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**T7-CYANO – PRODUCTION AND DEVELOPMENT OF A *SYNECHOCYSTIS*
STRAIN USEFUL FOR INDUCIBLE MEMBRANE PROTEIN EXPRESSION
AND CONTROLLED ANTISENSE RNA SYNTHESIS**

Direttore della Scuola: Ch.mo Prof. Giuseppe Zanotti

Coordinatore d'indirizzo: Ch.mo Prof. Fiorella Lo Schiavo

Supervisore: Dott.ssa Elisabetta Bergantino

Dottorando: Laura Giaretta

*The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!' but 'That's funny...'*

- Isaac Asimov -

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RIASSUNTO

Si ritiene che i cianobatteri siano stati i primi organismi in grado di produrre ossigeno attraverso la fotosintesi, il processo chimico che utilizza la luce solare come fonte di energia per fissare la CO₂ in molecole di carboidrati. I cianobatteri sono storicamente considerati un sistema modello per lo studio dei processi fotosintetici. Più recentemente questi organismi hanno acquisito un particolare interesse anche in ambito biotecnologico, in particolare come fonte alternativa di energia, per esempio nella produzione di bioetanolo o bioidrogeno, e nella produzione di molecole utili per l'industria farmaceutica. I cianobatteri, inoltre, sono interessanti organismi da usare come ospiti per l'espressione di proteine, in particolare per quelle di membrana. Infatti, il loro abbondante sistema membranale interno (i tilacoidi) dovrebbe fornire lo spazio adeguato per la localizzazione di questo tipo di proteine e permettere quindi una maggiore produzione delle stesse.

Synechocystis sp. PCC 6803 è a tutt'oggi uno dei ceppi cianobatterici più studiati ed è considerato un organismo modello per lo studio della fotosintesi. Le ragioni principali di questo suo ampio utilizzo risiedono in alcune sue caratteristiche: è spontaneamente trasformabile, può facilmente incorporare DNA esogeno nel suo genoma mediante ricombinazione omologa ed è in grado di crescere sia in fotoautotrofia sia in eterotrofia. Tuttavia, la mancanza di elementi episomali maneggevoli anche per il clonaggio e la presenza di 10-12 copie del genoma per cellula batterica hanno finora ostacolato un suo più vasto sfruttamento.

Lo scopo di questo studio è di sviluppare un ceppo di *Synechocystis sp. PCC 6803* che semplifichi l'utilizzo delle tecniche del DNA ricombinante in questo organismo e che possa essere utilizzato per l'espressione inducibile di proteine. Il *tool* consiste in un sistema a due componenti: i) un nuovo ceppo di *Synechocystis sp. PCC 6803*, chiamato "T7-cyano", che ospita il gene codificante la T7 RNA polimerasi sotto il controllo di diversi promotori inducibili; ii) un *set* di vettori, chiamati pSEV (*Synechocystis Expression Vectors*), per l'introduzione stabile nel genoma di un frammento di DNA di interesse sotto il controllo del promotore T7. Il sistema è analogo al ceppo BL21 di *Escherichia coli*: l'induzione della T7 RNA polimerasi conduce alla trascrizione della sequenza eterologa in modo controllato.

Il presente lavoro descrive il disegno sperimentale del sistema T7-cyano e i risultati preliminari conseguenti alla sua induzione. Dopo una breve introduzione sui cianobatteri (capitolo uno), nel secondo capitolo è descritto il nuovo *tool* cianobatterico e la costruzione di un vettore, *Transformation Vector*, per l'integrazione stabile del gene codificante la T7 RNA polimerasi nel genoma di

Synechocystis. In questo stesso capitolo, è inoltre illustrata la costruzione della seconda componente del sistema. Questa è rappresentata da tre diversi vettori di espressione aventi diverse applicazioni: pSEV1, per l'espressione di proteine in fusione con uno Strep-tag; pSEV2, per l'espressione di proteine ricombinanti endogene o eterologhe; pSAS, per la sintesi di molecole di RNA in antisenso.

La creazione e lo sviluppo del ceppo T7-ciano è riportata nel terzo capitolo. Tre diversi promotori inducibili sono selezionati per controllare l'espressione della T7 RNA polimerasi. I promotori scelti sono: P_{zia} , un promotore endogeno inducibile dallo zinco, P_{nrs} , un altro promotore endogeno inducibile da nickel e una variante del promotore P_{lac} di *Escherichia coli*. Utilizzando il *Transformation Vector* il gene della T7 RNA polimerasi è inserito in omoplasma nel genoma del cianobatterio. La produzione della T7 RNA polimerasi è, per la prima volta in *Synechocystis*, mostrata mediante analisi di immunoblotting nei ceppi con i promotori P_{zia} e P_{nrs} .

Il sistema è quindi testato nell'espressione di tre differenti proteine eterologhe (capitolo quattro): la proteina fluorescente eGFP; HydA, una [FeFe]-idrogenasi proveniente dall'alga *Chlamydomonas reinhardtii* precedentemente espressa in *Synechocystis sp. PCC 6803*; e una Baeyer-Villiger monoossigenasi (BVMO) dell'alga *Cyanidioschyzon merolae*, già precedentemente espressa in *Escherichia coli*. Le sequenze codificanti le tre proteine prescelte sono clonate in uno dei vettori di espressione sviluppati, pSEV1 e pSEV2, e i vettori ottenuti sono utilizzati per trasformare i ceppi di T7-ciano. In esperimenti preliminari di induzione è confermata la trascrizione dei geni eterologhi ad opera della T7 RNA polimerasi. Inoltre attraverso immunoblotting è stata con successo rilevata l'espressione dell'enzima BVMO. Tuttavia i promotori P_{zia} e P_{nrs} , sia mediante l'analisi dei trascritti che immunoblotting, sono risultati *leaky*. Inoltre la bassa quantità dell'enzima BVMO prodotta suggerisce una inefficiente traduzione del gene esogeno.

Nell'ultimo capitolo il sistema T7-ciano è proposto per la sintesi inducibile di RNA antisenso. *Synechocystis sp. PCC 6803*, infatti, possiede un numero elevato di piccoli RNA regolatori trascritti in diverse condizioni ambientali e di stress, tuttavia solo pochi RNA antisenso sono stati caratterizzati tutt'oggi. Il nuovo sistema può quindi essere un utile *tool* per lo studio e la caratterizzazione di questi piccoli RNA. Inoltre una interessante applicazione sarebbe il knockdown controllato di geni endogeni. Per testare il sistema T7-ciano nella sintesi di RNA antisenso, viene scelto IsrR, uno dei pochi RNA antisenso già caratterizzato in *Synechocystis sp. PCC 6803*, noto down-regolatore del gene *IsiA* attivato in condizioni di carenza di ferro. In aggiunta è disegnato un secondo RNA antisenso contro il 5'UTR dello stesso gene *IsiA*. Gli esperimenti preliminari di induzione sono stati svolti presso il dipartimento di biochimica della University of Turku (Finland) nel gruppo di ricerca Molecular Plant Biology guidato dalla Prof. Eva-Mari Aro, esperta nello studio degli organismi

fotosintetici. I primi risultati mostrano un fenotipo knockdown nei ceppi indotti, tuttavia maggiori esperimenti sono necessari per confermare il silenziamento del gene attraverso la sintesi di antisenso nei T7-cyano.

Il lavoro di questa tesi fornisce una visione di insieme del nuovo sistema mostrando, per la prima volta, la capacità di *Synechocystis sp. PCC 6803* di produrre la T7 RNA polimerasi che a sua volta è in grado di trascrivere il gene clonato a valle del promotore T7. Il lavoro ha inoltre evidenziato le due maggiori problematiche del sistema ovvero la *leakiness* dei promotori P_{zia} e P_{nrs} e una inefficiente traduzione del gene eterologo.

SUMMARY

Cyanobacteria are supposed to have been the first organisms able to evolve oxygen by photosynthesis, the process that uses solar light as energy source to fix CO₂ into carbohydrate molecules. Historically, they have been and are a model system for studying fundamental processes including and relating to photosynthesis. In more recent years cyanobacteria acquired interest also in biotechnology, in particular as an alternative energy resource, for instance in ethanol or hydrogen production, and as a source for the production of molecules useful in the pharmaceutical industry. Moreover cyanobacteria are interesting host organisms for proteins expression, in particular for membrane proteins. Indeed, the abundant internal membrane system (the thylakoids) typical of cyanobacteria would provide the proper compartment to harbour these kind of proteins, permitting higher production of these proteins.

Synechocystis sp. PCC 6803 is nowadays one of the most extensively studied species among all cyanobacteria and is considered a model organism for the study of photosynthesis. Some of the main reason are that it is spontaneously transformable, it can easily incorporate exogenous DNA into its genome by homologous recombination and it is able to grow both photoautotrophically and heterotrophically. However, the lack of handy episomal elements for cloning and the presence of 10-12 copies of the chromosome per cell have hampered its use so far.

The aim of the present study is to develop a *Synechocystis* strain suitable for a simplified use of DNA recombinant techniques within this organism and for the inducible expression of proteins. The genetic tool consists in a two component system: i) a new *Synechocystis* strain, called "T7-cyano", hosting the highly processive RNA polymerase from the bacteriophage T7 under the control of different inducible promoters; ii) vectors, called pSEV (*Synechocystis Expression Vectors*), for stable introduction of a DNA fragment of interest under the control of the T7 promoter. The system resembles therefore the BL21 strain of *Escherichia coli*: induction of the T7 RNA polymerase will lead to transcription of the heterologous sequence in a controlled manner.

The present work describes the design and the early development of the T7-cyano system. After a brief introduction on cyanobacteria (chapter one), the second chapter describes the design of the new cyanobacterial tool and the construction of a vector, called Transformation Vector, for the stable integration of the T7 RNA polymerase gene in the genome of *Synechocystis* sp. PCC 6803. In this same chapter, the construction of the second component of the system is also illustrated. This is represented by three different expression vectors having different applications: pSEV1, for Strep-tagged fusion proteins expression; pSEV2, for recombinant

expression of endogenous or heterologous proteins; pSAS, for the synthesis of RNA molecules in antisense.

The development of the T7-cyano strain is reported in the third chapter. Three different inducible promoters are chosen to control the expression of the T7 RNA polymerase gene: P_{zia} , a zinc-inducible endogenous promoter, P_{nrs} , an endogenous nickel-inducible one and variant of the *E. coli* promoter P_{lac} . By the developed Transformation Vector the T7 RNA polymerase gene is homoplasmically inserted in the genome of the cyanobacterium. The production of the bacteriophage polymerase in the T7-cyano strain is successfully confirmed by immunoblotting upon induction of the P_{zia} and P_{nrs} promoters.

The system is tested in the expression of three different heterologous proteins (chapter four): the eGFP fluorescence protein; HydA, a [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* previously expressed in *Synechocystis sp. PCC 6803*; and a Baeyer-Villiger monooxygenase, BVMO, from the algae *Cyanidioschyzon merolae*, previously expressed in *Escherichia coli*. The sequences of the chosen protein coding genes are cloned in one of the expression vectors developed, pSEV1 and pSEV2, and the obtained vectors are used to transform the T7-cyano strains. In preliminary induction experiments transcript analysis confirms transcription of the heterologous genes by the T7 RNA polymerase. Moreover by western blot, the BVMO enzyme is successfully confirmed expressed in T7-cyano. However the promoters P_{zia} and P_{nrs} are found both leaky and the low amount of the BVMO enzyme produced suggests translation inefficiency.

In the last chapter the system is proposed for the inducible synthesis of antisense RNAs. *Synechocystis sp. PCC 6803*, in fact, possesses a significant number of regulatory RNAs transcribed in a wide range of environmental changes and stress conditions. To date, however, only few of them have been characterized and new genetic tools are required for the study and identification of these small RNA molecules. In addition, an interesting application would be the controlled knockdown of endogenous genes. To test the T7-cyano system in the synthesis of antisense RNAs, the characterized IsrR, known to down-regulate the iron-starvation responding gene *isiA*, is chosen. In addition an antisense is designed against the 5' UTR of the same *isiA* gene. The experiments were performed at the Department of Biochemistry of the University of Turku (Finland) at the Molecular Plant Biology group headed by Prof. Eva-Mari Aro, one of the main experts in the study of photosynthetic organisms. Preliminary experiments shows a knockdown phenotype in induced cells, however more experiments are necessary to confirm the silencing of the gene by the synthesis of antisense RNAs in T7-cyano.

The present work gives a general view of the new system showing, for the first time, the capability of *Synechocystis sp. PCC 6803* to produce the T7 RNA polymerase that in

turn is able to transcribe the sequence downstream the T7 promoter. The work also pointed out the two major problematic issues found within the system that are the leakiness of the promoters, P_{zia} and P_{nrs} , and a translational inefficiency in proteins expression.

ABBREVIATIONS AND ACRONYMS

Amp^R: Ampicillin resistance

asRNA: antisense RNA

BVMO: Baeyer-Villiger monooxygenase

C. merolae: *Cyanidioschyzon merolae*

C. reinhardtii: *Chlamydomonas reinhardtii*

CFS: cell-free system

Cm^R: Chloramphenicol resistance

DGDG: digalactosyl diglyceride

E. coli: *Escherichia coli*

GOI: Gene Of Interest

Kan^R: Kanamycin resistance

LPS: lipopolysaccharides

MCS: multiple cloning site

MGDG: monogalactosyl diglyceride

MP: membrane protein

mRNA: messenger RNA

MW: molecular weight

ncRNA: non-coding RNA

OD: Optical Density

OM: Outer Membrane

o/n: overnight

PG: phosphatidylglycerol

PM: Plasma Membrane

pSEV: *Synechocystis* Expression Vector

RBS: ribosome binding site

RT: room temperature

RT-PCR: Reverse transcriptase - polymerase chain reaction

SD: Shine-Dalgarno

SQDG: sulfoquinovosyldiacylglycerol

sRNA: small RNA

Synechocystis: *Synechocystis* sp. PCC 6803

TM: Thylakoid membrane

TSS: Transcriptional start sites

CHAPTER 1

INTRODUCTION

1.1 Cyanobacteria

Among all microorganisms cyanobacteria are considered particularly relevant in the history of Earth because they are thought to have changed the early atmospheric composition by introducing gaseous oxygen. Indeed, a common ancestor of current cyanobacteria was the only organism that ever evolved the ability to use the energy of sunlight to drive photosynthesis, a process by which the solar light is used to split water molecules into oxygen, protons, and electrons. The process promoted such a change on our planet, giving rise to the current biodiversity of living organisms, that this event is sometimes called the oxygen catastrophe. Although there are still controversies about when the oxygenic photosynthesis had evolved, cyanobacteria might date back to 3.5 billion years ago. Indeed they are found in the sedimentary structure of stromatolites and oncolites, the most ancient fossil records on Earth¹.

Cyanobacteria get their name from their colour (Greek : κυανός (kyanós) = blue). Since they form visible blooms in fresh water lakes, they are also known as “blue-green algae”, although this is a misleading name since cyanobacteria are prokaryotic organisms, and not eukaryotic ones as algae. These microorganisms exhibit such a wide variety that they can be found in almost every terrestrial and aquatic environments, from the oceans to hot springs, in hypersaline bays, in temporarily moistened rocks of desert and even in the Antarctic rocks. Some of them are also endosymbionts in lichens, plants and sponges. Not surprisingly, they are a highly morphologically diverse group of organisms found as single cells or filaments, some of which are so large that they can be seen by naked eye. There is still little agreement about the number of cyanobacterial species, but they are traditionally classified into five sections according to their morphology and the plane of the cell division^{1,2}.

1.2 Characteristics of cyanobacteria cells

Cyanobacteria are photosynthetic prokaryotes classified as Gram-negative, ranging in diameter from about 1 to 10 μm . Their photosynthetic system, closely related to that of chloroplasts in eukaryotes, is composed of both the photosystems I and II, by which the oxygenic photosynthesis is carried out. The capture of light for the photosynthetic process is ensured by a set of pigments, in particular chlorophyll α , the carotenoids β -carotene and zeaxanthin are present. In addition they contain a specific class of light-harvesting antenna complexes called phycobilisomes, found only in cyanobacteria, Red algae and glaucophytes. The phycobilisomes are made of chromophotylated proteins, the phycobiliproteins, organized around a central core of

allophycocyanin anchored to the thylakoid membrane. These protein complexes allow the absorption of wavelengths of light comprised between 500-650 nm, inaccessible to chlorophyll and are therefore particularly advantageous in deep water where the light of longer wavelength, used from chlorophyll, is less available³. Photosynthetic pigments together with the electron transport chain components are located in the thylakoid membranes. In general, cyanobacteria use water molecules as an electron donor and produce oxygen as a byproduct, although some may also use hydrogen sulphide⁴. As in plants, the ATP and NADPH molecules produced during the light phase are used in the Calvin cycle and carbon dioxide is reduced to form carbohydrates. Although many cyanobacteria are obligate photolithoautotrophs, some can grow slowly in the dark as chemoheterotrophs by oxidizing glucose and few other sugars¹.

An important feature of many cyanobacteria is their ability to fix the atmospheric nitrogen, both under anaerobic and aerobic conditions. In the latter case the process usually takes place inside specialized cells, called heterocysts that are formed in harsh environmental conditions when both nitrate and ammonia are missing. The cells, in this case, synthesize a very thick new wall, reorganize the photosynthetic membranes and synthesize the nitrogen-fixing enzyme nitrogenase².

Although cyanobacteria lack flagella, many species are able to move along the surfaces by gliding motility that allows moving at a rate of few micrometers per second. The precise mechanism of this kind of motion has still to be established in cyanobacteria⁵, therefore a number of mechanisms and structural features have been proposed which include specific secretion processes, twitching and the presence of distinct surface proteins of the cells. To move vertically in the water they often use gas vesicles, that function by regulating the gas level inside a hollow structure made of proteins³.

Cyanobacteria can reproduce by vegetative and asexual methods. Vegetative reproduction occurs by fission or fragmentation or by the formation of hormogonia if exposed to environmental stress. Some species may also develop akinetes, thick-walled dormant cells resistant to desiccation and that can also store reserve food material^{2,6}.

1.3 The endosymbiotic theory

The endosymbiotic theory, first elaborated in 1910 by the botanist Mereschkowski⁷, is an evolutionary theory that explains the origin of eukaryotic cell. It suggests that the organelles of the eukaryotic cells have originated from a symbiotic event between two separate living organisms. In particular, mitochondria would have developed

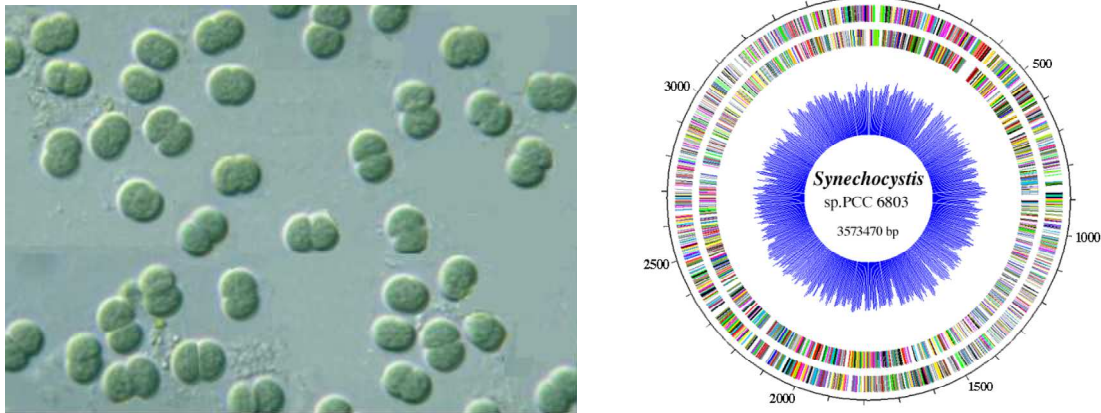
from proteobacteria and the chloroplasts from cyanobacteria³. The endosymbiotic theory is strongly supported by the physiological and biochemical similarities between the organelles and prokaryotic cells and has found even more evidence by the analysis of the 16S rRNA⁸. After the symbiotic event and during integration many genes were transferred from the original prokaryotic genome to the chromosome of its host. Indeed, the actual chloroplast genome encodes only for 100-200 proteins, a marked reduction in respect to the thousands of proteins encoded by cyanobacteria. In *Arabidopsis* it was estimated that about 18 % of the total protein-coding genes of the nuclear genome were acquired from the cyanobacterial ancestor plastids and that the product of these genes encompassed all functional classes⁹. The study of cyanobacteria has become therefore remarkably important to understand the evolutionary transition to current chloroplasts.

1.4 *Synechocystis* sp. PCC 6803

The cyanobacterial species *Synechocystis* sp. PCC6803 is one of the most popular organisms for the genetic and physiological studies of photosynthesis. Some of the main reasons are that it is naturally transformable and it can grow both autotrophically and heterotrophically, that is in the absence of photosynthesis if glucose is provided as source of carbon. Moreover it is able to integrate foreign DNA into its genome by homologous recombination and for this reason the study of this strain is often achieved by targeted modification of its genes.

Synechocystis sp. PCC6803 (from now on *Synechocystis*) is a unicellular, spherical cyanobacterium first isolated from a freshwater lake in California and deposited at the Pasteur Culture Collection (PCC) in 1968 (Figure 1.1, left). *Synechocystis* is commonly cultivated at 30 °C, using BG11 medium (pH = 7~8) both in solid and liquid. This non-nitrogen fixing specie has a dimension of $\approx 1.5 \mu\text{m}$ in diameter and can divide by binary fission at two or three successive planes. The strain was recognized to be polyploid, having an estimated number of 12 genome copies per cell¹⁰.

The entire genome sequence became available in 1996¹¹ (Figure 1.1, right) which placed it in the fourth position among the genomes completely sequenced and the first among phototrophic organisms. The publication of its sequence allowed not only to implement the study of its genes and their organization, but was also a caveat for the knowledge of the unique genetic characteristics of phototrophic organisms¹². In addition to the chromosomal DNA, *Synechocystis* contains seven plasmids, sequenced as well. The information on gene structure and gene function has been deposited in two genome databases, CyanoBase and CyanoMutants respectively.



1.1: Microscopy image of *Synechocystis* sp. PCC 6803 (right) (image modified from <http://protist.i.hosei.ac.jp>). Genome map of *Synechocystis* (left) (<http://www.kazusa.or.jp/cyano/Synechocystis/map/click/cmap.html>)

1.5 The genome and transcriptional regulation

According to the information reported by Kaneko et al.¹¹, the genome of *Synechocystis* is approximately 3.5 billion base pair long with an average GC content of 47.7 %. Open reading frames (ORFs) were assigned using a combination of similarity searches to other genomes and computer predictions. The results indicated a total of 3167 ORFs corresponding to 87 % of the entire genome. On the basis of sequence similarity with the genes of other organisms known at that time, 128 genes were found to be involved in various stage of photosynthesis, while 224 genes were found to be significantly homologous to the genes of algae and higher plant plastids. These data not only further supported the theory that cyanobacteria are candidates for the progenitors of plastids, but were also of essential importance for the subsequent studies regarding photosynthesis. One of the notable features found in the genome of *Synechocystis* was its high content of two types of repetitive sequences, insertion sequence (IS)-like elements and a HIP1 (highly iterated palindromic) sequence. While the origin and functional significance of the HIP1 element in cyanobacteria remains to be clarified¹³, the IS-like elements seem to play a role in the local and dynamic alteration of the genome through transposition¹¹.

Using differential RNA sequencing, a map of 3,527 transcriptional start sites (TSS) was established in the *Synechocystis* genome¹⁴. The analysis revealed the presence of a huge number of regulatory RNAs. Indeed, only one-third of all TSS were located

upstream of annotated genes while another third were set on the reverse complementary strand, suggesting significant antisense transcription. The remaining sites were located in intergenic regions, regulating transcription of noncoding RNAs (ncRNAs). Therefore, about the 64% of all TSS give rise to antisense or ncRNAs in a genome that is to 87% protein coding. The study also reported a significantly altered expression level of these ncRNAs in three different conditions (high light, CO₂ depletion, or dark), thus suggesting a relevant functional role of these small molecules in metabolic processes, photosynthesis included. Recently, deep sequencing analyses, focused on the identification of low molecular weight RNAs (≤ 200 nt), not only confirmed the presence of a high number of small RNAs (sRNA, putatively 5211) but also found them in plasmid regions¹⁵. Although until now only few sRNAs have been characterized, it is clear that these small molecules might play an important role in gene regulation in this organism.

1.5.1 Antisense RNAs

In prokaryotes the vast majority of the characterized sRNAs act by base pairing with the messenger RNA¹⁶. This category of regulatory RNAs are known as antisense RNAs (asRNA) and are generally divided into two categories: *trans*-encoded, if they are transcribed distant from the mRNA they regulate, and *cis*-encoded, when transcription occurs on the opposite DNA strand of their target mRNA. Although antisense RNAs were first observed in bacteria more than 30 years ago¹⁷, a systematic analysis to identify these RNA molecules started only in recent years. Illumina sequencing identified about 1,000 different asRNAs in *Escherichia coli*¹⁸, while in the pathogen *Helicobacter pylori* the percentage of asRNAs is reported to be around 46 % of all annotated open reading frames, revealing a huge percentage of antisense transcription¹⁹.

As previously mentioned, in *Synechocystis* one third of the TSS is found on the reverse complementary strand suggesting high presence of antisense RNA molecules. Massive antisense transcription is found also in other cyanobacteria. For instance in the nitrogen-fixing *Anabaena* sp. PCC 7120 the percentage is reported to be around the 39%, while the data for *Prochlorococcus* indicate that antisense transcription occurs for three-quarters of the genes, which is more than that observed in other bacteria²⁰. Recently, antisense transcription is reported also for plant plastids, the organelles evolved from cyanobacteria, where the percentage of asRNAs covers the 35% of all genes²¹.

This huge presence of antisense RNA transcription in cyanobacteria and in plant organelles suggests a significant role of these molecules in gene regulation. To date, however, the function of specific antisense transcripts in *Synechocystis* has scarcely

been defined and proper characterization has been reported just for few of them. The first one described at the functional level (in 2006), *IsrR*, negatively controls the expression of the iron-stressed induced protein *IsiA*²². This 177-nucleotide long antisense is transcribed from a constitutive promoter on the opposite strand of the *isiA* gene, whereas the *isiA* promoter is induced (derepressed) upon iron-, redox-, or light-stress conditions. Therefore, when the transcription of *isiA* is activated both transcripts are expressed simultaneously and the duplex formed is immediately degraded. As consequence, the *IsiA* protein is effectively translated only when the amount of *isiA* mRNA molecules exceeds that of *IsrR*.

Some years later, three asRNAs and one ncRNA were found associated with the *flv4-2* operon, whose genes are involved in photoprotection of PSII under inorganic carbon limitation and high-light conditions²³. It was described that one of these antisense, *As1_flv4*, is transiently expressed when the cells are shifted from high carbon to low carbon with a contemporary inverse accumulation of the mRNA transcribed from the *flv4-2* operon. The studies indicated that the antisense establishes a threshold for *flv4-2* expression in the early phase after a change in inorganic carbon conditions, thus preventing premature expression of the operon when the stress condition lasts for a short-time.

More recently two low abundant asRNAs, *PsbA2R* and *PsbA3R*, were described in *Synechocystis*²⁴. The two antisenses originate from the 5' UTR of the genes *psbA2* and *psbA3* respectively and act as positive regulators to achieve a stable level of D1 protein synthesis. It is interestingly to note that these antisense RNAs are present in small amount in the cell, providing evidence that also low-abundance transcripts might play a role in regulating important pathways like photosynthesis.

Although the information on the role of these regulatory RNAs in cyanobacteria are still low to date, it is very possible that these organisms, which can grow in very different environments and conditions, might have evolved a complex gene regulation system and that antisense molecules might play a consistent part in it.

1.6 The membrane system

Cyanobacteria are considered more complex than other prokaryotes since they contain three major differentiated membrane systems: the outer membrane (OM), the inner or plasma membrane (PM) and the interior thylakoid membrane system (TM), which is the site of the photosynthetic light reactions. The three membrane systems differ from one another with regard to their pigment, lipid and protein composition^{25,26}. The cell envelope comprises the OM, the PM, the periplasm, delimited by the two membranes, the peptidoglycan layer and the S-layer (surface layer), a monomolecular layer functioning as a protective coat.

