figure 3. Map of pUC118, an *E. coli* plasmid vector

Figure 3 shows the map of an *E. coli* plasmid vector called pUC118 that contains its multi-cloning site in the *lacZ* gene. This part of the *lacZ* gene codes for an amino-terminal fragment of β -galactosidase and is complemented by a mutant *lacZ* gene from the host cell. The products of the vector and host cell *lacZ* are individually inactive but can form a functional product after associating with each other. Cloning an insert into the multiple cloning site will cause inactivation of the vector *lacZ* gene and absence of β -galactosidase activity. This principle is utilized for the selection of colonies with the insert by blue-white screening. This plasmid vector pUC118 was used as an *E. coli* cloning vector in my present work.

Cloning of foreign genes in cyanobacteria is essentially done by homologous recombination of the insert into the host genome. pTCP2031V is a *Synechocystis* 6803 transformation vector that targets introduced DNA to the slr2031 site (Sato et al., 2001; Muramatsu et al., 2009). Transcriptomics studies reveal that this gene is not silent (data not shown) providing us an opportunity for improvement by selection of a neutral targeting site. There have also been developments in the field of shuttle vectors, plasmid DNA that can replicate autonomously in both *E. coli* and in various cyanobacterial strains. Some of the shuttle vectors that have been developed so far include pFC1, an expression vector that can replicate in both *Synechocystis* and *Synechococcus*, encoding a p_R promoter (Bouvier et al., 1994); pFF11, a shuttle vector system for *Synechocystis* 6803, based on which several more promoter-probe plasmids have been created (Ferino et al., 1989); and pFCLV7 (Chauvat et al., 1986).

1.4 Research objective and thesis outline

This report discusses the construction and function of an improved set of vectors pSL2034, pSL2035 and pSL2036. These vectors target the *psbA1* gene, and its promoter region in the genome of *Synechocystis* sp. PCC 6803. This gene is a member of the *psbA* gene family, which along with *psbA2* and *psbA3* encodes the D1 protein, a component of photosystem II (Mulo et al., 2009). In *Synechocystis* 6803, the *psbA1* gene has been found to be silent under most experimental conditions (Mohamed et al., 1989). This makes it a preferential site for introducing foreign as well as host genes. The vector system constructed also carries the *psbA2* promoter, which can be used to express inserted genes constitutively. These vectors can also be used for over expression of inserted genes by switching the culture to a high light condition (100 to 1000 $\mu\text{E m}^{-2} \text{s}^{-1}$) because of the high light promoter, *psbA2* (Voss et al., 2009). Such features make these a unique and improved set of vectors, for gene cloning in *Synechocystis* sp. PCC 6803.

Chapter 2 introduces the experimental materials and methods used in this study. Chapter 3 gives a detailed step by step approach to the construction of the vectors. Chapter 4 discusses the experiments that test the functional performance of the vector and experimental results. Chapter 5 concludes my thesis.

Chapter 2

Materials and methods

2.1 Cultures and conditions

A glucose tolerant wild type strain of *Synechocystis* sp. PCC 6803 was used for this study. *Synechocystis* 6803, PAL mutant, WT:2045 mutant and PAL:2045 mutant were grown at 30°C in liquid BG-11 medium or solid BG-11 medium (Stanier et al., 1971) at a light intensity of 50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ in ambient air. Iron deficiency was achieved by growing the cells in a medium deprived of all iron sources. Iron-deplete cultures were started by washing the cultures twice with iron-deplete BG11.

E. coli strain XL-1 Blue was the host for all plasmids constructed in this study. XL-1 Blue cells are *recA* deficient which improves stability of the incoming DNA. Cells were grown in test tubes containing Luria-Bertani (LB) medium at 37°C under continuous shaking. Whenever required, ampicillin (100 $\mu\text{g ml}^{-1}$), chloramphenicol (20 $\mu\text{g ml}^{-1}$), or kanamycin (20 $\mu\text{g ml}^{-1}$) was added to the LB medium for selection of plasmids in *E. coli*.

2.2 Transformation and mutant isolation

The cell density was measured as OD730 using a Biotech uQuant spectrophotometer. A calibration graph between OD730 and cell density was

used to calculate the concentration of cells from OD730. 2 ml of an exponentially growing *Synechocystis* 6803 culture ($1-3 \times 10^8$ cells ml^{-1}) was centrifuged at room temperature at a speed of 10,000xg for 2 minutes. The pellet was suspended in a fresh BG-11 medium to a density of $1-3 \times 10^9$ cells ml^{-1} and then mixed with plasmid DNA to a final concentration of 5-10 $\mu\text{g ml}^{-1}$ (Zang et al., 2007). The mixture was then incubated under normal light conditions ($50 \mu\text{E m}^{-2} \text{s}^{-1}$) for a time period of 6 to 12 hrs and then spread on BG-11 agar plates with a selected antibiotic. Mutant colonies started appearing between 7 to 10 days.

Synechocystis 6803 contains about 10 to 12 copies of genome, making it a challenge to have the mutant sequence in all genome copies. Wild type strain of *Synechocystis* 6803 is sensitive to the presence of antibiotics such as chloramphenicol or kanamycin. Hence mutant strains of *Synechocystis* 6803 were created with resistance to chloramphenicol or kanamycin. The mutants were grown on solid BG-11 medium containing the antibiotic to which the transformant was resistant.

E. coli transformation is a modification from Short protocols in molecular biology (Ausubel et al., 2002). Competent XL1-Blue cells were thawed on ice. The cells were mixed gently with 2 μl to 5 μl of plasmid DNA. The cells were placed on ice for 30 minutes. The cells were heat shocked at 42°C for 1 minute. This lets the cells be porous and allow the introduction of exogenous DNA molecules. The cells were kept on ice for 2 minutes followed by addition of 250 μl of a SOC medium. The cells were incubated at 37°C for an hour under

continuous shaking. Dilutions of the culture were then plated on LB Amp plates and incubated for 12 to 16 hrs at 37°C.

2.3 Converting an overhang to a blunt end

To convert a 5' or 3' overhang to a blunt end, T4 DNA polymerase with 5' to 3' polymerase activity was used to fill in 5' overhangs. In addition, T4 DNA polymerase possesses powerful 3' to 5' exonuclease activity with a single-stranded DNA as the substrate. The exonuclease activity of T4 DNA polymerase is used to trim 3'overhangs, converting them to blunt ends. The reaction must contain an excess of dNTPs, as in the absence of dNTPs, the 3' to 5' exonuclease activity of T4 DNA polymerase will degrade double-stranded DNA (Casali et al., 2003). T4 DNA polymerase from Fermentas was used and a standard protocol was followed.

2.4 Ligation

Ligation (Casali et al., 2003) is a process of combining linear DNA fragments together. This involves creation of phosphodiester bond between 3' OH group of one DNA fragment and the 5' phosphate group of another (Casali et al., 2003). The enzyme used is T4 DNA ligase (Fermentas, Canada). An insert to vector molar ratio of 3:1 or 6:1 was usually used for sticky or blunt end ligation respectively. Sticky end ligations were performed by leaving the reaction mixtures at room temperature for 30 minutes and blunt end ligations were performed at 16°C for 12 hours.

2.5 Plasmid DNA isolation

E. coli plasmid DNA was isolated by using a GeneJET plasmid miniprep kit (Fermentas, Canada). The basic principle is the following: bacterial cells are subjected to alkaline lysis to liberate DNA. Cell debris and SDS precipitates are pelleted by centrifugation and the supernatant contains the plasmid DNA. The plasmid DNA is then separated using silica based membrane technology. Plasmid DNA concentration was measured using Nanodrop ND-1000 (Thermo Scientific, USA).

2.6 Genomic DNA isolation

Harvested *Synechocystis* 6803 cells were homogenized by using glass beads on a vortex mixer for 2 minutes. The cell debris were pelleted by centrifugation at 10000xg, 4°C for 5 minutes. The supernatant containing DNA was washed repeatedly with phenol and chloroform mixture. DNA was then precipitated with isopropanol in the presence of 3M sodium acetate at -80°C for 30 minutes. The precipitates were further accumulated as a pellet by centrifugation at 10000xg, 4°C for 15 minutes followed by washing with 70% ethanol. The genomic DNA was then resuspended in 20 µl of distilled water. The concentration of DNA was then determined using Nanodrop ND-1000 (Thermo Scientific, USA).

2.7 RT PCR

Total RNA of *Synechocystis* 6803 was isolated using a RNeasy kit (Ambion, USA) following the manufacturer protocol with modifications (Singh et al., 2008). 1 ml of prewarmed RNeasy at 70°C was pipette directly into the frozen cells and vortexed immediately. The mixture was then incubated for 10 min at 70°C, after which 0.2 ml of chloroform was added; the mixture was mixed vigorously and incubated at room temperature for 10 min. Liquid phase partitioning was achieved by centrifugation at 10,000xg at 4°C. The aqueous phase containing RNA was transferred into a eppendorf tube and an equal volume of phenol and chloroform mixture was added. The aqueous phase was again separated, to which 0.5 ml of diethyl pyrocarbonate (DEPC) treated water was added. RNA was precipitated by the addition of isopropanol at room temperature. Total RNA was pelleted by centrifugation at 10,000xg at 4°C for 15 minutes followed by washing with 75% ethanol. The RNA was resuspended in 50 µl of DEPC treated water. The quantity and quality of RNA was then determined using Nanodrop ND-1000 (Thermo Scientific, USA).

Dnase was used to degrade genomic DNA if present in the RNA sample by incubating it at room temperature for 30 minutes. cDNA synthesis was performed by utilizing Reverse transcriptase II along with dNTPs, DTT, hexamers and reaction buffer. The mixture was incubated at 42°C for 90 minutes. cDNA from the reaction mixture was used as a template for PCR to detect the expression of mRNA of interest. *rnpb* (a constitutively expressed gene) was used as a control for this experiment.

2.8 Determination of chlorophyll content

Chlorophyll was extracted with 100% methanol from cell pellets. Chl *a* and Chl *b* have their wavelength maxima at 665 nm and 652 nm respectively. The absorbance values of the sample were recorded at 665 nm and 654 nm using DW2000 spectrophotometer (SLM Aminco, USA). As cyanobacteria possess no Chl *b*, the chlorophyll concentration was calculated with a modified formula using the absorbance values recorded at 665 nm and 654 nm (Porra et al., 1989; Lichtenthaler et al., 2001).

2.9 77 K fluorescence

Low temperature fluorescence emission spectra of cells were recorded in the presence of liquid nitrogen at 77 K using Fluoromax-2 (Horiba, Japan). Samples were diluted to a final chlorophyll concentration of 7 $\mu\text{g ml}^{-1}$ in BG-11. The cell suspension was frozen as a thin coating around an acrylic rod and placed in a liquid nitrogen optical Dewar flask and centered to the excitation beam. Care was taken to coat the samples uniformly around the acrylic rod. Fluorescence emission spectrum was recorded between 650 nm to 750 nm for an excitation wavelength of 420 nm. The fluorescence emission data obtained were then normalized to the fluorescence emission value at 730 nm.

