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Investigation of light-inducible P*cpeC* in *Nostoc punctiforme* ATCC 29133

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Abstract

The filamentous cyanobacterium *Nostoc puntiforme* ATCC 29133 is a promising strain for sustainable production of different compounds. The aim of this thesis is to investigate the native light-inducible promoter P*cpeC* as a tool for future biotechnological approaches. For this work the expression levels of different genes of *Synechocystis* sp. PCC 6803 and *Clamydomonas reinhardtii* under the regulation of P*cpeC* were analyzed. The genes were fused to fluorescence protein YFP or GFP to quantify the protein amount by determination of fluorescence. Three *N. punctiforme* strains containing the different constructs were tested under different green light conditions for induction, and under red light as repressing conditions. The results showed that there are different expression levels as well as different growth behavior of the tested strains. Furthermore, we were able to show that P*cpeC* in *N. punctiforme* ATCC 29133 is regulated by different intensities of green light and that this promoter is completely repressed by red light. Thus indicating its usefulness as a tool to regulate expression of recombinant proteins in cyanobacteria.

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Abbreviations:

DXS: 1-deoxy-D-xylulose 5-phosphate synthase

FBP: Fructose-1,6-bisphosphatase

HydA1: Hydrogenase of *Clamydomonas reinhardtii*

PSI: Photosystem I

PSII: Photosystem II

PE: Phycoerythrin

PC: Phycocyanin

SD: Shine-Dalgarno motif

qPCR: Quantitative Polymerase Chain Reaction

PCR: Polymerase Chain Reaction

YFP: Yellow fluorescence protein

GFP: Green fluorescence protein

2 INTRODUCTION

2.1 Fossil fuels vs. Biofuels

About 20 years ago the United Nations signed the Kyoto protocol to restrict the emission of greenhouse gases to reduce the greenhouse effect. The greenhouse effect is a major player in climate change and therefore responsible for the extinctions of several species, the melting of glacier and weather extremes.

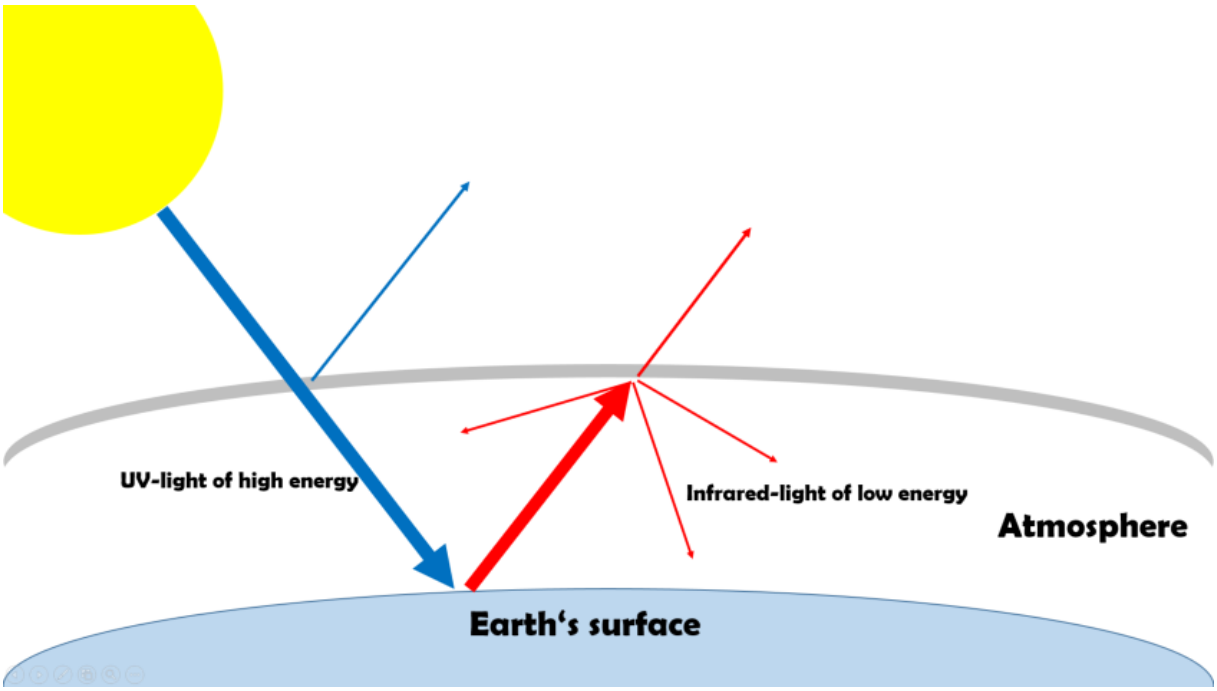

Figure 1: Schematic process of the greenhouse effect. Light, which is emitted by the sun enters the earth's atmosphere in form of UV-light of low wavelength and high energy. The energy is absorbed by the earth's surface and then reemitted in form of infrared light of long wavelength and low energy. The light can be absorbed by greenhouse gases and then emitted again into all directions. The result of this effect is an increasing temperature on the earth's surface.

One of the reasons of the increased greenhouse effect [1] is the CO_2 emission due to burning of fossil fuels. Fossil fuels were made and stored during the last 300 to 400 million years, when plants and small organism (for example plankton) were covered with sedimantary layers of sands, rocks and soils [2]. The high pressure of the layers created coal, oil and natural gas over the years. Because of this long producing period they are called non renewable fuels. An alternative to these environmental damaging fuels would be biofuels, which are produced by biological materials. Biological carbon based materials are produced through carbon fixation,

which means that the carbon needed to form the plant is taken from the CO_2 within the air. Therefore biofuels can be seen as CO_2 neutral, because they cannot emmit more CO_2 then they take out of the air. Because of the fast growth of plants compared to the formation of fossil fuels, biofuels are renewable fuels. A big problem, which arises with biofuels is the tremendous amount of space that is needed to grow the plants for biofuel production to fulfill the amounts desired by human. According to *Cai et al*. (2011) with the available land, only about 26 – 55 % of the current world liquid fuel consumption could be covered [3]. Due to the expected increase of the worlds population to 9,7 billion people until 2050, the demand of food and energy will further increase [4].

So there are fossil fuels, which are quite cheap, but release enormous amounts of CO_2, which is currently conserved in form of coal, oil and natural gas beneth the earth's surface. Furthermore, the unequal distribution of fossil fuels often lead to conflicts and wars, seen during the last decades in the Middle East [5].

On the other hand there are biofuels produced by plants, which keep the CO_2 level in the earths atmosphere constant, but also need agricultural land for their production. A solution for energy production which does not compete with agricultural land is already known: Cyanobacteria.

2.2 Cyanobacteria

Cyanobacteria are a group of gram-negative bacteria that can use solar energy, CO_2 and water to produce a large variety of industrial valuable commodities [6] as well as fuels [7]. The oldest known fossils – more than 3.5 billion years old – derive from cyanobacteria [2]. Over this timeframe they had an important impact on evolution through the fact that the oxygen atmosphere we breathe today was generated by numerous cyanobacteria during Archean (4000 to 2500 million years ago) and Proterozoic (2500 to 542 million years ago) eras [2].

They are also seen as the origin of plants. Plants use organelles called chloroplasts to harvest the energy of light and fix CO_2 to build up their structure. The endosymbiotic theory explains this as cyanobacteria living within plant's cells. Also, the eukaryotic mitochondria derive from symbiosis with heterotrophic bacteria [8].

Cyanobacteria are able to live in nearly every aquatic and terrestrial habitat [9]. Because of their ability to use photosynthesis, their aquatic attendance and their color they are often called “blue-green algae” although there is no relationship between cyanobacteria and algae, which belong to the domain eukaryote [2].

Cyanobacteria did not only play an important role in earth’s history, they are also seen as a promising platform for future biofuel production, but also for production of other industrial relevant chemicals, for example isoprene [10], mycosporines as well as therapeutics, insecticides and anti-fouling agents [6]. Through their ability to use CO_2 and light as their main carbon and energy source and the possibility to grow in non-agricultural land, they do not compete with food plants. Taking the growing world population into account and the agricultural land that will be needed for food production, the cultivation of cyanobacteria in oceans or lakes could be a cheap alternative to biofuel production via terrestrial plants.

Cyanobacteria occur free-living or in symbiotic relationships with plants or fungi. There are some unicellular forms as the widely used and well-studied *Synechocystis* sp. PCC 6803, but they also appear in filamentous forms as *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* ATCC 29133. Filamentous cyanobacteria can differentiate in three different forms: under favorable growing conditions they occur as vegetative cells, under environmental stressful conditions they form resistant akinetes and under nitrogen starvation they form heterocysts. In heterocysts nitrogen fixation takes place, in which the biofuel H_2 is produced [11].

2.3 Oxygenic photosynthesis

Oxygenic photosynthesis enables photoautotrophic organism (for example cyanobacteria) to harvest the energy of light and to use CO_2 as a carbon source instead of organic compounds as for example sugar. Photosynthesis is divided into a light dependent part, where the energy of light is consumed and high energetic compounds of adenosine triphosphate (ATP) and NADPH are produced, and a light independent part where the produced ATP and NADPH are consumed within the Calvin – Benson – Bassham cycle (2.3.2. Light independent reaction) to produce carbohydrates [12]. In cyanobacteria, the first part takes place in the thylakoid membrane (Figure 2), whereas the second part is located in the cytoplasm [12].