Despite their overall gram-negative structure, the envelope of cyanobacteria possesses a combination of Gram- and Gram+ features, like a thicker peptidoglycan layer and a higher degree of cross-linking²⁷. In addition, the cyanobacterial OM presents some unique constituents not usually found in gram-negative bacteria like carotenoids, uncommon fatty acids such as β -hydroxyplamitic acid or porin proteins, anchored to the underlying peptidoglycan layer. Furthermore the lipopolysaccharides (LPS) contain small amounts of bound phosphate and often lack ketodeoxyoctonate, a common LPS component of gram-negative bacterial outer membranes.

The membrane lipid composition of cyanobacteria resembles the lipid composition of chloroplast of higher-plants and algae, being therefore far from the simple phospholipid composition of other bacteria, such as *E. coli*. The most abundant lipids are the two neutral galactolipids monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG), while the charged lipids, the anionic glycolipid sulfoquinovosyldiacylglycerol (SQDG) and the acidic phospholipid phosphatidylglycerol (PG) are minor components. Whereas the MGDG content is similar in both thylakoids and the cell envelope, the percentage of the other lipids can vary between the two membranes. The lower amount in phosphorous-containing lipids, compared to other organisms as enterobacteria, might be due to adaptation of cyanobacteria to phosphate limiting environments. In *Synechocystis*, however, PG was found essential for biogenesis, since mutation in the CDP-diacylglycerol synthase gene requires supplementation of PG for growth²⁸. This underlined that the non-phosphorous galactolipids MGDG, DGDG and SDQG are not able to compensate all functions performed by the acidic phospholipid²⁹. Not much is known nowadays concerning fatty acid and lipid transport pathways in cyanobacteria. Recently, the gene *slr1045* of *Synechocystis* was proposed to be involved in this route, because of its high similarity to other genes from *E. coli* and *A.thaliana* associated with lipid transport across membrane. The *slr1045* mutant, moreover, showed a reduced PG content under acidic growth conditions, supporting the proposed involvement³⁰. However, experimental evidences on its role and function are still lacking.

Electron tomography of *Synechocystis* cells shows that the thylakoid membrane pair forms layered sheets that follow the periphery of the cell converging at various sites near the cytoplasmic membrane (Figure 1.2). This organization differs from other rod-shaped cyanobacteria like *Synechococcus* 7002 where the internal membranes is mainly arranged inside the cell as concentric cylinders creating a separate and isolated cytosolic compartment, and underlines that fundamental differences may exist in thylakoid organization among cyanobacteria. The sites where membranes converge are counted to be present with an average of five per cell and are marked as thylakoid centers. Although ultrastructure of cyanobacterial cells have been studied for decades there is still debate whether the thylakoid centers represent a physical

connection between thylakoid and plasma membrane or if the two membranes are completely separated^{31,32}.

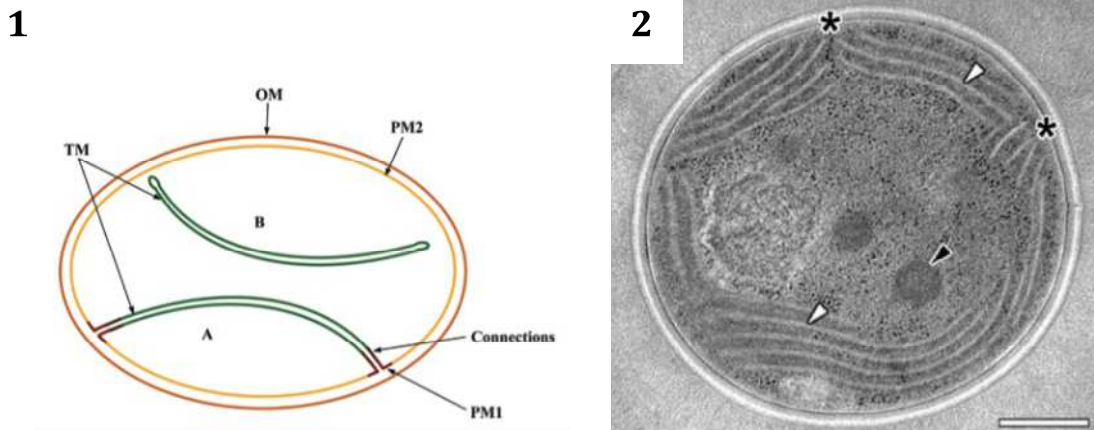


Figure 1.2: 1) Schematic representation of two models of membrane organization in *Synechocystis*: plasma and thylakoid membranes are continuous (A) or separated (B). Outer membranes (OM), thylakoid membranes (TM), major plasma membranes (PM2), minor plasma membranes (PM1)(image taken from Pisareva et al., 2011). 2) transmission electron microscopy (TEM) image of a *Synechocystis* cell, showing thylakoid membrane pairs (white arrowheads) converging at sites adjacent to the cytoplasmic membrane (black asterisks). Bar corresponds to 200 nm (image taken from van de Meene et al., 2005).

The complex subcellular membrane organization of cyanobacteria leads to the requirement of fine systems of targeting. It is believed that the transport system resembles the well-characterized one occurring in other enterobacteria, such as *E. coli*, via the Sec- or Tat- pathways. Indeed, proteomic studies of the periplasmic subfraction of *Synechocystis* report that all the proteins found have classical N-terminal signal peptides, the majority of which (82%) contains a Sec- signal and the remaining ones have a putative Tat- signal. Proteins in purified plasma-, outer-, and thylakoid membranes also contained Sec- or Tat- signals³³. However, the *Synechocystis* genome only contains single sets of genes encoding the necessary subunits of the bacterial Sec- and Tat- machinery, raising the question how and where proteins are specifically recognized and addressed to the different membranes and compartments. Multivariate sequence analysis suggests that the signal for the different extra-cytosolic compartments is contained in the different physicochemical properties of the signal peptide and mature N-terminal segments³³. Recently,

however, studies on uniquely localized integral membrane proteins suggest that the information for targeting is in the C-terminal tail of proteins and possibly in the protein 3D-structure. This latter model would imply, therefore, that sorting of integral membrane proteins occurs post-translationally, however, the mechanism of a post-translocon targeting was not yet defined³⁴

1.7 Current genetic and molecular tools in *Synechocystis*

1.7.1 Vectors for the introduction of foreign DNA

In *Synechocystis* two types of vectors are typically used for the introduction of foreign DNA sequences: integrative and replicative plasmids. Integrative plasmids allow the integration of a gene in the host genome by homologous recombination. Recombination sequences flanking the insert permit the integration of the foreign sequence in the genome by a double recombination event. Since *Synechocystis* contains an estimated number of 12 genome copies per cell, segregation by selective pressure is necessary to obtain a stable homoplasmic recombinant clone. The choice of a neutral site that would not cause significant disadvantages to the host is, therefore, very important for complete segregation of genome molecules.

Replicative plasmids allow a faster introduction of foreign sequences without the need of time-consuming restreaking steps needed when the foreign gene is integrated in the genome. In *Synechocystis* one of the most used replicative plasmid is the broad-host-range plasmid RSF1010, an *E. coli*-derived shuttle vector^{35,36}. Although their apparent easy use, the replicative plasmids exhibit several problematic issues. In particular they show genetic instability and high selective pressure is continuously required for their maintenance³⁷.

1.7.2 Transformation and Segregation

Since *Synechocystis* is naturally transformable, transformation is usually achieved by simply incubating the cells with the foreign DNA. Uptake of DNA shows higher efficiency during the exponential growth phase and the percentage of efficiency can be increased by 23 % by pretreating the cells for two days with EDTA, a chelating agent that limits the activity of Ca-dependent proteases³⁸. Several other factors have also been reported to affect transformation efficiency like the length of recombination fragment, the use of linear and circular DNA, time of incubation and light intensity^{38,39}. The selection of transformants is mainly achieved by the use of an antibiotic resistance, whose sequence is placed as marker in the introduced DNA fragment. Typical antibiotic resistance genes used in *Synechocystis* confer resistance

to kanamycin, spectinomycin and chloramphenicol. Restreaking the recombinant colony on progressively higher antibiotic concentration favours the obtainment of a homoplasmic recombinant clone.

1.7.3 Promoters

In *Synechocystis* both native and heterologous promoters have been used to drive expression of heterologous genes. Strong native promoters commonly used are the endogenous PrbcLS and PpsbA2, controlling the expression of the minor subunit of RuBisCO and the D1 protein of photosystem II respectively³⁵. Recently, a newly discovered promoter P_{cpc560} was used to express two heterologous genes, the first one from *Treponema denticola*, encoding the crotonyl-CoA-specific trans-enoyl-CoA reductase (Ter), and the second one from *E. coli* encoding D-lactate dehydrogenase⁴⁰. The promoter P_{cpc560} regulates the gene encoding the c-phycocyanin beta subunit, *cpcB*, one of the major soluble proteins in cyanobacteria. The sequence designated as P_{cpc560} consists of the genomic sequence of 560 nucleotides upstream of the initiation codon of the *cpcB* gene and contains two predicted promoters and 14 predicted transcription factor binding sites. Using P_{cpc560}, the level of the two heterologous expressed proteins was shown to be as high as the 15 % of total soluble proteins, a percentage comparable to those usually described for *E. coli*. However these strong promoters are constitutive and do not permit to control expression of the foreign gene. Native inducible promoters described in *Synechocystis* are unfortunately very limited in number; some of them respond to circadian rhythms⁴¹ and are therefore regulated by light, while others are metal-inducible⁴². The characterization of these promoters is scarce and a systematic investigation would be important to allow their utilization for controlling the expression of heterologous genes. The activities of heterologous inducible promoters, like those used in *E. coli* P_{trc}, P_{lac} and P_{tet}, were tested in *Synechocystis*; unfortunately it was shown that most of them loose their inducibility in the cyanobacterium⁴³.

1.7.4 Ribosome binding site (RBS)

The interaction of a ribosome to the RBS plays a crucial part in initiating translation. This is achieved by complementary pairing of the 3' terminal sequence of the 16S rRNA to the Shine-Dalgarno (SD) sequence (5'-GGAGG-3') contained in the RBS. However, the efficacy of a RBS depends also on the space between the core SD sequence and the start codon, as well as on the surrounding nucleotides. The efficiency of four different synthetic RBS has been recently tested in *Synechocystis* reporting that a designed RBS, containing the SD sequence and an optimal spacing of 9 nucleotides between the A of the core SD sequence and the first base of the start

codon, had an efficiency up to five fold higher in respect to other three BioBrick RBS tested (standard synthetic biological parts)³⁶. However, not much is known about translation efficiency in *Synechocystis* and most of the studies on heterologous expression use the RBS contained in the endogenous promoter selected for its expression. In this context it is interesting to notice that in *Synechocystis* only 26 % of the genes contain the core SD sequence suggesting that other sequences or mechanisms may be involved to achieve efficient translation in this organism^{44,45}.

1.8 Biotechnological applications of cyanobacteria

Cyanobacteria are emerging as organisms with potential applications in very diverse areas especially in the pharmaceutical industry and agriculture. Indeed, due to their wide diversity and distribution in different environments, these organisms can produce a huge variety of bioactive compounds, as secondary metabolites, with diverse biological activities including antiviral, antibacterial, antifungal and even anti-cancer, having medical interest as therapeutics⁴⁶. Industrial interest is mainly focused in the production of biopolymers, UV-absorbing compounds, useful for protecting biological samples or coating material surfaces, and sugars. For instance, isoprene, a volatile precursor used to generate synthetic rubber, was produced in *Synechocystis* by the expression of a codon optimized isoprene synthase from *Pueraria montana*⁴⁷. Scaled production of isoprene, as well as of other products, however, has not yet been described in cyanobacteria, mostly because their production is more efficient in other host organisms.

Cyanobacteria are also proposed in agriculture for wastewater treatment, for instance in the removal of heavy metals. To date, however, no technology has been developed for their use at commercial level, and research is still focused on the selection of the optimum pollutant tollerant cyanobacterial genus⁴⁸. In this same context, engineering of cyanobacteria was exploited to produce a luminescence strain useful to measure the concentration of pollutants, like herbicides and several heavy metals in aquatic environments. The growth or death of such a biosensor in different water samples can be an efficient indicator of the concentration of pollutants, although the main challenge to date is the selection of an efficient target-responsive promoter.

Oxygenic photosynthetic microorganisms like cyanobacteria and algae, are becoming promising producers of biofuels, because they represent a valid alternative to the production of bioethanol from, e.g., agricultural crop waste oils, that requires employment of wide land extensions and high-energy input. Microbial biofuels are generally produced from the lipid content of the microbial cells or from direct

biosynthesis of alcohols, like ethanol, butanol and isobutanol. It was proposed that synthetic biology could be used in cyanobacteria to modify the metabolic pathway of phototrophs via the addition of heterologous fermentative pathways, a concept called Photanol⁴⁹. However, genetic manipulation requires a deep understanding of the physiology of the organism for successful production of biofuels. Finally, H₂ is another renewable source of clean energy, which can be also produced by cyanobacteria directly from the native bidirectional hydrogenase or from nitrogenase contained in heterocysts. The extreme sensibility of hydrogenases to oxygen is, however, still an obstacle. Genetically engineering is again necessary for the construction of efficient hydrogen evolving cyanobacterial strains, for instance by the introduction of more efficient heterologous hydrogenases⁵⁰. In all these cases innovative biotechnology approaches are required to genetically modify metabolic pathways and improve yield of the target product. In this context cyanobacteria have some advantages to eukaryotic microalgae since they are more amenable to genetic manipulation for the introduction of genes and a more deep characterization of their genomes, transcriptomes and physiology is available.

1.9 Aim of the work

The present study describes the design and the first steps for the development of a new cyanobacterial tool useful for the inducible expression of recombinant proteins in *Synechocystis*, but also for the study of gene regulation within this organism.

Synechocystis, as described in this introduction, is considered the cyanobacterial model organism to study photosynthesis, since it is one of the most characterized genera among all cyanobacteria, it is spontaneously transformable and it is able to integrate foreign DNA into its genome by homologous recombination. However, some of its characteristics, like the presence of multiple copies of the genome, the lack of episomal vectors and absence of a wide knowledge of the molecular elements useful for proteins expression have hampered its use till now, especially when expression of foreign genes in the cyanobacterium is required.

Expression of proteins in this cyanobacterium would be, indeed, remarkably interesting, for instance for the study of gene functions. As described in the introduction, gene targeting is often used in this organism to produce knockout mutants and to study the effect that the deletion of a gene causes on important pathways like photosynthesis. The function of the deleted gene can be additionally investigated by complementation studies. In this case the same wild-type gene, a modified form of it or an heterologous one is reintroduced in the knockout mutant and the gene is studied by analysing the effect that the restoration of its function causes on the strain.

Complementation studies in the cyanobacterium are even more interesting when homologous genes from higher photosynthetic eukaryotic organisms, as plants or algae, are expressed. Cyanobacteria are, indeed, particularly interesting from an evolutionary point of view since, according to the endosymbiotic theory, they are considered the ancestor of current chloroplasts. The expression of proteins from higher organisms is therefore attractive, since it would provide considerable information about the evolutionary transition from prokaryotic cyanobacterial cells to the chloroplasts.

In addition to the study of gene functions, the aim of the present study is that of proposing *Synechocystis* as host organism for heterologous expression of membrane proteins. Membrane proteins (MPs) perform a wide range of essential biological functions and represent the 25 % of all genes in both prokaryotes and eukaryotes. However, the vast majority of MPs still do not have an assigned function and to date only approximately 500 of them among all organisms have been characterized at the structural level (<http://blanco.biomol.uci.edu/mpstruc/>). One of the reasons for this is due to their low abundance in the cells and to the difficulties encountered in their over-expression in heterologous systems⁵¹. These proteins, indeed, usually cause toxicity to the host organism when over-expressed in recombinant form and often

aggregate in insoluble inclusion bodies in an unfolded state. Nowadays, the bacterium *E. coli* is the most widely used host organism for proteins expression because of its simplicity of cultivation and the availability of many genetic tools. In this bacterium numerous strategies like the co-expression of specific chaperones, cultivation at lower temperatures or addition of tags have been used to allow the correct folding of these kind of proteins and to avoid the formation of inclusion bodies. The two mutated strains, C41 and C43, selected from the original BL21, are frequently used to express membrane proteins because they were found more tolerant to the toxicity typical of over-expressed MPs^{52,53}. However, all these strategies are not always successful and sometimes the cell-free system (CFS) is preferred for recombinant expression, so overcoming the problem of toxicity. The strength of this system is that the synthesis of the protein takes place *in vitro* and therefore does not depend on viable-host cells. Moreover in the reaction different compounds can be easily added, and in particular lipids, detergents and chaperones are usually supplied to assure the correct folding of MPs. However, the CFS still shows limitations as a low product yield, particularly relevant for structural studies, and a high cost⁵⁴. In this context cyanobacteria might be useful host organisms for the expression of membrane proteins: the presence of an abundant membrane system, the thylakoids, would provide an adequate compartment for their localization thus reducing the formation of inclusion bodies and would permit higher production of correctly folded proteins. Moreover they might permit to overcome the problem of toxicity. In *E. coli*, indeed, the over-expression of integral membrane proteins, such as ion channels, proteins transporters and receptors, usually causes the disruption of the integrity of its plasma membrane that in turn leads to cell lysis and to the death of the host. On the other hand, the possibility to grow *Synechocystis* heterotrophically by using glucose as a source of carbon, might overcome the problem of an eventual destabilization of the photosynthetic membrane due to over-expression of the recombinant protein therefore permitting the viability of the host cell. In addition, the cyanobacterial thylakoid membranes, having a different lipid composition and structure with respect to that of *E. coli* plasma membrane, might provide a more suitable environment for membrane proteins derived from photosynthetic organisms.

We would propose an engineered strain of *Synechocystis* not only as a host for recombinant protein expression, but also as a tool for the controlled synthesis of antisense RNAs, useful for the study of gene functions and regulation. As described in the introduction, the genome of *Synechocystis* encodes a huge amount of antisense RNA molecules that are considered important players in gene regulation. To date, however, the function of specific antisense transcripts in *Synechocystis* has scarcely been defined and proper characterization has been reported just for few of them. The antisense *IsrR*, the first one characterized in *Synechocystis* and shown to negatively

regulate expression of the gene *isiA*, was characterized also by analysing the effect that the over-expression of this small RNA molecule produced on the level of *isiA* mRNA²². Therefore, the over-expression of native antisense RNA molecules can be useful to study the role and function of these poorly characterized molecules and the development of a tool to induce the synthesis of asRNAs in *Synechocystis* might therefore simplify their study.

Another interesting application derived from the expression of antisense RNA molecules is the knockdown of endogenous genes. It is well known that in prokaryotes the majority of the antisense RNA molecules presenting a perfect pairing with the mRNA can inhibit the expression of the target gene⁵⁵. The synthesis of these antisense RNA molecules in *Synechocystis* might therefore be used to obtain conditional knockdown mutants. The advantage of using a knockdown strain resides in the possibility to induce the synthesis of the antisense only in specific conditions, overcoming the problems encountered in the removal of essential genes. In *Synechocystis* this would be particularly useful since the presence of numerous regulatory sequences and the high density of its genome often prevent the obtaining of completely homoplasmic knockout mutant, especially when essential genes are being studied.

The engineered strain of *Synechocystis* that we are proposing in this work consists of a “green” version of the BL21 strain of *E. coli*. The designed new tool comprises: i) a *Synechocystis* strain containing the gene encoding the T7 RNA polymerase homoplasmically inserted in the genome and controlled by an inducible promoter; ii) a set of three different vectors where the gene of interest can be cloned downstream the T7 promoter. The three vectors contain some specific elements useful for the different applications described in this section.

CHAPTER 2

THE PROJECT

2.1 Design of the T7-cyano system

Synechocystis sp. PCC 6803 as previously described is considered the cyanobacterial model organism to study photosynthesis. In the laboratory where this PhD work has been performed the cyanobacterium was extensively used to study the photosynthetic complexes and also used as host organism for heterologous protein expression⁵⁶⁻⁶³. Some characteristics of this organism, however, make difficult its use in the latter kind of applications. First of all, the presence of multiple copies of the genome rises up the necessity to perform several restreaking steps to select a homoplasmic clone carrying the foreign DNA sequence in each copy of its genome. The removal of all the wild-type copies of the cyanobacterium is not always successful and in many cases the strain used in the following experiments is heteroplasmic. Another reason that makes difficult working with this organism is the lack of handy episomal elements for the introduction of foreign genes. Although in *Synechocystis* seven natural plasmids have been found and sequenced, no origin of replication has been identified that can be used both in *E. coli* and *Synechocystis*, so allowing a more easy manipulation of vectors.

For all these reasons it was thought to develop a system to simplify the molecular biology within this organism. The adopted strategy is reminiscent of that used in the construction of the BL21 strain of *E. coli* developed by Studier & Moffatt⁶⁴. In this system the RNA polymerase gene of the bacteriophage T7 is inserted in *E. coli* genome under the control of the lacUV5 promoter. The polymerase directs high-level transcription of the foreign gene cloned in a multicopy plasmid downstream the T7 promoter, specifically recognized by the bacteriophagic enzyme.

Compared to prokaryotic and eukaryotic RNA polymerases the bacteriophage enzyme is characterized by a simpler structure consisting of a single subunit able to perform the complete transcription cycle in the absence of additional protein factors. The enzyme, first isolated from T7 infected *E. coli* cells in 1969⁶⁵, has been crystallized in several forms, allowing a deep understanding of its transcriptional mechanisms. The polymerase specifically recognizes its 17 nucleotides long promoter from which the downstream bacteriophage gene is transcribed. As for all the RNA polymerase, the initiation phase is characterized by abortive cycling, a process of repeated synthesis and release of short RNA products, that continues until a more stable elongation complex capable of transcribing the full template is formed⁶⁶. The characteristic high-level accumulation of RNAs directed by the T7 RNA polymerase led to the development of the *E. coli* BL21 expression system, one of the most used systems for the over-expression of proteins.

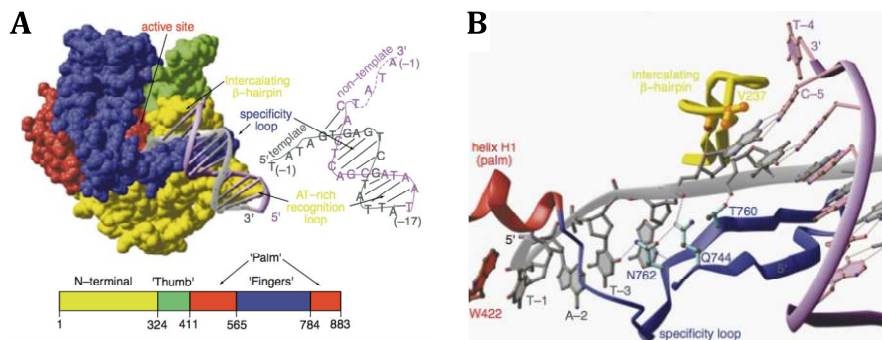


Figure 2.1: A) Representation of the T7 RNA polymerase-promoter complex structure. The promoter sequence is magnified on the right part of the figure, where colours specify the template strand (gray) and non-template strand (magenta). B) Interaction between the T7 promoter and the T7 RNA polymerase during the formation of the transcription bubble. (Image taken from Cheetham et al, 1999⁶⁷)

The cyanobacterial version of *E. coli* BL21 strain has been called T7-cyano. In the projected strain, the gene 1 of the T7 genome encoding for the bacteriophage polymerase would have been permanently and homoplasmically integrated in the genome of a wild-type strain of *Synechocystis* under the control of an inducible promoter and cloned together with the chloramphenicol resistance cassette as marker.

Together with the described cyanobacterial strain, vectors for the introduction of a gene of interest (GOI) under the control of the T7 promoter were designed. These vectors, called *Synechocystis* Expression Vectors (pSEV), would have contained some standard elements typical of expression plasmids for bacteria, as a multiple cloning site (MCS) for cloning and a resistance gene for selection of recombinant colonies. Transformation of the T7-cyano strain with pSEV would have permitted integration of the GOI into the chloramphenicol resistance cassette, disrupting it and concomitant with the introduction of the kanamycin resistance gene as new marker. Progressive increase of antibiotic concentration would then have permitted selection of a homoplasmic organism carrying the foreign gene in each copy of the T7-cyano genome.

The described T7-cyano system should thus work like the BL21 strain of *E. coli*: induction of the T7 RNA polymerase starts transcription, and consequently translation, of the GOI in a controlled manner (Figure 2.2).

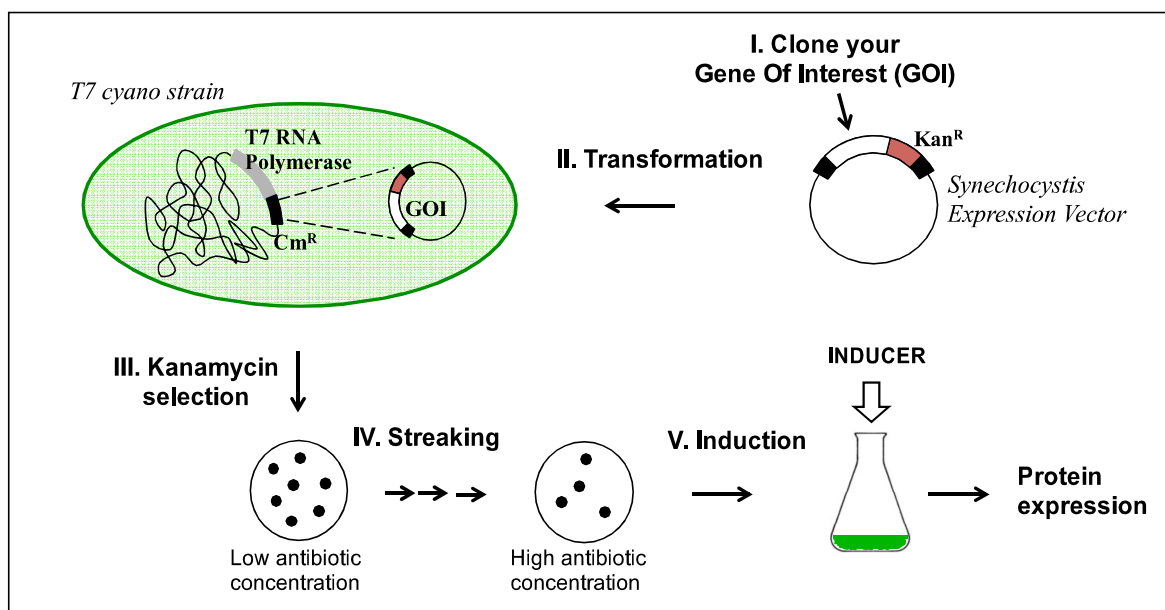


Figure 2.2: Schematic representation of the T7-cyano system. In the first step (I) a Gene Of Interest (GOI), white box, is cloned in one of the Synechocystis Expression Vectors. Black boxes are flanking regions homologous to the Cm^R gene ensuring recombination inside the genome of T7-cyano, while the red box is the gene conferring resistance to kanamycin (Kan^R). In the second step (II) the recombinant vector is used to transform the T7-cyano strain hosting the T7 RNA polymerase gene. Kanamycin is used to select recombinant colonies (III) carrying the foreign gene. The concentration of the antibiotic is progressively increased (IV) to select a homoplasmic organism carrying the recombinant gene in each copy of the cyanobacterial chromosomes. Finally (V) induction of the obtained strain will induce expression of the T7 RNA polymerase permitting transcription, and consequently translation, of the GOI in a controlled manner.

2.2 Construction of the Transformation Vector

The first step to develop the cyanobacterial system is to create the T7-cyano strain carrying the T7 RNA polymerase gene homoplasmically integrated in the genome. The *Synechocystis* genome is extremely compact, crowded with ORFs and, as it was shown in recent years, of regulatory sequences whose function has still to be completely investigated¹⁴. We looked for a neutral site where the introduction of the polymerase would not interfere with the normal activities of the organism and choose the region downstream the NADH dehydrogenase gene (*ndhB*). In the literature this site had been used several times for the insertion of foreign sequences in *Synechocystis*⁶⁸⁻⁷⁵ and was considered neutral site since in each case homoplasmy

had been obtained and no unexpected phenotype had been observed after recombination. It has to be specified, however, that insertion of a foreign sequence in this site interrupts the Open Reading Frame *ssl0410*, coding an unknown putative protein, 90 amino acids long (according to Cyanobase - <http://genome.microbedb.jp/cyanobase/Synechocystis>).