Chapter 3

Construction of vectors for chromosomal integration

3.1 Design of the flanking regions

We selected the *psbA1* open reading frame (ORF) and its promoter region as the target site for gene integration into the *Synechocystis* 6803 genome. Osiewacz et al., 1987 previously identified the nucleotide sequence for the *psbA1* ORF and its promoter. We also identified the flanking regions *psbA1u* (499 bp) and *psbA1d* (420 bp), located upstream and downstream of the *psbA1* gene. A nucleotide blast (http://blast.kazusa.or.jp/blast_search/cyanobase) of *psbA1u* and *psbA1d* confirmed that these sequences are unique to *Synechocystis* 6803 genome with no duplicates, avoiding the possibility of inaccurate recombination. The sequences are also sufficiently long (Zang et al., 2007) to cause a successful integration of the insert into the genome.

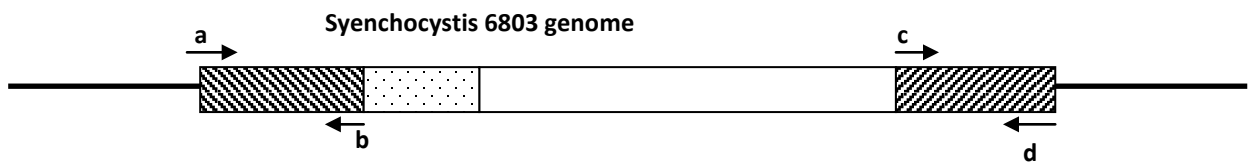
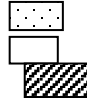





Figure 4. Location of *psbA1u* and *psbA1d* relative to the *psbA1* gene.  represents the upstream region to the *psbA1* ORF containing the promoter; represents the *psbA1* ORF;  represents the flanking region, *psbA1u*;  represents the flanking region *psbA1d*;  represents the primers (a) *ps1_up_fwd*, (b) *ps1_up_bwd*, (c) *ps1_down_fwd*, and (d) *ps1_down_bwd*

Primers ps1_up_fwd, ps1_up_bwd, ps1_down_fwd, and ps1_down_bwd (Supplementary Table 1) were designed to amplify psbA1u and psbA1d from the *Synechocystis* 6803 genome (Figure 4). Primers for psbA1u incorporated KpnI and MfeI as restriction sites, and primers for psbA1d incorporated HindIII and MfeI as restriction sites.

3.2 Cloning of the flanking regions

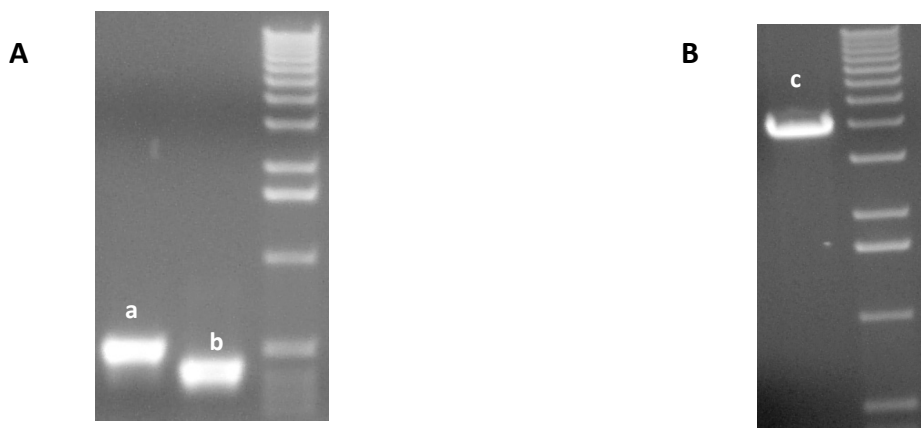


Figure 5. Agarose gel for cloning and confirmation of flanking regions psbA1u and psbA1d into pUC118. A. PCR amplified DNA sequence for psbA1u (a – 0.56 kb) and psbA1d (b – 0.42 kb). B. Digestion of pSL2078 with a single cutter MfeI confirming the presence of the flanking regions (c – 4 kb)

The flanking regions psbA1u and psbA1d were PCR amplified with their respective primers from the genome of *Synechocystis* 6803 (Figure 5A). The PCR product of psbA1u was digested with KpnI and MfeI, and the other PCR product of psbA1d was digested with HindIII and MfeI. The cloning plasmid pUC118 was digested with KpnI and HindIII. We purified the restriction digested sequences of psbA1u, psbA1d, and pUC118 using a purification kit (Fermentas, Canada). The purified fragments of psbA1u and psbA1d were cloned into

pUC118 using T4 DNA ligase. We performed the ligation at room temperature for 30 minutes, then transformed chemically competent XL-1 Blue *E. coli* cells with 5 μ l of ligation mixture and selected the transformants on plates containing 100 μ g/ml of ampicillin. Blue-white screening yielded one positive colony out of 26 colonies. The positive colony was grown overnight in LB amp100 medium and the plasmid was pSL2078 was isolated using plasmid miniprep kit (Fermentas, Canada). The presence of flanking regions in pSL2078 can be confirmed by digestion with MfeI, a single cutter for pSL2078. The digestion result also confirmed its predicted size of 4 kb (Figure 5B). Figure 6 shows the complete plasmid map of pSL2078.

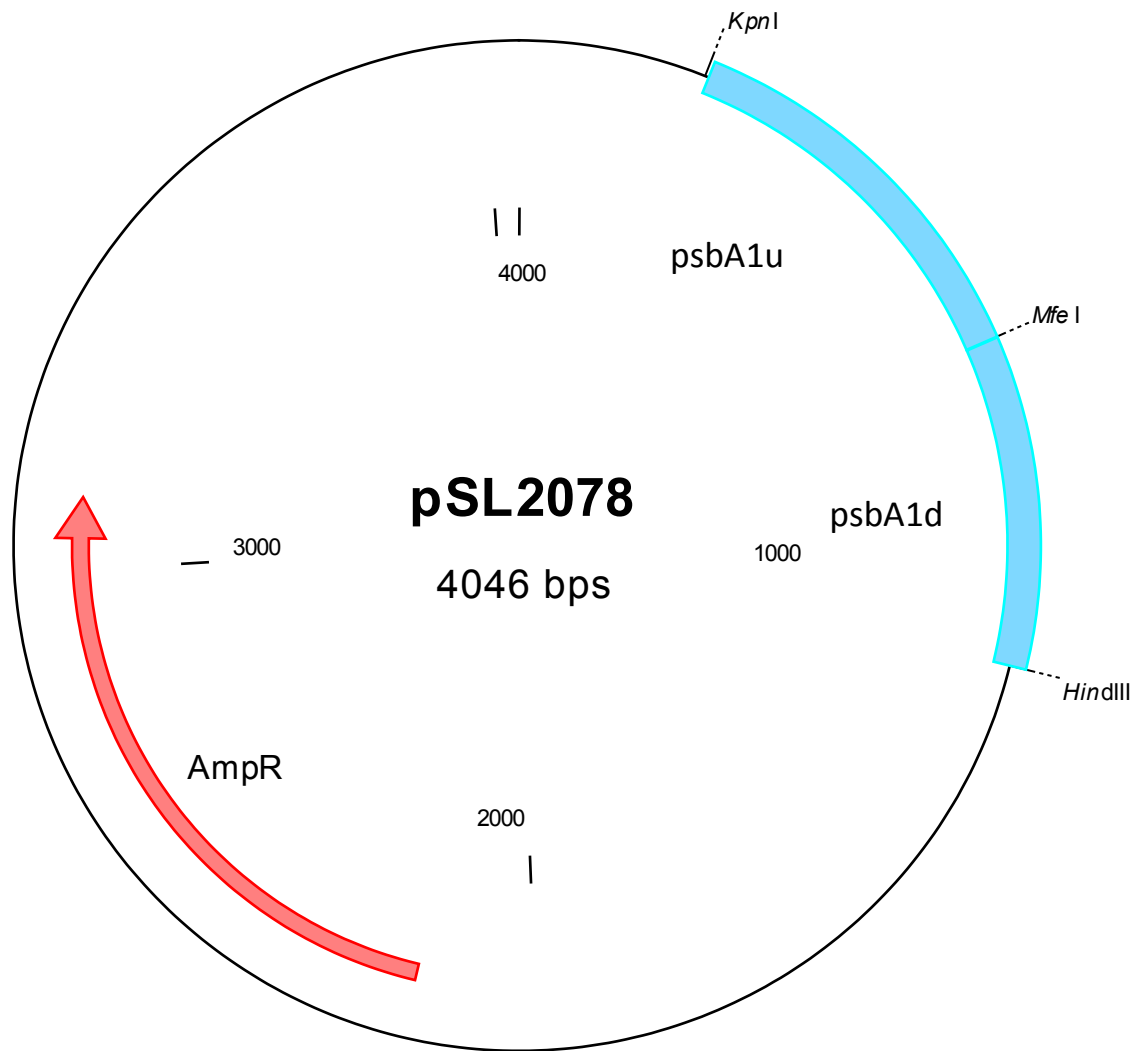


Figure 6. Map of pSL2078. pSL2078 is the resulting vector from cloning the two flanking regions into pUC118.

3.3 Cloning of the promoter and the selection marker

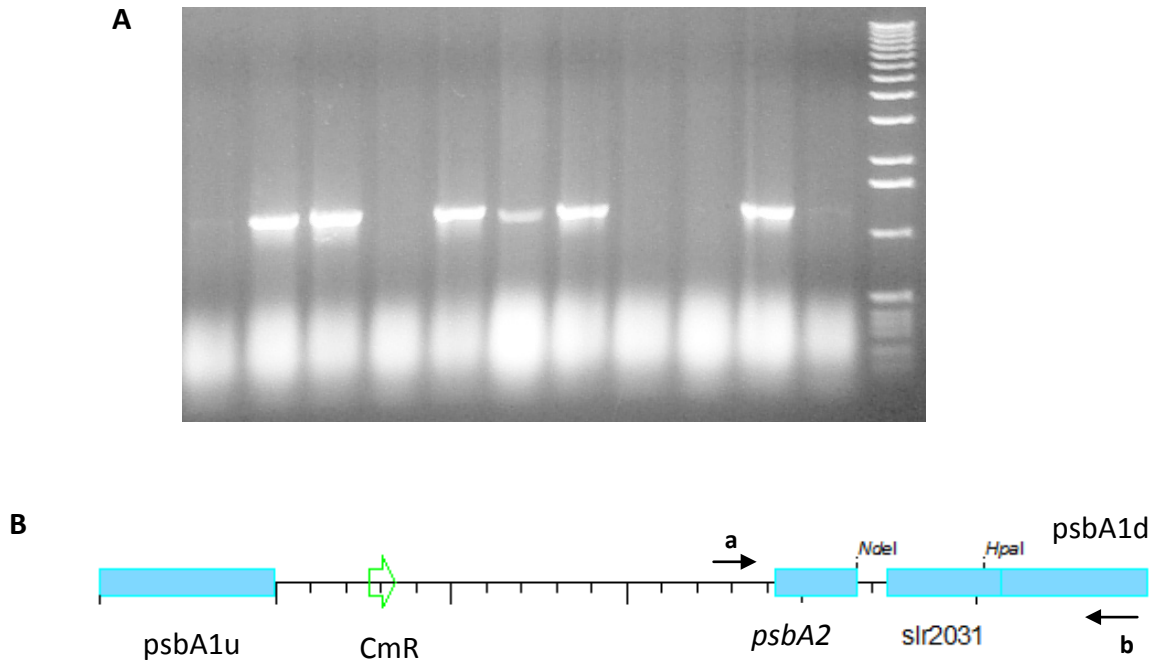


Figure 7. Selection of colonies for pSL2034 (vector pSL2078 with *psbA2* promoter and chloramphenicol resistance cassette as insert). A. Colony PCR for colonies with pSL2034. B. Region of amplification by primers (a) JC44 and (b) ps1_down_bwd, amplifying about 1.25 kb

We obtained a fragment including the chloramphenicol resistance cassette (CmR) and the promoter region of the *psbA2* gene by digestion of the plasmid pTCP2031v using MfeI. Dr. Ikeuchi (University of Tokyo) kindly provided the pTCP2031V (Sato et al., 2001; Muramatsu et al., 2009). The 2.2 kb region that includes the *psbA2* promoter and chloramphenicol resistance cassette was purified and then cloned into the MfeI site of pSL2078. We performed this ligation at room temperature for 30 minutes then transformed XL-1 Blue cells with 5 μ l of ligation mixture and selected the transformants on plates containing 20 μ g/ml chloramphenicol. The ligation was of a very high efficiency, yielding

approximately 1 positive colony out of 2 selected for colony PCR (Figure 7A). Primers JC44 and ps1_down_bwd were used for colony PCR. The forward primer, JC44, binds to the chloramphenicol resistance cassette and the reverse primer, ps1_down_bwd, binds to the downstream region of psbA1d. These two primers amplify a sequence of approximately 1.25 kb.