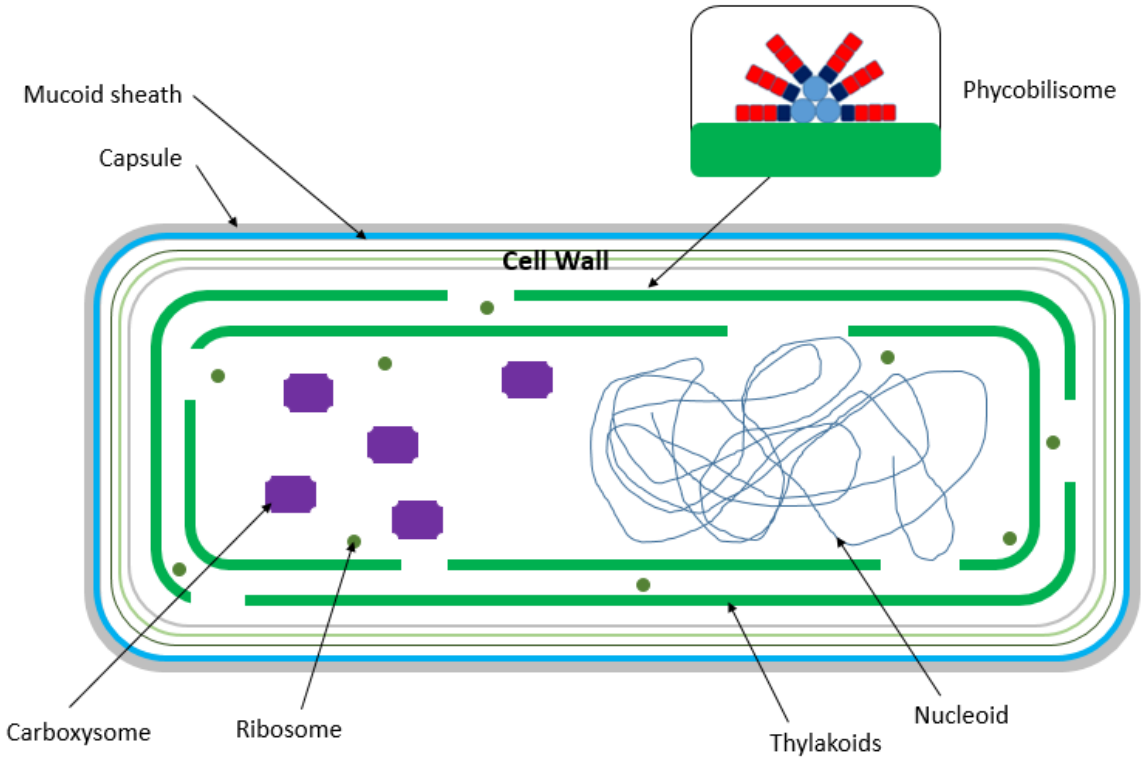

Figure 2: Structure of cyanobacteria and phycobilisome on thylakoid membrane. Cyanobacteria are surrounded by a thick cell wall. Above the cell wall there is a mucoid sheath and the capsule, which is a layer outside the cell envelope and consists mainly of polysaccharides. The thylakoid membranes are free in the cytoplasm and contain both photosystem I and photosystem II as well as the attached phycobilisomes as light harvesting complex for photosynthesis. The DNA lies coiled in a fibrillar structure in the center of the cytoplasm (Nucleoid). The ribosomes are responsible for protein translation. The carboxysomes contain RuBisCo for the Calvin-Benson-Bassham cycle [13].

2.3.1 LIGHT DEPENDENT REACTIONS

In the light dependent reactions [12] ATP and NADPH are produced for the fixation of CO_2 in the light independent part.

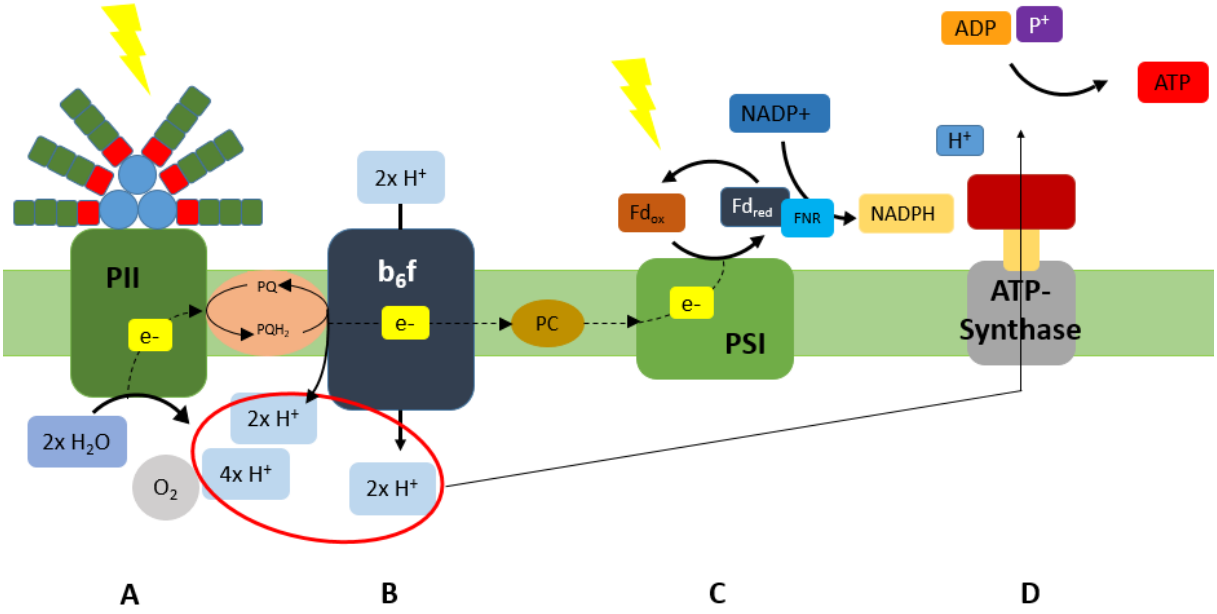

Figure 3: Electron transport chain. The energy of light is absorbed in photosystem II (PSII) and I (PSI) to create electrons by splitting water with high reducing potential. The electrons of PSII are transferred to plastoquinone (PQ) and via cytochrome b_6f to plastocyanin (PC). In the reaction center of PSI, electrons, which are transferred from PC are used to reduce ferredoxin and in a further step NADPH is created. The proton gradient produced during these processes is used by ATP-synthase to create ATP [12].

In Figure 3 the electron transport chain of photosynthesis in the thylakoid membrane is shown. It consists of 4 major complexes [12]:

1. Photosystem II (Figure 3 A): Photons are absorbed by the phycobilisomes on the surface of the thylakoids. The energy is then transferred to a chlorophyll pair in the center called special pair. Through a pheophytin and a plastoquinone, electrons are transferred to another plastoquinone (PQ) which picks up 2 protons and becomes plastoquinol (PQH_2). The oxidized special pair gains the missing electrons from a water molecule, which is split by a Mn - complex. Through this reaction 2 protons per H_2O are produced inside the lumen and a proton gradient is produced.

2. Cytochrome b_6f (B): Electrons are transferred from plastoquinol (PQH_2) to plastocyanin (PC) while 4 protons are transferred to the lumen which results in a proton gradient.
3. Photosystem I (C): Plastocyanin gets oxidized and the electrons are transferred to the reaction center of PS I, a modified chlorophyll a molecule. Through the absorption of photons by the phycobilisomes and energy transfer to the reaction center, electrons are excited and through several intermediate carriers ferredoxin is reduced. The electrons are then transferred from ferredoxin to $NADP^+$ to create NADPH by ferredoxin-$NADP^+$ reductase (FNR).
4. ATP-Synthase (D): Protons are pumped from the lumen to the stroma. The energy is used by ATP-synthase to create ATP out of adenosine diphosphate (ADP) and phosphate.

2.3.2 LIGHT INDEPENDENT REACTION

The energy that is created by the light dependent reaction is then consumed in the Calvin - Benson - Bassham cycle in the cytoplasm to convert carbon dioxide into hexoses. The enzymatic reactions of the Calvin - Benson - Bassham cycle are shown in Figure 4 [12].

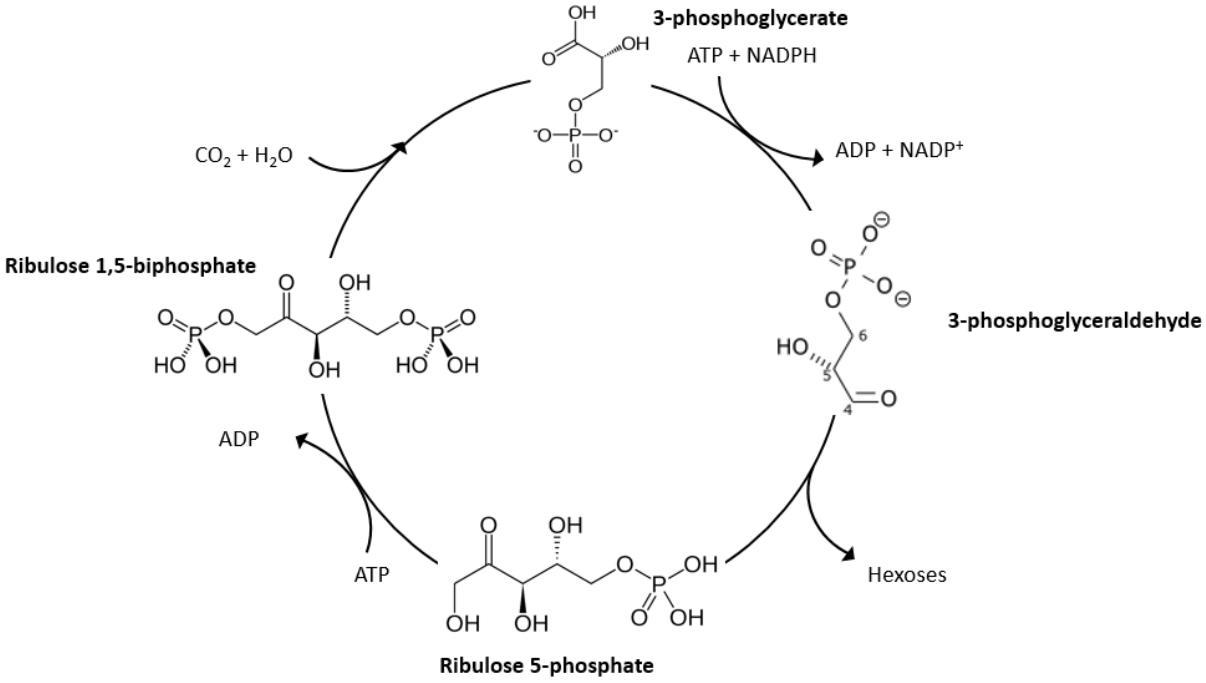

Figure 4: Calvin-Benson-Bassham cycle. The Calvin cycle explains the carbon fixation in photoautotrophic organism. In the first step ribulose-1,5-bisphosphate is carboxylated. Then 3-phosphoglycerate is phosphorylated and ATP is consumed. Through the reduction of 1,3-Bisphosphoglycerate (1,3BPGA), 3-phosphoglyceraldehyde (G3P) is produced and NADPH is oxidized. G3P is then used to produce Hexoses and to regenerate ribulose-1,5-bisphosphate via ribulose 5-phosphate and consumption of ATP.

The Calvin - Benson - Bassham cycle consist of several steps [12]:

1) 3x Ribulose-1,5-bisphosphate (RuBP) are carboxylated by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase to form 6x 3-phosphoglycerate (3PGA). In this step the CO_2-fixation takes place.
2) 6x 3-phosphoglycerate (3PGA) are phosphorylated by a phosphoglycerate kinase to 6x 1,-3-bisphosphoglycerate (1,3BPGA). 6x ATPs are consumed during this reaction.
3) The glyceraldehyde 3-phosphate dehydrogenase reduces 6x 1,3-bishposphoglycerate to 6x glyceraldehyde 3-phosphate (G3P). During this reaction 6x NADPH are oxidized to 6x $NADP^+$.

4) In several steps 5x glyceraldehyde 3-phosphate are converted into 3x ribulose 5-phosphate (R5P).
5) 3x ribulose 5-phosphate are phosphorylated to 3x ribulose-1,5-bisphosphate by consuming 3 ATP.

The net gain of the Calvin cycle is one G3P per three CO_2 molecules. The G3P is further converted to hexoses like glucose.