Once the neutral site was identified, a Transformation Vector containing all the elements needed to develop the T7-cyano strain was produced. The vector contains: two portions of the chosen neutral region (NR1 and NR2), an inducible promoter, the T7 RNA polymerase gene (T7 RNA pol) and the chloramphenicol resistance cassette (Cm^R) (Figure 2.3).

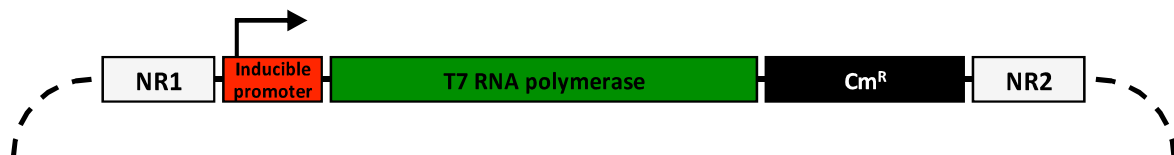


Figure 2.3: General scheme of the Transformation Vector containing: the two sequences for recombination inside the chosen neutral region of *Synechocystis* (NR1 and NR2); an inducible promoter; the T7 RNA polymerase gene; the chloramphenicol resistance cassette (Cm^R).

The mentioned sequences were amplified from the organism of origin or extracted from available plasmids and progressively transferred in a pUC18 vector using common molecular biology techniques. The strategy followed for the development of the Transformation Vector can be viewed in Figure 2.4 and is described as follows.

The sequence of the chosen neutral region (NR), necessary for homologous recombination, was amplified from *Synechocystis* genome by PCR using the mutagenic primers *Ndh_SmaI_for* and *Ndh_SmaI_rev*. These primers added at both end of the amplified sequence restriction sites *SmaI* that in the final Transformation Vector can be used to obtain a linear sequence with blunt ends to transform *Synechocystis*. The sequence was initially cloned inside a pGEM-T Easy Vector, then transferred into pUC18 by using *EcoRI* restriction sites.

For the cloning of the bacteriophage gene, we took advantage of two restriction sites, *BglII* and *ClaI*, naturally contained in the amplified NR sequence. Using this strategy

the NR sequence was interrupted generating the two flanking regions, NR1 and NR2, necessary for homologous recombination. These two sequences were 338 and 340 nucleotides long respectively. The sequence of the gene encoding the polymerase was amplified from the genome of *E. coli* BL21 strain using the specific primers T7pol_BglII_for and T7pol_ClaI_rev, containing the mentioned BglII and ClaI restriction sites for cloning. A special care was taken in manipulating all the vectors containing the sequence encoding the T7 RNA polymerase. The conventional commercial kits used for plasmid isolation, purification and gel extraction were inefficient in purifying them, and the DNA yield was very low or even zero. We can hypothesise that the sequence strongly interacts with proteins of *E. coli* so that binding to the column of the commercial kits is inhibited. Moreover, the difficulties that we encountered in amplifying and sequencing the gene, probably due to the formation of secondary structures, support the hypothesis of a very complex sequence. For these reasons, phenol:chloroform:isoamyl alcohol standard extraction was always used in place of the commercial kits for plasmid isolation and, in general, to further purify vectors containing the bacteriophage sequence after reactions of restriction or modification.

A BamHI restriction site naturally occurring downstream the coding sequence of the polymerase was used to clone the chloramphenicol resistance cassette. The gene was extracted by BamHI digestion from the plasmid pUC4CAT available in the laboratory. This vector is a modified pUC4K plasmid in which the kanamycin resistance cassette was replaced with the chloramphenicol marker.

After each cloning step the introduced inserts were sequenced to verify the absence of undesired accidental mutations.

The obtained vector containing the flanking regions for recombination (NR1 and NR2), the T7 RNA polymerase gene and the chloramphenicol marker gene was called Transformation Vector NP (No Promoter). A map of the vector is found in Appendix 1. The last step to complete the Transformation Vector was the cloning of the inducible promoter at the 5' side of the polymerase gene using a BglII restriction site. We will illustrate the selection of three inducible promoters and the cloning procedure in chapter 3.

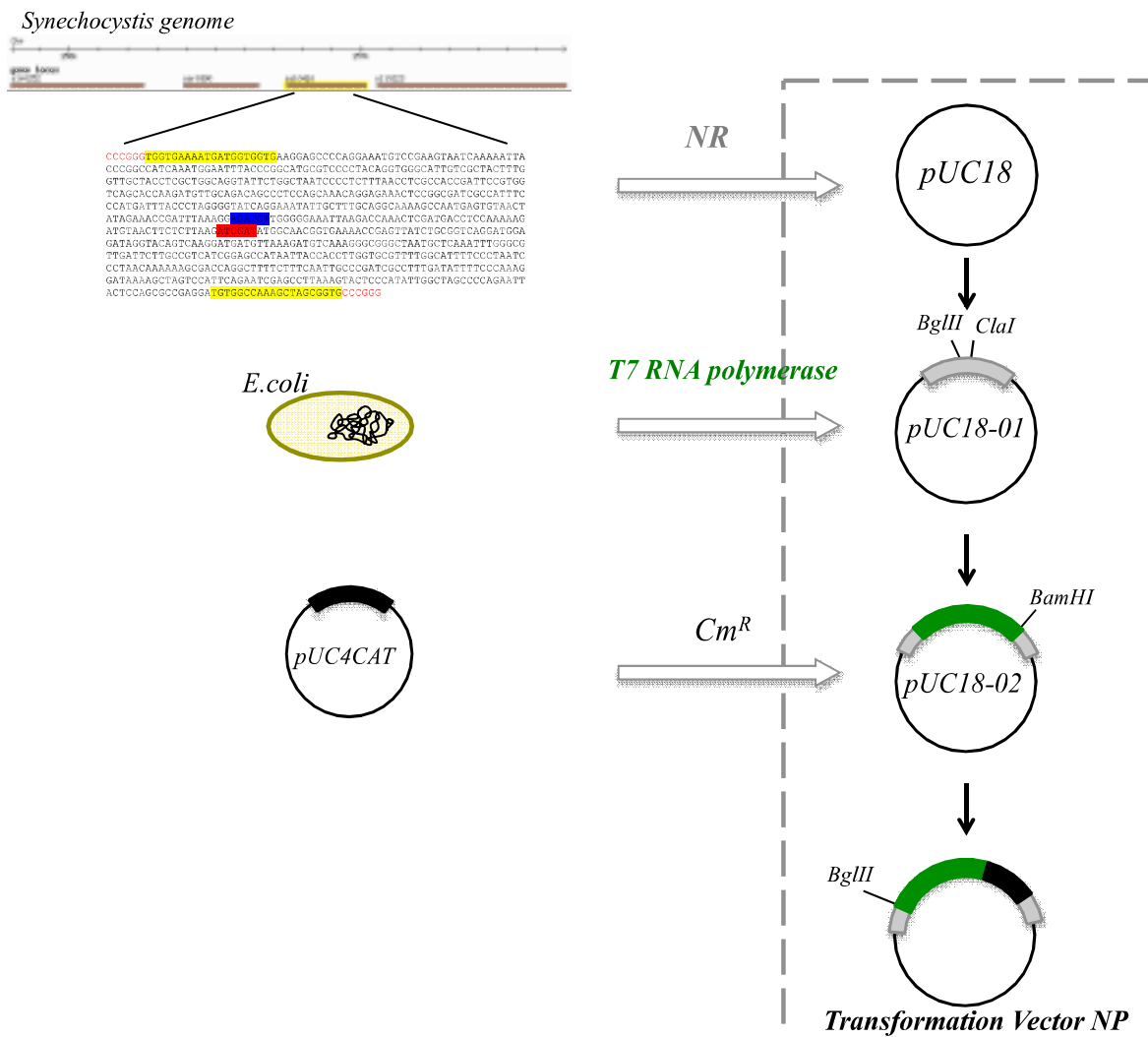


Figure 2.4: Schematic representation of the cloning strategy for the construction of the Transformation Vector. The restriction sites used for cloning are indicated. Inside the amplified sequence NR are highlighted respectively in blue and red the naturally contained restriction sites for *BglIII* and *ClaI*, the pairing of primers used to amplify the sequence (yellow) and the restriction sites for *SmaI* (red font) introduced with the same primers. In the final Transformation Vector NP (No Promoter) a *BglIII* site will be used to clone the inducible promoter. The sequences cloned are the neutral region (NR) (grey); the T7 RNA polymerase gene (green); the chloramphenicol resistance cassette (*Cm^R*) (black)

2.3 Construction of the *Synechocystis* Expression Vectors (pSEV)

The second component needed to complete the T7-cyano system are vectors, *Synechocystis* Expression Vectors (pSEV), for the insertion of a gene or a DNA fragment of interest under the control of the T7 promoter. The vectors were designed to integrate the foreign gene inside the chloramphenicol resistance cassette of T7-cyano by homologous recombination. This was considered a proper strategy to avoid the interruption of any other endogenous sequences of *Synechocystis*.

The obtained vectors contained a multiple cloning site (MCS) with properly chosen restriction sites. Upstream this site was inserted a portion of the chloramphenicol resistance gene for homologous recombination, the T7 promoter and the ribosome binding site (RBS) identical to that present in pET28 vector, a commercial expression plasmid normally used in *E. coli*. Downstream, it contains the T7 terminator for proper termination of transcription, the kanamycin resistance cassette as marker gene and the second portion of the chloramphenicol resistance gene.

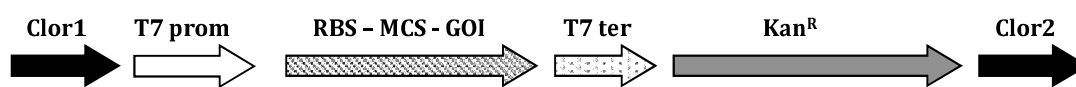


Figure 2.5: Schematic representation of the elements contained in the designed *Synechocystis* Expression Vectors: the recombination sites (Clor1 and Clor2); the T7 promoter (T7 prom), the ribosome binding site (RBS); a multiple cloning site (MCS) inside which a gene of interest (GOI) can be cloned; the T7 terminator (T7 ter); the kanamycin resistance cassette (Kan^R).

We have designed three different vectors that can be used for different applications. In addition to the sequences described above, each one contains some peculiar elements between the T7 promoter and the T7 terminator schematically represented in Figure 2.6.

In the first variant, called pSEV1, the multiple cloning site is flanked upstream and downstream by Strep-tags useful for the purification of the protein of interest by affinity chromatography. The sequence coding the first Strep-tag is followed by a sequence specifying the 4 aminoacids Ile-Glu-Gly-Arg. This small peptide sequence

is specifically recognized by protease Factor Xa and can be useful for the release of the expressed protein after purification. Moreover, since western blot is one of the most common and easy biochemical technique to detect proteins, we also verified in a previous experiment the utility of using an anti-Strep-tag antibody was checked with total cyanobacterial protein extract⁷⁶. This test showed that a common commercial antiserum does not cross-react with proteins of *Synechocystis*.

The untagged second vector, pSEV2, contains a MCS with few rare sites (BamHI, EcoRV, NotI). The vector was thought to express heterologous proteins or homologous wild-type ones, as a possible instrument for complementation studies by cloning any gene of interest after having obtained a classical knockout mutant.

In pSAS, the third vector, lacking the RBS sequence, the gene of interest can be cloned in the MCS in inverted orientation, so to permit the controlled transcription of its antisense (as) strand. As mentioned in the introduction, it has been shown that 64 % of the transcriptional start sites (TSS) code for regulatory RNAs, more than one half of them permitting synthesis of reverse complementary strands of identified ORFs¹⁴. Considering that only few cyanobacterial asRNAs have been studied up to date, the pSAS vector should be a useful tool for the study of these poorly characterized molecules. Moreover, another interesting application would be the controlled knockdown of endogenous proteins by suppression of translation.

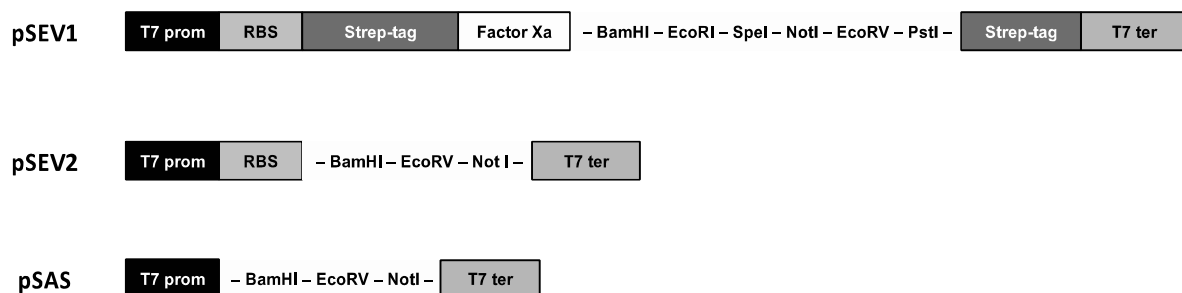


Figure 2.6: Schematic representation of the elements contained between the T7 promoter and the T7 terminator distinctive of the three *Synechocystis* Expression Vectors (pSEV1, pSEV2 and pSAS).

The strategy to develop the three expression vectors used common molecular biology techniques for cloning and will be briefly described.

Firstly, the elements common to the three expression vectors were cloned into the pUC19 plasmid. These elements are: the homologous recombination sites for the chloramphenicol gene (Clor1 and Clor2), the kanamycin resistance cassette (Kan^R) and the T7 terminator (T7 term).

The portions of the chloramphenicol resistance gene were amplified from the pUC4CAT vector previously mentioned using primers Clor1_for/rev for the first portion and Clor2_for/rev for the second portion. As described for the Transformation Vector, restriction sites at the extremities of the homologous regions for recombination, generating identical recombinant ends, can be useful to linearize the sequence before transformation of *Synechocystis*. For this reason, the naturally occurring PvuII site at the 5' side and ScaI site at the 3' side were included in the amplified sequence.

The kanamycin resistance gene, cloned in a second step, was extracted from a pUC4K vector together with its promoter using appropriate restriction sites, while the T7 terminator was cloned as a synthetic sequence.

Inside this first plasmid, pUC19+Clor1+T7ter+kan^R+Clor2, the elements distinctive of the expression vectors together with the T7 promoter were cloned and finally generated the three *Synechocystis* Expression Vectors described. The sequences have been all synthesized with appropriate restriction sites for cloning at their ends. In most of the cases, different restriction sites were chosen among those producing compatible ends, so to be abolished upon ligation of the insert into the vector. This strategy gave the possibility not only to use the same restriction sites more than once, simplifying the cloning procedures, but preserved also the integrity of the MCS in the final expression vectors. A scheme of the whole cloning strategy can be seen in Figure 2.7, while oligonucleotides used for amplifications and all the synthetic sequences are listed in the section "Materials and Methods".

After completing each cloning step the inserts introduced were sequenced to verify absence of undesired accidental errors. The maps of each vector can be seen in Appendix 1.

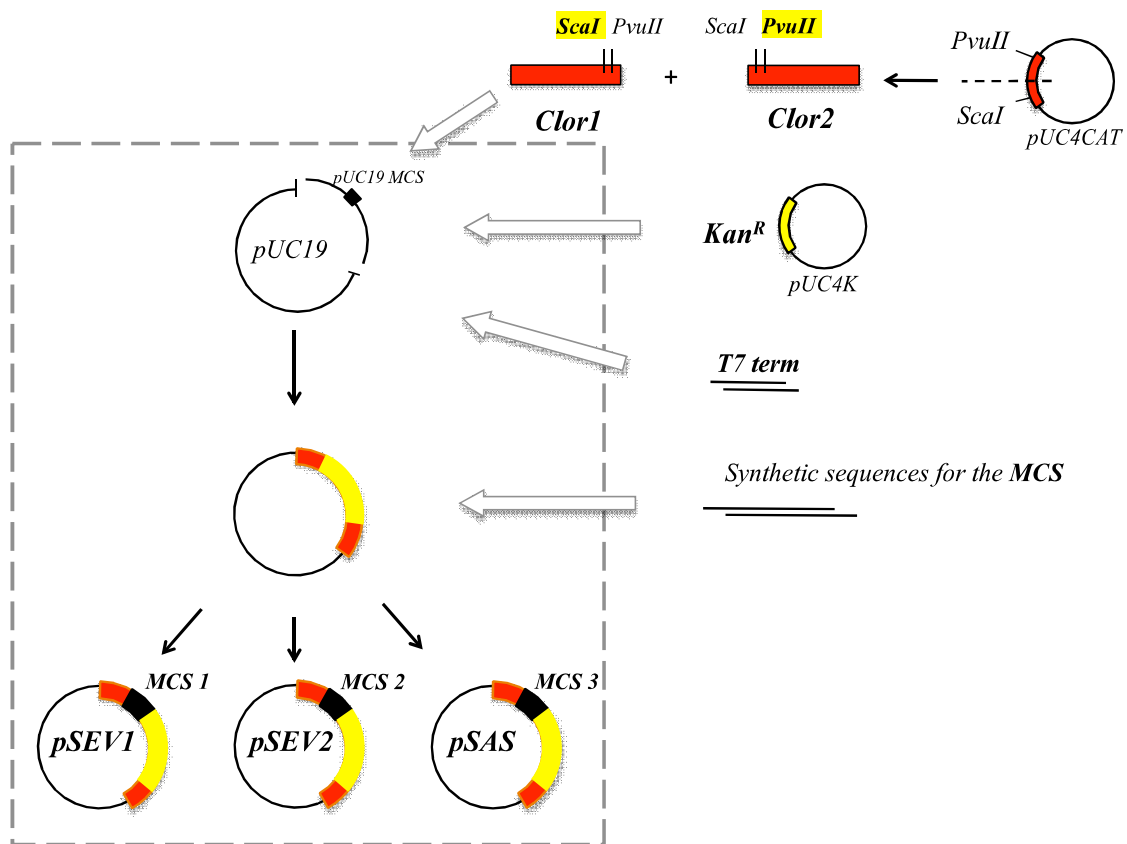


Figure 2.7: Schematic representation of the cloning strategy for the construction of the three *Synechocystis* Expression Vectors. The restriction sites, *ScaI* and *PvuII*, are naturally contained in *Cm^R* resistance while sites introduced during amplification are highlighted in yellow.

2.4 Construction of the Direct Expression Vectors (pDEV)

To better evaluate the transcription efficiency of T7-cyano system additional vectors called Direct Expression Vectors (pDEV) were developed. In these vectors the same inducible promoters used for controlling the expression of the T7 RNA polymerase (see chapter 2) were engineered so to directly control expression of the GOI. We developed the pDEV vectors using the same genetic elements contained in pSEV1 that are: the coding sequence for two Strep-tags, for the amino acid site recognized by Factor Xa, a MCS and the kanamycin resistance gene. A *NdeI* restriction site downstream the promoter region can be used to clone the foreign gene without the tag at the 5' end.

The vector was developed starting from the pUC18-01 plasmid previously constructed (containing the 3' untranslated sequence of the cyanobacterial *ndhB* gene, see figure 2.4), thus allowing introduction of the GOI to be expressed into the same neutral region (NR) chosen for the development of the T7-cyano strain. The two restriction sites *Bgl*II and *Cla*I, naturally contained inside that sequence were again used to clone the genetic elements described. These were amplified from pSEV1 using appropriate primers, listed in the section “Materials and Methods”, containing *Bgl*II and *Cla*I restriction sites for cloning.

Figure 2.8 shows a schematic representation of cloning steps leading to the construction of the pDEV_NP (No Promoter) vector; its map can be found in Appendix 1.

The inducible promoters were cloned afterwards upstream the MCS using a *Bgl*II site. The promoters and the cloning strategy are the same used for the production of the Transformation Vectors; these details are illustrated in chapter 3.

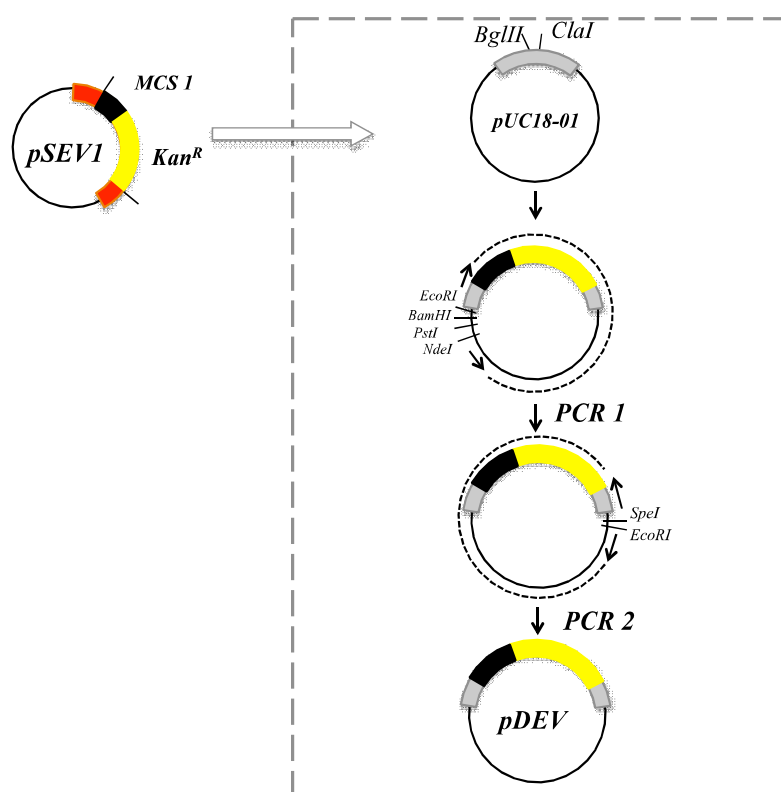


Figure 2.8: Schematic representation of the cloning strategy for the construction of pDEV. The MCS and the Kanamycin resistance cassette were amplified from the previously developed pSEV1. Two mutagenic PCR amplifications were performed to remove the restriction sites contained in the original MCS of pUC18 vector.

CHAPTER 3

DEVELOPMENT OF THE T7-CYANO STRAIN

3.1 Selection of the inducible promoters

One of the reasons that brought to the development of the T7-cyano system is the lack of reliable promoters suitable for the inducible expression of proteins. Secondly, transcription by the highly processive T7 RNA polymerase should amplify the level of messengers for the recombinant gene, thus assuring good yield of expressed protein. For this reason the choice of the most suitable promoter to control the bacteriophage gene was mainly focused on a tight regulation and possibly easy induction. Until today, the number of inducible promoters characterized in *Synechocystis* is quite low and includes some natural and few other heterologous ones.

Acclimation of microorganisms to different environmental conditions induces the expression of a number of different genes. Those responding to an increase of inorganic ion concentration can be considered interesting in a biotechnological perspective since their promoters are easily activated by the addition of a particular ion in the growth medium. Among these promoters, we chose two different ones induced by metals: the first one is induced by zinc and controls *ziaA* gene; the second one regulates the operon *nrsBACD* and is nickel inducible.

The *ziaA* gene (ORF slr0798) encodes an ATPase efflux pump involved in zinc homeostasis, as shown by the disruption of its sequence⁷⁷. In the same cluster, the *ziaR* gene (slI0792) is divergently transcribed with respect to *ziaA* and encodes a negative regulator protein homologous to the zinc responsive repressor SmtB of *Synechococcus* PCC 7942⁷⁸ (Figure 3.1, above). ZiaR functions by binding to the *ziaA* operator-promoter and represses the transcription of *ziaA* in the absence of the metal ion. In proximity to *ziaR* another ORF, slI0793, was identified (Figure 3.1, above): the hydrophobicity profile found in the predicted product of this ORF suggests that it could be a membrane bound protein and possibly contributing to Zn²⁺ transport. It was consequently suggested that these two genes, both involved in Zn²⁺ homeostasis, could be co-transcribed. The *ziaA* operator-promoter found in the intergenic region between *ziaA* and *ziaR* shows a degenerate 12-2-12 inverted repeat. The structure is similar to the proposed site for SmtB-DNA interaction within the *smtA* operator-promoter of *Synechococcus* and thus is proposed as a candidate for ZiaR-DNA interaction. We chose this region as first inducible promoter for the designed constructs.

The *nrsBACD* operon involved in Ni²⁺ resistance (Figure 3.1, middle) is found in the proximity of the *zia* genes, integrated into a *Synechocystis* metal resistance cluster⁷⁹. The function of each one of the four genes of the operon has not been fully elucidated, but it has been hypothesized that *nrsA* and *nrsB* products form a Ni²⁺ efflux system, while NrsD seems to be a putative member of the superfamily of permeases involved in Ni²⁺ efflux. The products of the divergently transcribed genes *nrsR* and *nrsS* seem

to form a two-component system controlling the nickel-dependent expression of *nrsBACD* operon. Lopez-Maury and coworkers⁸⁰ suggest that the carboxy-terminal part of NrsS is a histidine kinase that transfers a phosphate group to NrsR in the presence of Ni²⁺ to the medium. The phosphorylated NrsR probably binds as a dimer to the *nrsRS-nrsBACD* intergenic region activating the transcription of *nrsBACD* genes and positively autoregulating its own synthesis. The 118 bp *nrsRS-nrsBACD* intergenic region contains the promoter and the regulatory sequences controlling the response to Ni²⁺.

A study analysing the transcriptional activity of several metal inducible promoters shows that a very low basal expression level is found within the two described Zn²⁺ and Ni²⁺ inducible promoters⁴². Moreover, the ion concentration that can cause significant activation of these promoters, without concomitant unspecific stress responses, was defined in the same work. It is reported that by using the recommended maximal ion concentration (i.e. 4 µM of Zn²⁺ for *ziaA* promoter and 5 µM of Ni²⁺ for *nrsBACD* promoter) the level of the regulated gene is increased to 40-fold for the *ziaA* promoter, and to 400-fold for the one inducible by nickel.

Both promoters have been already used for directing expression of foreign proteins in *Synechocystis*. The zinc inducible one was previously used in our research group to express a Fe-Fe hydrogenase of the algae *Chlamydomonas reinhardtii*⁶³, while the nickel-inducible one was adopted for the development of a bioreporter strain responding to a variety of metal salts in polluted soils or wastewater samples⁸¹.

In addition to the two native ones, heterologous promoters were also described in *Synechocystis*. Huang et al. characterized a series of inducible promoters commonly used in *E. coli* finding that most of them lose their inducibility when they are used in the cyanobacterium⁴³. In *Synechocystis*, P_{trc} led to high expression levels in the absence of its inducer, while P_{lac}, P_{tet}, and P_R showed low expression levels of the regulated gene even with high inducer concentrations.

Recently, however, the P_{A1lacO-1} promoter (Figure 3.1, below), a variant of P_{lac}, was used in *Synechocystis* to express the ethylene-forming enzyme (EFE) from *Pseudomonas syringae* that catalyzes the synthesis of ethylene⁸². In contrast to other promoters tested in this same work, P_{A1lacO-1} showed a stronger expression with contemporary fine-tuned regulation as was suggested by measuring ethanol accumulation in the medium. This P_{lac} variant, like its natural counterpart, is induced by IPTG, while the constitutively expressed LacI^q repressor blocks the binding of the polymerase to the promoter in the absence of the inducer. It was found, however, that the degree of control becomes progressively more relaxed when the culture density increases, probably due to the binding of endogenous sugars to the repressor.

Since the latter problem can be resolved by monitoring the culture density, the

described P_{lac} variant was found interesting for our project and we decided to consider it, and its $LacI_q$ repressor, as third choice to control the transcription of the T7-polymerase gene.

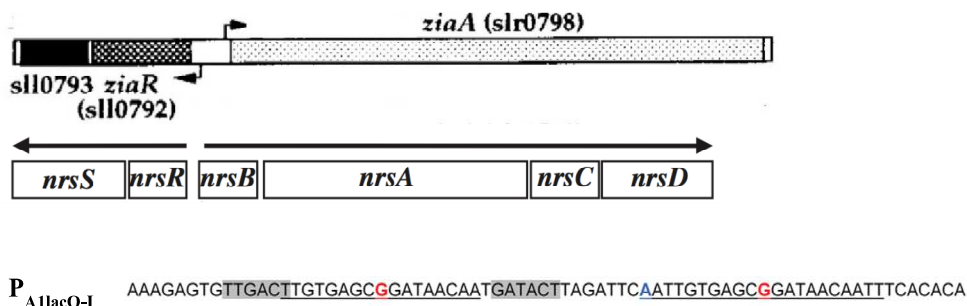


Figure 3.1: Physical map of the *zia* genes (above) and of the *nrs* operon (middle). Arrows indicate direction of transcription. The sequence of $P_{A1lacO-I}$ promoter reported (below) contains the -35 and -10 regions (grey), the *lac* operators (underlined) and its predicted center (red), and the transcriptional start site (blue).