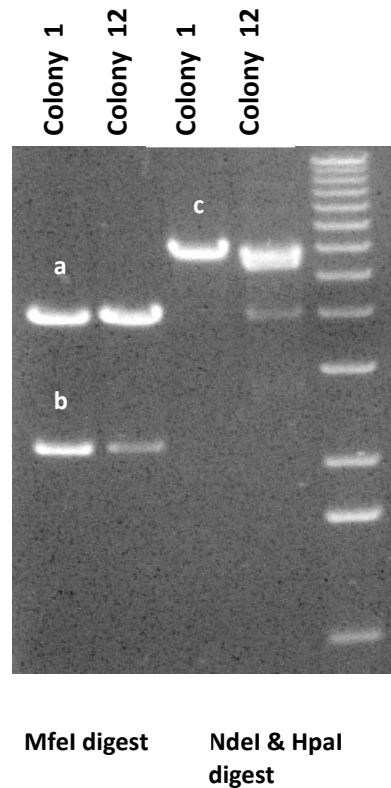


Figure 8. Digestion results of pSL2034. MfeI is a double cutter for pSL2034 and gives fragments of sizes (b) 2.2 kb and (a) 4 kb. NdeI and HpaI are single cutters for pSL2034 and gives fragments of sizes (c) 5.9 kb and 0.37 kb (not shown).

The new plasmid, designated pSL2034, was isolated from 2 colonies (colony 1 and colony 12). We performed two restriction digestions with pSL2034 obtained from both the colonies. The digestion results for the plasmid from colony 12 showed a band with unexpected size (4 kb in Lane 4), whereas the band

sizes for the vector from colony 1 matched the predicted size. We selected colony 1 for further study (Figure 8). The plasmid map of pSL2034 is shown in Figure 9.

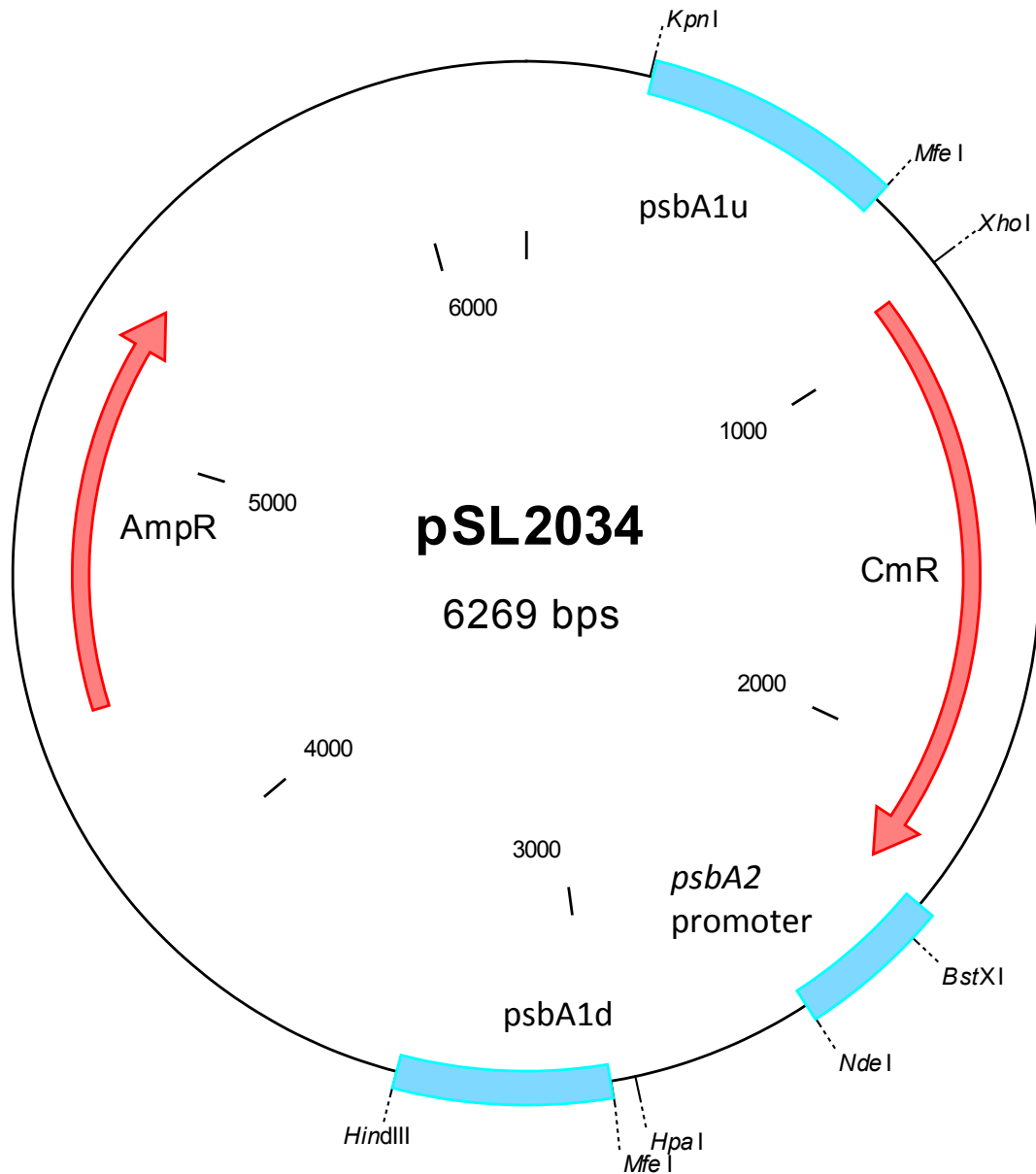


Figure 9. Map of pSL2034. pSL2034 is the result of cloning the chloramphenicol resistance cassette and the sequence with *psbA2* promoter into pSL2078 at the *Mfe*I restriction site.

3.4 Replacing the chloramphenicol resistance cassette with kanamycin resistance cassette in pSL2034

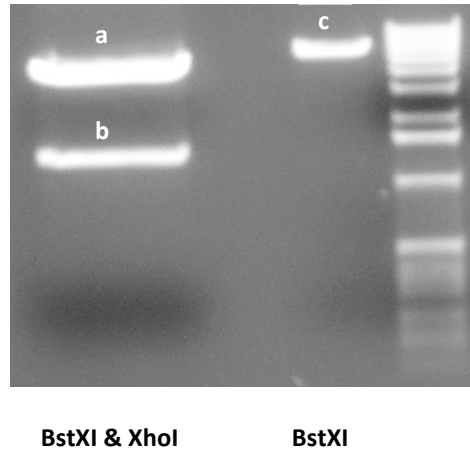


Figure 10. Releasing the chloramphenicol resistance cassette (CmR) from pSL2034. pSL2034 was digested with BstXI and XhoI, releasing the CmR cassette of (b) 1.4 kb. pSL2034 was digested with BstXI as a control, giving a linear fragment of (c) 6.3 kb.

For replacing the chloramphenicol resistance cassette (CmR) with kanamycin resistance cassette (KmR), the initial step was the deletion of CmR from pSL2034. We identified the XhoI and BstXI sites to remove the CmR region from pSL2034. However, it can be seen from Figure 9 that BstXI cuts a part of the *psbA2* promoter. Studies have shown that replacing the sequences upstream of position -167 relative to the ATG site did not affect the *psbA2* gene activity (Eriksson, 2000). Digestion of the *psbA2* promoter with BstXI still leaves 232 base pairs of the original *psbA2* promoter sequence intact. Hence it was concluded that this digestion and elimination of 64 base pairs of the *psbA2* promoter region will still maintain the *psbA2* promoter activity. pSL2034 was digested with XhoI and BstXI, releasing the CmR (Figure 10). The 4.85 kb

fragment from pSL2034 without the CmR was purified and the ends blunted with T4 DNA polymerase.

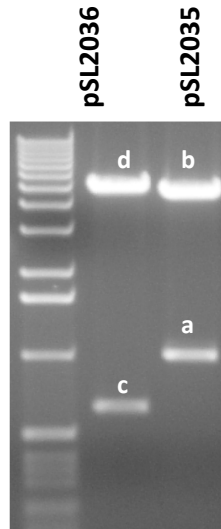


Figure 11. Digestion results for selection of pSL2035 and pSL2036. Plasmid (pSL2035) with the kanamycin resistance cassette (KmR) in clockwise orientation digested with SmaI and NdeI gives fragments of size (a) 1 kb and (b) 5 kb. Plasmid (pSL2036) with the KmR in counter-clockwise orientation digested with SmaI and NdeI gives fragments of sizes (c) 0.67 kb and (d) 5.4 kb.

A kanamycin resistance cassette (KmR) isolated by restriction digestion of pUC4k with HincII was cloned into the 4.85 kb fragment from pSL2034. We performed colony PCR, selected two positive colonies and isolated the recombinant plasmid DNA. Plasmids were digested with SmaI and NdeI to confirm the orientation of KmR (since this plasmid was created by cloning blunt ends). Based on the size of digestion fragments, we confirmed the orientation of KmR (Figure 11) and called the plasmids with different orientations pSL2035 and pSL2036 (Figure 12 and Figure 13).