2.4 Phycobilisomes

Cyanobacteria harvest the energy of light through light harvesting complexes called phycobilisomes which are located on the thylakoid membrane on PSII but are also associated with PSI [14]. Cyanobacteria and red algae evolved phycobilisomes to use the energy of wavelengths between 500 and 600 nm, which cannot be absorbed by chlorophyll a and chlorophyll b of PSI and PSII, which are the main light absorbing molecules (Figure 5) [14].

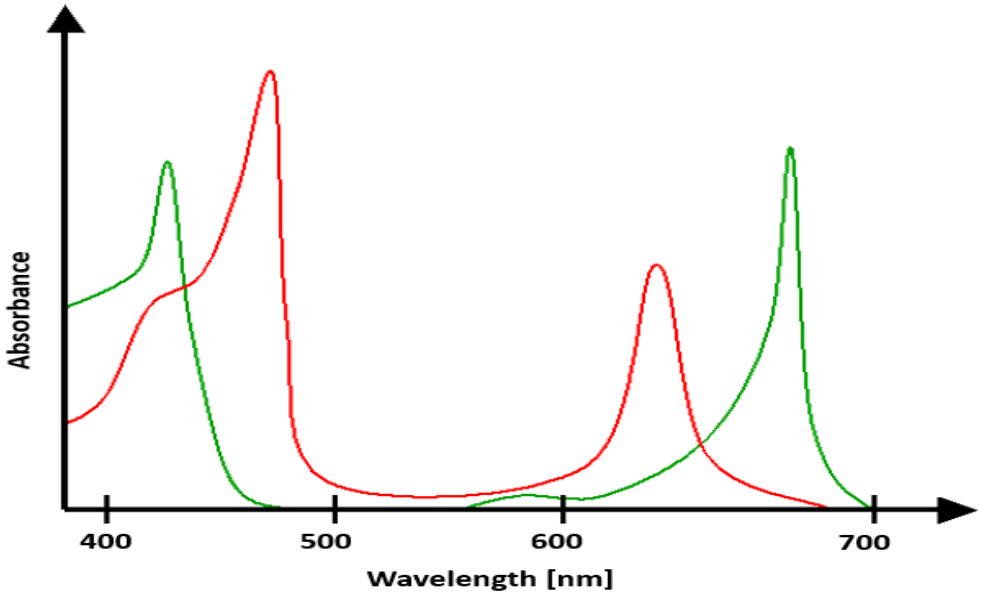

Figure 5: Absorption spectra of Chlorophyll a and Chlorophyll b.

Phycobilisomes consist of a core structure which consists of allophycocyanin (AP) and several structures build by phycoerythrin (PE), phycocyanin (PC) and several linker proteins (Figure 6) [15]. The rods are responsible for the light absorption and transfer of the energy to the core structure and then to the reaction center of PSII [16].

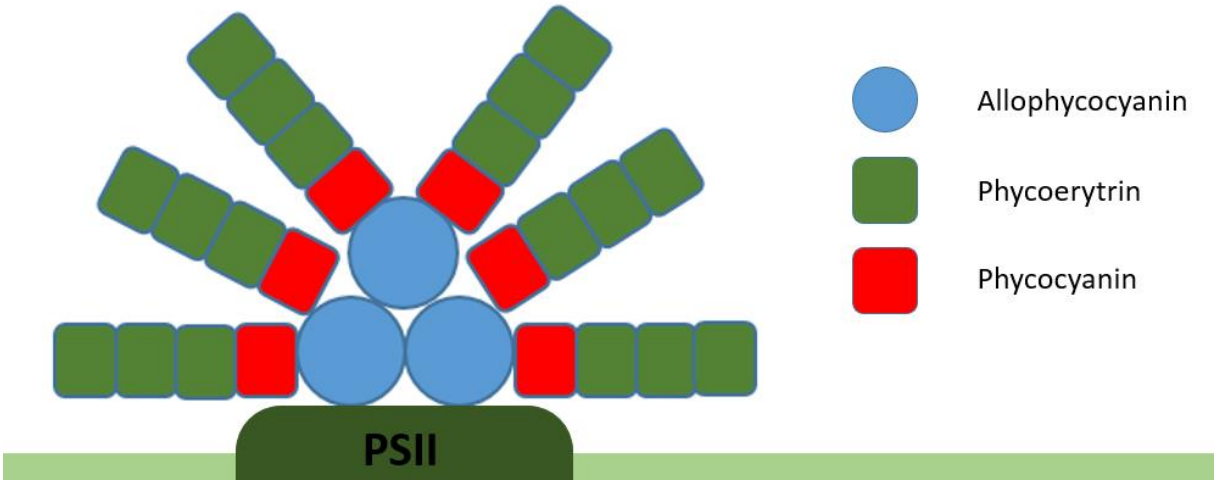

Figure 6: Structure of phycobilisomes. Phycobilisomes are light harvesting complexes located on the thylakoid membrane of cyanobacteria and red algae. They consist of a core structure (blue circles) of allophycocyanin and a rods structure of phycocyanin (blue squares) and phycoerythrin (red squares).

In Table 1 the absorption maxima of the phycobiliproteins are shown [17] [18] [19] [20].

Table 1: Absorption wavelength of phycobilisome components.

Phycobiliprotein	Absorption maxima [nm]
Allophycocyanin	633
Phycocyanin	~ 550, ~615
Phycoerythrocyanin	~575
Phycoerythrin	496, 529-534, 555

2.5 Promoters & Gene expression

For biotechnological applications, it is of big importance to change the producing organism genetically to optimize the cultivation of the producing strain and the production of the desired product. It is also important to knock out genes of pathways, which compete with the production pathway, but also to insert the gene for the biosynthesis of the product of interest in single or multiple copies. The genes are inserted under either a constitutive promoter, which leads to a constant transcription levels, or an inducible promoter, which expresses the gene after induction [21]. Which promoter is used depends on the effect of the expressed protein on the cells. If the protein has no negative effect on the cell, it is no problem to express it under a constitutive promoter, because the growth of biomass is not inhibited, whereas in most cases it is important to first gain biomass and then produce the desired protein in a second step where the protein of interest is produced by inducing gene expression. A high amount of biomass is needed to have lots of producing cells. Usually the induction of gene expression is achieved by addition of an inducing agent to the cultivating media (for example IPTG for the lac Operon [22] (Figure 7)), which will activate the promoter and the gene of interest will be expressed. It is important that the inducing agent is not toxic to the cell, only affects the expression of the gene of interest, and is easy to apply or remove [23].

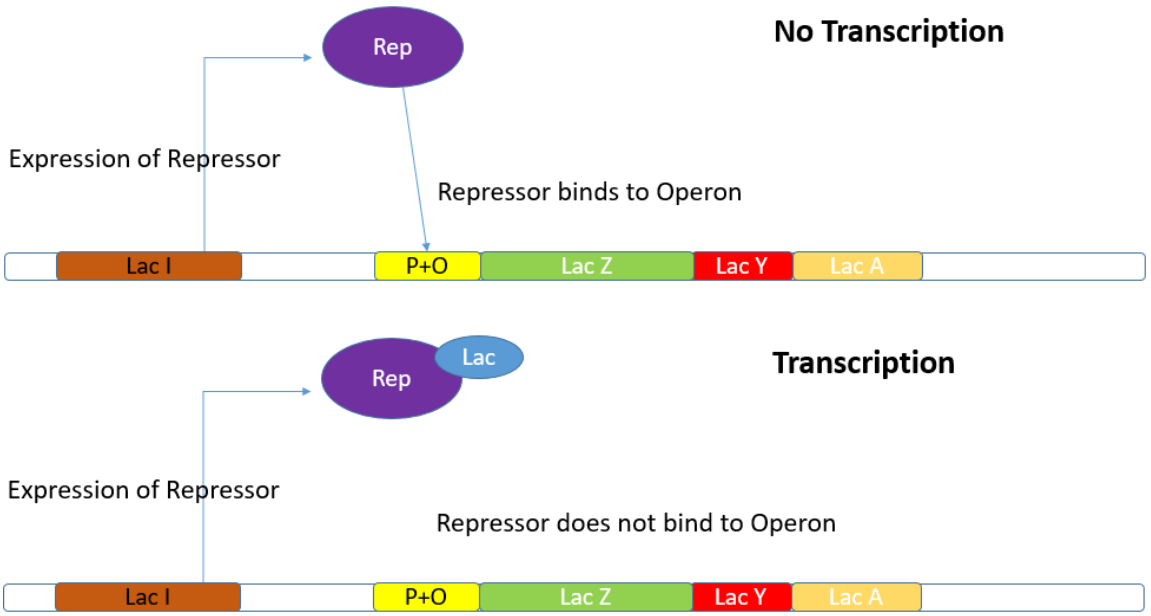

Figure 7: Lac Operon. *LacI* is the gene encoding the *lac* repressor, which binds to the operator of the lac-operon and stops transcription if no Lactose is available. In presence of Lactose, it binds to the lac repressor and transcription of the lac operon is started [16].

A protein is produced through two different steps: First the DNA is transcribed by the RNA-polymerase into mRNA and in the second step the mRNA is translated by the ribosome into a protein, which is an amino acid chain (Figure 8).

Figure 8: Steps of protein expression. DNA is transcribed into messenger RNA (mRNA) by the RNA-Polymerase in a step called transcription. In a second step called translation, mRNA is translated into an amino acid chain by a protein-RNA-complex called the ribosome.

The transcription level of a gene under a specific promoter depends on the nucleotide sequence of the promoter [21]. Therefore in theory, there should be no difference on transcription level of different constructs expressed under the same promoter. The variability of protein amount within the cell that may occur, even by using the same promoter for the different genes, derives from posttranscriptional regulation that depends on the ribosome binding probability to the mRNA or the stability of the mRNA [24]. This in turn determines the translation level. The mRNA is a single stranded nucleotide chain and is able to form different secondary structures, which is an important factor for posttranscriptional regulation including mRNA stability [24].

3 ABOUT THE PROJECT

3.1 Motivation and aim of the project

The aim of the project was to investigate the regulation of the light-inducible promoter P*cpeC* in the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133, and to investigate if the expression levels of different genes of interest (GOI) are differentially regulated by this promoter. The project was divided into two major tasks defined by two questions:

1) Is the expression level of a downstream gene under P*cpeC* in *Nostoc punctiforme* ATCC 29133 depending on the expressed gene or is there a constant expression level for all genes?

To answer this question I investigated the expression levels of different genes under the P*cpeC* in *N. punctiforme*.

5'UTR	Gene of interest	3'UTR

Figure 9: Structure of a mRNA. A mRNA consist of a 5' untranslated region (5' UTR) where the ribosome binds to start translation. The gene of interest describes the amino acid sequence in a 3 nucleotides based code, and the 3' untranslated region (3' UTR) contains the termination sequence for the transcription. Neither the 5' UTR nor the 3' UTR are part of the protein.