3.2 Generation of the T7-cyano strains

The three selected promoters, P_{zia} , P_{nrs} and $P_{A1lacO-1}$ (from now on P_{lac}), were cloned in the Transformation Vector NP upstream the polymerase gene using the previously mentioned BglII restriction site. The two endogenous promoters, P_{zia} and P_{nrs} , were amplified from the *Synechocystis* genome by PCR using primers T7_Zia_BamH1_for and T7_Zia_BglII_rev for amplification of P_{zia} and primers *nrs_for* and *nrs_rev* for P_{nrs} BACD, all containing appropriate restriction sites for cloning. The modified variant of the P_{lac} *E. coli* promoter was obtained by annealing the synthetic oligonucleotides *Plac_for* and *Plac_rev*, designed to expose the appropriate sticky ends for cloning. The cloning strategy used was the same for all the promoters: the Transformation Vector was cut by the enzyme BglII, while the BamHI and BglII restriction sites contained in the promoter sequences were used for cloning.

Concerning the repressor proteins, the endogenous regulatory system of *Synechocystis* should already provide an appropriate control of the activity for P_{zia} and P_{nrs} . On the contrary for the modified variant of P_{lac} promoter the addition of the $LacI_q$ repressor gene was necessary⁸². The sequence for the repressor was amplified

together with its own promoter from the *E. coli* genome using the oligonucleotides LacI_for and LacI_rev and cloned upstream P_{lac} in the opposite orientation.

The three Transformation Vectors obtained were called as follows, depending on the different promoters they are carrying: Transformation Vector P_{zia}, Transformation Vector P_{nrs}, and Transformation Vector P_{lac}. A map of the each vector is reported in Appendix 1.

To finally generate the T7-cyano strains with the three different promoters chosen a wild-type strain of *Synechocystis* was transformed with one of each Transformation Vector. The vectors were linearized by SmaI digestion, so excising the linear, full sequence to be introduced and improving the recombination efficiency. Recombinant colonies plated on 5-10 µg/ml of chloramphenicol started to be visible 10-15 days after transformation and single colonies were progressively transferred to higher antibiotic concentrations, until the final 50 µg/ml concentration was reached.

Correct insertion of the gene was then checked by PCR using primers that pairs outside the recombination sequence, in the neutral region downstream *ndhB* gene, or combined with primers pairing inside the inserted sequence. As examples, the PCR obtained by using external primers on the template DNA from the T7-cyanoP_{zia} strain and the PCR for the T7-cyanoP_{lac} strain, obtained by combining external primers with the internal ones, are shown in Figure 3.2.

Although the PCR using external primers suggests absence of wild-type copies of the genome a second PCR was performed to give more evidence of the homoplasmy of the strains. This second PCR was set up using external primers and the same conditions used in the first one except for the extension time that was reduced to promote amplification only of potential wild-type copies. Total absence of bands having the size attended for the wild-type chromosome, even in the second PCR, confirmed complete segregation of the strains.

In accordance to the different promoters controlling the T7 RNA polymerase gene the three recombinant cyanobacterial strains were called: T7-cyanoP_{zia}, T7-cyanoP_{nrs}, T7-cyanoP_{lac}.

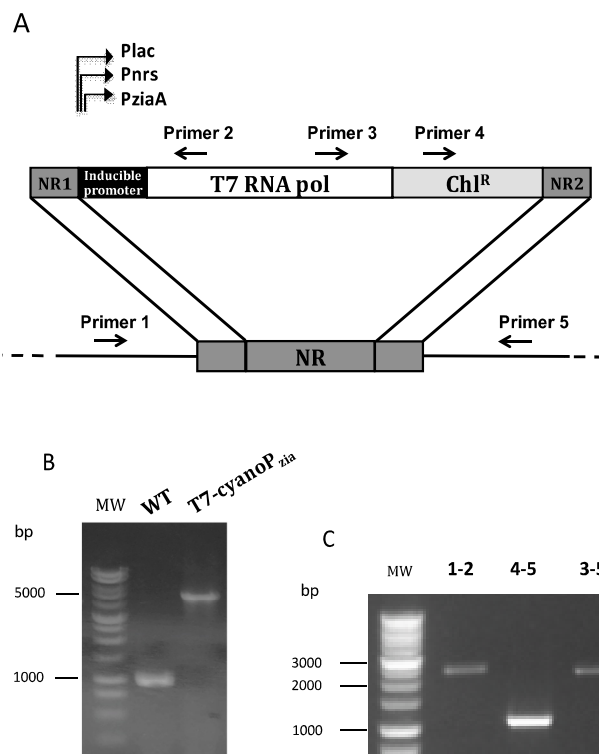


Figure 3.2: (A) Schematic diagram of the construction of T7-cyano strains. Positions of PCR primers used to verify correct insertion of the cassette and flanking regions by site-specific recombination are indicated. (B) Agarose gel electrophoresis of analytical PCR amplification performed on genomic DNA of T7-cyanoP_{zia} strain using external primers 1 and 5. Absence of a band corresponding to the size attended for the wild-type chromosome indicates complete segregation of the strain. (C) PCR performed on T7-cyanoP_{lac} using a combination of internal and external primers to verify correct insertion of the sequences. The combination of used primers is indicated above each lane.

3.3 Preliminary induction/expression experiments

In order to define the capability of *Synechocystis* to express the T7 RNA polymerase preliminary experiments of expression upon induction were performed. Cells were cultivated in liquid to a final OD₇₃₀ = 0.5 and the relative inducers, according to the different strain used, were directly added to the medium. For the T7-cyanoP_{zia} a concentration of 4 μM of Zn²⁺ was chosen, while for T7-cyanoP_{nrs} the concentration of Ni²⁺ was equal to 5 μM. These are considered the maximum amount of inducer not toxic or stressful for the organism as was shown by transcript analysis⁴². In the case of T7-cyanoP_{lac}, on the contrary, the best concentration of IPTG has never been tested in *Synechocystis*. We might consider, however, that the range of concentration between 100 μM and 1.5 mM used in *E. coli* can be suitable for *Synechocystis* too and we will use 1 mM a concentration previously used for this same promoter in *Synechocystis*⁸². Cells were cultivated for up to 48 h in the presence of the inducer and a specific antibody against the T7 RNA polymerase was used to detect the protein on a total cell extract.

As shown in Figure 3.4, synthesis of the polymerase was observed by western blotting in T7-cyanoP_{nrs} 30 hours after induction, confirming expression of the bacteriophage gene in *Synechocystis*. The protein was detected at the expected

molecular weight of 99 kDa by the specific antibody. By western blot the polymerase was not detected in the samples collected from the T7-cyanoP_{zia} strain, however its expression was confirmed also for this strain by dot blot 24 hours after induction. However, in both cases production of the protein was observed by dot blot also in non-induced cells: the basal level of expression indicated that the promoters were not completely switched off before induction.

For T7-cyanoP_{lac} induction experiments are still in progress to verify expression and inducibility of the promoter.

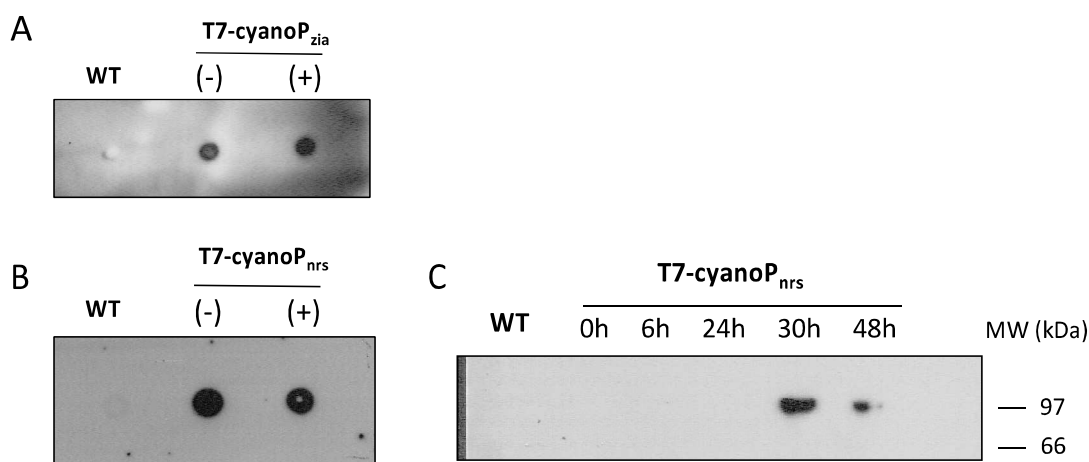


Figure 3.3: Dot blot performed on total extract of T7-cyanoP_{zia} (A) and T7-cyanoP_{nrs} (B) cells, using a specific antibody against the T7 RNA polymerase. Expression of the bacteriophage gene 24 hours after induction, by Zn²⁺ and Ni²⁺ respectively, was shown. Signals were detected both in non-induced (-) and induced (+) cells. (C) Western blot analysis of total extracts of induced T7-cyanoP_{nrs} cells, showing accumulation of the polymerase 30 hours after induction. The protein was detected at the expected molecular weight of 99 kDa.

3.5 Concluding remarks

Three different *Synechocystis* strains, T7-cyanoP_{zia}, T7-cyanoP_{nrs} and T7-cyanoP_{lac} carrying the T7 RNA polymerase under the control of three different promoters were produced. The strains contain the bacteriophage gene homoplasmically inserted into the *Synechocystis* genome.

Initial induction experiments on these strains confirmed expression of the polymerase in *Synechocystis*. The activity of these promoters was found to be low, as reported in the literature⁴². For T7-cyanoP_{zia} detection of the polymerase only by dot

blot suggests lower expression level compared to the nickel-inducible promoter. In any case, other expression experiments should be done, for example by testing the best concentration of inducer or the level of cell growth preferable for induction. However, since our aim was the expression of the downstream foreign gene, we chose to consider the tightness of the induction step after having verified the expression of test genes upon transcription by the exogenous T7 RNA polymerase. To our knowledge, indeed, the activity of the bacteriophage enzyme in *Synechocystis* had never been shown before.

It is known that promoters, including the inducible ones, show a basal expression activity that can be of different extent for different regulatory sequences. The leakiness observed for the chosen promoters (P_{zia} and P_{nrs} , in particular) will be examined and discussed in Chapter 4.

CHAPTER 4

PROTEINS EXPRESSION

4.1 Selection of protein coding genes to validate the system

In order to verify and define the expression capability of T7-cyano system, some selected genes were cloned in one of the expression vectors developed.

In the literature a limited number of proteins are described to function as reporter in *Synechocystis*. In many cases heterologous gene expression in cyanobacteria was targeted to create novel biosynthetic pathways to produce valuable chemicals such as ethanol, butanol or isoprene^{47,82,83}. For the majority of them the success of expression was ascertained by an indirect measurement of the produced target chemical, but expression level of the synthesized proteins was not reported.

In other papers studying promoters and regulatory sequences, fluorescent proteins have been described to function as reporter^{43,84,85}. In these studies, the spectra of GFP, some of its mutated versions and EYFP have been measured and compared to the spectra of wild-type cells, reporting that the endogenous pigments of the cells don't influence the measurement of the light emitted by the fluorescent protein. According to this evidence we decided to use as first protein a mutated version of the wild-type GFP, eGFP, available in our laboratory. The major difference with respect to the native GFP is a single point mutation (S65T) that results in increased fluorescence and a shift of the major excitation peak from 475 nm to 488 nm, while the emission peak is kept at 509 nm. A second point mutation (F64L) enhances the folding efficiency at 37 °C. The protein was often used in our laboratory as a fusion product for localization studies in plants^{86,87}.

A second protein selected for validation of the T7-cyano system is HydA, a Fe/Fe hydrogenase from *Chlamydomonas reinhardtii*. Hydrogenases, key enzymes involved in hydrogen metabolism of microorganisms, are biotechnologically interesting as catalysts for hydrogen production, an attractive alternative to fossil fuels⁸⁸. In our research group the algal enzyme had previously been expressed in *Synechocystis* in active form under the direct control of the *ziaA* promoter⁶³, i.e. the same one used in the T7-cyanoP_{zia} strain. Even though this protein has a complex structure that needs several accessory proteins to be correctly folded, we considered the enzyme a good candidate to compare its expression directly driven by the promoter with that controlled by the T7 RNA polymerase.

The third chosen protein is a Baeyer-Villiger monooxygenase from the algae *Cyanidioschyzon merolae*, a flavin-containing enzyme able to catalyze a remarkable wide variety of oxidative reactions. This soluble protein was previously expressed in *E. coli* and characterized for its use in biocatalytic reactions after purification by IMAC chromatography⁸⁹. It was also reported that the presence of the flavin cofactor is

required not only for the activity of the enzyme but also for the correct folding of BVMO, as evidenced by increase of its solubility upon addition of riboflavin, the precursor of FAD, in the growth medium. The choice of this enzyme, currently studied in our laboratory, was also favoured by the availability of a good specific antibody previously produced in house (against the recombinant protein expressed in *E. coli*), that would facilitate its detection on western blot.

The results obtained by expressing in T7-cyano the described selected proteins, after cloning their genes in one of the developed expression vectors, will be reported in the following sections.

4.2 Cloning and transformation of the T7-cyano strains by the reporter genes for expression

The sequence for eGFP was taken from the available plasmid pGREAT-eGFP (provided by Dr. Szabò from our Department), in which the sequence of the fluorescent protein was previously cloned downstream the multiple cloning site of a pGreen vector. The sequence, extracted by digestion with BamHI/NotI, was cloned by using the same restriction enzymes in pSEV2, to express the protein without any tag. The obtained plasmid was called pSEV2_eGFP. The gene was also cloned in a mutated pSEV2 vector containing the zia operator sequence between the T7 promoter and the RBS. The latter vector was used only in the T7-cyanoP_{zia} strain, in which the binding of the repressor ZiaR to this site should provide a more tight control of the transcription mediated by the T7 RNA polymerase in the absence of the inducer. The same zia operator sequence was added also to pSEV1 and these engineered vectors were respectively called pSEV1zia and pSEV2zia (the maps are presented in Appendix 1). The plasmid containing the eGFP gene was called pSEV2zia_eGFP.

The sequence for the hydrogenase enzyme HydA was taken from the available recombinant vector TA-TOPO_HydA⁶³ (provided by Dr. P. Costantini, from our Department) in which the ORF coding for the mature HydA1 isoform was cloned⁶³. Primers HydA_BamHI_for and HydA_NotI_rev were used to amplify the sequence that was afterwards cloned between the BamHI and NotI restriction sites in pSEV2zia. The vector containing the hydrogenase gene was called pSEV2zia_HydA.

The available plasmid pET28-CmBVMO containing the ORF of the BVMO from *C. merolae* was used as template to amplify the sequence of the enzyme. This sequence was cloned inside pSEV1, since all sites contained in the MCS of pSEV2 were also

present in the sequence of the BVMO coding gene. The obtained plasmid was called pSEV1_BVMO. The sequence was cloned only in this vector, and not in pSEVzia, since, doing the experiments, the zia operator sequence was not found to affect large transcriptional control. The primers used, BVMO_Nde_for and BVMO_EcoRI_rev were designed to amplify the sequence together with the his-tag coding region (already present at its 3' side) and the restriction sites useful for cloning. The tag was included so to permit the eventual purification of the expressed protein.

Once sequenced, the obtained plasmids pSEV2zia_eGFP, pSEV2zia_HydA and pSEV1_BVMO were used to transform the T7-cyanoP_{zia} strain, while plasmids pSEV2_eGFP and pSEV1_BVMO were used for the T7-cyanoP_{nrs} strain.

Recombinant colonies started to easily grow on 10 µg/mL of kanamycin 10-12 days after transformation of the strains by the vectors. Single colonies were picked up and transferred to Petri dishes containing progressively higher antibiotic concentration, 20 µg/mL and 50 µg/mL, to allow selection of homoplasmic clones. The whole procedure, from transformation to the last restreaking step, took an average of three weeks. Correct insertion of each construct in the genome was verified by PCR using internal and external primers in respect to the recombination site. As an example, the result of the PCR performed by using internal primers on the T7-cyanoP_{zia} strain transformed by the eGFP-containing vector is shown (Figure 4.1). Absence, in the transformed strains of a band of the size expected for the original T7-cyano chromosome is an indication that the homoplasmicity was successfully obtained.

The final recombinant strains were called T7-cyanoP_{zia}_eGFP, T7-cyanoP_{zia}_HydA, T7-cyanoP_{zia}_BVMO, T7-cyanoP_{nrs}_eGFP and T7-cyanoP_{nrs}_BVMO in dependence of the strain and plasmid used for transformation.

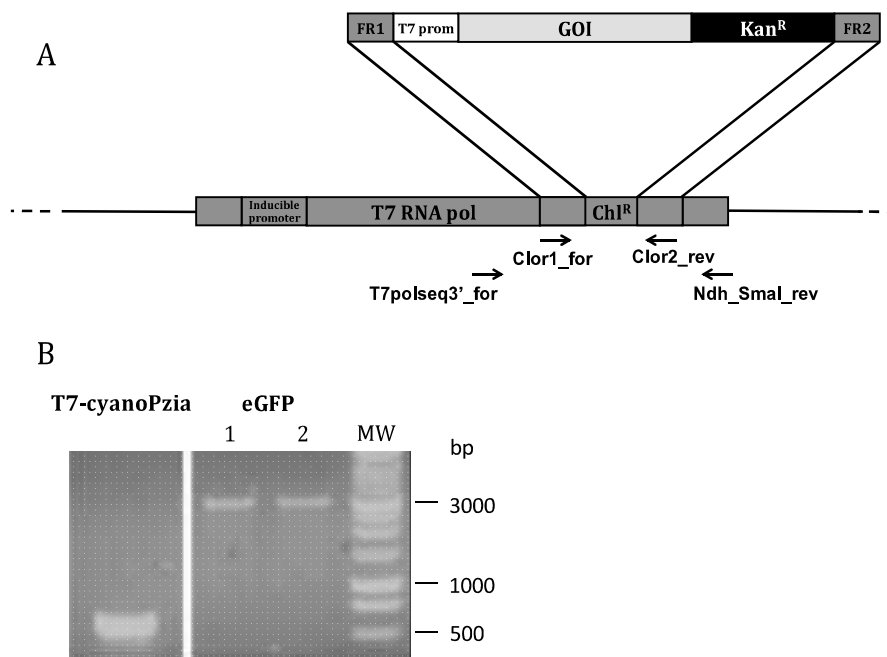


Figure 4.1: (A) Schematic diagram of the construction of T7-cyano strains for heterologous protein expression. Positions of PCR primers used to verify correct insertion of the cassette and flanking regions through recombination are indicated. (B) Agarose gel electrophoresis of analytical PCR amplification performed on genomic DNA of T7-cyanoP_{zia} strain using internal primers Clor1_for and Clor2_rev on two recombinant colonies (1 and 2) carrying eGFP sequence. Absence of a band corresponding to the size expected for the original T7-cyano strain indicates complete segregation of the new recombinant strain.

4.3 Preliminary expression experiments in T7-cyanoP_{zia}

As first induction experiment, we decided to use the same conditions and concentration of inducer, 4 μM of Zn^{2+} , previously used to check expression of the T7 RNA polymerase in the T7-cyanoP_{zia} strain. Cells were cultivated in liquid to a final $\text{OD}_{730} = 0.5$ and the inducer was directly added to the medium. For each recombinant strain, T7-cyanoP_{zia}_eGFP, T7-cyanoP_{zia}_HydA and T7-cyanoP_{zia}_BVMO, carrying one of the selected reporter genes encoding eGFP, HydA or BVMO, the samples of cultured cells were collected every 24 h for a maximum of 6 days.

Expression of the recombinant proteins was checked in total cell extracts by SDS-PAGE and western blot using specific antibodies against the selected proteins. As shown in Figure 4.2, showing the western blot analysis of samples recovered from the T7-cyanoP_{zia}_BVMO strain, the BVMO protein (with a calculated MW of 60 kDa) is expressed at 24 and 48 hours after induction. A basal expression level is observed in

non-induced cells, indicating that the system is leaky. This is not surprising since a basal expression of the polymerase had previously been described in T7-cyanoP_{zia} strain (see chapter 3, Figure 3.3A). Absence of detection before induction suggests that the protein does not accumulate in sufficient amount in the starting culture (grown to a cell density corresponding to OD₇₃₀ = 0.5), however, as noticed by SDS-page gel stained with Coomassie Brilliant Blue, proper quantification of the samples is needed for adequate comparison. Moreover, by observing the Coomassie stained gel, we can conclude that synthesis of the protein is achieved, not over-expression.

In each sample, a band is detected below that corresponding to the expressed BVMO, probably due to a non-specific recognition of a protein of the cyanobacterium by the polyclonal primary antibody, previously produced in our laboratory against the protein. By a tBlastn search in the genome of *Synechocystis* for proteins having homologies to the protein from *Cyanidioschyzon merolae*, we propose that the band could belong to the product of the *pntA* gene (slr 1239), encoding the alpha subunit of a pyridine nucleotide transhydrogenase of *Synechocystis*. This protein indeed contains a potential epitope of 8 aminoacids also present in the primary sequence of the BVMO and has a predicted molecular weight of 53 kDa, compatible with the position of the band detected in the blot.

Expression of the hydrogenase from *Chlamydomonas* and the eGFP in the T7-cyanoP_{zia}_HydA and T7-cyanoP_{zia}_eGFP strains respectively, was not confirmed by western blot in the same experimental conditions for induction. For the fluorescent protein, a good antibody working in *Synechocystis* has still to be found since those ones used, a monoclonal and a polyclonal one, didn't allow its detection probably because of the low amount of expressed protein. Even though the fluorescence of this kind of protein was reported in cyanobacteria, in our strain the measurement at the fluorescence microscope was not successful. The huge amount of pigments contained in the cells makes difficult the detection of a probable low amount of expressed protein by fluorescence.

In regard to HydA, the specific polyclonal antibody against it had been previously successfully used in *Synechocystis*; in our experiment the hydrogenase was not detected by western blot. Again we can hypothesize that the protein was expressed in a too low amount in the T7-cyano system. Transcription of the HydA gene 24 h after induction was in any case confirmed by reverse transcriptase-PCR (RT-PCR). Total RNA was extracted from induced cells and complementary DNA (cDNA) was produced by reverse transcription using random primers: a small fragment of the sequence was amplified by PCR using the internal primers HydA1_for and HydA1_rev. Figure 4.3 shows that also in this case transcription of the foreign gene was detected in non-induced cells, confirming the leakiness of the promoter system.

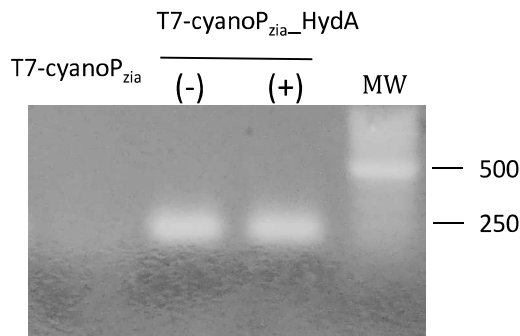


Figure 4.3: RT-PCR on T7-cyanoP_{zia} and on non-induced (-) and induced (+) cells of T7-cyanoP_{zia}HydA strain showing transcription of HydA.

4.4 Preliminary expression experiments in T7-cyanoP_{nrs}

The same inducing conditions, 5 μ M Ni²⁺, used previously to verify the expression of the polymerase from the T7-cyanoP_{nrs} strain, were applied to switch on expression of eGFP and BVMO respectively in the nickel-inducible strains T7-cyanoP_{nrs}_eGFP and T7-cyanoP_{nrs}_BVMO.

Expression of BVMO was not detected after 24 h and 48 h by western blot suggesting a lower or even absent expression of the gene.

Also in the case of eGFP, expression could not be confirmed by western blot. Transcription of the heterologous gene was verified by semi-quantitative RT-PCR. Random primers were used to synthesise the cDNA from a total RNA extracted from non-induced and induced cells, and amplification of a small fragment of eGFP was obtained by using internal primers eGFP_for and eGFP_rev. The result, shown in Figure 4.4, points out a significant increase in the transcript content in induced compared to non-induced cells. A low transcription of the fluorescent protein in non-induced cells is not surprising since a basal expression of the polymerase was previously described within the T7-cyanoP_{nrs} strain (see chapter 3, Figure 3.3B).

A possible explanation of this probable low amount of protein expression in both strains can be due to the RBS contained in the expression vectors and therefore to an inefficient translation of proteins.

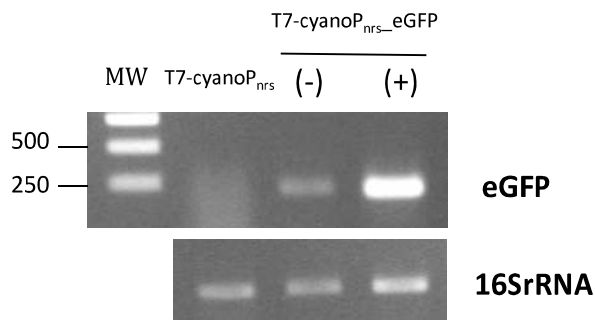


Figure 4.4: RT-PCR on T7-cyanoP_{nrs} cells and on non-induced (-) and induced (+) cells of T7-cyanoP_{nrs}-eGFP strain showing enhanced transcription of eGFP upon induction.

4.5 Substitution of the RBS

The expression vectors pSEV1 and pSEV2 were originally produced with a ribosome binding site identical to that present in commercial expression plasmids for *E. coli*. In prokaryotes, in general, the translation is initiated by the binding of a ribosome to the mRNA at the RBS, which contains a core Shine-Dalgarno (SD) sequence, however the effectiveness of the RBS sequence depends also on the surrounding nucleotides which may affect base-pairing potential of the anti-SD sequence (3'- terminal sequence of the 16SrRNA) or form secondary structures between the SD sequence and the translational start codon. When the project for this thesis began, studies about the genetics requirements for efficient translation in cyanobacteria were quite few, while some ones appeared in these last years. Since a reliable RBS has not yet been found in cyanobacteria, we planned to test four different sequences, two endogenous and other two synthetic. The RBSs of the genes *rbcl* (slr0009) and *psbA2* (slr1311) were chosen in consideration of the very high expression in *Synechocystis* of the encoded proteins, respectively the large subunit of the ribulose biphosphate carboxylase (RuBisCo) and the D1 protein of photosystem II.

A synthetic RBS already tested in *Synechocystis* and indicated as very efficient³⁶ was selected as third sequence.

The fourth one was designed using a bioinformatic tool developed by the Penn State University⁹⁰, that permits identification of the (most probable) best consensus for ribosome binding by analysis of the whole bacterial genome.

The sequences of these RBSs, containing BglII and NdeI restriction sites for cloning at their extremities (underlined), are reported below and substituted the RBS previously present in the pSEV expression plasmid. We decided to test their efficiency in promoting translation by cloning them into the vector pSEV1_BVMO, that is at present the only one for which protein expression could be verified by western blot.

(evaluated by density of the cell culture), to identify the ones that maximise expression of the foreign gene.

In addition we point out that the T7-cyanoP_{lac} strain, although already obtained in the laboratory, was not further transformed by the pSEV vectors. Consequently, the efficiency of the third promoter in controlling transcription or enhancing expression of the foreign gene remains to be investigated. This part of the work is still in progress.

CHAPTER 5

SYNTHESIS OF ANTISENSE RNAs

5.1 Selection and design of antisense RNAs for cloning in pSAS vector

In *Synechocystis* a genome-wide map of transcriptional start sites (TSS) predicted the existence of a huge number of non-coding RNAs estimated to cover the 65 % of the total sites identified. Half of these sites were found on the reverse complementary strand of ORFs, suggesting a high degree of antisense transcription¹⁴. However, the function of specific antisense transcripts in *Synechocystis* has been scarcely defined and to date proper characterization has been reported just for few of them^{22,23,24}.

The pSAS vector, as already mentioned, could become a useful instrument not only for the study of these non-coding RNA molecules, but also as a tool to switch on and off endogenous genes in an effort to characterize them. Testing of the T7-cyano system for the synthesis of antisense RNA molecules was performed at the Department of Biochemistry of the University of Turku (Finland), in collaboration with Prof. Eva-Mari Aro, one of the main expert in the study of photosynthetic organisms, cyanobacteria included. For this purpose, different antisense molecules, natural or artificial, have been cloned in the expressly designed vector.