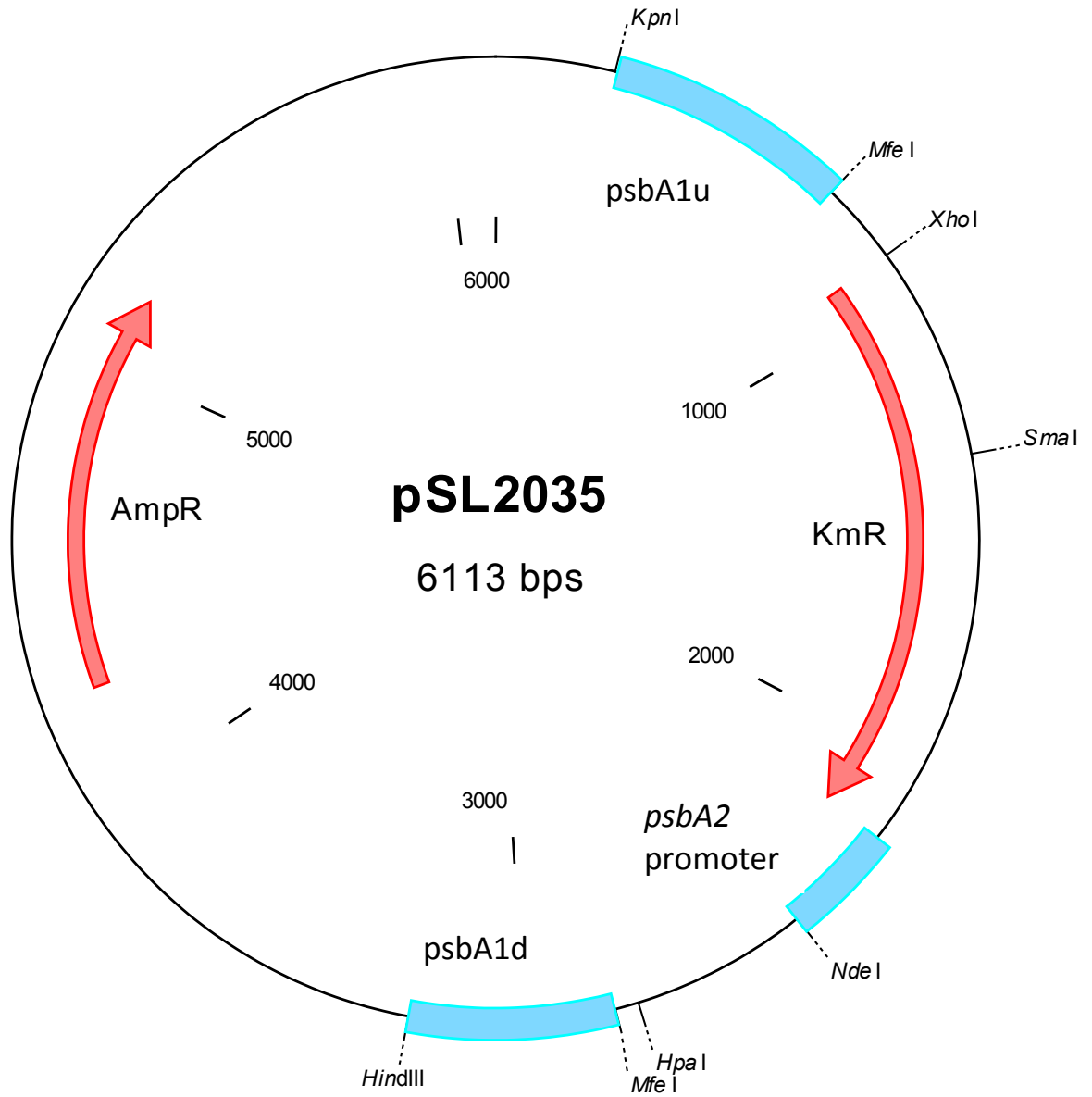


Figure 12. Map of pSL2035. pSL2035 was created by replacing the CmR sequence in pSL2034 with KmR in a clockwise orientation.

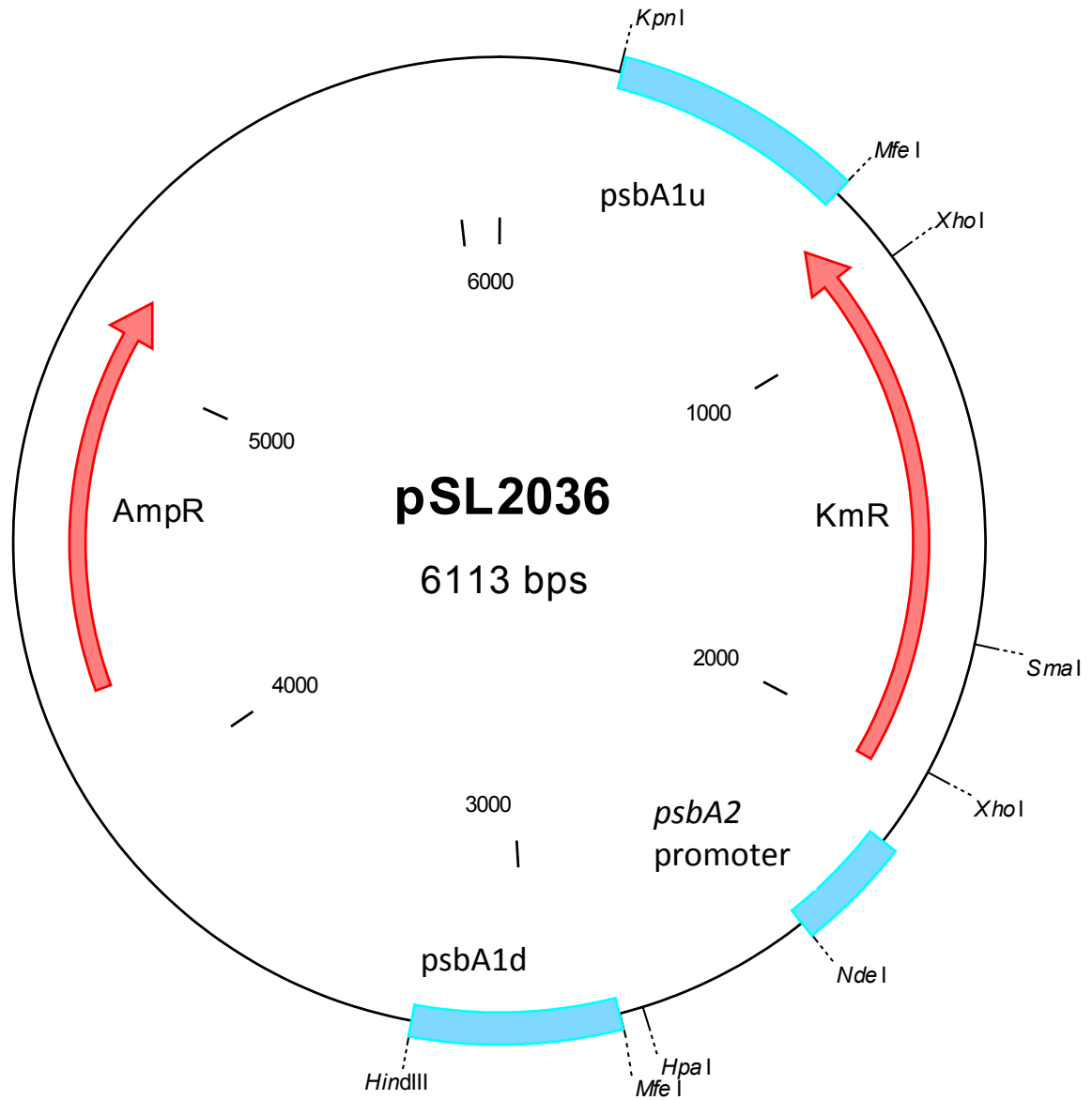


Figure 13. Map of pSL2036. pSL2036 was created by replacing the CmR sequence in pSL2034 with KmR in a counter-clockwise orientation.

Chapter 4

Genetic engineering of *Synechocystis* 6803

4.1 Construction of pSL2045

The *isiA* ORF was amplified from the genome of *Synechocystis* 6803 using primers *isiA*-NdeI-F, *isiA*-HpaI-R (Supplementary Table 1). The amplified sequence of the *isiA* ORF was purified using the PCR kit and was digested with NdeI and HpaI restriction enzymes. The purified fragments were then cloned into the NdeI and HpaI restriction sites of pCR2.1 resulting in the vector pSL2043.

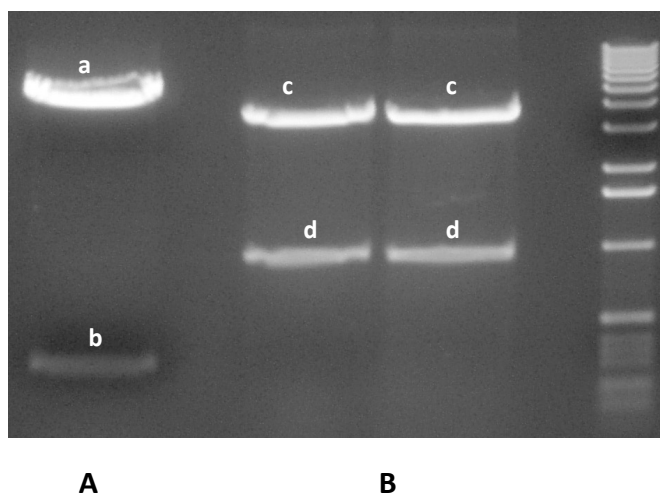


Figure 14. Digestion results of the two pSL2035 and pSL2043. A. NdeI and HpaI are single cutters for pSL2035 and the two fragments size (a) 5.9 kb and (b) 0.37 kb. B. NdeI and HpaI are single cutters for pSL2043 and the two fragments size (c) 3.9 kb and (d) 1 kb.

The NdeI and HpaI sites in pSL2035 and pSL2043 were digested using the respective enzymes. The restriction digested fragments were then purified to obtain a 5.9 kb fragment of pSL2035 and the *isiA* ORF of 1 kb from pSL2043

(Figure 14). The purified *isiA* ORF was cloned into the 5.9 kb fragment from pSL2035 using T4 DNA ligase. The ligation was performed at 16°C for 12 hours with a vector to insert molar ratio of 1:6. This was a very low efficiency ligation, resulting in just 2 colonies. A colony PCR was performed with pSL2043 as a positive control and one positive colony was obtained (Figure 15A). The positive colony was grown overnight in LB Km 20 media, and the plasmid designated as pSL2045 was isolated. The plasmid pSL2045 was digested with NdeI and HpaI to further confirm the presence of the *isiA* ORF. This digestion resulted in fragments of the predicted size: 5.9 kb and 1 kb (the *isiA* ORF), further confirming the presence of the *isiA* ORF in the isolated plasmid (Figure 15B).

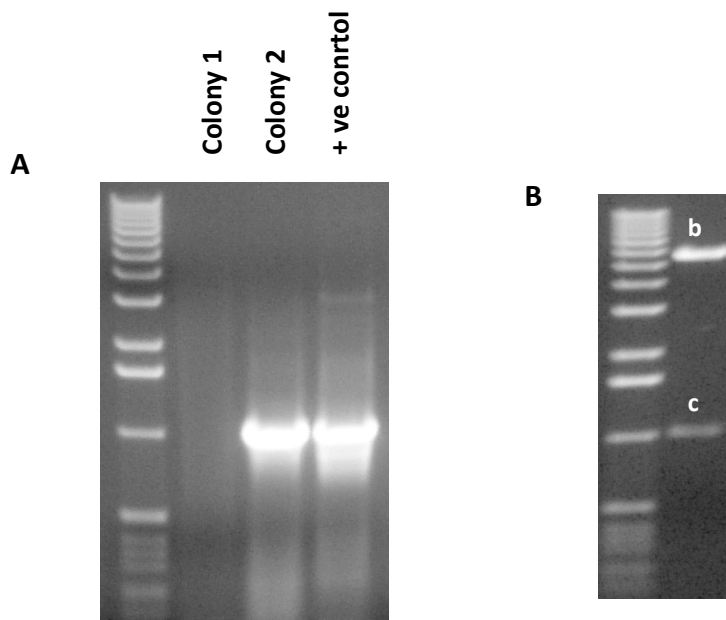


Figure 15. Selection for pSL2045. A. Results of a colony PCR showing the presence of one positive colony (colony 2). B. NdeI and HpaI digested fragments of pSL2045 matched the predicted size of (b) 5.9 kb and (c) 1kb.