In Figure 9 the main parts of the mRNA are shown. The ribosome binding site is located in the 5' untranslated region (5' UTR). In case of highly conserved secondary structures within the 5' UTR the probability of the ribosome to bind should be always the same, and the expression level should be similar [25]. If there is any difference in expression level of the different proteins this would lead to the assumption that the GOI part of the mRNA might form different secondary structures with the 5' UTR which leads to different binding probabilities of the ribosomes to the ribosomal binding site [25].

One attempt to solve the problem of different expression levels is to insert a short fragment of DNA after the 5' UTR region. The interaction between the short inserted fragment and the

5’ UTR region is predictable because it is always the same, independent on the GOI. This construct is called bicistronic design (BCD) (Figure 10) [25]:

Figure 10: Structure of a bicistronic design (BCD). A BCD is a translational regulation approach. Two peptides are expressed on the same mRNA under a promoter (red). The transcription start point is shown as ‘+1’. The ribosome binds to the ribosomal binding site (SD1) of the first gene and melts the secondary structure of the mRNA while the translation, which is started at ATG (Start codon). The ribosomal binding side (SD2) for the second gene is within the sequence of the first gene and therefore no secondary structure is present, which would influence the binding probability of the ribosome. The binding probability of the ribosome to SD1 is constant because of its constant secondary structure. The ribosome starts translation at the second start codon (ATG) which overlaps with the stop codon of the first protein (TAA) [25].

The ribosome binds to the Shine-Dalgarno motif (SD1) on the mRNA and starts translation of the inserted fragment (Figure 10, bright green). During the translation of the mRNA, the secondary structure of the mRNA gets melted. The following Shine-Dalgarno motif (SD2) inside the cistron of the leader peptide is now free for another ribosome to bind and does not have any secondary structure which would hinder the ribosome to bind. The mRNA is constructed so that the last nucleotide of the stop codon of the leader protein is the first nucleotide of the start codon, ATG, of the GOI (dark green). The translation rate is just dependent on the free energies of the binding 16S rRNA and the free SD2 sequence. The short peptide produced in this construct should show no further impact on the cells [25].

2) Can the expression level of different genes under P*cpeC* in *Nostoc punctiforme* ATCC 29133 be regulated by different light intensities?

For biotechnological applications, not only the induction of a promoter is of big importance but also the ability to regulate the expression to different levels. I was interested to study the regulation potential of P*cpeC* and therefore, the potential to regulate protein expression under P*cpeC* in *N. punctiforme* under different light intensities was investigated. Furthermore, the ability to repress gene expression is of big interest, because genes regulated by leaky promoters can lead to cultivation- and experimental problems [26]. For this reason the repression capability of P*cpeC* in *N. punctiforme* was also investigated.

3.2 Experimental Realization

To quantify the protein levels there are several different methods, for example Western blot, mass spectrometry, enzyme specific reactions and by using a promoter-reporter gene construct. We decided to transform *N. punctiforme* with genetic constructs in which the gene of interest was fused to the gene encoding a Yellow fluorescence protein (YFP) at the 3' end, and the P*cpeC*-promoter was introduced 5' of the GOI, to express the genes encoding the fusion protein. The amount of protein was determined by the level of fluorescence signal of the YFP protein.

3.3 P*cpeC*

It is known that many cyanobacteria species are able to regulate the PE/PC ratio of the phycobilisomes under different light conditions in a process called 'complementary chromatic adaption' [15]. Under green light the PE/PC ratio is increased, whereas it is reduced under red light. Some cyanobacteria are just able to regulate the PE content of the rods (group II) whereas others are able to regulate both, PE and PC (group III). The 'complementary chromatic adaption' in *N. punctiforme* is regulated by a complex system shown in Figure 11 [15].

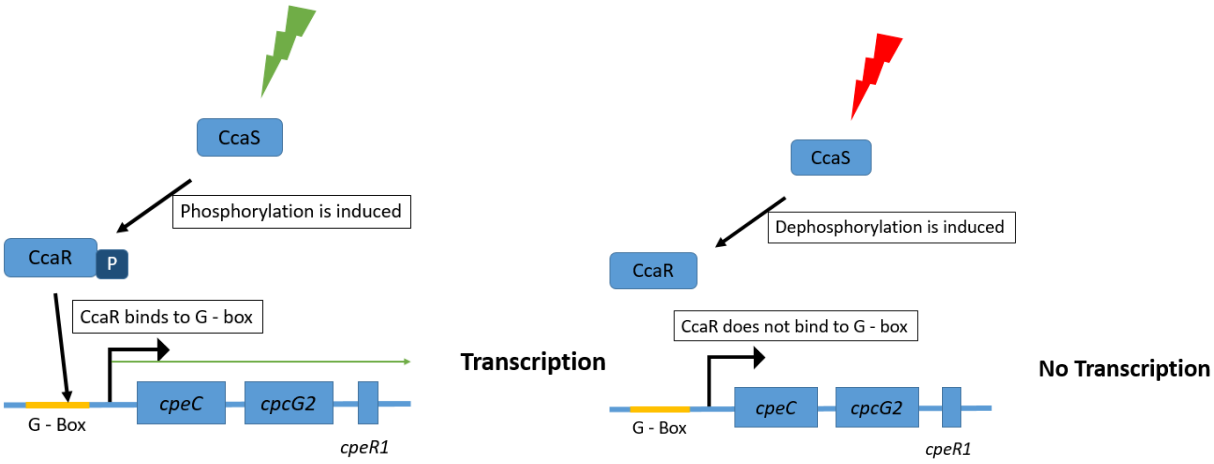

Figure 11: Mechanism of light inducible P*cpeC*. Induction (Left): The histidine kinase, which is activated by green light, phosphorylates the transcription factor CcaR. CcaR binds to the G-box of P*cpeC* and the transcription of *cpeC* is started. Repression (right): The histidine kinase, which is deactivated by red light, does not phosphorylate the transcription factor CcaR. CcaR cannot bind to the G-box of P*cpeC* and the transcription of *cpeC* is repressed. Modified from Hirose et al. (2010) [15].

P*cpeC* is the promoter in which the G-box is located and thus regulates the transcription of the operon *cpeC-cpcG2*. This operon is regulated by the two regulators, CcaS and CcaR, and the P*cpeC*. The cyanobacteriochrome CcaS of cyanobacteria consists of several parts, including a histidine kinase and a GAF domain. In attendance to green light, the chromophore of CcaS changes its conformation in the GAF domain, so that CcaS is able to phosphorylate the transcription factor CcaR, which then binds to the G-Box in the P*cpeC* and the transcription is started. The G-box is a conserved direct-repeat motif of [CTTTNCNATTT] x 2, which is a common structure of a binding site of transcription factors, where the protein binds as a dimer. In attendance to red light the chromophore changes its conformation to a state that CcaS cannot phosphorylate CcaR any longer and thus the CcaR cannot bind to the G-Box any longer and the transcription is repressed [15].

3.4 Nostoc punctiforme ATCC 29133

The cyanobacterium used in this study is *Nostoc punctiforme* ATCC 29133, a filamentous cyanobacterium, which was isolated from a symbiotic association with the gymnosperm cycad *Macrozamia* sp. roots in Australia. *N. punctiforme* is a photoautotrophic organism, but can also grow rapidly in complete dark conditions, if sucrose, glucose or fructose are available [27].

4 MATERIAL & METHODS

4.1 Instruments

- Thermocycler: - Biorad MJ mini gradient thermal cycler
- Spectrophotometer: - Varian Cary® UV-vis spectrometer
  - Thermo scientific NanoDrop 2000 UV-vis spectrometer
- Plate reader: - Hidex Chameleon 4.47
- Sonicator: - Sonics Vibra-cell VC 130
- Electroporator: - Bio Rad Gene Pulser Xcell Electroporation System
- Confocal microscope: - Leica DM6000 CS confocal microscope

4.2 Bioinformatics

The sequences of the Promoter and the Genes for the Fusion proteins where obtained from *Cyanobase – Kazusa Genome Resources*. For the P*cpeC* a sequence of 264 nt upstream the *cpeC* in *N. punctiforme* was used (sequences shown in appendix).

To design primers and align sequences the two free softwares *ApE- A plasmid Editor* (http://biologylabs.utah.edu/jorgensen/wayned/ape/) and *SnapGene Viewer* (http://www.snapgene.com/products/snapgene_viewer/) were used.

4.3 Construction of the plasmids

4.3.1 GENES OF INTEREST

The genes which were used for the fusion protein with YFP were:

- ***Slr1192*** (1008 bp): Alcohol dehydrogenase of *Synechocystis* sp. PCC 6803
- ***HydA1*** (1296 bp): Hydrogenase of *Clamydomonas reinhardtii*; HydA1 is a promising hydrogenase for H_2 production which is used in several projects in cyanobacteria.

- ***DXS*** (1920 bp): 1-deoxy-D-xylulose 5-phosphate synthase of *Synechocystis* sp. PCC 6803; DXS is an enzyme of the MEP (methylerythritol-4-phosphate) pathway for terpenoid production.
- ***FBP*** (1035bp): Fructose-1,6-bisphosphatase of *Synechocystis* sp. PCC 6803; FBP is an enzyme of the gluconeogenesis, the pentose phosphate pathway and the Calvin cycle

The constructs P*cpeC* – GOI (gene of interest) were produced by overlap extension PCR with the sequence of P*cpeC* in *N. punctiforme* and the GOI (sequences of are shown in appendix).

4.3.2 PLASMID

The plasmid that was used to transform *Escherichia coli* and *N. punctiforme* was pSAW which derives from pSCR119 [28], is a plasmid constructed by Adam Wegelius in our lab (Figure 12).