The antisense of *isiA*, *IsrR*, was used as first asRNA to validate the T7-cyano system. The *IsiA* protein, expressed under iron-limiting conditions, forms a giant additional antenna ring around PSI enhancing light absorption and thus helping to compensate the reduction of PSI trimeric complexes that occurs under iron limitation⁹¹. The gene is activated also under high light, oxidative stress and salt-stress conditions, but the physiological role of the protein in these cases is more ambiguous⁹². The *isiA* gene is co-transcribed together with the downstream *isiB*, encoding a flavodoxin believed to act as an alternative electron carrier under iron-limiting conditions, substituting almost all the functions of the iron-containing ferredoxin⁹³.

The promoter of *isiAB* operon was mapped by primer extension analysis that assigned the start of transcription to the adenine located 211 nt upstream the GTG start codon. Comparison with known promoters predicted the possible -10 and -35 regions, while a putative Fur binding sequence was found 40 nt upstream the start codon⁹³. Fur proteins are found in diverse prokaryotes and acts as a repressor by binding to specific Fur box elements under iron-replete conditions. In conditions of iron deficiency, removal of the repressor Fur permits transcription of the operon; however the accumulation of the transcript has been shown to be controlled also by a second factor, that is the specific antisense RNA *IsrR*.

The constitutively expressed *IsrR* negatively regulates expression of *IsiA* by targeting the *IsrR/isiA* mRNA duplexes to degradation. Once the transcription of *isiA* is

activated, its concentration starts to increase but its accumulation (and accumulation of the translated protein) becomes significant only when its amount exceeds that of IsrR. It has been shown that artificial over-expression of the antisense under iron depleted or oxidative stressed conditions causes a diminished *IsiA* transcript level and consequently a reduced protein accumulation²². The antisense-mediated knockdown of *isiA* is also detectable in the absorption spectra of whole cells, indicated by a shift of the red chlorophyll absorption peak toward the blue part of the spectrum^{22,94}. This easy method to monitor *IsiA* expression together with a consistent knowledge on *isiAB* regulation was found convenient to test the T7-cyano system in the synthesis of asRNAs for the knocking down of endogenous genes. The cis-encoded antisense was therefore included in this study and was called T7IsrR (being synthesized by the T7 RNA polymerase), to distinguish it from the endogenous one.

A second antisense was also designed against the 5' UTR of *isiA* in order to try the knockdown of the same gene in an alternative way. This idea came from the knowledge that frequently in prokaryotes the direct interaction of antisense molecules to the SD sequence and/or to the upstream part of the ORF prevents the binding of the 30S ribosomal subunit, thus leading to inhibition of translation. According to the information derived from a 5' RACE analysis^{14,55}, the sequence corresponding to the 5' UTR of *isiA* gene was reported to cover the 231 nucleotides upstream the start codon. As a preliminary approach, therefore, the antisense was designed to pair against the entire 5' UTR of *isiA* gene and was named as5UTR_IsiA.

The second target chosen to test the T7-cyano system was SynK (slr0498), a thylakoid potassium channel that contributes to ion homeostasis and affects the efficiency of the photosynthetic and respiratory electron transfer. In order to study its physiological role, a SynK-less mutant had previously been generated and characterized in our research group⁶⁰. Fluorimetric experiments indicated that the mutant cannot build up a proton gradient as efficiently as the wild type and it was suggested that the channel might be involved in the regulation of the electric component of the proton motive force. As a consequence, the mutant exhibits enhanced photosensitivity; indeed, at high light intensities, a modified ratio between the photosystem II/photosystem I and a decreased growth of the strain were reported. An antisense was designed complementary to the 5' UTR of the SynK gene, in order to knockdown the gene by repressing the binding of the ribosome to the RBS. Inhibition of translation would lead to a reduction in SynK protein expression, thus producing a phenotype comparable to that described for the SynK-less mutant. Based on the information on the transcriptional start sites of *Synechocystis* provided by Mitsche and co-workers, the 5' UTR of SynK gene was reported to be located 76

nucleotides upstream the coding sequence¹⁴. The antisense was therefore designed against this sequence, designated as the 5' UTR of SynK gene, and was named as5UTR_SynK.

In the article by Mitsche et al. (2011)¹⁴, it was also reported the existence of a 103 nt long antisense pairing the coding sequence of SynK gene. The physiological role of this antisense has never been investigated, nor it is known if this cis-encoded antisense is a real player in the regulation of the expression of the SynK channel. Although in prokaryotes several diverse mechanisms have been described for asRNAs^{16,55}, the majority of the cis-encoded asRNAs negatively regulate their target. The induction of this endogenous antisense, named asSynK, in T7-cyano, might therefore decrease the transcriptional level of SynK gene and consequently produce a phenotype comparable to the SynK-less mutant. The asSynK was therefore included in this study.

The light-dependent NADPH:protochlorophyllide oxidoreductase (LPOR) catalyses the penultimate step in chlorophyll biosynthesis. It was recently reported that a LPOR-KO mutant accumulates only 20 % chlorophyll with respect to the wild type, while its carotenoid content dramatically increases⁹⁵. Moreover, when compared to the wild type, the KO mutant grows very slowly at low light conditions (30 μ E), while proliferation stops completely at high light (100 μ E). This clear phenotype was thought to facilitate observation and screening in validation of knocking down experiments with pSAS. Any information about the presence of endogenous antisense RNAs or about the length of the 5' UTR was found for LPOR gene (slr 0506)¹⁴. An artificial antisense, named asLPOR, was therefore designed against the full coding sequence of the gene; again, the knockdown of the gene by the expression of this antisense should consequently produce a clear phenotype comparable to the KO mutant.

Some features of the selected asRNAs are briefly reported in Table 5.1, while the sequences and pairing to their targets are reported in Appendix 2.

Name	Length (nt)	Target	Type
T7lsrR	175	isiA (slr 0247)	endogenous
as5UTRisiA	231	isiA (slr 0247)	artificial
asSynK	103	SynK gene (slr0498)	annotated
as5UTRSynK	88	SynK gene (slr0498)	artificial
asLPOR	969	por gene (slr 0506)	artificial

Table 5.1: Characteristics of the antisense RNAs chosen in this study

5.2 Cloning and transformation of the T7-cyanoP_{zia} strain by the selected antisense RNAs

The sequences for the five asRNAs selected, i.e. T7IsrR, as5UTR_IsiA, asSynK, as5UTR_SynK, asLPOR, were cloned in pSAS between the restriction sites BamHI and NotI. Each sequence was amplified from *Synechocystis* genome by PCR using primers listed in section “Materials and Methods”. Each primer contained the appropriate BamHI and NotI restriction sites for cloning. Once sequenced, the obtained vectors, called pSAS_T7IsrR, pSAS_as5UTRIsiA, pSAS_asSynK, pSAS_as5UTRSynK and pSAS_asLPOR in accordance to the selected antisense cloned, were used to transform the T7-cyanoP_{zia} strain.

After 10-12 days from transformation, recombinant colonies started to grow on 10 µg/ml of kanamycin. Single colonies were picked up and transferred to progressively higher antibiotic concentration, until the final concentration (50 µg/ml) was reached. Using internal and external primers with respect to the recombination site, correct insertion of each construct in the genome was verified by PCR. As an example, the results of the PCR performed by using the internal primers Clor1_for and Clor2_rev on the T7-cyanoP_{zia} strain transformed by the vectors pSAS_asLPOR, pSAS_T7IsrR and pSAS_asSynK are shown (Figure 5.1). In the transformed strains absence of a band of the size corresponding to the original T7-cyanoP_{zia} strain confirmed that homoplasmy was successfully obtained. The five obtained strains were called T7-cyanoP_{zia}_T7IsrR, T7-cyanoP_{zia}_as5UTRIsiA, T7-cyanoP_{zia}_asSynK, T7-cyanoP_{zia}_as5UTRSynK and T7-cyanoP_{zia}_asLPOR in accordance to the different antisense inserted in the genome of T7-cyanoP_{zia} strain.

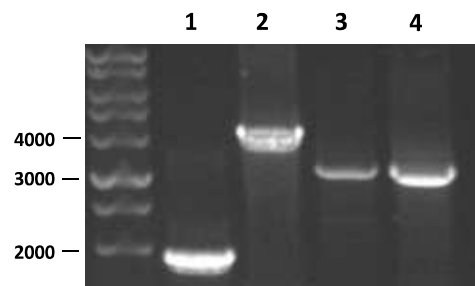


Figure 5.1: Agarose gel electrophoresis of analytical PCR amplification performed on genomic DNA of T7-cyanoP_{zia} strain (1) using internal primers Clor1_for and Clor2_rev and on extracts from the recombinant colonies of T7cyanoP_{zia}_asLPOR (2), T7cyanoP_{zia}_T7IsrR (3), T7cyanoP_{zia}_asSynK (4). Absence of a band corresponding to the size expected for the original T7-cyanoP_{zia} strain indicates complete segregation of the new recombinant strains.

5.3 Preliminary experiments for the synthesis of the selected antisense RNAs in T7-cyanoP_{zia}

As previously described, the accumulation of IsiA in the thylakoid membranes under iron-stressed conditions produces a characteristic shift of the chlorophyll *a* peak towards the blue part of the spectrum. This shift was exploited to rapidly evaluate the capacity of controlling the knockdown of *isiA* by induction of T7IsrR and as5UTRIsiA transcription. Therefore, whole-cells absorption spectra were recorded in induced T7-cyanoP_{zia}_T7IsrR and T7-cyanoP_{zia}_as5UTRIsiA strain respectively, to obtain preliminary data on the efficacy of the system. Cells were cultivated in normal BG11 medium and then transferred in iron-free medium for up to 72 h. Induction of the strains was performed by adding a final concentration of 4 μM Zn²⁺, previously used to induce expression of the T7 RNA polymerase (see chapter 3). Absorption spectra were recorded before transferring the culture in iron-depleted conditions (0 hour) and after 24, 48 and 72 hours.

In the T7-cyanoP_{zia}_T7IsrR strain the shift of the chlorophyll *a* peak started to be visible 24 hours after cultivation in iron-free medium and became more consistent at 48 and 72 hours. In induced T7-cyanoP_{zia}_T7IsrR cells a less prominent shift was observed. At 48 hours after cultivation in iron-depleting condition the maximum absorption peak of chlorophyll *a* in non-induced T7-cyanoP_{zia}_T7IsrR strain was 676.7 nm, while in induced cells the absorbance was 680.4 nm (Figure 5.2, left). The experiment was repeated three times giving comparable results, with slight changes in the wavelength of the maximum absorption peak. This less prominent shift suggested that a reduced amount of IsiA protein was present in induced T7-cyanoP_{zia}_T7IsrR cells that in turn would be attributed to the synthesis of the artificial antisense T7IsrR in the strain.

The T7-cyanoP_{zia}_as5UTRIsiA was tested under the same iron limiting conditions and absorption spectra were measured in induced and non-induced cells. In contrast to what observed for the T7-cyanoP_{zia}_T7IsrR strain, the shift of maximum absorption peak for chlorophyll *a* remained stable around 683.4 nm, both in non-induced and induced cells. Also in this case, the experiment was repeated three times giving comparable results. According to these measurements IsiA would seem not or poorly expressed, thus suggesting that as5UTRIsiA strongly down-regulates the target gene *isiA*. Absence of the shift also in non-induced cells might be explained by a basal transcription of the artificial antisense, already sufficient to switch off translation of the *isiA* messenger.

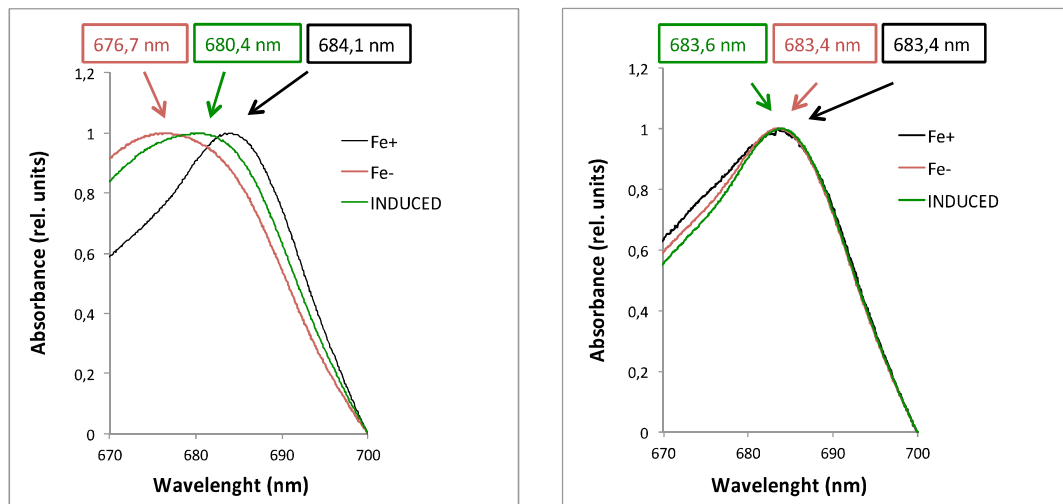


Figure 5.2: Absorption spectra of whole cells of *T7cyanoP_{zia}T7IsrR* (left) and *T7cyanoP_{zia}as5UTRIsiA* (right). Spectra were recorded after 48 hours of growth in normal BG11 medium (Fe+) iron-depleted conditions (Fe-) and in iron-depleted conditions in presence of Zn²⁺. In induced *T7cyanoP_{zia}T7IsrR* cells a less prominent shift was observed compared to non-induced cells, suggesting partial silencing by *T7IsrR*. In *T7cyanoP_{zia}as5UTRIsiA* any significant change was recorded suggesting strong silencing by the antisense. Spectra were normalized to 1 at their maximum absorbance value while the absorbance value at 700 nm was taken as 0.

The *T7-cyanoP_{zia}asSynK*, *T7-cyanoP_{zia}as5UTRSynK* and the *T7-cyanoP_{zia}asLPOR* strains were also preliminary tested in the expression of the corresponding antisenses, *asSynK*, *as5UTRSynK* and *asLPOR* respectively. The strains expressing antisense against *SynK*, i.e. *T7-cyanoP_{zia}asSynK* and *T7-cyanoP_{zia}as5UTRSynK*, were grown under high light conditions to check if a phenotype comparable to the one described for a *SynK*-less mutant (consisting in a visible decrease in the growth rate⁶⁰) could be detected. However, by analysing the growth on solid medium, any evident change in the growth rate was observed in these strains upon induction.

For the *T7-cyanoP_{zia}asLPOR* strain the chlorophyll content was measured in samples collected from induced cells. Neither in this case, a strong decrease in the chlorophyll content, as expected from what reported for the KO mutant⁹⁵, was detected in this first experiment.

It has to be underlined that the experiments on the *T7-cyanoP_{zia}asSynK*, *T7-cyanoP_{zia}as5UTRSynK* and the *T7-cyanoP_{zia}asLPOR* strains were performed only once to gain preliminary indications. Since a clear phenotype was not immediately observable, we decided to delay further and more accurate experiments necessary to

assess the actual behavior of these strains upon induction of the antisense molecules. Those preliminary results are therefore not reported in this manuscript. On the other hand, we decided to continue the analysis of the more promising T7-cyanoP_{zia}_T7IsrR and T7-cyanoP_{zia}_as5UTRIsiA strains.

5.4 Analysis of transcripts and proteins in T7-cyanoP_{zia}_T7IsrR under iron-depleted condition

In the previous experiment, measurement of whole-cell absorbance of T7-cyanoP_{zia}_T7IsrR strain showed a shift of the chlorophyll peak upon induction less prominent than that observed in the iron-depleted wild-type strain, suggesting that a reduced amount of IsiA protein was synthesized.

In order to confirm the decrease in IsiA protein content a total cell extract was analysed by SDS-PAGE and western blot. The cells were cultivated in the same iron-depleted conditions used in the previous experiment and samples were collected after 48 hours. The lower part of the membrane was incubated with a specific antibody against IsiA (apparent MW of 28 kDa), while the upper part of the same membrane was incubated with a specific antibody against the subunit b of ATP synthase (AtpB) as internal standard. As shown in Figure 5.3 the western blot analysis of the samples recovered from the induced T7-cyanoP_{zia}_T7IsrR strain (in the absence of iron) didn't accumulate a significantly higher quantity of IsiA protein in comparison to the non-induced strain or to the control T7-cyanoP_{zia} strain grown in iron-depleted conditions. Although an equal amount of total cell extract was loaded in each lane, the saturated signal of the internal standard AtpB makes difficult a proper comparison between the samples and should therefore be considered in the analysis. The analysis of the transcripts could help in explaining what observed at the protein level, at least in terms of rough comparison in relative concentration of the transcripts *isiA* and *isrR*.

Total RNA was therefore extracted from non-induced and induced T7-cyanoP_{zia}_T7IsrR cells grown under the iron-depleted condition and the transcripts were analysed by northern blot. Samples were collected before transferring the culture in iron-free medium (0 hour), as well as 6, 24, 48 hours after establishing the iron depleting condition. The radioactive probe developed against *isiA* mRNA, probe_IsiA, paired in a central region of the transcript, while the probe against T7IsrR, and therefore also to the endogenous IsrR, covered almost the full sequence of the antisense and was called probe_isrR (primers used to prepare the probes were listed in section "Materials and Methods"). As reported in the literature²² IsrR is constitutively expressed in normal iron replete conditions (Fe+).

The *isiA* mRNA was detected at 24 h and 48 h after transferring the culture in iron-limiting conditions with a contemporary decrease in the level of the antisense *IsrR*. The level of *isiA* transcript seemed comparable between non-induced and induced samples. It has to be considered, however, that the signal went rapidly to saturation, therefore more time points would be needed to follow the level of the mRNA between 24 and 48 hours of iron-depletion. In conclusion, by western blot or northern blot experiments a decrease level of *IsiA* protein or *isiA* transcript respectively was not observed in these analyses, even though the absorption spectra suggested that a reduced amount of *IsiA* protein was synthesized in induced T7cyanoP_{zia}_T7IsrR strain. In conclusion, neither western blot nor northern blot analysis confirmed reduction in the synthesis of *isiA* protein as it was suggested by a reproducible measurement of the absorbance of chlorophyll *a*.

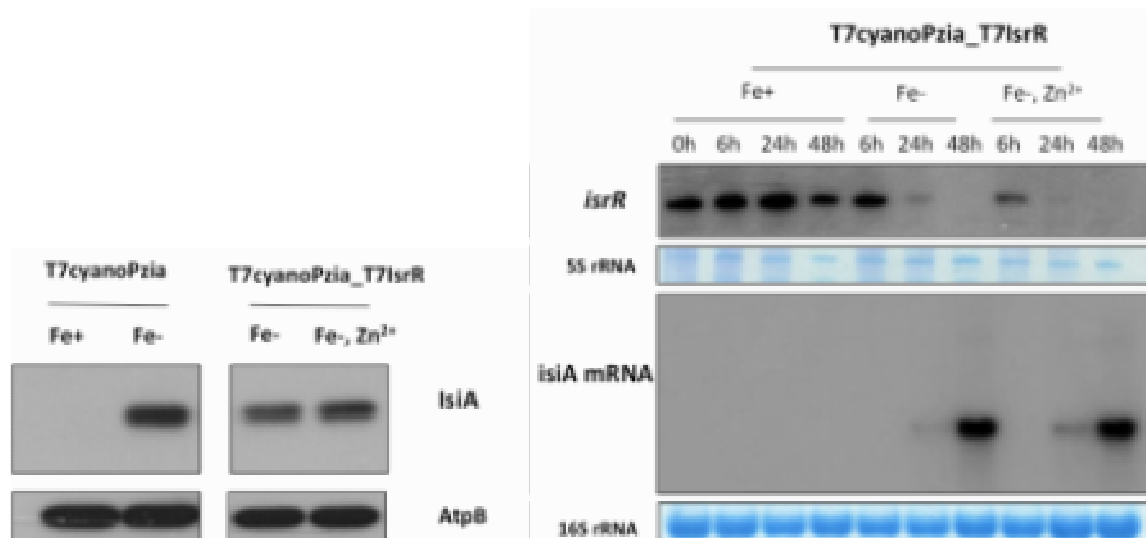


Figure 5.3: Western blot (left) and northern blot (right) analysis of T7-cyanoP_{zia}_T7IsrR strain grown in normal BG11 medium (Fe⁺), iron-depleted condition in absence (Fe⁻) or presence of the inducer (Fe⁻, Zn²⁺). The samples for the western blot analysis were collected 48 hours after establishing the iron depleted condition, while for the northern blot, samples were collected before (0h) and after 6, 24, 48 hours. The membrane was stained before hybridization with the radioactive probes and the bands corresponding to the 5s rRNA and 16S rRNA were used as internal control of RNA loading.

5.5 Analysis of the transcripts under oxidative stress condition in T7-cyanoP_{zia}_T7IsrR and T7-cyanoP_{zia}_as5UTRIsiA strains

The T7-cyanoP_{zia}_T7IsrR and the T7-cyanoP_{zia}_as5UTRIsiA strains were tested also under oxidative stress, another condition by which *isiA* is induced. The strains were cultivated in normal BG11 medium to OD₇₃₀ = 1. A final concentration of 2 mM H₂O₂ was added to the medium to induce the stress condition and contemporary 4 μM of Zn²⁺ was added to induce expression of the antisense. Samples were collected before the application of the oxidative stress and after 0.5, 1, 3 and 24 hours. The same radioactive probes used in the previous experiment, i.e. probe_{isiA} and probe_{isrR}, were used to detect respectively *isiA* mRNA and *IsrR* antisense by northern blot. In the samples recovered from the strain T7-cyanoP_{zia}_as5UTRIsiA a third radioactive probe, probe_{as5UTRIsiA}, was used to detect as5UTRIsiA (primers are listed in section “Materials and Methods”).

As reported in the literature²², induction of *isiA* was expected to be faster under oxidative stress than in iron-depleting condition. Indeed, in the samples from the control T7-cyanoP_{zia} strain the transcript of *isiA* was immediately detected 0.5 hour after the application of H₂O₂ and its level decreased very rapidly within 24 hours (Figure 5.4). Accordingly, a contemporary appearance and a subsequent decrease in the level of the antisense *IsrR* were detected. Unfortunately, on the other side of the T7-cyanoP_{zia}_T7IsrR strain, no different results could be observed in the samples collected from (Zn²⁺) induced cells. (The induced sample collected at 0.5 hour exhibited degradation, as noticed by looking at the 16SrRNA standard in the stained membrane; nevertheless it clearly showed the presence of both the *isiA* and *isrR* transcripts, in quantities comparable to the correspondent non-induced and control samples.)

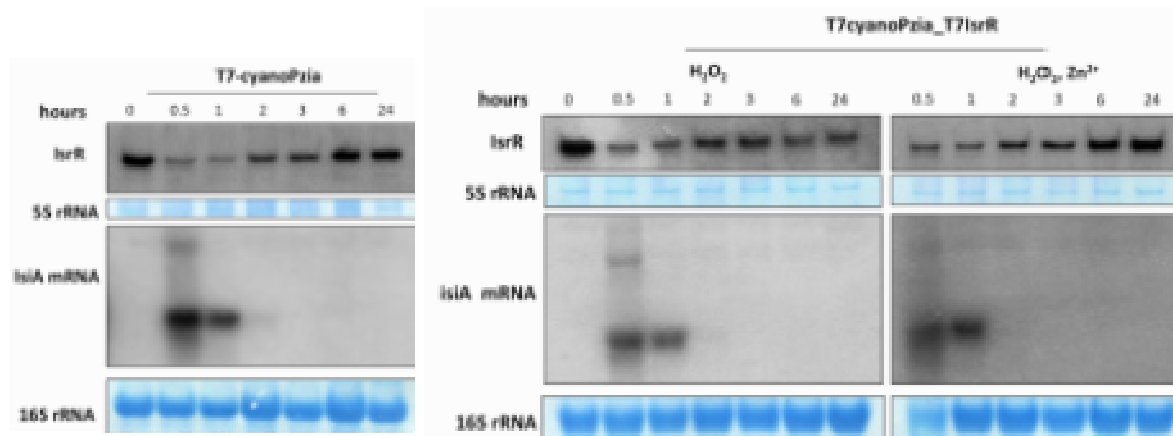


Figure 5.4: Northern Blot analysis of T7-cyanoP_{zia}-IsrR (right) strain grown under oxidative stress condition in the absence (H₂O₂) or presence of the inducer (H₂O₂, Zn²⁺). Samples were collected before the application of 2 mM H₂O₂ (0h) and after 0.5, 1, 2, 3, 6 and 24 hours. Control samples were also collected from T7-cyanoP_{zia} strain, grown under the same oxidative condition, and analysed (left). Before hybridization with the radioactive probes, the membranes were stained and the bands corresponding to the 5s rRNA and 16S rRNA were used as internal standard for loading.

For the T7-cyanoP_{zia}-as5UTR strain, transcripts were analysed only in induced cells, at the same time points chosen for the previous experiments. The as5UTR_{isiA}, detected by the specific radioactive probe_{as5UTR_{isiA}}, was found in comparable amount in each sample, independently from the time of sampling. Detection of the transcript also before induction confirmed the presence of basal transcription, definitively confirming that the promoter *ziaA* is not strictly controlled. The *isiA* mRNA was transcribed 0.5 and 1 hour after the application of the stress, as it was observed in the control. The presence of *isiA* mRNA together with a constant amount of as5UTR_{isiA} didn't suggest the knockdown of the gene.

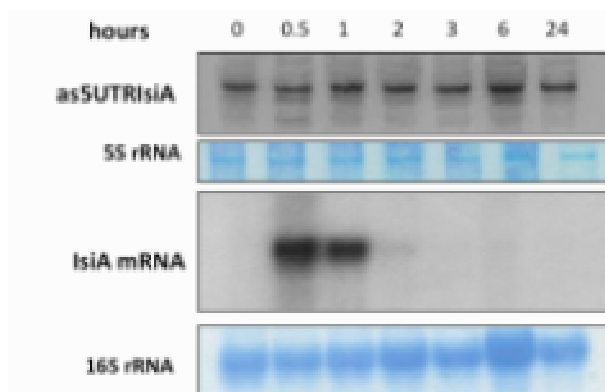


Figure 5.5: Northern Blot analysis of T7-cyanoP_{zia}-as5UTR_{isiA} strain grown under oxidative stress condition and in presence of Zn²⁺. Samples were collected before (0h) and after 0.5, 1, 2, 3, 6 and 24 hours after induction by addition of 2 mM H₂O₂. The membranes were stained before hybridization and the bands corresponding to the 5S rRNA and 16S rRNA were used as standard.

5.6 Concluding remarks

We selected and cloned in the vector pSAS the five antisenses T7IsrR, as5UTRIsiA, asSynK, as5UTRSynK and asLPOR to be used for the transformation of the strain T7-cyanoP_{zia}. The obtained strains were preliminary examined in experiments aiming to emphasize knockdown phenotypes obtained by silencing of the correspondent target genes.

This was (at least partially) observed only in the case of the *isiA* gene, when a reduced lateral shift of the maximum absorption peak of chlorophyll *a* was reproducibly registered in induced T7-cyanoP_{zia}_T7IsrR cells. This behavior, compared to that of control cells, suggested that a lower amount of IsiA protein was synthesized, due to the partial silencing of its messenger by the overexpressed T7IsrR antisense. However, this was not confirmed by the western experiment or by northern blot analysis, where a decreased level of *isiA* transcript could not be observed. We can tentatively hypothesize that the antisense is able to simply hamper translation (resulting in a slightly reduced accumulation of the IsiA protein) without directing the messenger to degradation. On the base of the few data collected, we cannot support nor rule out this hypothesis.

Absence of the characteristic shift in the maximum absorption of the chlorophyll *a* peak under iron-depleted conditions suggested that the as5UTRIsiA was able to silence the *isiA* gene. Again, this was not confirmed by the analysis of the transcripts produced under oxidative stress in the T7-cyanoP_{zia}_as5UTRIsiA cells.

It should be noticed that the absorption spectra were recorded three times in three independent experiments, while the western blot and the northern blot analysis were performed in only once. As reported in the literature, *isiA* is a gene highly transcribed under iron-limiting and oxidative stress conditions, therefore small differences in the transcript and protein level should be evaluated through experiments set to permit adequate quantification

However, it is maybe not worth performing such experiments before having solved the most relevant problem encountered in this project: the evident leakiness of the promoter(s) in charge to turn on and off the system (i.e. the transcription mediated by the T7 enzyme).