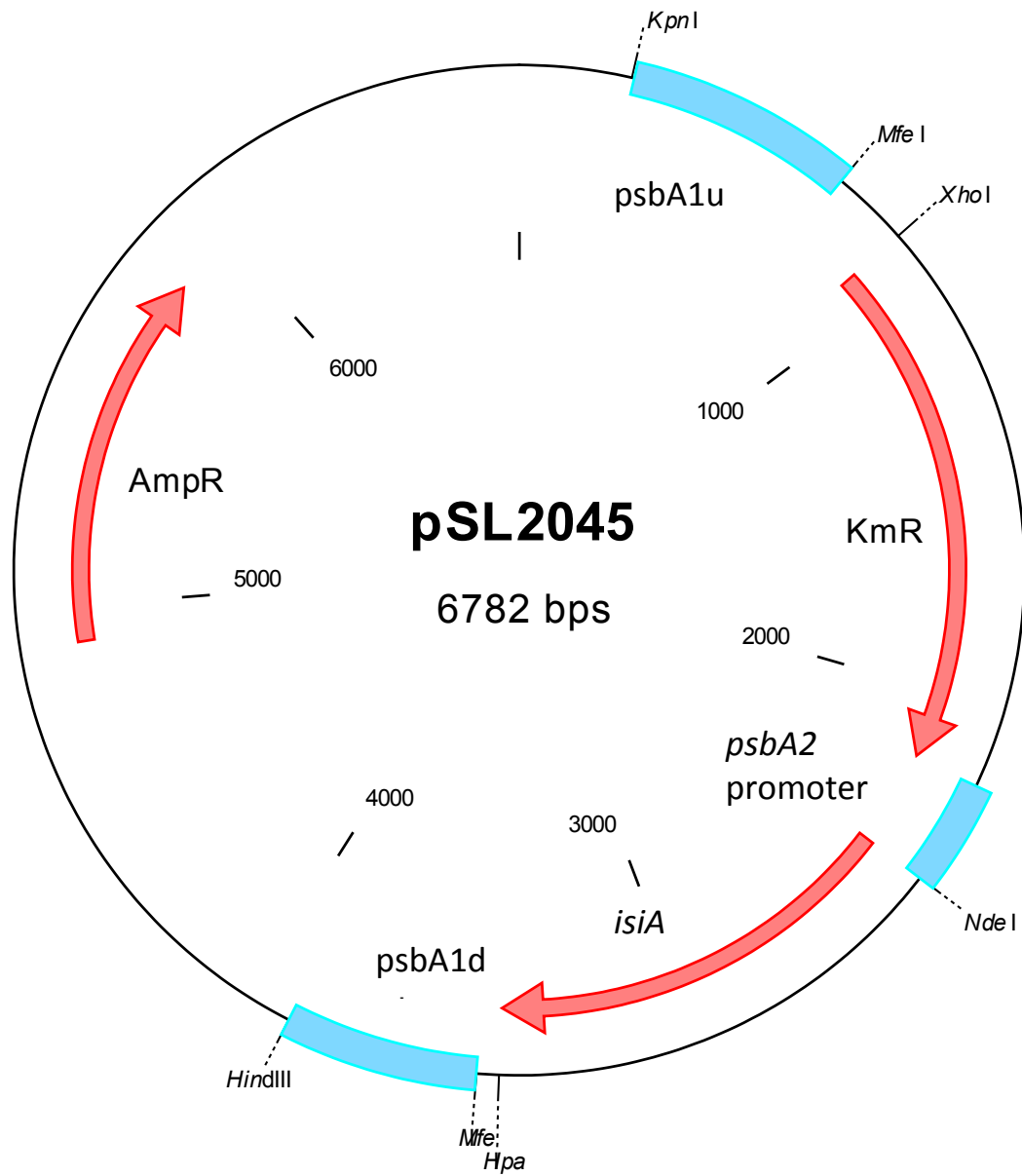


Figure 16. Map for pSL2045. pSL2045 was obtained by cloning the *isiA* ORF into pSL2035

4.2 Sequencing the *psbA2* promoter region and the *isiA* gene in pSL2045

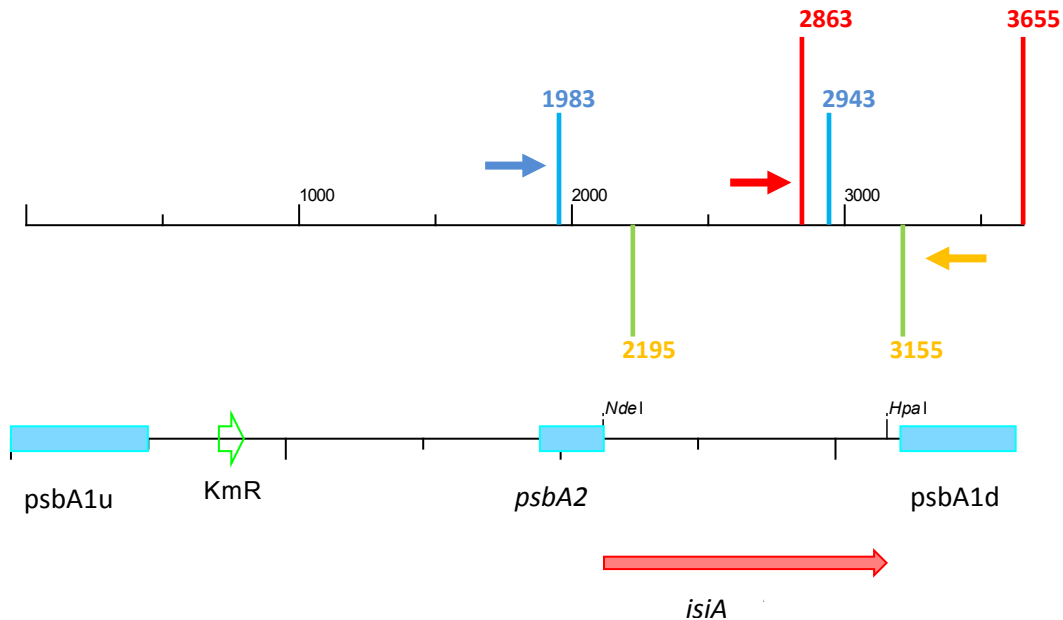


Figure 17. Location of primers used for sequencing. → KmpA2F sequence of the *psbA2* promoter region and a part of the *isiA* gene; → iARTF, located in the intergenic region of the *isiA* gene; ← p1dslR, partially located in the *psbA1d* flanking region.

It is essential to verify the sequence of the cloned gene before further experiments. Hence, pSL2045 was used as a template for sequencing the *isiA* ORF and the *psbA2* promoter region. Figure 17 shows a partial segment of pSL2045, with the various locations marked: *psbA2* (promoter region) - 1925 bp to 2157 bp, the *isiA* ORF - 2158 bp to 3186 bp, *psbA1d* (downstream flanking region) - 3236 bp to 3655 bp. Primers KmpA2F, iARTF and p1dslR (Supplementary Table 1) were used for sequencing. KmpA2F was located in the downstream region of the kanamycin cassette; iARTF was located in the

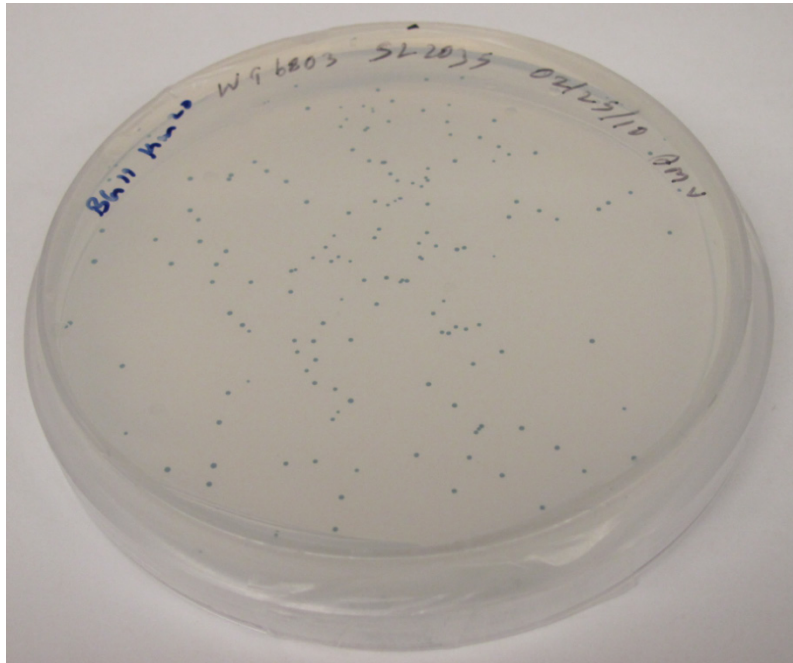
intergenic region of the *isiA* gene; p1dslR was designed to be located partially in the *psbA1d* flanking region and its adjoining sequence.

The plasmid pSL2045 and the primers discussed above were mixed at a standard concentration and were submitted to PNACL (Protein & Nucleic Acid Chemistry Laboratory, St. Louis) for sequencing. The sequencing results from the primer KmpA2F, covered bases from 1983 bp to 2863 bp and was 100% identical to the original sequence. The sequencing results cover -174 bp upstream of 'ATG' in the *psbA2* promoter region. Previous studies have shown that replacing the sequences upstream of position -167 relative to the ATG site did not affect the *psbA2* gene activity (Eriksson, 2000). Our sequencing results also indicated that the functional part of the *psbA2* region containing the promoter was completely intact.

The sequencing results from the primer iARTF covered regions from 2863 bp to 3655 bp but gave a single mismatch at the location 2478 bp. The sequencing results from the primer p1dslR covered regions from 3155 bp to 2195 pb and gave the mismatch at the same location: 2478 bp. Since both sequencing results showed the same mismatch, it was concluded that the mismatch at location 2478 bp is certain (Appendix). Further analysis was done from the nucleotide blast data, and it was found that this mutation is a result of 'C' replacing 'T'. This mutant location at 2478 bp belongs to the original codon 'GCU' and the mutant codon at this location is 'GCC'. It was also verified and found that both these codons code for the same amino acid, 'alanine'. Hence it was concluded that the mutation at this single base has not changed the functionality of the *isiA* ORF.

4.3 Transformation of *Synechocystis* 6803 by pSL2035 derivatives

A



B

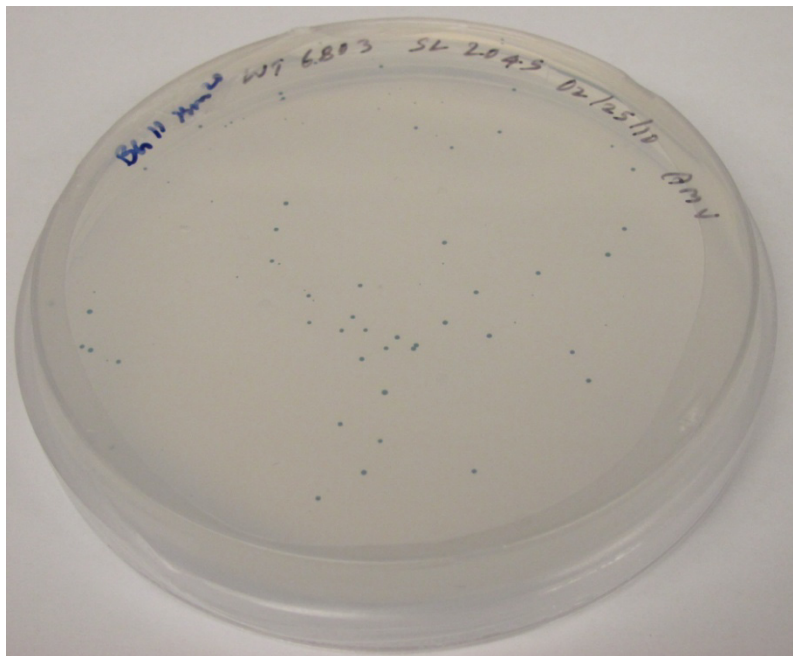


Figure 18. Transformants of pSL2035 and pSL2045. A. *Synechocystis* 6803 transformants of pSL2035 (Δ psbA1). B. *Synechocystis* 6803 transformants of pSL2045 (WT:2045).