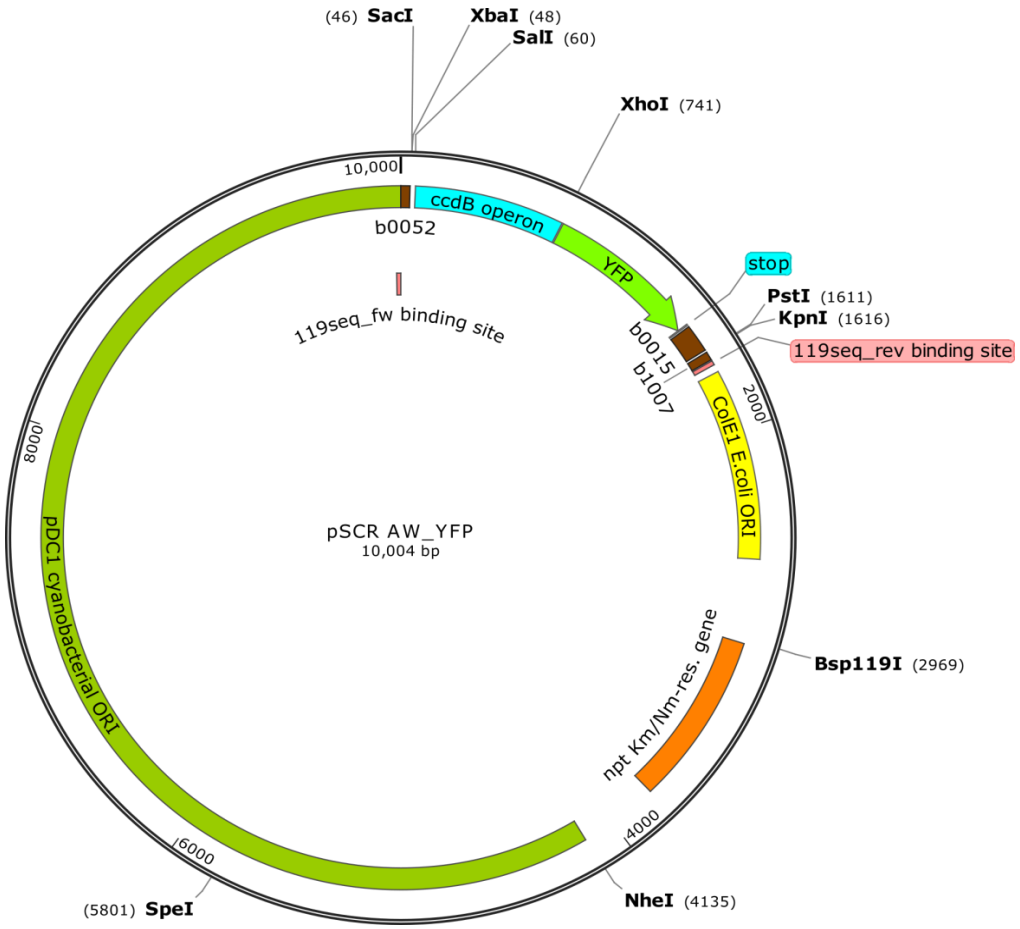

Figure 12. Self-replicative vector pSAW. pSAW is a 10kb-plasmid with an origin of replication (oriC) for *Escherichia coli* and *Nostoc puntiforme*. It has a kanamycin resistance cassette, multiple restriction sites and a lethal *ccdB* operon.

4.3.3 CONSTRUCTION OF THE PLASMIDS

Between the genes and YFP a 4 amino acid-linker (GSGS) was put to ensure the right folding of YFP. To the primer sequence the nucleotide-sequence of the restriction sites and the linker were added. The constructs and the plasmid were cut with Sac1 and Xho1 enzymes of FastDigest by Thermo Scientific. The cut fragments were purified by the Thermo Scientific GeneJET PCR Purification kit. The ligation into pSAW was performed by the manufacturing instruction with the New England Biolabs QuickLigase. The final ligation mix was used for transformation into *E. coli*.

4.4 Culture conditions of *Escherichia coli*

E. coli was cultivated in 20 ml of lysogeny broth media (LB), supplemented with 50 µg of kanamycin. The cultures were grown over night at 37 °C on a shaking Table.

4.5 Transformation in *Escherichia coli*

For transformation of *E. coli* dH5α, one vial with 100 µl of competent cells were put on ice and 5 µl of ligation mix (produced in 4.3.3) were added. After 30 minutes the cells were put into a water bath (42 °C) for 40 s and filled up with LB-media to 1 ml. The suspension was incubated at 37 °C for 1 h and spun down for 3 min at 6000 rpm. A volume of 800 µl of supernatant was removed and the pellet in the remaining media diluted. The final 200 µl were plated on LB agar plates, which were supplemented with 50 $\mu g \cdot ml^{-1}$ kanamycin.

4.6 Culture conditions of *Nostoc punctiforme*

4.6.1 LIQUID CULTURE

The wild type of *N. punctiforme* and the transformants were grown in Erlenmeyer flasks in BG11 (Appendix) with 5 mM NH_4Cl and 10 mM HEPES pH 7.5. For the transformants - with the plasmid pSAW - kanamycin (25 µg·ml^{-1}) was added.

4.6.2 PLATES

For growing the different strains on plates, the media was prepared equal to liquid culture. Additionally, 10 g·L^{-1} agar were added.

4.6.3 LIGHT CONDITIONS & TEMPERATURE

The wild type of *N. punctiforme* was always grown under 15 µmol photons·m^{-2}·s^{-1} white light. After transformation, the strains were grown under non-inducing 15 µmol photons·m^{-2}·s^{-1} red light (LED panel; Green Energy Star).

For testing the constructed strains on induction, different light intensities of green light where set with a multispectral LED lamp (Heliospectra). The emission spectra are shown in Figure 13.

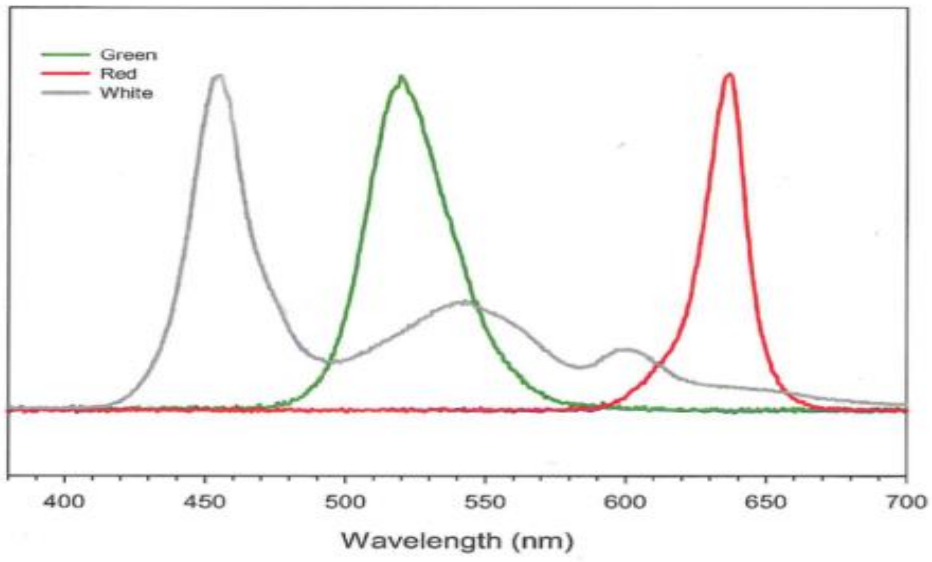

Figure 13: Emission spectra of white, red (LED panel; Green Energy Star) and green light (multispectral LED lamp; Heliospectra).

The cultures were all kept at 25 °C. Additionally, all liquid cultures were kept on a shaker to get homogenous conditions.

4.7 Determination of Chlorophyll a content

To determine the chlorophyll content 1 ml of sample was taken and spun down at maximum speed (13 000 rpm) at room temperature. Then 90 % of the supernatant was removed and the same amount of 100 % methanol were added. After shaking on a micromixer (vortex) and fully suspending of the pellet the tube was put into dark for at least 5 min. After extraction, the solution was spun down to remove the cell debris. The supernatant was put into a cuvette and the absorbance at 665 nm was measured against a 90 % Methanol blank with the UV-vis spectrometer.

According to the extinction coefficient of 78.74 $L^{-1} \cdot g^{-1} \cdot cm^{-1}$ for Chlorophyll a, the Abs_{665} value is multiplied with 12.7 to get µg chlorophyll $a \cdot ml^{-1}$. The calculation can be adjusted for different dilutions of the culture.

4.8 Transformation by electroporation of *Nostoc punctiforme*

30 ml of exponentially growing *N. punctiforme* wild type was concentrated by centrifugation (4000 rpm, 5 min). The supernatant was removed to get a volume of 5 ml. The concentrated culture was sonicated 2 times with a Sonics Vibra-cell CV130 (Amp 20-30, pulse 1p 1.0 s, 30 s). On the light microscope, the cells were observed to be sure they were healthy and single cells were available. In case the cells were not separated, the sonication was done a 3rd time. The sonicated cells were diluted with media to 25 ml into Erlenmeyer flasks. After recovering for 4 h at 30 °C and weak illumination they were spun down (3500 rpm, 5 min) and suspended in 20 ml HQ. This washing step was repeated 4 times before they were suspended with HQ to a final volume of 1 ml. The chlorophyll a content was measured (measurement according 4.7) and the concentration of the cells was adjusted to 50 – 100 µg Chlorophyll $a \cdot ml^{-1}$. A volume of 40 µL was mixed with 1 - 3 µg plasmid DNA in an Eppendorf tube and kept on ice. The suspension was transferred to a pre-cooled electroporation-cuvette. The electroporation was

done with a Bio Rad Gene Pulser Xcell Electroporation System (2400 V, 5.0 ms exponential decay pulse).

The cells were suspended in 20 ml media and incubated overnight in Erlenmeyer flasks at 25 °C and 10 µmol photons·m^{-2}·s^{-1} white light. The next day, the cells were concentrated (4000 rpm, 10 min) to 200 µl. The concentrated cells were then plated onto cellulose filter on ager plates supplemented with 25 µg kanamycin·ml^{-1}.

The plates were put at 10 µmol photons·m^{-2}·s^{-1} white light and 25 °C for 25 – 35 days until colonies appeared.

Due to problems with the transformation of *N. punctiforme*, the transformation protocol above was modified for several approaches. Therefore, the transformation was done without sonication, just one sonication step as well as 4 sonication steps. Also to break the filaments, different sizes of needles was tried.

4.9 Fluorescence measurement

4.9.1 MEASUREMENT WITH PLATE READER (HIDEX CHAMELEON 4.47)

The Fluorescence measurement was used to determine the expression level of the fusion proteins. The different strains were grown under red light until the exponential phase was reached. Afterwards, the chlorophyll content was measured and triplicates of 5 ml cultures were prepared in 6-well plates with a concentration of 1 µg of chlorophyll a·ml^{-1} of culture. Duplicates were taken at time point 0 h, 24 h and 48 h.

200 µl of culture were taken and put into a well of a transparent 96-well plate. The excitation filter was set to 485 nm while the emission filter was at 535 nm. With the chlorophyll a concentration (measurement according 3.7) the Fluorescence signal per µg of Chlorophyll a could be calculated.

The whole experiment was done in a 25 °C growth room.

4.9.2 CONFOCAL MICROSCOPY

To image the YFP amount which was expressed, a DM6000 TM fluorescence confocal microscope (Leica Microsystem) was used. 20 µl of culture were put on glass slides and illuminated with an argon laser light source at 20 % power. The emission wavelength was set to 488 nm and 20 % power. The pictures were taken with a HCX PL Fluotar 40x objective. The wavelength of the two detectors were set (Table 2) and the bright field image was set to "Scan – BF".

Table 2: Detector Settings.

Detector	Wavelength [nm]
PMT1 (GFP/YFP fluorescence)	525 – 570
PMT2 (Chlorophyll auto fluorescence)	645 – 705

The objective was adjusted to a maximum signal of PMT2 to be sure to be in the mid layer of the cell. This procedure was done for every sample to make them comparable. The sensitivity of the detectors was set to a level, where no saturation was reached.