DISCUSSION AND CONCLUSIONS

The work of this PhD thesis describes the design and the first steps in the development of a new a T7 RNA polymerase-based expression system in *Synechocystis*, called T7-cyano system. This would be a cyanobacterial version of the BL21 strain of *E. coli*, useful for the inducible expression of foreign genes in *Synechocystis*.

Due to its high efficiency and specificity in directing transcription of genes from the T7 promoter, the T7 RNA polymerase was used, besides *E. coli*, also in other prokaryotic organisms⁹⁶⁻⁹⁹ and in plants¹⁰⁰ in order to enhance protein expression. One of the main reasons that brought to the choice of such a system for the utilization in *Synechocystis* was the lack of well-known, regulated promoters permitting good yield of recombinant proteins upon induction. Although the use of the T7 RNA polymerase to amplify gene expression in this cyanobacterium was already suggested⁸⁵, to our knowledge this is the first time that a system based on the use of the bacteriophage enzyme has been constructed in *Synechocystis*.

To develop such a tool, we have inserted the gene encoding the T7 RNA polymerase, controlled by an inducible promoter, in the genome of a wild-type strain of *Synechocystis* and we have called it T7-cyano. Contemporary, we have constructed three vectors, pSEV1, pSEV2 and pSAS, where the foreign sequence can be cloned in a MCS downstream the T7 promoter and then introduced inside the chloramphenicol resistance cassette present in the T7-cyano genome (Chapter 2). Once developed, the system was tested in order to analyse its capacity to (i) produce the T7 RNA polymerase (Chapter 3), (ii) transcribe and express an heterologous proteins (Chapter 4), or (iii) synthesize a sequence cloned in antisense (asRNA) (Chapter 5), upon induction of the polymerase.

In preliminary results presented in this work, we showed that the T7 RNA polymerase was successfully produced in this organism. We succeeded, indeed, not only in showing the production of the bacteriophage enzyme in *Synechocystis*, but also in providing evidences that the polymerase recognizes its specific promoter and transcribe the sequence cloned downstream of it in the pSEV vectors. We don't have yet defined the extent of the enhancement in transcription and expression of the foreign gene directed by the T7 polymerase with respect to endogenous RNA polymerase. However, we have developed additional vectors, pDEV, to retrieve such information. These vectors will allow the expression of the foreign gene directly controlled by one of the promoters, P_{zia} , P_{nrs} , P_{lac} , used to switch on and off the T7 RNA polymerase and will be tested in the future.

The presence of the T7 RNA polymerase in *Synechocystis* did not seem to alter the growth of the T7-cyano strain in comparison to the wild-type (results are shown in Appendix 3). However, we do not have yet verified if the polymerase recognizes sequences homologous to the T7 promoter present in *Synechocystis* genome and consequently activate inappropriate transcription. By a rapid search using the bioinformatic tool BLAST, the sequences present in the genome having the highest homology with the T7 promoter were mainly found inside coding sequences. We cannot exclude that some of them might be functioning, leading to the transcription of RNAs altering the expression of the (incorrectly and/or partially) transcribed gene. This aspect will be further investigated in the future, since it may be relevant when using the system for the study of gene function and regulation.

The main task of the work was the development of an inducible system. To this purpose we selected three different inducible promoters, P_{zia} , P_{nrs} and P_{lac} to control the T7 RNA polymerase gene. Unfortunately, both the promoters P_{zia} and P_{nrs} were found to be leaky in the expression of the T7 RNA polymerase and, consequently, also in the expression of the foreign sequence downstream the T7 promoter (both in protein expression and asRNAs synthesis). Although these inducible promoters were chosen among those described to have the lowest basal transcriptional activity in *Synechocystis*, we could verify that they were not sufficient to switch off transcription by the bacterial RNA-polymerase. Consequently, also transcription of the sequences controlled by the T7 promoter is never switched off, since the T7 RNA polymerase is a highly processive enzyme and small amount of it results in considerable transcription of the foreign gene from the T7 promoter.

To improve the inducibility of the promoter P_{zia} , we could suggest the introduction in the system of a second copy of the gene coding for the repressor ZiaR, since the endogenous repressor produced by *Synechocystis* may be not enough to control both the endogenous promoter and the promoter of the T7-cyano P_{zia} strain. The leakiness of P_{zia} may also be due to the low amount of zinc (0.22 $\mu\text{g}/\text{ml}$) contained in the growth medium (BG11), since a precise titration of the concentration of Zn^{2+} required for removal of the repressor nor homeostatic concentration of the same ion in the cell have ever been measured. We can additionally suggest the use of a modified growth medium lacking this metal that will be added only to induce transcription from P_{zia} .

In this line of thought, the leakiness of nickel-inducible promoter P_{nrs} cannot be due to nickel, since this ion is absent in BG11, but possibly to the presence of other metals such as Co^{2+} that have been described to be able to induce this same promoter⁷⁹. Also in this case, the use of a modified growth medium when using the T7-cyano P_{nrs} strain might also be suggested. These metals are however essential elements and we should verify if their absence affects the viability of the engineered *Synechocystis*. Preliminary tests performed on the P_{zia} -containing strains show that the lack of zinc

does not alter growth, at least up to 5 days on solid medium (results are shown in Appendix 3).

Before testing all these strategies, however, we will examine the third promoter chosen, P_{lac} , controlled by the LacIq repressor, by which we have already produced the respective T7-cyano P_{lac} strain.

The second part of the thesis was focused on the use of pSEV vectors to introduce a foreign sequence in the genome. Among all cyanobacteria *Synechocystis* is one of the strains most amenable to genetic modification. However the insertion of foreign genes in this organism presents some challenges due to its intrinsic characteristics. In particular, the genome rich of ORFs and, as shown in recent years, of ncRNAs involved in gene regulation, makes difficult the selection of an appropriate neutral site. On the other side, the polyploid genome rises up the necessity of time-consuming restreaking passages to look for homoplasmic clones. The insertion of the desired DNA segment into the chloramphenicol resistance cassette of T7-cyano was found to be a good strategy to avoid the injury of *Synechocystis* genome. Indeed, by using the pSEV vectors for transformation we always obtained recombinant colonies as well as complete segregation.

We have then tested the system for the inducible expression of proteins using the vectors pSEV1 and pSEV2. We showed that the enzyme BVMO from the algae *C. merolae* was successfully expressed for the first time in *Synechocystis* using the T7-cyano system, confirming its ability to translate the reporter gene. The BVMO enzyme was, however, the only one detected by western blot, while only transcription could be verified for the other two heterologous proteins (eGFP and HydA) chosen to test the system.

The latter consideration and the low amount of protein produced in the T7cyano P_{zia} _BVMO strain suggested to examine the step of translation. To check if the formation of the ribosomal initiation complex (30S), by recognition and interaction with the RBS, could be critical for the start of translation, we planned the substitution of the RBS originally contained in pSEV1 and pSEV2. As previously mentioned, not much is known about the translational efficiency in relation to the RBS in *Synechocystis*. A study addressed to understand the evolutionary changes of the mechanisms of translation initiation⁴⁵, underlined that, among the 277 prokaryotes examined, cyanobacteria contained substantial differences in the upstream sequence of coding genes. It was even suggested that new mechanisms of translation initiation may exist within these organisms. In this study, and also in a previous one⁹, it was shown that the Shine-Dalgarno sequence is infrequently present upstream the coding sequences of cyanobacteria, and it was also speculated that a cytosine bias immediately before the initiation codon may be involved in translation⁴⁵. Although this hypothesis was not experimentally supported, it seems that cyanobacteria differs

from other prokaryotic organism concerning translation initiation suggesting that this may be an important point to work on to improve expression in cyanobacteria.

In the last part of the thesis, we used the third vector, pSAS, to examine the system for the synthesis of asRNA molecules in order to silence endogenous genes. A partial knockdown phenotype was observed in the case of *isiA* gene, upon the expression of the artificial antisenses T7IsrR and as5UTR*isiA*. Comparison of the absorption spectra showed a reduced shift of the chlorophyll *a* peak when the antisense T7IsrR was expressed, while the shift was practically abolished by the expression of as5UTR*isiA*. Other experiments are necessary to demonstrate that the expression of these artificial antisenses may be used to knockdown the gene. For this kind of study, however, the leakiness of the system remains the real problem to be solved. The use of antisense RNAs was thought as an alternative to the construction of knockout mutants (very often heteroplasmic), in particular for the study of essential genes and for the controlled switch-off of the genes in different experimental conditions. Although the system is not ready for this kind of investigations, we have presented here preliminary results suggesting that the inducible expression of asRNAs in *Synechocystis* might be a useful instrument for the knockdown of genes in *Synechocystis*.

Various aspects of this project were not exhaustively investigated and the results that have been achieved deserve anyway further insights: the controlled production and the activity of the T7 RNA polymerase in *Synechocystis*, the inducibility of the chosen promoters, the insertion of foreign genes by the pSEV vectors into the T7-cyano genome, the expression of coding sequences and the synthesis of asRNAs by using the vectors developed. The information acquired gave a general view of the system and pointed out the two major problematic issues found within it. These were the leakiness of the promoters, P_{zia} and P_{nrs} , and an inefficient translation of proteins. Compared to *E. coli*, up to few years ago the studies on *Synechocystis* and other cyanobacteria were principally focused on the characterization of physiological processes, particularly photosynthesis. The research focused on the characterization of biological tools useful to drive gene expression started only in recent years, when these organisms acquired interest from a biotechnological point of view^{49,50}. The problems found during the development of the T7-cyano system underlined some aspects, emerging more frequently also in the literature^{35,36,43,101}, that hinder the complete exploitation of *Synechocystis*; these are the absence of known well-regulated promoters and the scarce knowledge on the molecular basis driving translation.

MATERIALS AND METHODS

MATERIALS

List of oligonucleotides

Oligonucleotides for the construction of vectors and screening

Name	Sequence	Target vector	Purpose of oligo
CLASSICpSEV1_for	CGTGTCAGATCTCATATGTGGAGCCACC	pDEV	forward primer for amplification of pSEV1 elements - fragment 1
CLASSICpSEV1KAN_rev	ATCTATCGATTGTATGGGAAGCCCG	pDEV	reverse primer for amplification of pSEV1 elements - fragment 1
CLASSICpSEV1KAN_for	TACAATCGATAGATTGTCGCACCTG	pDEV	forward primer for amplification of pSEV1 elements - fragment 2
CLASSICpSEV_rev	CGTCATCGATTTTACCCTAACACG	pDEV	reverse primer for amplification of pSEV1 elements - fragment 2
pUC18_fromPvull_for	CTGCATTAATGAATCGGCCAACGCGCG	pDEV	forward primer for full vector amplification - PCR2
pUC18_fromNde_rev	GGTGCCTCTCAGTACAATCTGCTCTG	pDEV	reverse primer for full vector amplification - PCR1
Clor2_for (PRIMER 4)	TATACTGCAGTATAATGCATCGTGTACG GTGAAACCTG	Expression Vectors	forward primer for Clor2 amplification
Clor2_rev	TATACAGCTGCCACTCATCGAGTACT	Expression Vectors	reverse primer for Clor2 amplification
Clor1_for	TATAGACGTCAGTACTGGATATACCACCG TTGAT	Expression Vectors	forward primer for Clor1 amplification
Clor1_rev	TATAGAATTCACATCTTGCAATATATGTG TA	Expression Vectors	reverse primer for Clor1 amplification
T7term_for	GTAGCATAACCCCTTGGGGCCTTAAACG GGTCTTGAGGGGTTTTTGTATGCA	Expression Vectors	synthetic oligo of the T7 terminator (plus strand)
T7term_rev	TCAAAAACCCCTCAAGACCCGTTTAGAG GCCCAAGGGGTTATGCTACTGCA	Expression Vectors	synthetic oligo of the T7 terminator (negative strand)
T7prom_Zia_for	AATTGTAATACGACTCACTATAGGGAGAA ATATCTGAGCATATCTCAGGTGTG	Expression Vectors	synthetic oligo of the T7 promoter and zia operator (plus strand)
T7prom_Zia_rev	GATCCAACACCTGAAGATATGCTCAGATA TTTCTCCCTATAGTGAGTCGTATTAC	Expression Vectors	synthetic oligo of the T7 promoter and zia operator (negative strand)
MCS1.1_for	GATCTAAGGAGATTTTCATATGTGGAGCCA CCCGCAGTTCGAAAAAATCGAAGGGCGC G	pSEV1	synthetic oligo of the RBS + MCS of pSEV1 (plus strand) - fragment 1
MCS1.1_rev	GATCCGCGCCCTTCGATTTTTTGAATGCG GGGTGGCTCCACATATGAAATCTCCTTA	pSEV1	synthetic oligo of the RBS + MCS of pSEV1 (negative strand) - fragment 1
MCS1.2_for	GATCCGAATTCAGTAGTGGCCGCGATA TCCTGCAGTGGAGCCACCCGCGATTCGAA AAATAATAATGCA	pSEV1	synthetic oligo of the RBS + MCS of pSEV1 (plus strand) - fragment 2
MCS1.2_rev	TTATTATTTTTTGAATGCGGGTGGCTCCA CTGCAGGATATCGCGGCCGCACTAGTGAA TTCG	pSEV1	synthetic oligo of the RBS + MCS of pSEV1 (negative strand) - fragment 2
MCS2_for	GATCTAAGGAGATTTTCATATGGGATCCGA TATCGCGGCCGCTAATAATGCA	pSEV2	synthetic oligo of the RBS + MCS of pSEV2 (plus strand)
MCS2_rev	TTATTAGCGGCCGCGATATCGGATCCCAT ATGAAATCTCCTTA	pSEV2	synthetic oligo of the RBS + MCS of pSEV2 (negative strand)
MCS3_for	AATTGTAATACGACTCACTATAGGGAGAG GATCCGATATCGCGGCCGATGCA	pSAS	synthetic oligo of the T7 promoter and MCS of pSAS (plus strand)
MCS3_rev	TGCGGCCGCGATATCGGATCCTCTCCCTAT AGTGAGTCGTATTAC	pSAS	synthetic oligo of the T7 promoter and MCS of pSAS (negative strand)
Ndh_Smal_for	CCCGGGTGGTGAATGATGGTGGTG	Transformation Vector	forward primer for ndhB amplification
Ndh_Smal_rev	CCCGGGCACCGCTAGCTTTGGCCACA	Transformation Vector	reverse primer for ndhB amplification

Name	Sequence	Target vector	Purpose of oligo
T7_Zia_BamHI_for	CTAGGATCCAGGTTGGCGTTAGGAGCTAG	Transformation Vector	forward primer for ziaA promoter amplification
T7_Zia_BglII_rev	TAGAGATCTGGCCAACGTGATTTAAAGAA	Transformation Vector	reverse primer for ziaA promoter amplification
nrs_for	CTAGGATCCCGCCTCGCCTTTTTATAACG	Transformation Vector	forward primer for nrsBACD promoter amplification
nrs_rev	CTAAGATCTACCACCTCAAATTGGGAATTTG	Transformation Vector	reverse primer for nrsBACD promoter amplification
Plac_for	GATCCAAAGAGTGTGACTTGTGAGCGGATAACAATGATACTTAGATTCAATTGTGAGCGGATAACAATTTACACAA	Transformation Vector	synthetic oligo of PA1lacO-1 promoter (plus strand)
Plac_rev	CTAGAACACACTTTAACAATAGGCGAGTGTTAACTTAGATTATAGTAACAATAGGCGAGTGTTCAGTTGTGAGAAAC	Transformation Vector	synthetic oligo of PA1lacO-1 promoter (negative strand)
LacI_for	ATTAGATCT GAAGGCGAAGCGGCATGC	Transformation Vector	forward primer for LacI repressor amplification
LacI_rev	GATAAGGGATCCTCACTGCCCGCTTTC	Transformation Vector	reverse primer for LacI repressor amplification
T7pol_BglII_for	CTAAGATCTATGAACACGATTAACATCGC	Transformation Vector	forward primer for T7 RNA polymerase amplification
T7pol_ClaI_rev	TGAATCGATAATTGCATCTACTCGTCGCG	Transformation Vector	reverse primer for T7 RNA polymerase amplification
CyanoT7_recomb1_for (PRIMER 5)	ACCGCCGTTCCCGCCACCCT		forward primer for screening of T7-cyano
CyanoT7_recomb2_rev (PRIMER 1)	GGGGCATTCTCCTCTGGCGGGC		reverse primer for screening of T7-cyano
AF6_rev (PRIMER 2)	GAGGAACAACCTCAACAAGCG		reverse primer for screening of T7-cyano
T7pol_seq_3'_for (PRIMER 3)	CAGCTACCATTAAACCAAC		forward primer for screening of T7-cyano

Oligonucleotides for cloning of genes and asRNAs

Name	Sequence	Purpose of oligo
BVMONde_for	TTGTACATATGGGAGCGGAGCTCAACCAATTTC	forward primer for BVMO cloning
BVMOEcoRI_rev	GATCTAGAATTCTCAGTGGTGGTGGTGGTGGTG	reverse primer for BVMO cloning
HydA_BamHI_for	CTAGGATCCGCCGACCCGCTGC	forward primer for HydA cloning
HydA_NotI_rev	GATGCGGCCGCTCACTTCTTCTCGTCTTCTCC	reverse primer for HydA cloning
T7IsrR_BamHI_for	CTAGGATCCGATAACCATAAATAACCAAAAGGAT	forward primer for T7IsrR cloning
T7IsrR_NotI_rev	GATGCGGCCGCGAAAACCCAGCAACTA	reverse primer for T7IsrR cloning
as5UTRIsrR_for	CTAGGATCCAGAATTGCCTCTTAATTG	forward primer for as5UTRIsrA cloning
as5UTRIsrR_rev	GATGCGGCCGCTGCCCTATAATAATTGGG	reverse primer for as5UTRIsrA cloning
asLPOR_BamHI_for	CTAGGATCCCTAAACCAGACCCACTAACTTTTCGC	forward primer for asLPOR cloning
asLPOR_NotI_rev	GATGCGGCCGATGGAACAACCGATGAAACCCAC	reverse primer for asLPOR cloning
asSynK_for	CTAGGATCCAGAGTAAAAACAAAAGACTCC	forward primer for asSynK cloning
asSynK_rev	GATGCGGCCGACCATTAGCCAACAATTGAA	reverse primer for asSynK cloning
as5UTRSynK_for	CTAGGATCCGGTAAAAATAGTAATAAAACAATTG	forward primer for as5UTRSynK cloning
as5UTRSynK_rev	GATGCGGCCGCTATGTTTGCAAATATCGAC	reverse primer for as5UTRSynK cloning

Oligonucleotides for the RT-PCR and construction of probes for northern blot

Name	Sequence	Purpose of oligo
HydA1_for	GCCGACCCACCCTGCGCCAG	RT-PCR
HydA1_rev	GCCGGTGGTGCCGAACAGCAC	RT-PCR
eGFP_for	ATGGTGAGCAAGGGCGAGGAGC	RT-PCR
eGFP_rev	CACAAGTTCAGCGTGTCCGGCGA	RT-PCR
16SrRNA_for	CGGTACCTGATGAATAAGCATCG	RT-PCR
16SrRNA_rev	GATGTCAAGCCTTGTAAGG	RT-PCR
IsrRfor_probe	ATTTAGGTGACACTATAGAAGTGTGGGT CATCATCTCCTGTTT	forward primer for T7IsrR probe preparation
IsrRrev_probe	CCAAAGGATCTAATGTTGGCTG	reverse primer for T7IsrR probe preparation
IsiAfor_probe	ATCAGTAGCCTAGAAGATCTGG	forward primer for IsiA probe preparation
IsiArev_probe	ATTTAGGTGACACTATAGAAGTGAAAAT TCTGGCGGATAGGCC	reverse primer for IsiA probe preparation
as5UTRIsiAfor_probe	ATTTAGGTGACACTATAGAAGTGTGGGA GTAAGTGCTAAAGATTC	forward primer for as5UTRIsiA probe preparation
as5UTRIsiArev_probe	ATTATGGCAATAAGTGCCGAG	reverse primer for as5UTRIsiA probe preparation

List of plasmids

- **pUC18** and **pUC19** (ThermoScientific)
- **pUC4K** (Pharmacia)
- **pUC4CAT** - modified pUC4K vector in which the kanamycin resistance gene was replaced by the chloramphenicol resistance cassette (provided by Dr. P. Costantini, Department of Biology, University of Padova)
- **pGEM®-T Easy Vector** (Promega)
- **TA-TOPO_HydA** – TOPO®-TA cloning containing the ORF coding sequence for the mature HydA1 isoform from *C. reinhardtii* (provided by Dr. P. Costantini, Department of Biology, University of Padova)
- **pGREAT_eGFP** – pGreen vector containing the eGFP coding sequence (provided by Prof. I. Szabò, Department of Biology, University of Padova)
- **pET28_Cm-BVMO** – pET28 vector containing the BVMO sequence from *C. merolae* (available in our laboratory)

BG11 medium for *Synechocystis*STOCK SOLUTIONS

1000X solution	g/100ml
K ₂ HPO ₄ (175 mM)	3.05
Na ₂ CO ₃ (189 mM)	2.00

Trace mineral solution	g/1L
H ₃ BO ₄	2.86
MnCl ₂ x 4 H ₂ O	1.81
ZnSO ₄ x 7 H ₂ O	0.22
NaMoO ₄ x 2 H ₂ O	0.39
CuSO ₄ x 5 H ₂ O	0.08
Co(NO ₃) x 6 H ₂ O	0.05

100X Macronutrient	g/1L
NaNO ₃	149.58
MgSO ₄ x 7 H ₂ O	7.49
CaCl x 2 H ₂ O	3.60
Citric Acid	0.92
0,25 M NaEDTA pH=8,0	1.12
Trace mineral solution	100 ml

0.25 M Na-EDTA pH=8	EDTA 46.5 g/500 ml deionized H ₂ O Adjust the pH with NaOH
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1 M TES-KOH pH=8.2	TES g/500 ml deionized H ₂ O Adjust the pH with KOH
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PREPARATION

LIQUID MEDIUM	1L
100X Macronutrient	10 ml
1 M TES-KOH, pH 8.2	10 ml
Trace mineral solution	1 ml
1000X solution	1 ml
Ferric ammonium citrate (6 mg/ml)	1 ml
MQ H ₂ O	to 1L

SOLID MEDIUM	g/1L
LIQUID MEDIUM	
Na ₂ S ₂ O ₃ • 5H ₂ O	3
Agar	15

Restriction enzymes

Restriction enzymes used for cloning and for screening of recombinant colonies were obtained from New England Biolabs (NEB) or from Promega.

Other enzymes used for cloning

TSAP (Thermosensitive Alkaline Phosphatase) for dephosphorylation reaction was purchased from Promega. T4 DNA Ligase for DNA ligase reactions was NEB T4 DNA ligase.

T4 polynucleotide Kinase from Promega was used for phosphorylation of synthetic oligonucleotides.

Polymerase Chain Reaction (PCR)

Phusion™ High-fidelity DNA polymerase from Finnzymes was used in the amplification of sequences when high performance was needed. GoTaq® DNA polymerase was used in occasional PCR for fast screenings of recombinant colonies.

Primary Antibodies

T7 RNA polymerase Monoclonal Antibody (Novagen)

Anti-IsiA (gently provided by Prof. Eva-Mari Aro, Department of Biochemistry, University of Turku)

Anti-BVMO polyclonal antibody (available in the laboratory)

Anti-HydA polyclonal antibody (gently provided by Dr. P. Costantini, Department of Biology, University of Padova)

METHODS

Molecular biology methods

Annealing of synthetic oligonucleotide

Some sequences required for the construction of vectors were synthesised because they were not available or were of new design. Each synthesised strand was brought to a final concentration of 100 μ M. In the same eppendorf tube were mixed together equimolar quantities of the two oligonucleotides and the tube placed in boiled H₂O until the temperature decreased to RT. This method allowed a slow decreasing of the temperature enhancing annealing efficiency.

Plasmid extraction using phenol:chlorophorm:isoamyl alcohol (25:24:1)

SOLUTIONS

Isotonic solution	volume
Glucose 1M	250 μ L
TRIS HCl 1M	125 μ L
Na ₂ EDTA 0,25 M pH8	200 μ L
MQ H ₂ O	To 5 mL

Lysis solution	volume
NaOH 1 M	1 mL
10 % SDS	0.5 mL
MQ H ₂ O	To 5 mL

Neutralization solution	volume
KC ₂ H ₃ O ₂ 5 M	6 mL
CH ₃ COOH	1.15 mL
MQ H ₂ O	To 10 mL

PROCEDURE

This procedure for plasmid DNA extraction was used instead of the commercial kits in case of protein contamination, in particular for the vectors containing the T7 RNA polymerase gene.

From *E. coli* liquid culture cells were pelleted at 14,000 rpm for 2 min and

resuspended in 100 μL of isotonic solution, vortexed and then 200 μL of alkaline lysis solution were added. The tubes were inverted roughly 2-3 times for proper mixing and homogeneous disruption of the cells. After 5 min of incubation 150 μL of neutralization solution were added, vortexed, and centrifuged at 14,000 rpm for 5 minutes at 4 $^{\circ}\text{C}$. The supernatant was transferred in a new tube and one volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) was added. The mixture was vortexed until becoming white and then centrifuged at 14,000 rpm at 4 $^{\circ}\text{C}$ for 2 minutes. The upper phase containing the DNA was transferred to a new eppendorf tube. The DNA was then precipitated by addition of 2 volumes of 100 % ethanol and centrifugation at 14,000 rpm for 5 minutes at 4 $^{\circ}\text{C}$. The resulting pellet was washed once with 1 mL of ethanol 70 %, let dry under the fume hood and then resuspended in 20 μL of sterile MQ H_2O .

Strains and culture conditions

Escherichia coli strains XL1-Blue and K12 ER2925 (dam⁻ cells) were grown with LB medium at 37 $^{\circ}\text{C}$ rotary shaking or on agar plates, supplemented with antibiotics depending on the plasmid.

The wild-type and recombinant strains of *Synechocystis* sp. PCC 6803 were grown at 30 $^{\circ}\text{C}$ in BG11 medium (receipt is reported in section "Materials") in heterotrophic condition with 5 mM glucose. Light intensity was adjusted to 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of white light. Growth was monitored by the increase in optical density at 730 nm. In the T7-cyano recombinant strains cultures (T7-cyanoP_{zia}, T7-cyanoP_{nrs}, T7-cyanoP_{lac}) chloramphenicol was included in the medium at a final concentration of 50 $\mu\text{g mL}^{-1}$, while for the recombinant strains transformed with one of the expression vectors pSEV (T7-cyanoP_{zia}-eGFP, T7-cyanoP_{zia}-HydA, T7-cyanoP_{zia}-BVMO, T7-cyanoP_{nrs}-eGFP, T7-cyanoP_{nrs}-BVMO, T7-cyanoP_{zia}-T7IsrR, T7-cyanoP_{zia}-as5UTRIsiA) kanamycin was added at the same final concentration of 50 $\mu\text{g mL}^{-1}$.

Iron starvation was induced using a modified BG11 medium lacking Ferric ammonium citrate (Iron-free medium). Cells were cultivated under iron-repleted conditions to a final OD₇₃₀ = 0.3, washed three times in iron-free medium, and growth further in iron-depleted conditions. Glassware for iron-depleted growth was treated o/n with 10 % HCl, washed with mQ H_2O and further treated o/n with 10 μM Na/EDTA, washed and autoclaved, to assure complete absence of iron.

Transformation procedure for *Synechocystis*

Since *Synechocystis* is naturally competent any pretreatment is necessary for transformation.

The day before cyanobacteria were inoculated in BG11 medium with the addition of 5

mM glucose and antibiotics if needed at a final $OD_{730} = 0.2$. The day of transformation OD_{730} should be comprised between 0.6 and 0.8. The amount of culture required for transformation was centrifuged at 5,000 g for 5 minutes at RT and cyanobacteria were resuspended in BG11 medium at a final density of 10^{10} cell/mL. The cell density was calculated considering that at $OD_{730} = 0.25$ the cell density is 10^8 cell/mL. For each transformation 200 μ L of resuspended cyanobacteria were used and transferred to new tubes. At this point DNA was added from a minimum of 200 ng to a maximum of 1 μ g. Cyanobacteria were incubated under shaking at 30 °C for 5 hours in low light conditions, approximately around 4-6 μ E. Cells were plated in BG11 medium with the addition of 5 mM glucose, without antibiotics above a sterile laboratory filter paper. Plates were placed for 24 h at 30 °C under low light (4-6 μ E). The next day filter paper was moved to a plate containing 5 or 10 μ g/mL of antibiotic. To obtain a homoplasmic clone antibiotic concentration was progressively increased until the final 50 μ g/mL.