The transformation of Wild type *Synechocystis* 6803 was done with pSL2035 and pSL2045 generating transformants Δ psbA1 and WT:2045 respectively. Both transformation processes were done by adding equal moles of plasmid into the cell cultures of the same age, which were incubated for the same time interval. The transformants were selected for kanamycin resistance on BG 11 plates containing 20 $\mu\text{g ml}^{-1}$. Mutant colonies started appearing after about 10 days. But the plasmid pSL2035 without the *isiA* ORF generated more transformants than pSL2045 and a possible reason for this low efficiency could be expression of the *isiA* gene. Also, studies on the transformation of *E. coli* show that transformation efficiency decreased with an increase of plasmid size (Szostkova et al., 1998). In this study, the size of pSL2045 was 1 kb larger than that of pSL2035, so it is possible that transformation efficiency with pSL2045 could be limited with increased size.

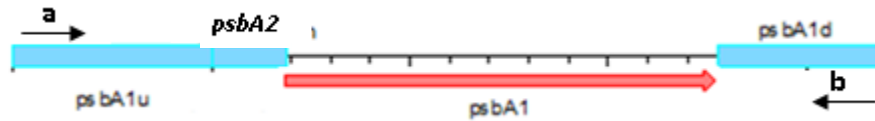
Another mutant was generated by transforming these plasmids into PAL mutant. PAL is a mutant strain of *Synechocystis* 6803 devoid of phycobiliproteins, a most important component of phycobilisomes (the light harvesting antenna complex for cyanobacteria) (Ajlani et al., 1998). The mutant PAL: 2045 was generated by transforming the PAL mutant with pSL2045. Because, the PAL mutants lack the light harvesting complex, their doubling time is very long; in our experiment it took 20 days for the mutants to begin to appear. To generate more cells for further experiments we grew the PAL:2045 mutants on BG 11 plates containing 5mM glucose. Supplementing the medium with glucose

made the mutant strains grow at the same rate as the wild type strains (data not shown).

4.4 Segregation studies with the pSL2045 transformants



Fragment from pSL2045 (3655 bps)



Fragment from WT (2189 bps)



Fragment from pSL2035 (2986 bps)

Figure 19. Amplification regions of primers (a) ps1_up_fwd and (b) ps1_down_bwd in pSL2045 (3.6 kb), Wild Type (2.1 kb) *Synechocystis* 6803 and pSL2035 (2.9 kb).

A segregation experiment was performed to check the integration of the foreign *isiA* gene along with the *psbA2* promoter region and the kanamycin

cassette into the target site, the *psbA1* gene. Genomic DNA of WT:2045, PAL:2045 and wild type (WT) *Synechocystis* 6803 were isolated and used as a template for PCR. *ps1_up_fwd* and *ps1_down_bwd* were used as primers for the PCR reaction, and they amplified the regions shown in Figure 19.

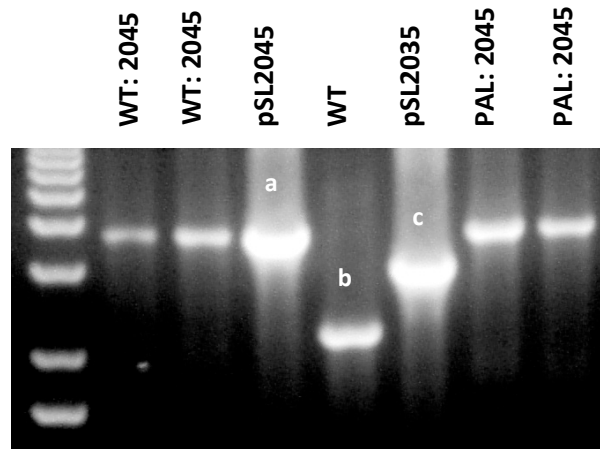


Figure 20. Genome PCR for segregation of mutants. This agarose gel picture confirms the recombination of foreign sequence from pSL2045 into the genome of mutants. (a) 3.6 kb; (b) 2.2 kb; (c) 2.9 kb.

pSL2045 was used as a positive control. Wild type genomic DNA was used as a negative control. The PCR results in Figure 20 indicate that the band size from the mutants matched exactly with that of pSL2045 (the positive control). This indicates that the foreign sequence containing the Kanamycin cassette, the *psbA2* promoter region and the ORF for *isiA* has integrated into the mutant genome. There was no other band observed for the mutants, whose size was equivalent to the one obtained from the wild type (negative control). This also confirms that the mutants were fully segregated.

pSL2035 was the vector to which *isiA* was cloned, resulting in pSL2045. pSL2035 was used as the other control for the PCR reaction. We predicted that the band resulting from pSL2035 would be slightly smaller than that from pSL2045 and slightly larger than that from the wild type band. Our predictions can be verified in Figure 20.

4.5 RT-PCR for the expression of the *isiA* ORF

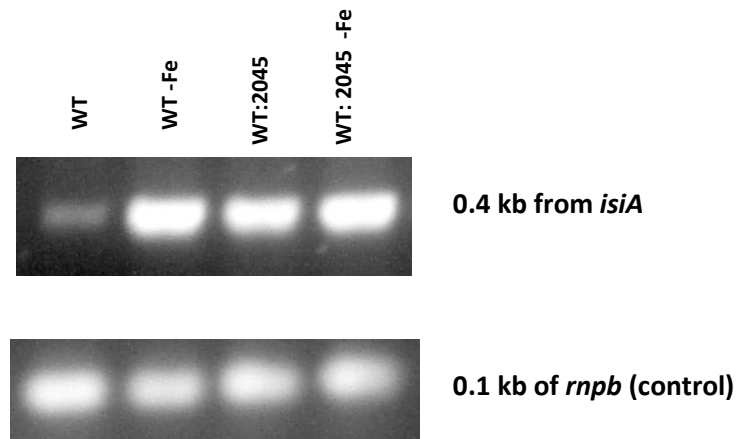


Figure 21. RT- PCR for the WT:2045 mutants. WT:2045 shows the relative abundance of *isiA* mRNA even under iron-replete conditions.

The aim of this experiment was to verify the expression of *isiA* under the effect of *psbA2* promoter. The wild type (WT) and mutant (WT:2045) cultures were grown in iron-replete and iron-deplete BG11 media under continuous normal light ($50 \mu\text{E m}^{-2} \text{s}^{-1}$). mRNA was isolated from wild type (WT) cultures grown in normal BG 11 (iron-replete) as well as iron-deplete BG 11 (positive control). DNase was used to degrade any DNA that was present with the isolated

mRNA. cDNA was synthesized following a standard protocol. PCR was conducted with the synthesized cDNA as a template using the primers that amplified a 400 bp region in the *isiA* gene. Another set of primers were also used that would target a constitutively expressed gene (*rnpb*), which performed as a positive control for the whole procedure. Duhring et al (2006) provide the evidence that *isiA* was expressed under iron-deplete conditions, Hence wild type cells grown under iron-deplete conditions were used as a positive control. Figure 21 shows that for the wild type of cells, a relatively higher expression of *isiA* occurs only under the iron-deplete conditions whereas for the mutants, a relatively higher expression of *isiA* occurs under the iron-replete conditions itself. This confirms that *isiA* was getting expressed in the mutants due to the action of the *psbA2* promoter.

A small band of mRNA for *isiA* in the wild type cells was seen even under iron-replete conditions. Singh et al (2007) have shown that *isiA* does get expressed under few more stress conditions other than iron-depletion; it is possible that the wild type cells in our study were under some stress that we did not intend. Also, the primers were designed to amplify only part of *isiA* transcript; hence it is possible that the whole of the *isiA* transcript was not degraded.

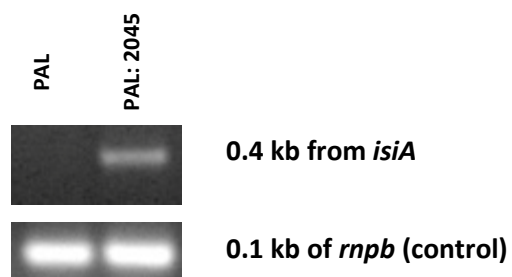


Figure 22. RT- PCR for the PAL:2045 mutants. PAL:2045 shows the relative abundance of *isiA* mRNA even under iron-replete conditions.

The RT- PCR experiment was with PAL:2045 using PAL as a negative control. Since it was confirmed that the *isiA* expression was present in the mutant WT:2045, it was not necessary to use the iron-deplete cultures in this experiment. Figure 22 clearly reveals the presence of *isiA* mRNA in PAL:2045, whereas it is not present in the PAL mutant. This proves that the constitutive promoter of *psbA2* introduced by the plasmid vector pSL2045 was active.

4.6 77 K Fluorescence measurement with the PAL mutant

Cyanobacteria grown under iron-deplete conditions show a dominant 77 K fluorescence emission peak at around 685 nm (Pakrasi et al., 1985, Wilson et al., 2007). This peak has been directly related to the presence of the IsiA complex.