5 RESULTS

5.1 Transformation of *Escherichia coli*

After transformation of *E. coli* DH5α with pSAW with the 5 different constructs under P*cpeC* (Figure 14), see also 4.3.1., the strains were cultivated and the plasmids were extracted. The sequencing results of the plasmids showed that all 5 constructs were correct. The plasmids were purified and transformed into the wild type of *N. puntiforme*.

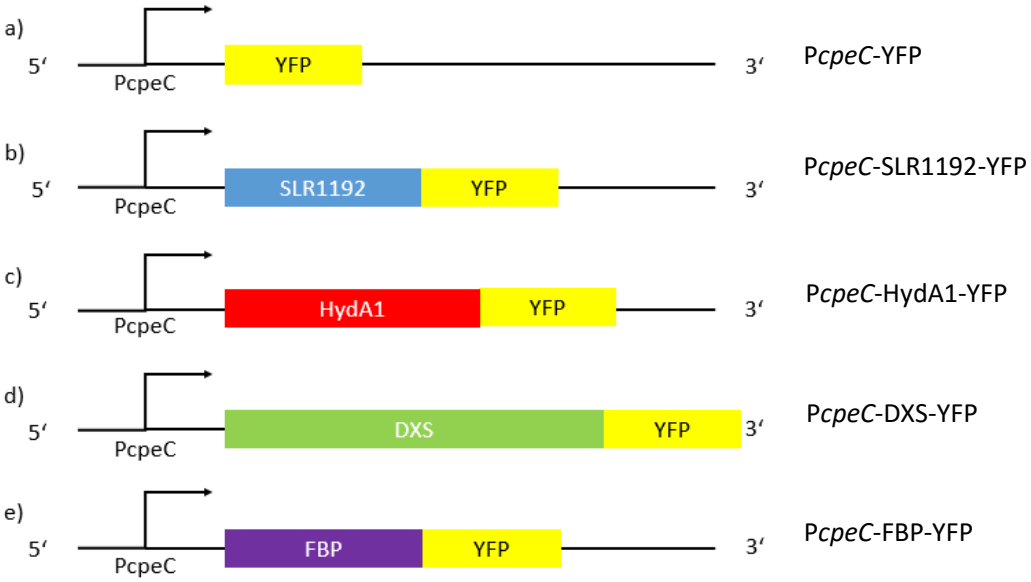

Figure 14: Successful Transformants into E.coli. . 5 different constructs (P*cpeC*-YFP, P*cpeC*-SLR1192-YFP, P*cpeC*-HydA1-YFP, P*cpeC*-DXS-YFP, P*cpeC*-FBP-YFP) were successful transformed into *E. coli*.

5.2 Transformation of *Nostoc punctiforme*

The transformation into *N. punctiforme* led to colonies for three of the five constructs with P*cpeC* (Figure 14). The constructs with *fbp*, *dxs* and without any GOI showed positive results, whereas the constructs with *hydA1* and *slr1192* did not show any colonies. The transformation was repeated several times without detection of any positive colonies. The three successful transformations (control, *fbp* and *dxs*) were checked for the inserted fragment via polymerase chain reaction (PCR) and gel electrophoresis.

During the fluorescence measurements, we noticed that the construct P*cpeC*-YFP did not show any fluorescence signals. Therefore, only the strains P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP were used. Additionally, an available strain P*cpeC*-GFP in pAW in *N. puntiforme* (Figure 15), which was created by Marcel Llavero Pasquina was used for the fluorescence measurements to answer the questions in 3.1. (Figure 15) [29].

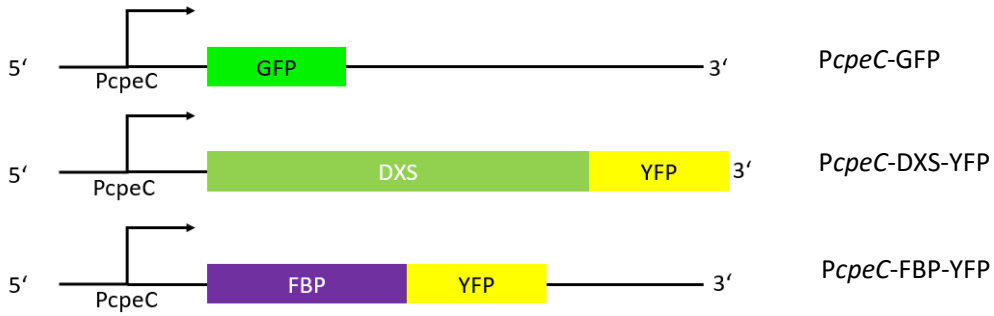

Figure 15: Successful Transformants of *Nostoc punctiforme* ATCC 29133. Two different constructs (P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP) were successfully inserted into pSAW and transformed into *N. punctiforme* ATCC 29133. Third construct was available from former experiments in pAW (same vector as pSAW, only difference is that it contains a GFP instead of YFP) in *N. puntiforme* ATCC 29133.

5.3 Fluorescence measurements

To study the effect of different light intensities on the expression level of P*cpeC* in *N. punctiforme* the fluorescence signals of the fluorescence proteins GFP of P*cpeC*-GFP, and YFP of PcpeC-DXS-YFP and PcpeC-FBP-YFP in *N. punctiforme* were measured after 0, 24 and 48 h in cells cultivated under four different light conditions. The strains were treated without any green light under 15 µmol photons·m^{-2}·s^{-1} red light (A), with 1.5 µmol photons·m^{-2}·s^{-1} green light (B), with 7.5 µmol photons·m^{-2}·s^{-1} green light (C) and with 15 µmol photons·m^{-2}·s^{-1} green light (D) (Figure 16).

5.3.1 FLUORESCENCE MEASUREMENT OF P*cpeC*-GFP

The fluorescence results of the treatment (treatments shown in 5.3) under red light (Figure 16A) show that there is no significant increase in fluorescence after 24 and 48 h. For all three treatments with green light, there is already a significant increase in fluorescence after 24 h and a further increase until 48 h. Under 1.5 µmol photons·m^{-2}·s^{-1} green light (Figure 16B) the fluorescence after 24 and 48 h is smaller compared to the treatments under higher green light intensities (Figure 16 C and D). Under higher green light intensities the strain shows a similar fluorescence signal at 24 h, but after 48 h the treatment with 15 µmol photons·m^{-2}·s^{-1} green light leads to a 1.5 times higher fluorescence signal as compared to the treatment with 7.5 µmol photons·m^{-2}·s^{-1} green light after 48 h.

Furthermore, it is visible that the increase of fluorescence over time at different green light intensities is not linear.

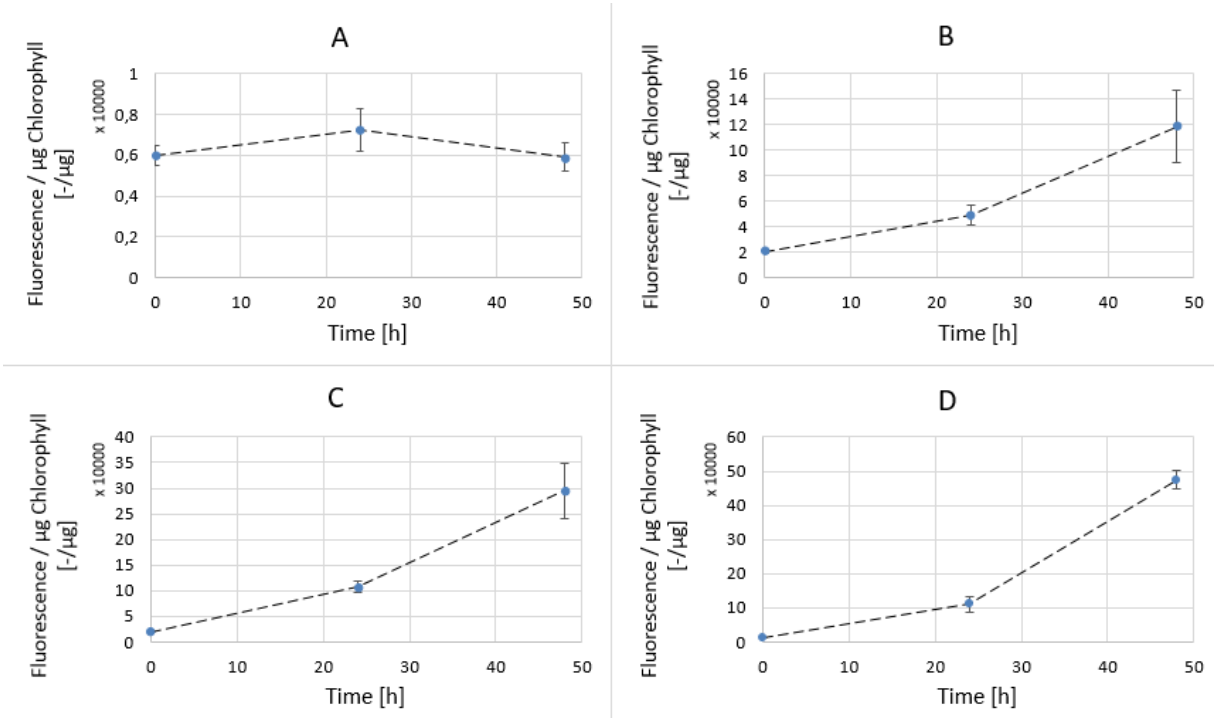

Figure 16: Fluorescence signal of P*cpeC*-GFP under different light conditions. P*cpeC*-GFP in pAW transformed into *N. punctiforme* ATCC 29133 was tested under repressing conditions with 15 µmol photons·m^{-2}·s^{-1}red light (A), under 1.5 µmol photons·m^{-2}·s^{-1} green light (B), under 7.5 µmol photons·m^{-2}·s^{-1} green light (C), under 15 µmol photons·m^{-2}·s^{-1} green light (D). The fluorescence signal was determined as Signal per µg Chlorophyll.

5.3.2 FLUORESCENCE MEASUREMENT OF P*cpeC*-DXS-YFP

The fluorescence results of the treatment (treatments shown in 5.3) of P*cpeC*-DXS-YFP under red light (Figure 17A) show that there is no significant increase in fluorescence after 24 and 48 h. The three treatments with different green light conditions show a significant increase after 24 h and a further increase after 48 h. The fluorescence increase of the strain under treatment with 1.5 µmol photons·m^{-2}·s^{-1} green light (Figure 17B) shows a linear increase over time, whereas the slopes at treatment with higher green light intensities (Figure 17 C and D) seem to have a higher slope between 24 and 48 h. The fluorescence signals increase with increasing green light intensities (D>C>B).