Genomic DNA isolation from *Synechocystis*

Cyanobacteria were cultivated for 48 h or to a final $OD_{730} = 1/1.6$ and then centrifuged 10 min at 6,000 g. Pellet was resuspended in 2.5 mL of saturated NaI and samples incubated at 37 °C for 30 min under shaking. After a second centrifugation of 10 min at 6,000 g samples were washed with deionized H₂O and then frozen at -80 °C o/n. The day after 2.5 mL of lysis buffer (50 mM glucose, 50 mM Tris-HCl pH 8, 10 mM EDTA) was added together with lysozyme to a final concentration of 15 mg/mL. Samples were incubated at 37 °C for 1 h under shaking and then 5 mL of NaOH 0.2% and SDS 1% were added. After 10 min of incubation in ice 3.75 mL of NaOAc 3 M was added, samples were incubated other 30 min on ice and then centrifuged at 12,000 g for 10 min at 4°C. Supernatant was transferred to new tubes and after addition of 1 vol of phenol:chlorophorm mixture the samples were centrifuged at 14,000 rpm for 2 min. The supernatant containing the DNA was precipitated by addition of 1/10 volume NaAcO 3 M and 2 volume of EtOH 100 %. Pellet was washed twice with EtOH 70 %, dried under the fume hood and resuspended in mQ H₂O. Samples were treated with RNase to avoid presence of RNA contamination.

Spectral measurements

Absorbance spectra of whole cells to track the shift in the chlorophyll *a* absorbance were measured between 650 and 750 nm. For proper comparison between samples, recorded spectra were normalized to 1 at their maximum absorbance value, while the absorbance at 700 nm was taken as 0.

Protein isolation from *Synechocystis* and immunoblot analysisSOLUTIONS

Washing buffer
50 mM Hepes·NaOH, pH 7.5 30 mM CaCl ₂

Resuspension buffer
50 mM Hepes·NaOH, pH 7.5 30 mM CaCl ₂ 800 mM Sorbitol 1 mM ϵ -amino-n-caproic-acid

Storage buffer
50 mM Hepes ·NaOH, pH 7.5 600 mM Sucrose 30 mM CaCl ₂ 1 M glycinebetaine

PROCEDURE

Proteins were isolated starting from 20-60 mL of cell culture ($OD_{730}=1.0$) using cells centrifuged at 6000 g for 8 min at 4°C. Pellet was washed with with 2 mL of washing buffer and then resuspended in 200 μ L of resuspension buffer. Glass beads (150-212 μ m, Sigma G-1145) were added to the samples and vortexed 6 times for 1 min each, cooling on ice between intervals. After addition of 0.4 mL of resuspension buffer, samples were vortexed and centrifuged briefly for 1 min at 1500 g at 4 °C. Extraction with resuspension buffer was repeated once and the supernatant, representing a total cell extract, transferred in new tubes. From the addition of glass beads procedure was carried on in the cold room under very dim light. Protein quantification was achieved by Lowry method.

In some cases a more fast procedure was used: cells were directly lysed in LB (SDS-PAGE loading buffer) and then boiled. Samples were run on SDS-PAGE with a 12% acrylamide-0.4% bisacrylamide gel in Tris/glycine buffer. Quantification was performed by measuring OD at 730 nm (OD_{730}). Proteins were blotted on PVDF and decorated with primary antibodies.

For dot blot analysis cells were disrupted by French press treatment at 2.7 kBar for three times. Samples were spotted on nitrocellulose blotting membrane and decorated with primary antibody.

RNA isolation from *Synechocystis*

RNA isolation using TRIzol Reagent

For RT-PCR analysis total RNA was isolated from *Synechocystis* using TRIzol® Reagent (Life Technologies) following manual instructions with some few modifications. Briefly, 1 mL of TRIzol Reagent was added to 1×10^{10} pelleted cells and resuspended. The mixture was incubated for 5 min at 100 °C and then shaken vigorously. Samples were centrifuged 5 min at 12,000 g and 0.2 mL of chlorophorm was added to the supernatant. After 10 min incubation at RT, samples were centrifuged 5 min at 12,000 g and RNA precipitated using 0.7 volumes of isopropanol. Pellet was washed once with NAOAc 3M pH 5.2 and then washed twice with ethanol 70 %. RNA pellet was then resuspended in DEPC H₂O.

RNA isolation using hot phenol/chlorophorm

SOLUTIONS

RNA resuspension buffer

0.3 M saccharose
10 mM sodiumacetate (pH 4.5)

RNA lysis buffer

2% SDS
10 mM sodium acetate (pH 4.5)

Phenol:Chloroform (1:1)

20 ml saturated phenol pH 4.5 (Amresco 0981)
20 ml Chloroform
About 200 µl Buffer from the phenol bottle.
Mixture was mixed well and phenol and water phase were let separate overnight.

PROCEDURE

The amount of cells required were pipetted into precooled centrifuge bottle and centrifuged at 6000 rpm for 6 min at 4 °C. From this point on samples were kept on ice and precooled bottles and solutions were used. Pellet was resuspended in 1 mL of RNA resuspension buffer and pipetted in 1.5 mL Eppendorf tube. Cells were centrifuged at 19,500g for 2 minutes at 4 °C. The supernatant was removed by

pipetting and pellet was frozen in liquid nitrogen and stored at -80 °C until further isolation (if needed).

Pellet was resuspended in 200 µl of RNA resuspension buffer and 60 µl of 250 mM EDTA (pH 8). To the resuspended cells 300 µl of lysis buffer were added, followed by addition of 500 µl phenol:chloroform (1:1 ACID PHENOL). The mixture was well vortexed, incubated 3 min at 60°C and centrifuged at 18,500g for 5 min. The upper phase containing the isolated RNA was transferred to a new tube. Isolation steps with phenol:chloroform was repeated 2 or 3 times until disappearance of the white protein phase. Isolation step was repeated once with chloroform and then RNA was precipitated by addition of 1/5 volume of ice cold 10 M LiCl and 2X volume of -20 °C ethanol. Samples were incubated overnight at -20 °C and then centrifuged at 19,500g for 15 min at 4 °C Pellet was washed with 1 mL of 70 % ethanol in DEPC H₂O was stored in -20 °C and let get dried for 10/15 minutes under the fume hood at room temperature. Pellet was resuspended in DEPC H₂O and frozen in liquid nitrogen and samples were stored at -80 °C.

Reverse Transcriptase PCR (RT-PCR)

The Reverse Transcriptase PCR allowed the amplification of DNA starting from a total RNA extraction. The technique consisted in two phases: in the first one RNA was converted into the complementary DNA (cDNA) by SuperScript™ II Reverse Transcriptase (Invitrogen) following manuals instructions and using random primers. In the second phase the cDNA was amplified by PCR using primers specific for the gene of interest.

Preparation of DEPC H₂O

The DEPC treated water was used in manipulating RNA samples to reduce the risk of degradation by RNases. The procedure consisted in diluting Diethylpyrocarbonate (DEPC) 1:1000 directly in the bottle containing mQ H₂O; the bottle was shaken vigorously and left open o/n under the fume hood. The next day the full bottle was autoclaved.

Northern blot

Total RNA was isolated by Phenol/chlorophorm extraction (protocol is reported in this same section) and separated on 10 % polyacrylamide-urea gel in 10X SSC buffer (for sRNA separation) or 1.2 % agarose gel in phosphate buffer (for mRNA separation). In each lane 4 µg of total RNA was loaded on the gel. PAA gels were electroblotted to Hybond-N+ membranes (Amersham), while for agarose gels

capillary transfer was used for transferring. After RNA was cross-linked to the membranes under UV-light for 3 minutes at both sides, verification that the same amounts of RNA samples were loaded in each lane was verified by direct comparison of rRNA band intensities after staining the membranes with 0.04 % methylene blue solution in 0.5 M sodium acetate. Following prehybridization for at least 30 minutes at 68 °C by PerfectHyb™ Plus hybridization Buffer (Sigma Aldrich), oligonucleotides probes labelled by α -³²P-dUTP (Perkin Elmer) were added and hybridized at 68 °C over-night. All RNA probes were prepared using MAXIscript® Kit (Ambion) following manual instructions. As DNA template, PCR products containing the SP6 promoter were prepared using primers listed in section “Materials”. The membranes were washed for 5 minutes at room temperature in low stringency wash buffer (2X SSC, 0.1% SDS) and then for 20 minutes in high stringency wash buffer (0.5X SSC, 0.1% SDS), before exposure to X-ray films.

Appendix 1

Map of vectors

Transformation Vectors

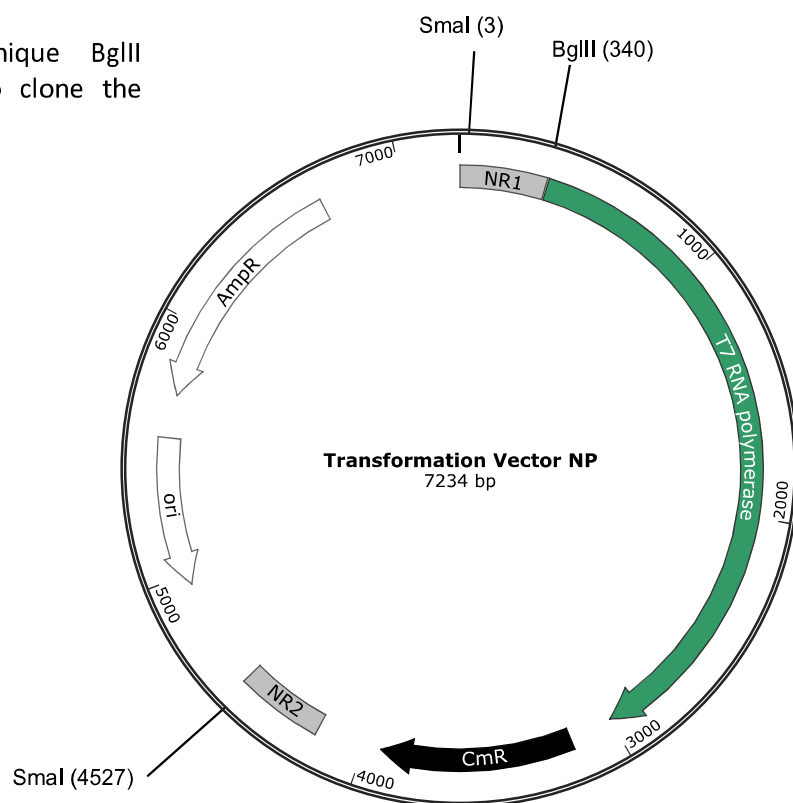
On the map of each vectors restriction sites *Sma*I to extract a linear sequence to transform *Synechocystis* are indicated. The sequence of the promoter is shown below the map.

Transformation Vector NP

Transformation Vector NP sequence landmarks

NR1	1-339
T7 RNA polymerase	346-2997
Cm ^R	3171-3933
NR2	4192-4530
ori	4957-5545
Amp ^R	5716-6681

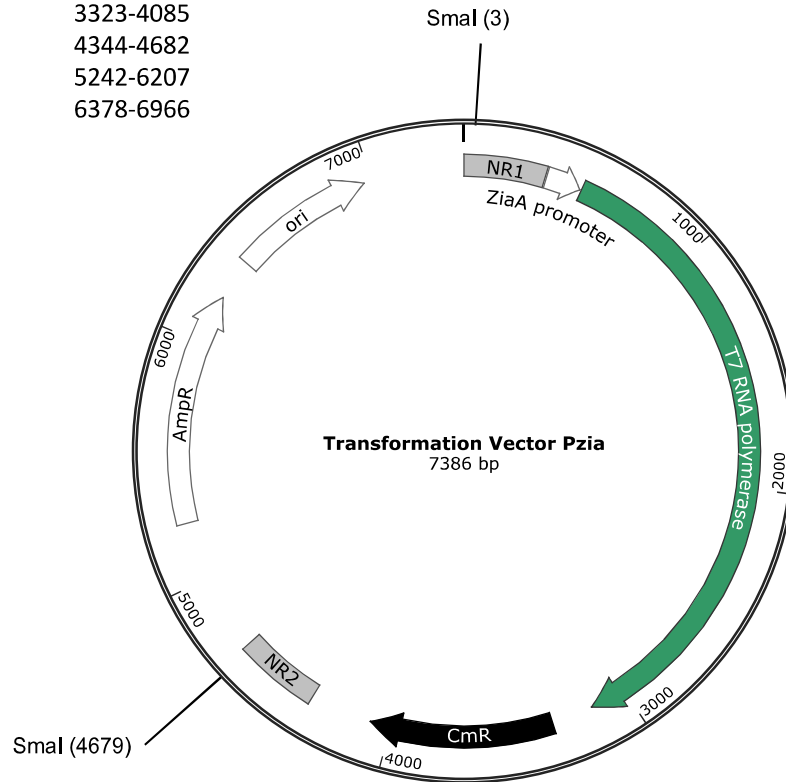
In the map the unique *Bgl*III restriction site used to clone the promoter is shown.



Transformation Vector P_{zia}

Transformation Vector P_{zia} sequence landmarks

NR1	1-339
ZiaA promoter	346-491
T7 RNA polymerase	498-3149
Cm ^R	3323-4085
NR2	4344-4682
Amp ^R	5242-6207
ori	6378-6966



Pzia

Transformation Vector Pzia
 GATCCAGGTTGGCGTTAGGAGCTAGGGAAAAATTTAAACTGGATTTAGAAA
 CTAGGTCCAACCGCAATCCTCGATCCCTTTTAAATTTGACCTAAATCTTT

Transformation Vector Pzia
 ATGATTTTCATCCTAACATCTTTAATATCTGAGCATATCTTCAGGTGTTTC
 TACTAAAAGTAGGATTGTAGAAATTATAGACTCGTATAGAAGTCCACAAAG

Transformation Vector Pzia
 AAGATTTGTGCTACGGTTCAAGGAGGTTTTCTTTAAATCACGTTGGCCAG
 TTCTAAACACGATGCCAAGTTCCTC AAAAAGAAATTTAGTGCAACCGGTC

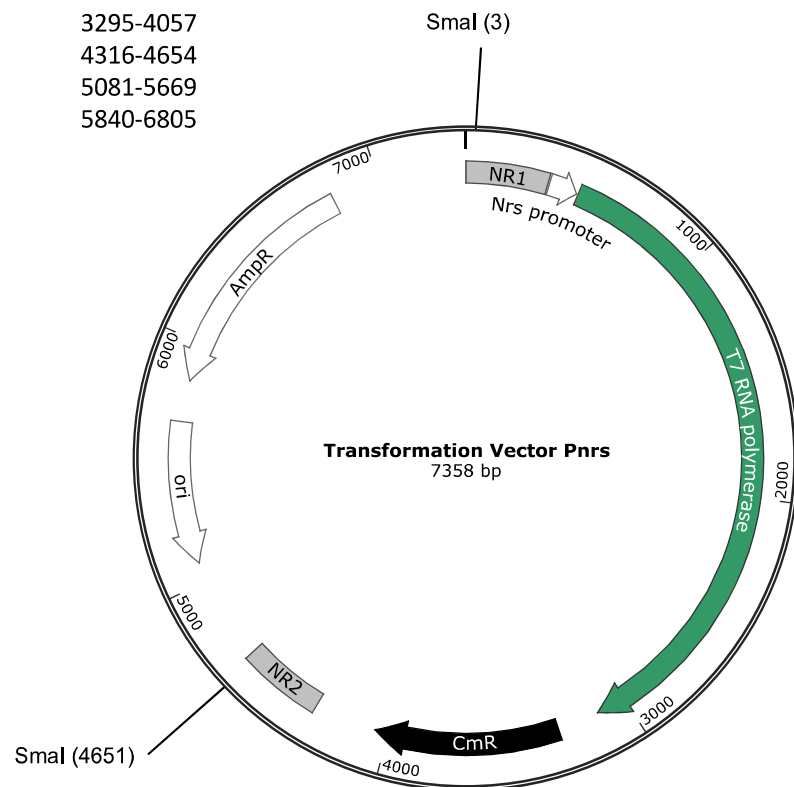
T7 RNA pol

Transformation Vector Pzia
 ATCTATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAACT
 TAGATAGTTGTGCTAATTGTAGCGATTCTTGCTGAAGAGACTGTAGCTTGA

Transformation Vector P_{nrs}

Transformation Vector P_{nrs} sequence landmarks

NR1	1-339
Nrs promoter	346-463
T7 RNA polymerase	470-3121
Cm ^R	3295-4057
NR2	4316-4654
ori	5081-5669
Amp ^R	5840-6805



P_{nrs}

Transformation Vector P_{nrs} GATCCCGCCTCTGCCTTTTTTATAACGGTCTGATCTTAGCGGGGAAGGA
CTAGGGCGGAGACGGAAAAAATATTGCCAGACTAGAATCGCCCCCTTCT

Transformation Vector P_{nrs} GATTTTCACCTGAATTTTCATACCCCTTTGGCAGACTGGGAAAATCTTGG
CTAAAAGTGGACTTAAAGTATGGGGAAACCGTCTGACCCTTTTAGAAC

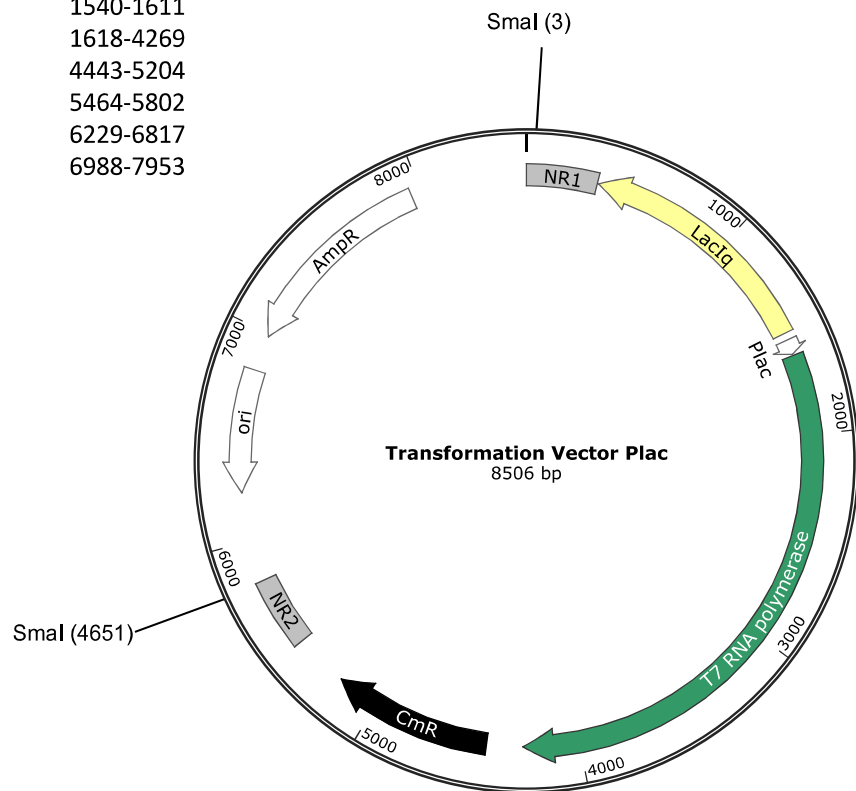
Transformation Vector P_{nrs} ACAAATCCCAATTTGAGGTGGTAGATCTATGAACAGATTAACATCGCT
TGTTTAAGGGTTAAACTCCACCATCTAGATACCTGTGCTAATTTGAGCGA

T7 RNA pol

Transformation Vector Plac

Transformation Vector Plac sequence landmarks

NR1	1-339
LacIq	346-1506
Plac	1540-1611
T7 RNA polymerase	1618-4269
Cm ^R	4443-5204
NR2	5464-5802
ori	6229-6817
Amp ^R	6988-7953



Plac

Transformation Vector Plac GATCCAAAGAGTGTGGACTTGTGAGCGGATAACAATGATACTTAGATTCAAT
CTAGGTTTCTCACAACCTGAACACTCGCCTATTGTTACTATGAATCTAAGTTA

T7 RNA pol

Transformation Vector Plac TGTGAGCGGATAACAATTTACACAAGATCTATGAACACGATTAACATCGCT
ACACTCGCCTATTGTTAAAGTGTCTAGATACCTTGTGCTAATTGTAGCGA

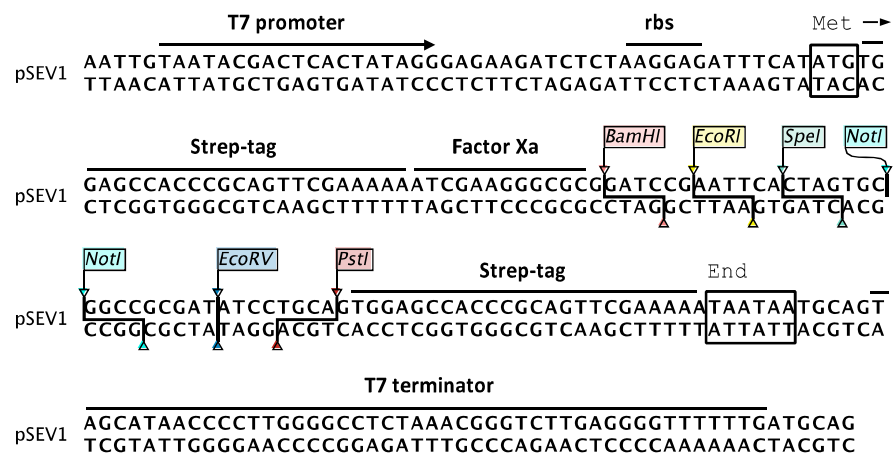
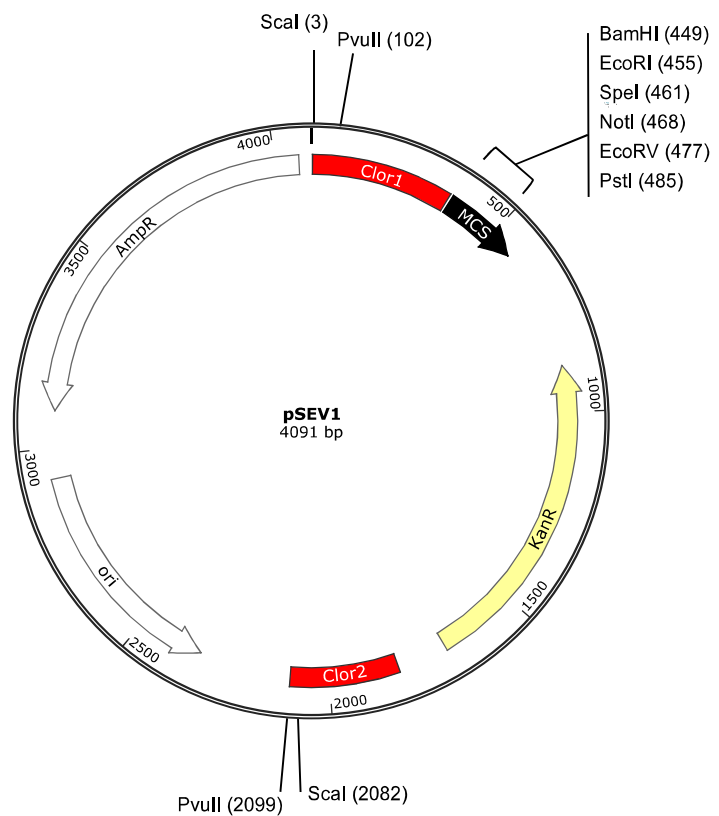
Synechocystis Expression Vectors

On the map of each vectors the restriction sites PvuII and Scal to extract a linear sequence to transform *Synechocystis* are indicated. The unique restriction sites of the MCS are also shown. The cloning/expression region is shown below the map.

pSEV1

pSEV1 sequence landmarks

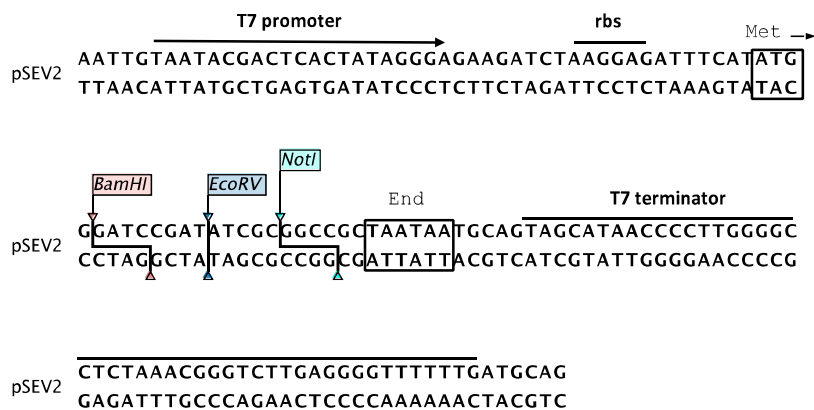
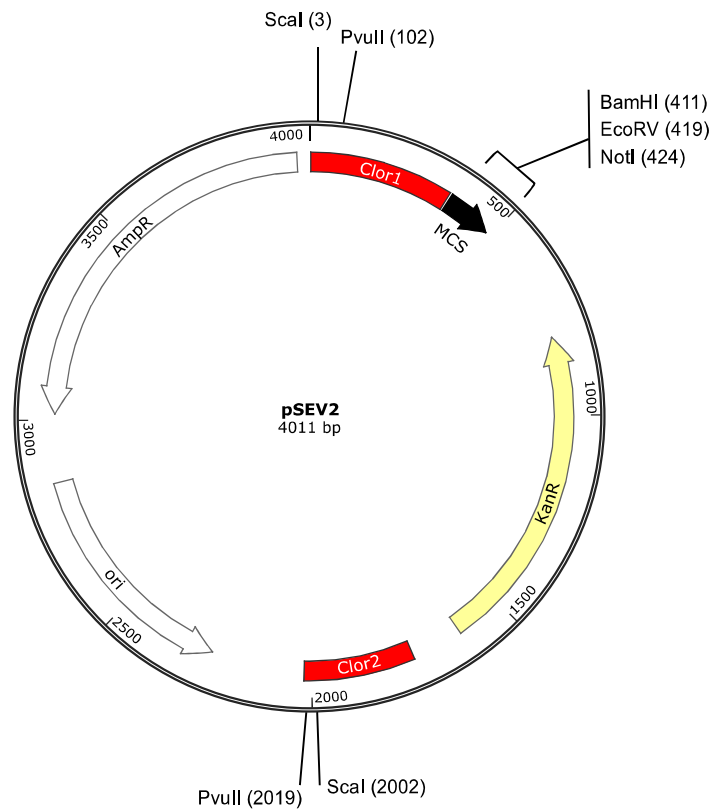
Clor1	4-359
T7 promoter	366-384
RBS	397-402
Strep-tag I	413-436
Factor Xa	437-448
MCS (<i>Bam</i> HI- <i>Pst</i> I)	449-486
Strep-tag II	487-510
T7 terminator	522-568
Kan ^R	882-1697
Clor2	1825-2099
Ori	2336-2924
Amp ^R	3095-4060



pSEV2

pSEV2 sequence landmarks

Clor1	4-359
T7 promoter	366-384
RBS	395-400
MCS (<i>Bam</i> HI- <i>Not</i> I)	411-430
T7 terminator	442-488
Kan ^R	802-1617
Clor2	1745-2019
Ori	2256-2844
Amp ^R	3015-3980