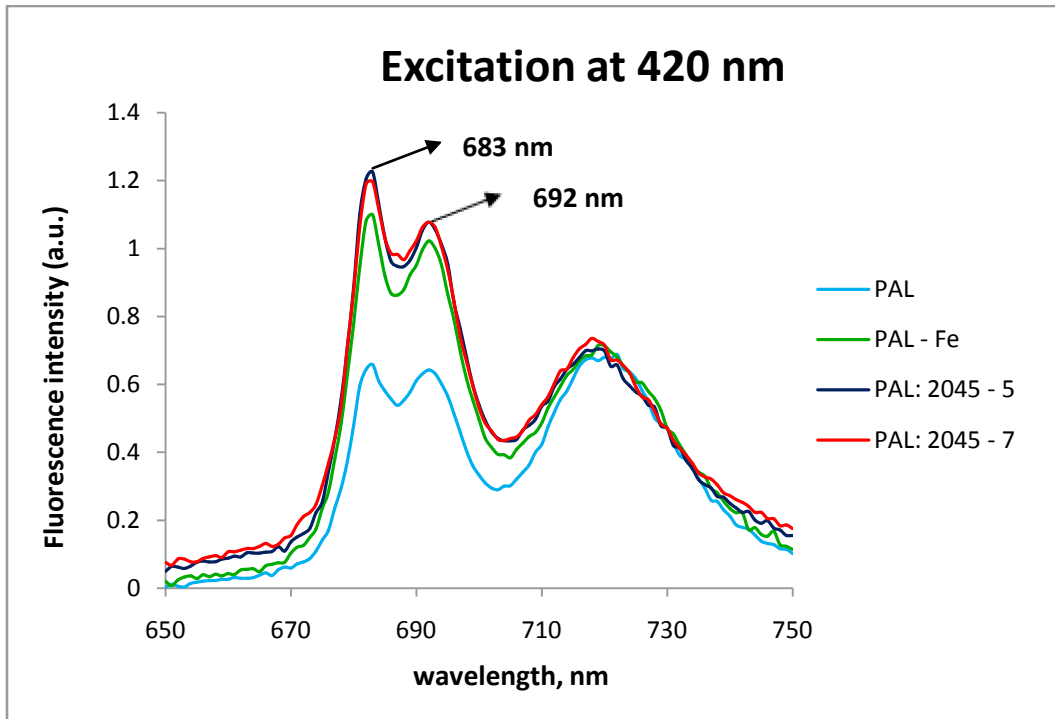


Figure 23. 77 K Fluorescence spectra of the PAL mutant. Excitation wavelength was 420 nm, sensitizing chlorophyll preferentially, and normalized at 730nm. All cells were diluted to the same chlorophyll concentration of 7 $\mu\text{g/ml}$

We performed 77 K Fluorescence spectra for the PAL mutant PAL:2045 expecting that the absence of a functional light harvesting antenna complex would make the presence of the IsiA complex easily detectable even under iron-replete conditions because of the absence of phycocyanin in the PAL mutant. PAL (the

negative control) and PAL:2045 cultures were grown in iron-replete BG11 under normal light ($50 \mu\text{E m}^{-2} \text{s}^{-1}$). PAL:2045 cells picked from two different mutant colonies was used for this experiment. The PAL mutant grown in iron-deplete BG11 was used as a positive control. Cultures at their logarithmic phase of growth were picked for 77 K fluorescence. 77 K fluorescence was performed at an excitation wavelength of 420 nm. Figure 23 shows the normalized fluorescence spectra from all the four cultures. PAL:2045 mutants behaved much in the same way as the PAL mutants grown under iron-deplete conditions, showing a large peak at 683 nm. This confirms the presence of IsiA complex in the PAL:2045 mutants.

4.7 Discussion on PAL:2045

In this chapter the creation of a mutant PAL:2045 for constitutive expression of the *isiA* gene was described. 77 K fluorescence spectra on the PAL:2045 mutant grown under iron replete conditions indicated the presence of IsiA. Previous studies on IsiA have shown that it forms a light harvesting antenna complex for photosystem I (PSI) in *Synechocystis* 6803 (Bibby et al., 2001). It is possible that the IsiA complex synthesized for PAL:2045 forms a light harvesting complex as well. This would mean that more light energy could be harvested by PAL:2045 to increase the flux for the CO₂ fixing reaction. Although IsiA is seen for the PAL mutants grown under iron starved conditions, the cells do not grow well. If the IsiA complex of PAL:2045 indeed acts a light harvesting antenna,

genetic engineering has allowed us to create a mutant that forms an IsiA antenna under normal growth conditions. Our preliminary experiments on PAL suggest that they can grow better than wild type *Synechocystis* 6803 under high light conditions without becoming photo-inhibited. Therefore PAL:2045 might grow under high light conditions with the advantage of having additional antenna for PSI because of the high light promoter *psbA2* used in the creation of PAL:2045. This can make it an interesting mutant to study for the production of high value metabolites, as the supply of high light for growth in itself is energy intensive.

Chapter 5

Conclusions

In this project we have successfully constructed three vectors (pSL2034, pSL2035 and pSL2036) that are designed to integrate into the *psbA1* gene as the target site. This work generated an improved vector system that can be used for engineering *Synechocystis* 6803, an important model organism for various studies. In order to test the construct a gene (*isiA*) was cloned to one of the vectors, pSL2035 and *Synechocystis* 6803 transformants were generated. This transgenic line expressed *isiA* constitutively as opposed to the WT where the gene is expressed only under iron-deplete conditions. RT-PCR results confirmed constitutive expression of this gene in the mutant line, thereby validating the activity of the *psbA2* promoter. In agreement with previous studies (Pakrasi et al., 1985; Wilson et al., 2007) our 77 K fluorescence analysis with one of the mutants showed a peak at 683 nm when excited at 420 nm, confirming the presence of IsiA in the mutant.

An advantage of this vector system is the presence of the NdeI site next to the *psbA2* promoter which provides an ATG codon when digested, allowing direct translation of the introduced gene and eliminating unnecessary bases in between the gene and the promoter region. However the vector only has two cloning sites, NdeI and HpaI. The next step in upgrading this vector system will be to introduce a multiple cloning site in this region which will allow more flexibility in introducing foreign genes.

Appendix

A.1 Sequence results with primer iARTF

Query – Data from sequencing centre

Subject – Linear sequence from pSL2045

Score = 1465 bits (793), Expect = 0.0
Identities = 793/793 (100%), Gaps = 0/793 (0%)
Strand=Plus/Plus

```
Query 1      TTTTCCGGGGAAGCCATTTTGTCTATTCCCTGGGGGGCATTGCCCTAGCTGGATTTGTG 60
            |||
Sbjct 2863    TTTTCCGGGGAAGCCATTTTGTCTATTCCCTGGGGGGCATTGCCCTAGCTGGATTTGTG 2922

Query 61     GCGGCCTATTTCTGTGCAGTCAACACCCTGGCCTATCCGCCAGAATTTTACGGCCCGCCT 120
            |||
Sbjct 2923    GCGGCCTATTTCTGTGCAGTCAACACCCTGGCCTATCCGCCAGAATTTTACGGCCCGCCT 2982

Query 121    TTGGCAATTA AATTGGGCATTTTCCCTATTTTGCCGATACGGTGGA ACTGCCCATGCAT 180
            |||
Sbjct 2983    TTGGCAATTA AATTGGGCATTTTCCCTATTTTGCCGATACGGTGGA ACTGCCCATGCAT 3042

Query 181    GCCCACACCAGCCGGGCTTG GTTAGCCAACGCTCACTTCTTCTGGCCTTTTCTTCCTC 240
            |||
Sbjct 3043    GCCCACACCAGCCGGGCTTG GTTAGCCAACGCTCACTTCTTCTGGCCTTTTCTTCCTC 3102

Query 241    CAAGGCCACCTCTGGCAGCACTGCGGGCTTTGGGTTTTGACTTCAAACGGGTCGAACAA 300
            |||
Sbjct 3103    CAAGGCCACCTCTGGCAGCACTGCGGGCTTTGGGTTTTGACTTCAAACGGGTCGAACAA 3162

Query 301    GCGTTTGATTCTTGCAAACCTAGGTTAACCTCACATTGGATTCTTCCACTGTCTGAGT 360
            |||
Sbjct 3163    GCGTTTGATTCTTGCAAACCTAGGTTAACCTCACATTGGATTCTTCCACTGTCTGAGT 3222

Query 361    TATTTTGACCGTCAATTGTGCCATTGCCATAACTGCTTTCGGTTAGACTTCGTTTCATTT 420
            |||
Sbjct 3223    TATTTTGACCGTCAATTGTGCCATTGCCATAACTGCTTTCGGTTAGACTTCGTTTCATTT 3282

Query 421    GGTTAATCAAGGGCACTCTCGCAATGGGGTGCCTTTTATGGTCCAAGGTTAAAGTTAAGC 480
            |||
Sbjct 3283    GGTTAATCAAGGGCACTCTCGCAATGGGGTGCCTTTTATGGTCCAAGGTTAAAGTTAAGC 3342

Query 481    CAGTAAAGTTTAAAGTCTATTTCTAGGGTGAAATGTAATGAATCAATTTAGGGACTGGGGT 540
            |||
Sbjct 3343    CAGTAAAGTTTAAAGTCTATTTCTAGGGTGAAATGTAATGAATCAATTTAGGGACTGGGGT 3402

Query 541    TTTTCCACTGATTGGTGGCAAGGCCAAAAAGGGGAATATTGGGTACTTGGGCAAAACAATT 600
            |||
Sbjct 3403    TTTTCCACTGATTGGTGGCAAGGCCAAAAAGGGGAATATTGGGTACTTGGGCAAAACAATT 3462

Query 601    CTCTCCGTTGGATTTGACTGTTACCGGTTTATACTCCTACTAATTTACAGTTACTAGCT 660
            |||
Sbjct 3463    CTCTCCGTTGGATTTGACTGTTACCGGTTTATACTCCTACTAATTTACAGTTACTAGCT 3522

Query 661    GAACAAAATCAGTGGATTGCCTGGGCTGGTACTCTGTTTTTTGGCATGATTGCGGCAGTT 720
            |||
Sbjct 3523    GAACAAAATCAGTGGATTGCCTGGGCTGGTACTCTGTTTTTTGGCATGATTGCGGCAGTT 3582
```

```

Query 721  TTGCTGATTggggggggTCTGCATTTAGGGGAAAATCTGACTCCCTTGCCCCATCCTAAA 780
          |||
Sbjct 3583  TTGCTGATTGGGGGGGTCTGCATTTAGGGGAAAATCTGACTCCCTTGCCCCATCCTAAA 3642

Query 781  AAGGATAGTCAAA 793
          |||
Sbjct 3643  AAGGATAGTCAAA 3655

```

A.2 Sequence results with primer KmpA2F

Query – Data from sequencing centre

Subject – Linear sequence from pSL2045

Score = 1921 bits (1040), Expect = 0.0
 Identities = 1058/1068 (99%), Gaps = 4/1068 (0%)
 Strand=Plus/Plus

```

Query 1      CTGTTTACCCATGGAAAAACGACAATTACAAGAAAGTAAAACCTTATGTCATCTATAAGC 60
          |||
Sbjct 1983    CTGTTTACCCATGGAAAAACGACAATTACAAGAAAGTAAAACCTTATGTCATCTATAAGC 2042

Query 61     TTCGTGTATATTAACCTTCTGTACAAAGCTTTACAAAACCTCATTAAATCCTTTAGACT 120
          |||
Sbjct 2043    TTCGTGTATATTAACCTTCTGTACAAAGCTTTACAAAACCTCATTAAATCCTTTAGACT 2102

Query 121    AAGTTTAGTCAGTTCCAATCTGAACATCGACAAATACATAAGGAATTATAACCATATGCA 180
          |||
Sbjct 2103    AAGTTTAGTCAGTTCCAATCTGAACATCGACAAATACATAAGGAATTATAACCATATGCA 2162

Query 181    AACCTATGGCAACGACACCGTTTACGACGAATGGTGGGCCGGAATGCCCGTTTCGCTGA 240
          |||
Sbjct 2163    AACCTATGGCAACGACACCGTTTACGACGAATGGTGGGCCGGAATGCCCGTTTCGCTGA 2222

Query 241    TCAGTCTGGGCTTTTTATTGCGGCCCATGTGGCCAGGCGGCTTTAACAGCATTTTGGGC 300
          |||
Sbjct 2223    TCAGTCTGGGCTTTTTATTGCGGCCCATGTGGCCAGGCGGCTTTAACAGCATTTTGGGC 2282

Query 301    CGGAGCCTTTACCTGTTTGAATTTCCCGATTTGATCCCACCCAGGCCATGGGAGACCA 360
          |||
Sbjct 2283    CGGAGCCTTTACCTGTTTGAATTTCCCGATTTGATCCCACCCAGGCCATGGGAGACCA 2342

Query 361    GGGATTAATTCTACTGCCCATTTGGCTACCCTCGGTTGGGGTGTGGGCGATGGCGGACA 420
          |||
Sbjct 2343    GGGATTAATTCTACTGCCCATTTGGCTACCCTCGGTTGGGGTGTGGGCGATGGCGGACA 2402

Query 421    AATTGTGGATACCTATCCCTACTTTGTCATTGGTAGTATTACCTGATTGCTTACGCGGT 480
          |||
Sbjct 2403    AATTGTGGATACCTATCCCTACTTTGTCATTGGTAGTATTACCTGATTGCTTACGCGGT 2462

Query 481    ACTGGGGGCTGGGGCCTTGTTTCATACCCTGCGGGCCCCGCTGACCTCAGTACCCTTAA 540
          |||
Sbjct 2463    ACTGGGGGCTGGGGCCTTGTTTCATACCCTGCGGGCCCCGCTGACCTCAGTACCCTTAA 2522

Query 541    GGGCCAGGGCAAAAAATCCATTTACAGTGGGAAAACCCCAAGCAACTAGGGATAATTTT 600
          |||
Sbjct 2523    GGGCCAGGGCAAAAAATCCATTTACAGTGGGAAAACCCCAAGCAACTAGGGATAATTTT 2582