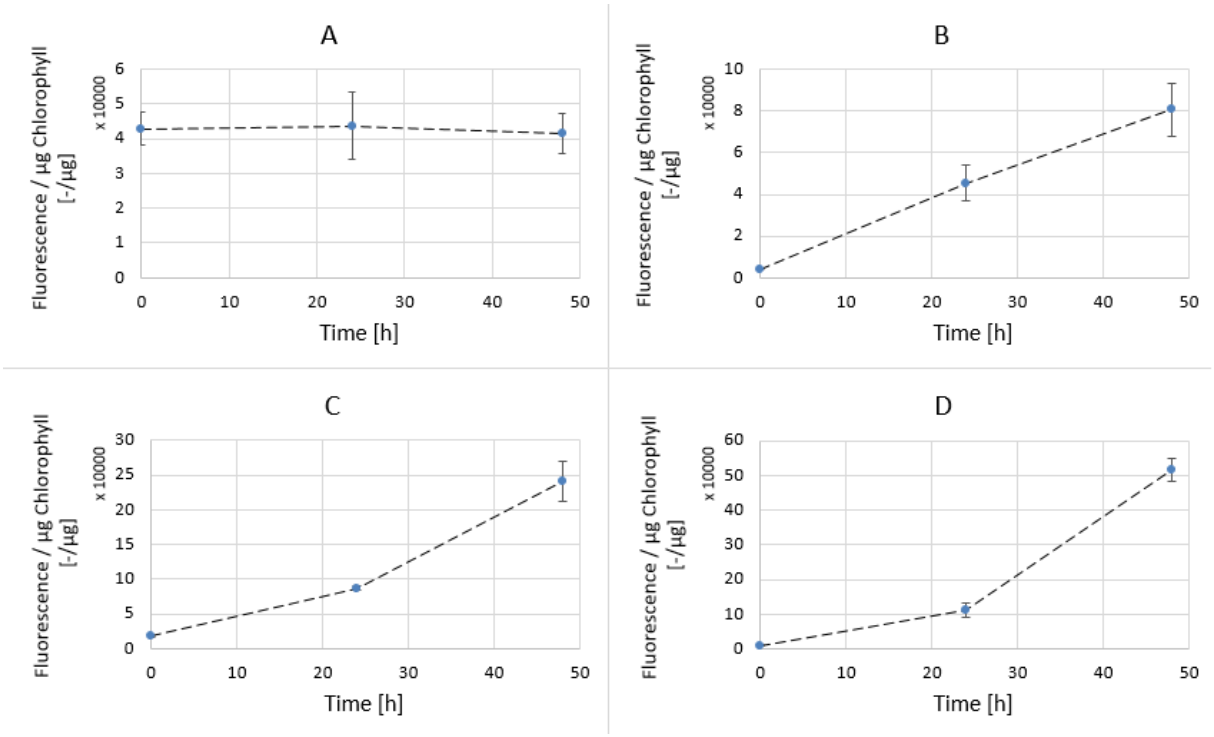

Figure 17: Fluorescence signal of P*cpeC*-DXS-YFP under different light conditions. P*cpeC*-DXS-YFP in pSAW transformed into *N. punctiforme* ATCC 29133 was tested under repressing conditions with 15 µmol photons·m^{-2}·s^{-1} red light (A), under 1.5 µmol photons·m^{-2}·s^{-1} green light (B), under 7.5 µmol photons·m^{-2}·s^{-1} green light (C), under 15 µmol photons·m^{-2}·s^{-1} green light (D). The fluorescence signal was determined as Signal per µg Chlorophyll.

5.3.3 FLUORESCENCE MEASUREMENT OF P*cpeC*-FBP-YFP

The fluorescence results of the treatment (treatments shown in 5.3) under red light (Figure 18A) show that there is no significant increase in fluorescence after 24 and 48 h. The three treatments with different green light conditions show a significant increase after 24 h and a further increase after 48 h. The fluorescence increase of the strain under treatment with 7.5 µmol photons·m^{-2}·s^{-1} green light (Figure 18C) shows a linear increase over time, whereas the slopes at treatment with 1.5 and 15 µmol photons·m^{-2}·s^{-1} green light (Figure 18 B and D) seem to have a higher gradient between 24 and 48 h. The fluorescence signals increase with increasing green light intensities (D>C>B).

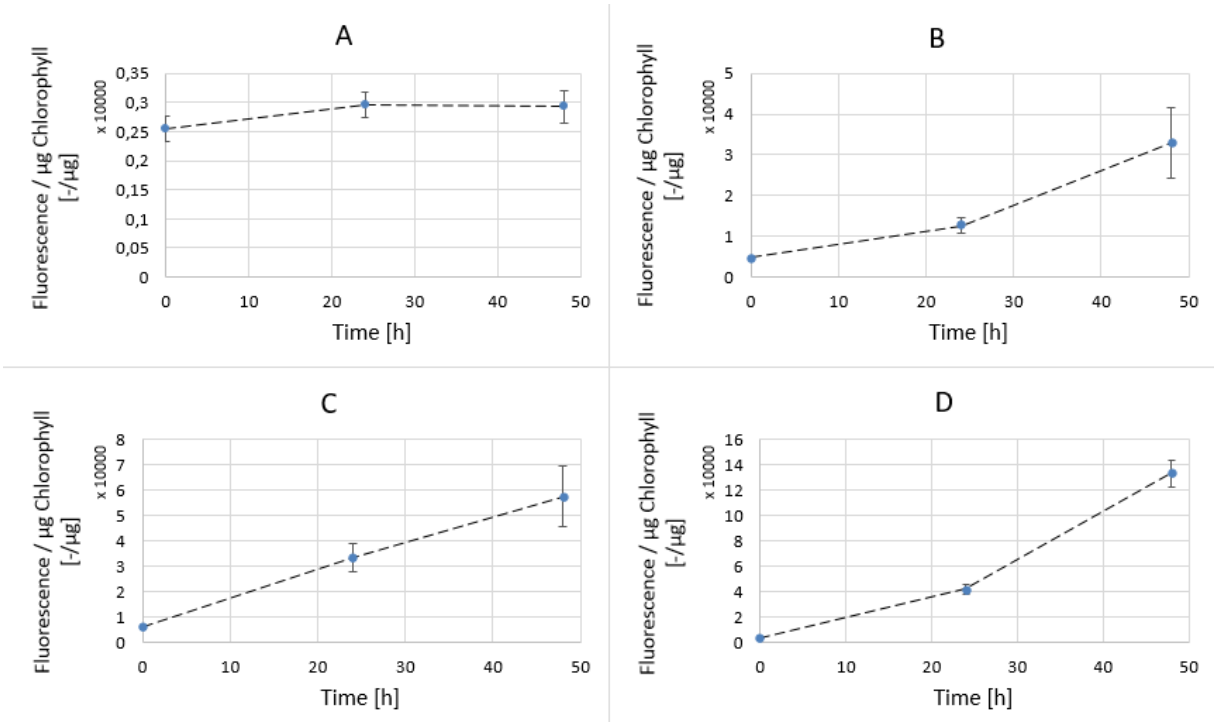

Figure 18: Fluorescence signal of P*cpeC*-FBP-YFP under different light conditions. P*cpeC*-FBP-YFP in pSAW transformed into *N. punctiforme* ATCC 29133 was tested under repressing conditions with 15 µmol photons·m^{-2}·s^{-1} red light (A), under 1.5 µmol photons·m^{-2}·s^{-1} green light (B), under 7.5 µmol photons·m^{-2}·s^{-1} green light (C), under 15 µmol photons·m^{-2}·s^{-1} green light (D). The fluorescence signal was determined as Signal per µg Chlorophyll.

5.3.4 COMPARISON OF THE EXPRESSION OF THE DIFFERENT GOI-YFP CONSTRUCTS

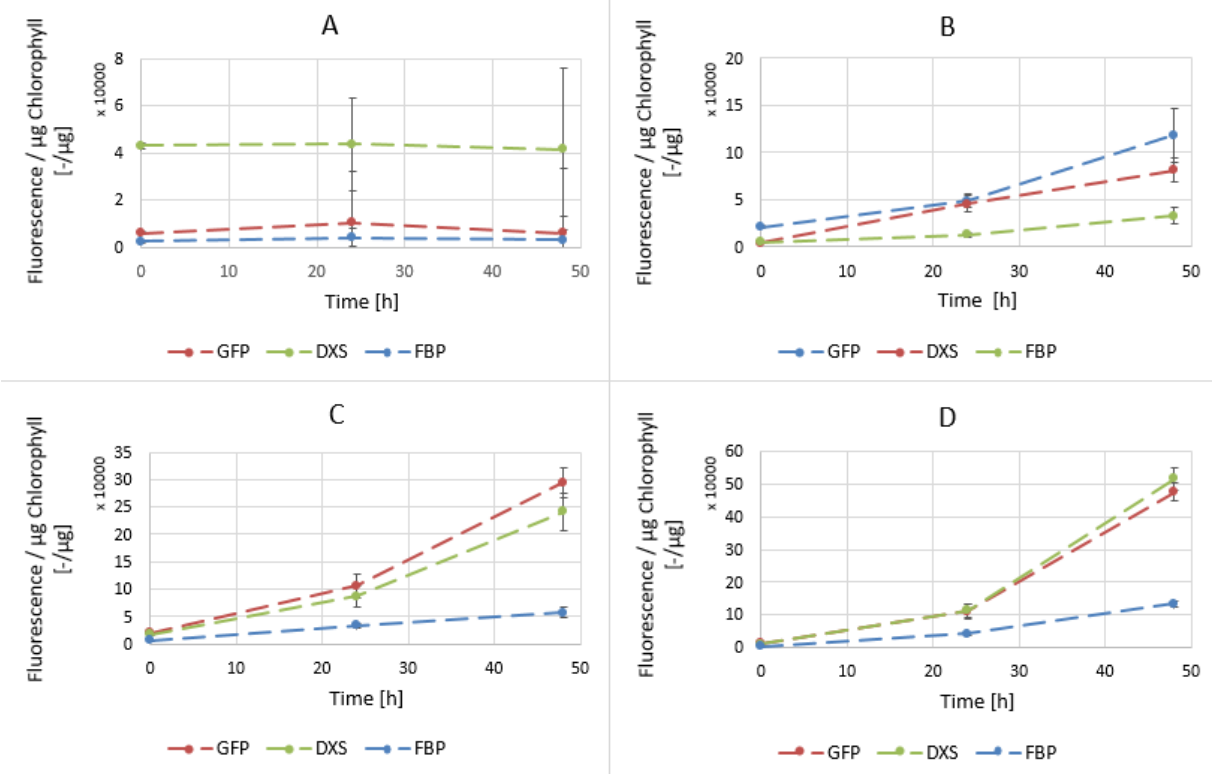

Figure 19: Comparison of fluorescence increase of PcpeC-GFP, PcpeC-DXS-YFP and PcpeC-FBP-YFP in *N. punctiforme* ATCC 29133 under different light conditions. P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP in pSAW transformed into *N. punctiforme* ATCC 29133 were tested under repressing conditions with 15 µmol photons·m^{-2}·s^{-1} red light (A), under 1.5 µmol photons·m^{-2}·s^{-1} green light (B), under 7.5 µmol photons·m^{-2}·s^{-1} green light (C), under 15 µmol photons·m^{-2}·s^{-1} green light (D). The fluorescence signal was determined as Signal per µg Chlorophyll.