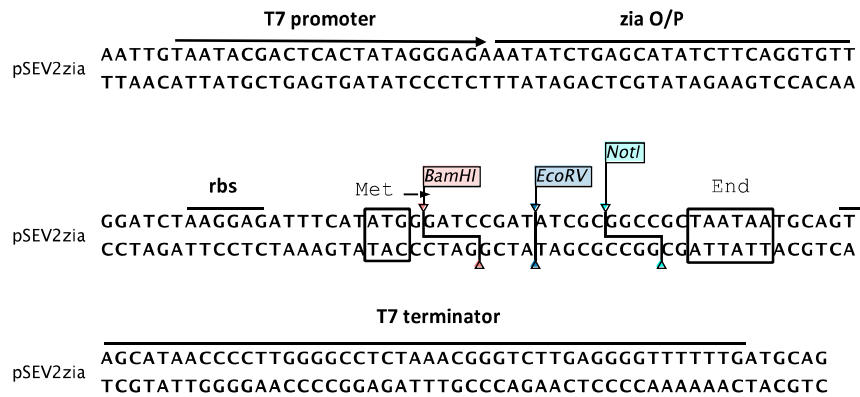
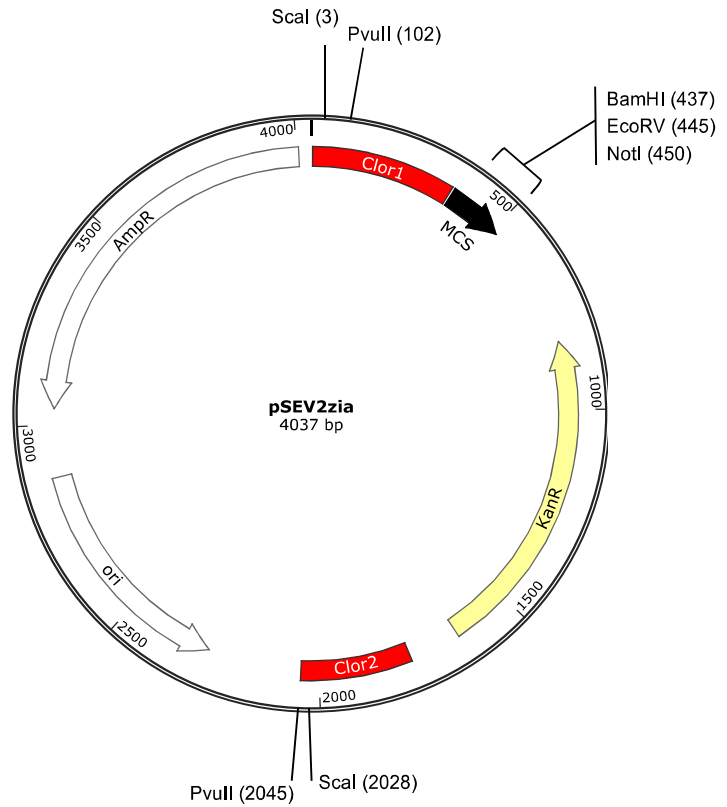


pSEV2zia

pSEV2zia sequence landmarks

Clor1	4-359
T7 promoter	366-384
Zia O/P	389-414
RBS	421-426
MCS (<i>Bam</i> HI- <i>Not</i> I)	437-456
T7 terminator	468-514
Kan ^R	828-1643
Clor2	1771-2045
Ori	2282-2870
Amp ^R	3041-4006

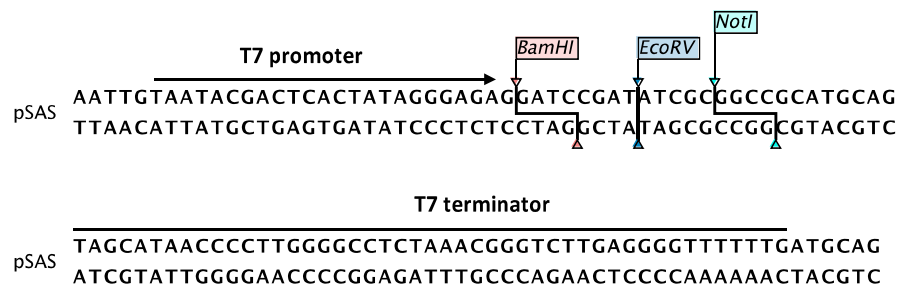
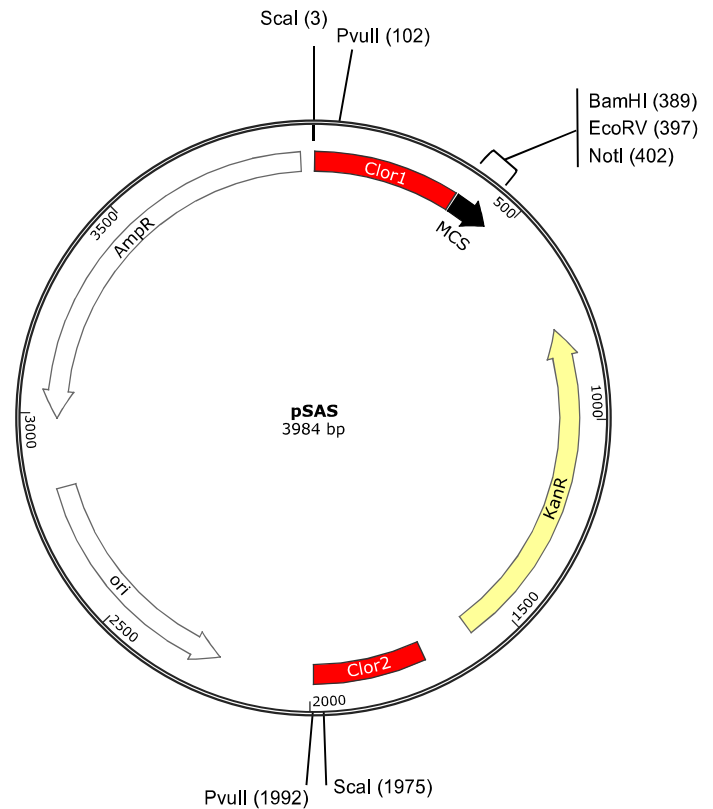
The pSEV2zia contains the same elements of pSEV2 except for the addition of zia operator for usage in T7-cyanoPzia strain.



pSAS

pSAS sequence landmarks

Clor1	4-359
T7 promoter	366-384
MCS (<i>Bam</i> HI- <i>Not</i> I)	389-408
T7 terminator	415-461
Kan ^R	775-1590
Clor2	1718-1992
ori	2229-2817
Amp ^R	2988-3953



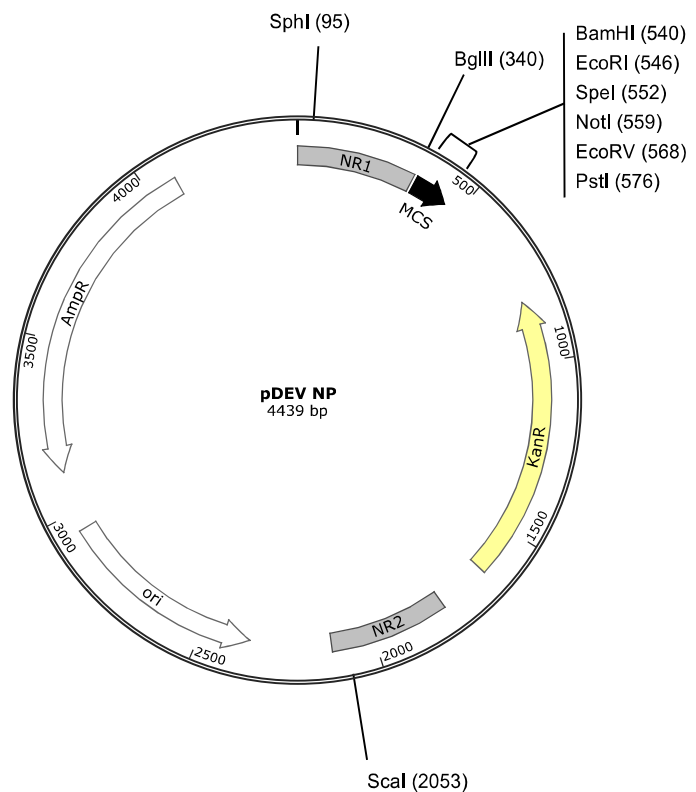
Direct Expression Vectors (pDEV)

On the map of each vectors the restriction sites *SphI* and *Scal* to extract a linear sequence to transform *Synechocystis* are indicated. The unique restriction sites of the MCS are also shown. The cloning/expression region and the sequence of the promoter are shown below the map.

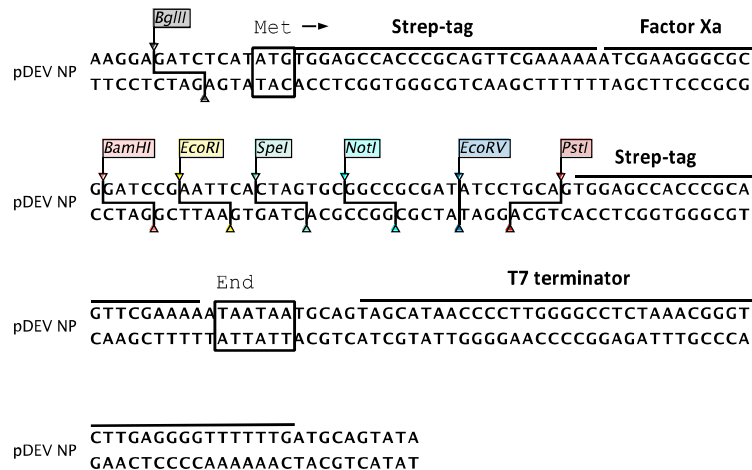
pDEV NP

pDEV NP sequence landmarks

NR1	1-359
Strep-tag I	352-375
Factor Xa	376-387
MCS (<i>BamHI-PstI</i>)	388-425
Strep-tag II	426-449
T7 terminator	461-507
Kan ^R	821-1636
NR2	1783-2122
ori	2359-2947
Amp ^R	3118-4083



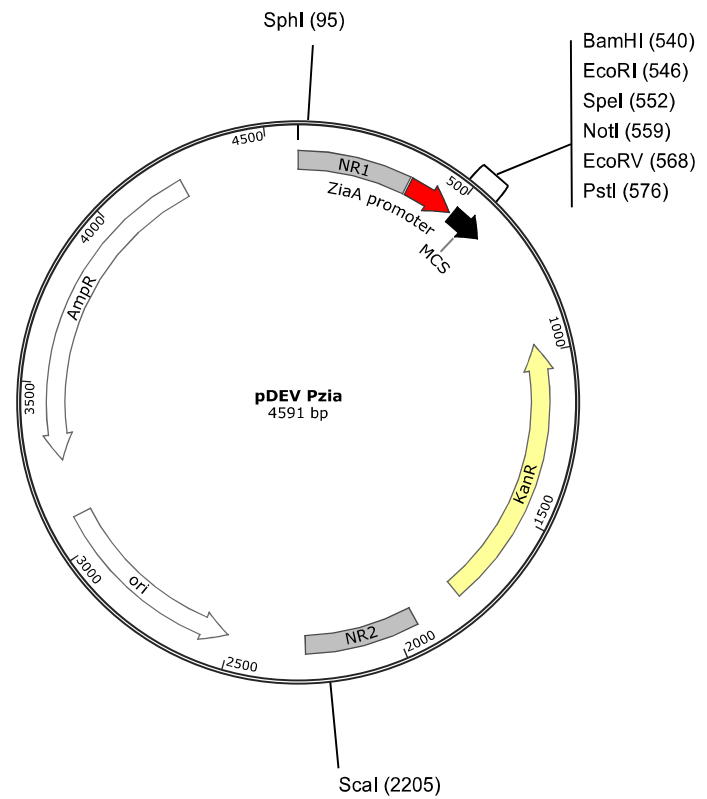
In the map the unique *BglII* restriction site used to clone the promoter is shown.



pDEV Pzia

pDEV Pzia sequence landmarks

NR1	1-339
Pzia	346-491
Strep-tag I	504-527
Factor Xa	528-539
MCS (<i>Bam</i> HI- <i>Pst</i> I)	540-577
Strep-tag II	578-601
T7 terminator	613-659
Kan ^R	973-1788
NR2	1935-2274
ori	3118-4083
Amp ^R	3270-4235



Pzia

pDEV Pzia GATCCAGGTTGGCGTTAGGAGCTAGGGAAAAATTTAAACTGGATTTAGA
CTAGGTCCAACCGCAATCCTCGATCCCTTTTTAAATTTGACCTAAATCT

pDEV Pzia AAATGATTTTCATCCTAACATCTTTAATATCTGAGCATATCTTCAGGTG
TTTACTAAAAGTAGGATTGTAGAATTATAGACTCGTATAGAAGTCCAC

pDEV Pzia TTTCAAGATTTGTGCTACGGTTCAGGAGGTTTTCTTTAAATCACGTT
AAAGTCTAAACACGATGCCAAGTTCCTCAAAAAAGAAATTTAGTGCAA

pDEV Pzia GGCCAGATCTCATATGTTGGAGCCACCCGCAGTTCGAAAAAATCGAAGGG
CCGGCTAGAGTATACACCTCGGTGGGCGTCAAGCTTTTTAGCTTCCC

Met → Strep-tag Factor Xa

pDEV Pzia CGCGATCCGAATTCACTAGTGGGCCGCGATATCCTGCAGTGGAGCCA
GCGCCTAGGCTTAAAGTGATACGCCGGCGCTATAGGACGTCACCTCGGT

*Bam*HI *Eco*RI *Spe*I *Not*I *Eco*RV *Pst*I

pDEV Pzia CCCGCAGTTCGAAAAATAATAATGCAGTAGCATAACCCCTTGGGGCCTC
GGGCGTCAAGCTTTTTATTATTACGTCATCGTATTGGGGAACCCCGGAG

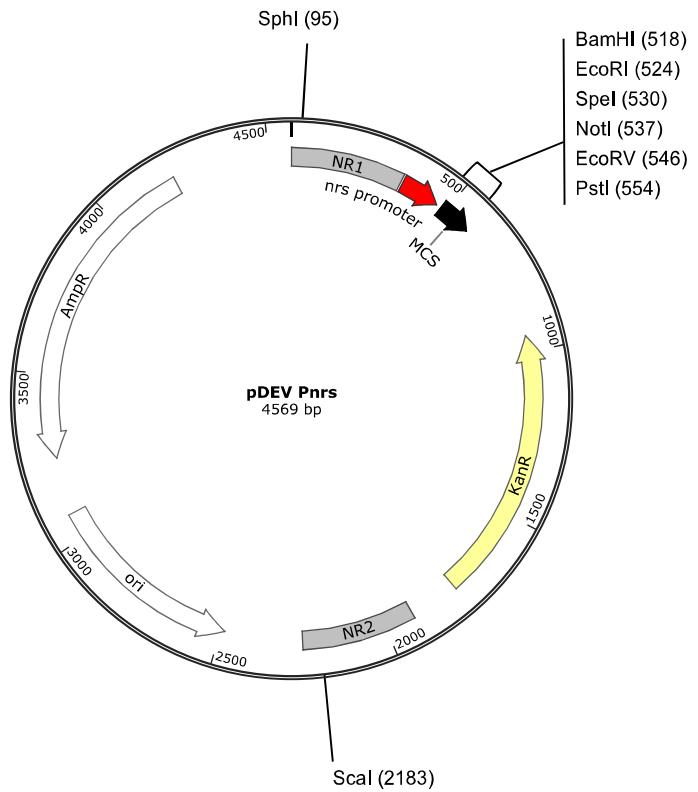
Strep-tag End T7 terminator

pDEV Pzia TAAACGGTCTTGAGGGTTTTTTGATGCAGTATA
ATTTGCCAGAACTCCCCAAAAACTACGTCATAT

pDEV Pnrs

pDEV Pnrs sequence landmarks

NR1	1-339
Pnrs	346-466
Strep-tag I	482-505
Factor Xa	506-517
MCS (<i>Bam</i> HI- <i>Pst</i> I)	518-555
Strep-tag II	556-579
T7 terminator	591-637
Kan ^R	951-1766
NR2	1913-2252
ori	2489-3077
Amp ^R	3248-4213



Pnrs

pDEV Pnrs
 GATCCGCGCTCTGCCTTTTTTATAACGGTCTGATCTTAGCGGGGAAGG
 CTAGGGCGGAGACGGAAAAATATTGCCAGACTAGAATCGCCCCCTTC

pDEV Pnrs
 AGATTTTCACCTGAATTTTCATACCCCTTTGGCAGACTGGGAAAATCTT
 TCTAAAAGTGGACTTAAAAGTATGGGGGAAACCGTCTGACCCTTTTAGAA

pDEV Pnrs
 GGACAAATTCCTCAATTTGAGGTGGTGTGATGAGATCTCAATATGGGAGC
 CCTGTTAAGGGTTAAACTCCACCACACTACTCTAGAGTATACACTCGG

pDEV Pnrs
 CACCCGCGAGTTCGAAAAATCGAAGGGCGCGGATCCGAATTCATAGTG
 GTGGGCGTCAAGCTTTTTAGCTTCCCGGCCTAGGCTTAACTGATCAC

pDEV Pnrs
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 GCCGCCTAGGACGTCACCTCGGTGGGCGTCAAGCTTTTTATTATT

T7 terminator

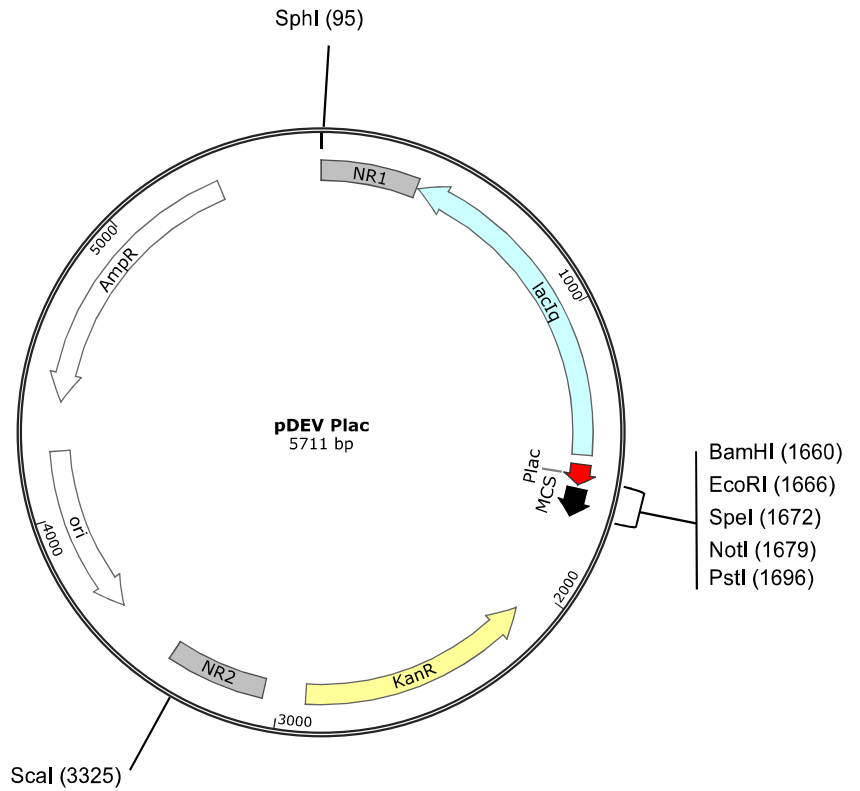
pDEV Pnrs
 TGCAGTAGCATAACCCCTTGGGCCTCTAAACGGTCTTGAGGGTTTT
 ACGTCATCGTATTGGGAAACCCGGAGATTGGCCAGAACTCCCAAAA

pDEV Pnrs
 TTGATGCAGTATA
 AACTACGTCATAT

pDEV Plac

pDEV Plac sequence landmarks

NR1	1-339
lacIq	346-1506
Plac	1540-1611
Strep-tag I	1624-1647
Factor Xa	1648-1659
MCS (<i>Bam</i> HI- <i>Pst</i> I)	1660-1697
Strep-tag II	1698-1721
T7 terminator	1733-1779
Kan ^R	2093-2908
NR2	3055-3394
ori	3631-4219
Amp ^R	4390-5355



Plac

pDEV Plac GATCCAAAGAGTGTGACTTGTGAGCGGATAACAATGATACTTAGATTCA
CTAGGTTTCTCACAACCTGAACACTCGCCTATTGTTACTATGAATCTAAGT

pDEV Plac ATTGTGAGCGGATAACAATTTACACAAGATCTCAATGTGGAGCCACCC
TAAACTCGCCTATTGTTAAAGTGTGTTCTAGAGTATACACCTCGGTGGG

NdeI → Met →

pDEV Plac GCAGTTCGAAAAATCGAAGGGCGCGGATCCGAATTCACTAGTCCGGCCG
CGTCAAGCTTTTTAGCTTCCCGCGCCTAGGCTTAACTGATCACGCCGGC

BamHI EcoRI SpeI NotI

pDEV Plac CGATATCCTGCAGGTGGAGCCACCCGAGTTCGAAAAATAATAATGCAGTA
GCTATAGGACCGTCACTCGGTGGCGTCAAGCTTTTATTATTACGTCAT

PstI Strep-tag End

T7 terminator

pDEV Plac GCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGTTTTTTGATGCA
CGTATTGGGGAACCCCGGAGATTTGCCAGAACTCCCCAAAAAACTACGT

pDEV Plac GTATA
CATAT

Appendix 2

Sequence of *isiA* and pairing of the antisenses T7IsrR and as5UTR*isiA*

CATATATACACCAATCTAAATAAGATCTTTTACACCGCTACTGCAATCAACCTCATCAACAAAATTC
 CCTCTAGCATCCCTGGAGGCAAATCCTCACCTGGCCATGGGTTCACCCTGCTTAACATTTCTTAATAA
 TTTTAGTTGCTATAAATTCTCATTTA**TGCCCTATAATAATTCGGGAGTAAGTGCTAAAGATTCTCAAC**
TGCTCCATCAGTGGTTTGAGCTTAGTCCTAGGGAAAGATTGGCGATCGCCGTTGTGGTTAAGCCAGAAT
AGGTCTCGGGTGGACAGAGAACGCTTATTCTTTGCCTCCATGGCGGCATCCACCTAGGTTTCTCGGC
ACTTATTGCCATAATTATTATTGTGCTCTCAATTAAGGAGGCAATCTGTGCAAACCTATGGCAACG
ACACCGTTCAGTACGAATGGTGGGCCGGCAATGCCGTTTCGCTGATCAGTCTGGGCTTTTTATTGCGG
CCCATGTGGCCAGGCGGCTTTAACAGCATTTTGGGCCGGAGCCTTACCCTGTTTGAATTTCCCGAT
TTGATCCACCCAGGCCATGGGAGACCAGGGATTAATCTACTGCCCATTTGGCTACCCTCGGTTGGG
GTGTGGGCGATGGCGGACAAATTGTGATACTATCCCTACTTTGTCATTGGTAGTATTCACCTGATTG
CTTCAGCGGTACTGGGGGCTGGGGCTTTGTTTCATACCTGCGGGCCCCGCTGACCTCAGTACCCTTA
AGGGCCAGGGCAAAAAATTCATTTACCGTGGGAAAACCCCAGCAACTAGGGATAATTTGGGTATC****
ATCTCCTGTTCCTGGGGCCGGAGCTCTACTGTTGGCAGGCAAAGCCATGTATTGGGGGGATTGTACG****
ACGCCACCACCAAACGGTGCCGCTAGTCAGTCAGCCAACATTAGATCCTTTGGTTATTTATGGTTATC****
AAACCATTTTGCAGCATCAGTAGCCTAGAAGATCTGGTGGGGGACATATTTTGTGGTTTTCTGC
TCATCGGTGGAGGCATTTGGCACATCTGGTGCCCCCTCTGGGTGGGCTAAAAAAGTTCTGCTCTTTT
CCGGGAAGCCATTTTGTCTATTCCCTGGGGGGCATTGCCCTAGCTGGATTGTGGCGGCCTATTTCT
GTGCAGTCAACACCTGGCCTATCCGCCAGAATTTACGGCCCGCCTTTGGCAATTAATTTGGGCATTT
TTCCCTATTTTGCCGATACGGTGGAACTGCCCATGCATGCCACACCAGCCGGGCTTGGTTAGCCAACG
CTCACTTCTTCCTGGCCTTTTTCTTCC**TCCAAGGCCACCTCTGGCACGCACTGCGGGCTTTGGGTTTTG**
ACTTCAAACGGTCGAACAAGCGTTGATTCTTGCAAACCTAGTCAT**TAGCCATCAGCTTTTCTCTC**
 TTAAGCAAGCATCGGGGTTACTTTGCCTAGCTCTGCCAGCCTGAAATTAA

In bold: *isiA* gene (sll0247)

In red: T7IsrR (pairing sequence)

In blue: as5'UTR (pairing sequence)

Sequence of *SynK* and pairing of the antisenses asSynK and as5UTRSynK

GGCCAATTATCTCCCCACCATCGCTTGAAAGCCGTTTATTAGCTTTACAAATGGCCGCCGCTGCA
 TCAATGTTGATTAGATGCTTGATGGCCACGGAGCCAGGGATTATGTTTGGCAAATATCGACAGAAAA
 ATCTTGATAACTGGCATTACCGCAATCTGTTTTGGTCAATTGTTTTATTACTATTTTTTACC**ATGTTT**
GTGAAAACGAGAATGGGGGAACTATTACTTCCATCTTGTTCACGGTGACTATTTTGGTAATGGTAAA
AAATATGGCCATCAGCTCTCTTTGGAAAACCTTTCTACGGGGACTAGTGGCGATCGCCTTGGGCTGTG
ATTTATTAACCTTGTGATTAGTAATCCACCATTCCCAAAGACTCTTTACTTGGGCGGATATAGTC
TATGCGGTGTTCTTCGGGGCGGCAGTGATTACCATTAGCCAACAATTGAATAAGGTCAGAAAGTAGA****
CCAAAATGCTTTATTAGGAGCTATCAGTGTCTATTTACTGATTGGAGTCTTTTGGTTTTTACTCTACC
GGATATCCTATATTATTTCCCCACTAATTTTAACGAATTACAAAGTGATGGGATTAATAATTTTCATC
TTGCTCTATTTTAGCTTTACGACCTTGACCACCCTGGGCTACGGTGACATAACTCCTACGGATTCCAT
TGCCATGGGATTGTCCAACATGGAAGCCATTGTCGGTCAAATGTACTCGGTTATTTTTGTTGCTCGCC
TGGTTAGTCTTTACACGACTGACCTCAATTGCCACAGTCAGATCCGGGAAGAACAGGAATCTGATTAG
 AGCCCATGCGAGAAAATTCATCCCTTGGCACAATGGTAAAAGCGAAACCAAGCCCAAACAGTATTACT
 TAACTTATTTTCATGGT

In bold: SynK gene (slr0498)

In red: asSynK (pairing sequence)

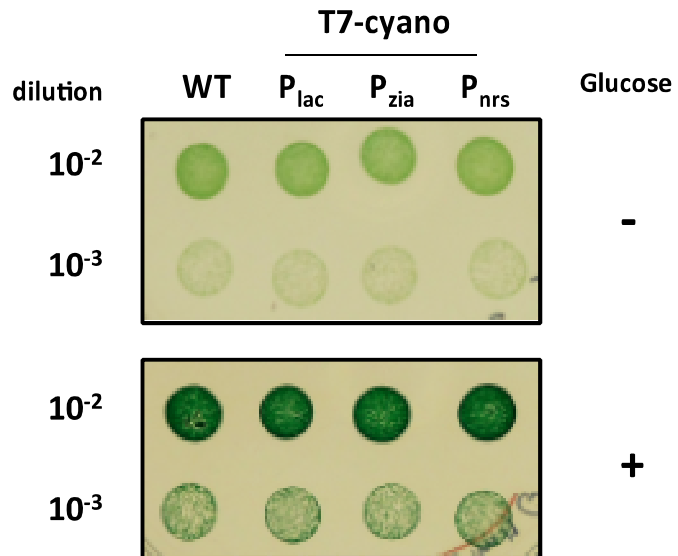
In blue: as5'UTRSynK (pairing sequence)

The sequence of the *antisense asLPOR* covers the full length of LPOR gene

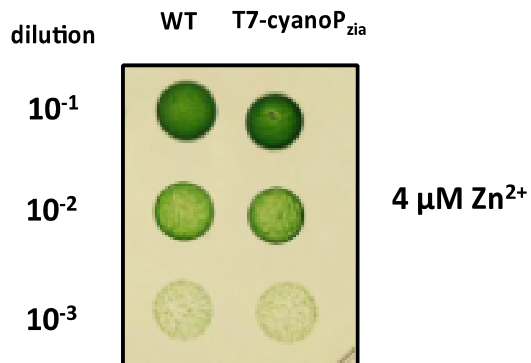
(slr 0506) - not shown

Appendix 3

Growth of WT and T7-cyano strains under standard conditions



Growth of WT and T7-cyanoP_{zia} in presence of 4 μM Zn²⁺



Strains were grown on solid BG11 medium for 120 days under standard conditions (30 °C, at 25 μE). Different conditions are indicated. The experiments were repeated two times.

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