```

```

Query 601 GGGTCATCATCTCCTGTTCTGGGGGCGGAGCTCTACTGTTGGCAGGCAAAGCCATGTA 660
          |||
Sbjct 2583 GGGTCATCATCTCCTGTTCTGGGGGCGGAGCTCTACTGTTGGCAGGCAAAGCCATGTA 2642

Query 661 TTgggggggATTGTACGACGCCACCACCCAAACGGTGCGCCTAGTCAGTCAGCCAACATT 720
          |||
Sbjct 2643 TTGGGGGGGATTGTACGACGCCACCACCCAAACGGTGCGCCTAGTCAGTCAGCCAACATT 2702

Query 721 AGATCCTTTGGTTATTTATGGTTATCAAACCCATTTTGCCAGCATCAGTAGCCTAGAAGA 780
          |||
Sbjct 2703 AGATCCTTTGGTTATTTATGGTTATCAAACCCATTTTGCCAGCATCAGTAGCCTAGAAGA 2762

Query 781 TCTGGTGGGGGACATATTTTGTGCGTTTTCTGCTCATCGGTGGAGGCATTTGGCACAT 840
          |||
Sbjct 2763 TCTGGTGGGGGACATATTTTGTGCGTTTTCTGCTCATCGGTGGAGGCATTTGGCACAT 2822

Query 841 TCTGGTGCCCCCTGCGTTGGGCTAAAAAAGTTCTGCTCTTTCCGGGGAAGCCATTTT 900
          |||
Sbjct 2823 TCTGGTGCCCCCTGCGTTGGGCTAAAAAAGTTCTGCTCTTTCCGGGGAAGCCATTTT 2882

Query 901 GTCCTATTCCCTGGGGGGCATTGCCCTAGCTGGATTTGTGGCGGCCTATTTCTGTGCAGT 960
          |||
Sbjct 2883 GTCCTATTCCCTGGGGGGCATTGCCCTAGCTGGATTTGTGGCGGCCTATTTCTGTGCAGT 2942

Query 961 CAACACCTGGCCTATCCGCCAGAANTTTACGGCCNCNTT-GGCAAT-AAATGGGGCAT 1018
          |||
Sbjct 2943 CAACACCTGGCCTATCCGCCAGAANTTTACGGCCCGCCTTTGGCAATTAATTTGGGGCAT 3002

Query 1019 TTTTCCCTATTTTGCN-ATACGGTGA-CTGCCATGCATGCCACAC 1064
          |||
Sbjct 3003 TTTTCCCTATTTTGCN-ATACGGTGA-CTGCCATGCATGCCACAC 3050

```

A.3 Sequence results with primer p1dslR

Query – Data from sequencing centre

Subject – Linear sequence from pSL2045

Score = 1899 bits (1028), Expect = 0.0
 Identities = 1074/1095 (98%), Gaps = 16/1095 (1%)
 Strand=Plus/Minus

```

Query 1 ACCCGTTTGAAGTCAAACCCAAAGCCCGCAGTGCGTGCCAGAGGTGGCCTTGAGGAAG 60
          |||
Sbjct 3155 ACCCGTTTGAAGTCAAACCCAAAGCCCGCAGTGCGTGCCAGAGGTGGCCTTGAGGAAG 3096

Query 61 AAAAAAGGCCAGGAAGAAGTGAGCGTTGGCTAACCAAGCCCGCTGGTGTGGGCATGCATG 120
          |||
Sbjct 3095 AAAAAAGGCCAGGAAGAAGTGAGCGTTGGCTAACCAAGCCCGCTGGTGTGGGCATGCATG 3036

Query 121 GGCAGTTCACCGTATCGGCAAAATAGGGAAAAATGCCCAATTTAATTGCCAAAGGCGGG 180
          |||
Sbjct 3035 GGCAGTTCACCGTATCGGCAAAATAGGGAAAAATGCCCAATTTAATTGCCAAAGGCGGG 2976

Query 181 CCGTAAATTTCTGGCGGATAGGCCAGGGTGTGACTGCACAGAAATAGGCCGCCACAAAT 240
          |||

```

Sbjct 2975 CCGTAAAATTCTGGCGGATAGGCCAGGGTGTGACTGCACAGAAATAGGCCGCCACAAT 2916
 Query 241 CCAGCTAGGGCAATGCCCCCAGGGAATAGGACAAAATGGCTTCCCCGGAAAAGAGCAGA 300
 |||
 Sbjct 2915 CCAGCTAGGGCAATGCCCCCAGGGAATAGGACAAAATGGCTTCCCCGGAAAAGAGCAGA 2856
 Query 301 ACTTTTTTAGCCCAACCCAGAGGGGGCACCAGAATGTGCCAAATGCCTCCACCGATGAGC 360
 |||
 Sbjct 2855 ACTTTTTTAGCCCAACCCAGAGGGGGCACCAGAATGTGCCAAATGCCTCCACCGATGAGC 2796
 Query 361 AGAAAACCGACAAAATATGTCCCCCACCAGATCTTCTAGGCTACTGATGCTGGCAAAA 420
 |||
 Sbjct 2795 AGAAAACCGACAAAATATGTCCCCCACCAGATCTTCTAGGCTACTGATGCTGGCAAAA 2736
 Query 421 TGGGTTTGATAACCATAAATAACCAAAGGATCTAATGTTGGCTGACTGACTAGGCGCACC 480
 |||
 Sbjct 2735 TGGGTTTGATAACCATAAATAACCAAAGGATCTAATGTTGGCTGACTGACTAGGCGCACC 2676
 Query 481 GTTTGGGTGGTGGCGTCGTACAATccccccAAATACATGGCTTTCCTGCCAACAGTAGA 540
 |||
 Sbjct 2675 GTTTGGGTGGTGGCGTCGTACAATCCCCCAATACATGGCTTTCCTGCCAACAGTAGA 2616
 Query 541 GCTCCGGCCCCAGGAACAGGAGATGATGACCCAAAATTATCCCTAGTTGCTGGGGGTTT 600
 |||
 Sbjct 2615 GCTCCGGCCCCAGGAACAGGAGATGATGACCCAAAATTATCCCTAGTTGCTGGGGGTTT 2556
 Query 601 TCCCACGTGAAATGGAATTTTTGCCCCTGGCCCTTAAGGGTACTGAGGTGAGCGGGGGCC 660
 |||
 Sbjct 2555 TCCCACGTGAAATGGAATTTTTGCCCCTGGCCCTTAAGGGTACTGAGGTGAGCGGGGGCC 2496
 Query 661 CGCAGGGTATGAAACAAGGCCCCAGCCCCAGTACCCTGAAGCAATCAGGTGAATACTA 720
 |||
 Sbjct 2495 CGCAGGGTATGAAACAAGGCCCCAGCCCCAGTACCCTGAAGCAATCAGGTGAATACTA 2436
 Query 721 CCAATGACAAAGTAGGGATAGGTATCCACAATTTGTCCGCCATCGCCACACCCCAACCG 780
 |||
 Sbjct 2435 CCAATGACAAAGTAGGGATAGGTATCCACAATTTGTCCGCCATCGCCACACCCCAACCG 2376
 Query 781 AGGGTAGCCAAATGGGGCAGTAGAATTAATCCCTGGTCTCCCATGGCTGG-TGGGATCA 839
 |||
 Sbjct 2375 AGGGTAGCCAAATGGGGCAGTAGAATTAATCCCTGGTCTCCCATGGCTGGGTGGGATCA 2316
 Query 840 AATCGGGAAATTTCAAACAGGGTAAAGGCTCCGGCCAAAATGCTGTTAAAGCCGCTGG 899
 |||
 Sbjct 2315 AATCGGGAAATTTCAAACAGGGTAAAGGCTCCGGCCAAAATGCTGTTAAAGCCGCTGG 2256
 Query 900 GCCACATGG-CCGCAATAAAAAGCCAGACTGATCAGCGAAACGGGCATTGCCGGCCAC 958
 |||
 Sbjct 2255 GCCACATGGGCCGCAATAAAAAGCCAGACTGATCAGCGAAACGGGCATTGCCGGCCAC 2196
 Query 959 CATTCTACTGAACG-TGTCGTTGCCATAGGTTTGCATATGGT-ATAAT-CNT-ATGTAT 1014
 |||
 Sbjct 2195 CATTCTACTGAACGGTGTGTCGTTGCCATAGGTTTGCATATGGTTATAATTCCTTATGTAT 2136
 Query 1015 TTGTCGATGT-CAGAT-GN--CTGACTAA-CT-AGTCTAA-GGATTAATGANN-TTT-GT 1065
 |||
 Sbjct 2135 TTGTCGATGTTTCAAGATTGGAAGTAACTGACTAACTTAGTCTAAAGGATTAATGAGAGTTTTGT 2076
 Query 1066 AA-GCTTTGTAACAG 1079
 |||
 Sbjct 2075 AAAGCTTTGTAACAG 2061

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Supplementary Table 1

Oligonucleotides used for PCR and RT PCR

| Primer name | Primer sequence | T _m , °C |
|--------------|---|---------------------|
| ps1_up_fwd | CGCGGGTACCGGAACAGGACCAAGCCTT | 61 |
| ps1_up_bwd | CGCGCAATTGGATGGTTTCTCAGATTGCA GTTGGTTTCC | 60 |
| ps1_down_fwd | CGCGCAATTGTGCCATTGCCATAACTGCT TTCGGTTAG | 60 |
| ps1_down_bwd | CGCGAAGCTTTGACTATCCTTTTTAGGAT GGGGCAAGG | 60 |
| isiA-NdeI-F | GGCGACCATATGCAAACCTATGGCAACG | 49 |
| isiA-HpaI-R | GGCGAGGTTAACCTAGGTTTGCAAGGAA | 41 |
| iArtF | GGTGGGGGGACATATTTTTGTCG | 57 |
| iArtR | CTAGGTTTGCAAGGAATCAAACGC | 56 |
| p1dslR | GGCAATGGCACAATTGACGGTC | 57 |
| kmpA2F | CCTTCTTCACGAGGCAGAC | 55 |
| JC44 | CAGTGTGACCGTGTGCTTCT | 54 |

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