The light intensity dependent fluorescence signals of the three different constructs show similar patterns (Figure 19). Under 15 µmol photons·m^{-2}·s^{-1} of red light (Figure 19A) they do not show a significant increase in fluorescence after 24 and 48 h.

In contrast after induction with 1.5 µmol photons·m^{-2}·s^{-1} green light (Figure 19B), they show significant increase of fluorescence signal already after 24 h although P*cpeC*-FBP-YFP shows less increase than P*cpeC*-GFP and P*cpeC*-DXS-YFP which show similar values. After 48 h the difference is clearly visible with high fluorescence values for P*cpeC*-GFP and P*cpeC*- DXS-YFP.

After induction with 7.5 µmol photons·m^{-2}·s^{-1} green light (Figure 19C) the strains show similar patterns as during the treatment with lower green light intensity, although they show a steeper increase of the fluorescence signal between 24 and 48 h. P*cpeC*-FBP-YFP remains

linear over time. All three strains show higher fluorescence signals for 24 and 48 h compared to induction with 1.5 µmol photons·m^{-2}·s^{-1} green light.

For induction with 15 µmol photons·m^{-2}·s^{-1} green light (Figure 19D) the increase in fluorescence signal shows the same pattern as with 7.5 µmol photons·m^{-2}·s^{-1} green light. It is also visible that the fluorescence signals are higher compared to the induction with lower green light intensities.

5.3.5 FLUORESCENCE SIGNAL UNDER DIFFERENT LIGHT INTENSITIES

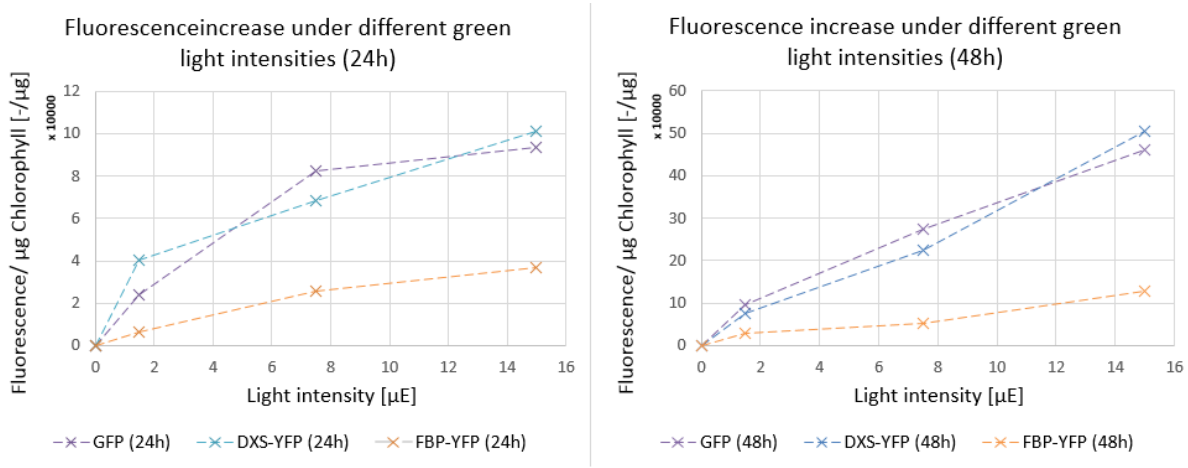

Figure 20: Fluorescence signal compared to the green light intensity. The fluorescence signals of P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP in *N. punctiforme* ATCC 29133 are compared with their inducing light intensities after 24 h (left) and 48 h (right).

The results of the fluorescence signals of P*cpeC*-GFP, P*cpeC*-DXS-YFP, P*cpeC*-FBP-YFP against their different light intensity treatments are shown in Figure 20. It is clearly visible that in both cases (24 and 48 h) the fluorescence signal increases for all three strains at higher light intensities. The fluorescence signal under higher green light intensity raises to a fourfold signal as compared to under low green light. The fluorescence signal of P*cpeC*-FBP-YFP shows lower absolute increase compared to the other two strains at both time points. Its graph shows a nearly linear course, whereas the fluorescence signals of P*cpeC*-GFP and P*cpeC*-DXS-YFP have a steep increase at lower intensities and gets flatter at higher intensities.

5.4 Determination of relative codon usage

To analyze the effect of codon usage of *dxs* and *fbp* the relative frequency a codon was used to produce its amino acid was determined and compared with the codon bias of *Kazusa DNA Research Institute* [30] for *N. punctiforme.* In Figure 21 the result of this comparison is shown. The codon usage of *dxs* shows a distribution around 1, whereas the codon usage of *fbp* more often uses codons, which are less common in *N. punctiforme*, shown by the codons with a frequency greater than 1.

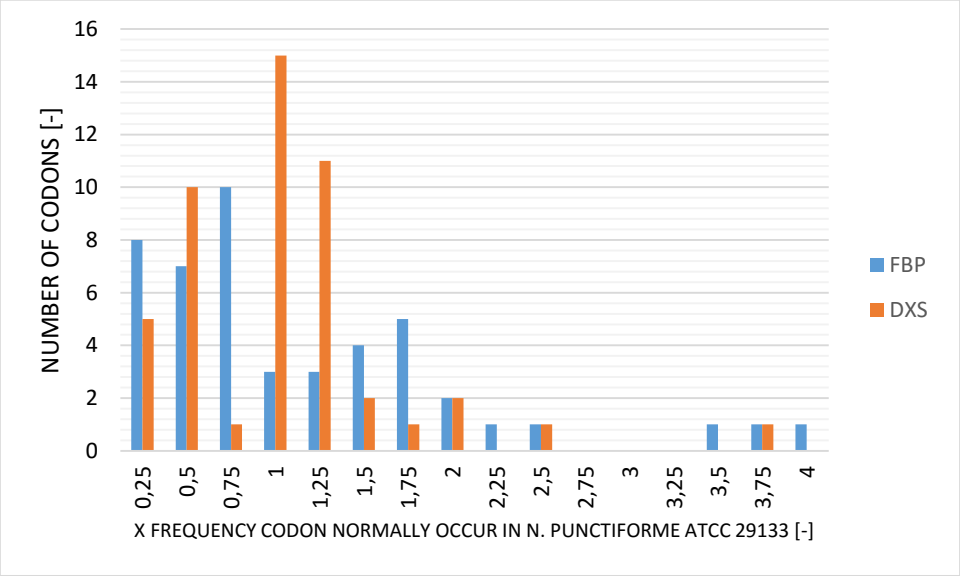

Figure 21: Determination of relative codon usage. The diagram shows the relative use of codons. Codons which are used around a frequency of 1 show a good match to the codon usage of *N. punctiforme* ATCC 29133 and therefore, should not slow down the speed of translation, because the availability of tRNA.

5.5 Growth measurement

To study the effect of different light intensities on the growth of the three strain of *N. punctiforme* (P*cpeC*-GFP; P*cpeC*-DXS-YFP; P*cpeC*-FBP-YFP), the chlorophyll a content was measured after 0, 24 and 48 h during the treatment with four different light conditions.

The strains were treated without any green light under 15 µmol photons·m^{-2}·s^{-1} red light (A), with 1.5 µmol photons·m^{-2}·s^{-1} green light (B), with 7.5 µmol photons·m^{-2}·s^{-1} green light (C) and with 15 µmol photons·m^{-2}·s^{-1} green light (D). The concentration at 0 h was set to 1 µg chlorophyll a·ml^{-1}.

5.5.1 GROWTH CURVE OF THE STRAIN P*cpeC*-GFP

P*cpeC*-GFP shows significant growth under treatment (treatments shown in 5.5) with red light (Figure 22A) after 48 h, although there is no growth after 24 h. The strain treated with 1.5 µmol photons·m^{-2}·s^{-1} green light and 7.5 µmol photons·m^{-2}·s^{-1} green light (Figure 22, B and C) do not show any significant growth after 48 h. Under 15 µmol photons·m^{-2}·s^{-1} green light (Figure 22D) there is a small increase in culture density to about 1.3 µg·ml^{-1}, although there is a higher density at 24 h, which could be seen as a dying of the cells between 24 and 48 h.

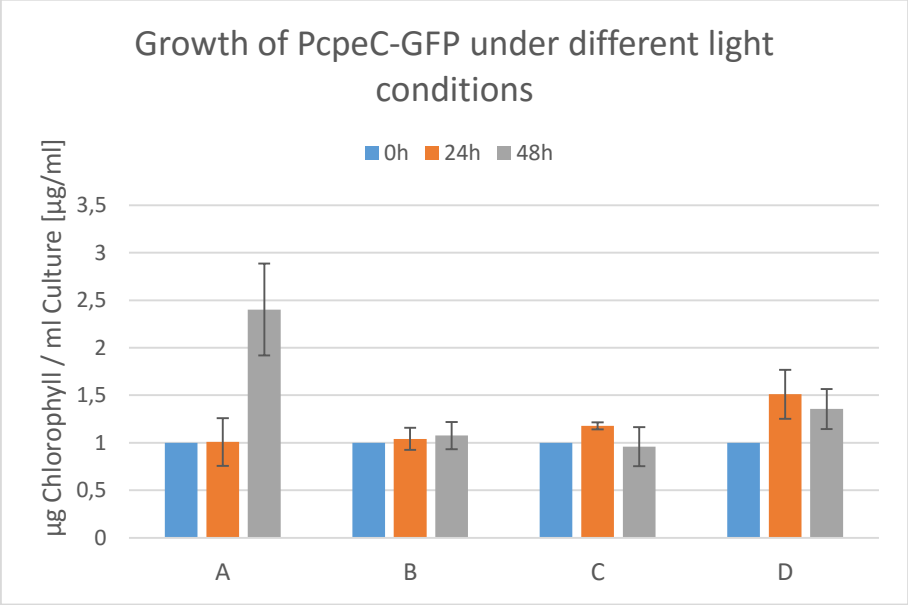

Figure 22: Growth of P*cpeC*-GFP. P*cpeC*-GFP in pSAW transformed into *Nostoc punctiforme* ATCC 29133 was treated with different µmol photons·m^{-2}·s^{-1} of green light (0/1.5/7.5/15) and the chlorophyll content was measured at 0h (blue), 24h (orange) and 48h (grey). The chlorophyll concentration at time point 0h was set to 1 µg·ml^{-1} culture.

5.5.2 GROWTH CURVE OF THE STRAIN P*cpeC*-DXS-YFP

Under red light (treatments shown in 5.5) P*cpeC*-DXS-YFP (Figure23A) shows growth after 24 h and a further increase after 48 h to about 1.7 . At 1.5 µmol photons·m^{-2}·s^{-1} green light and 7.5 µmol photons·m^{-2}·s^{-1} green light (Figure 23 B and C) the strains seem to decrease cell density after 24 h and stay constant the second half of the observation period. Under 15 µmol photons·m^{-2}·s^{-1} green light (Figure 23D) there is an increase in cell density after 24 h but a lower density at the end of the observation period.

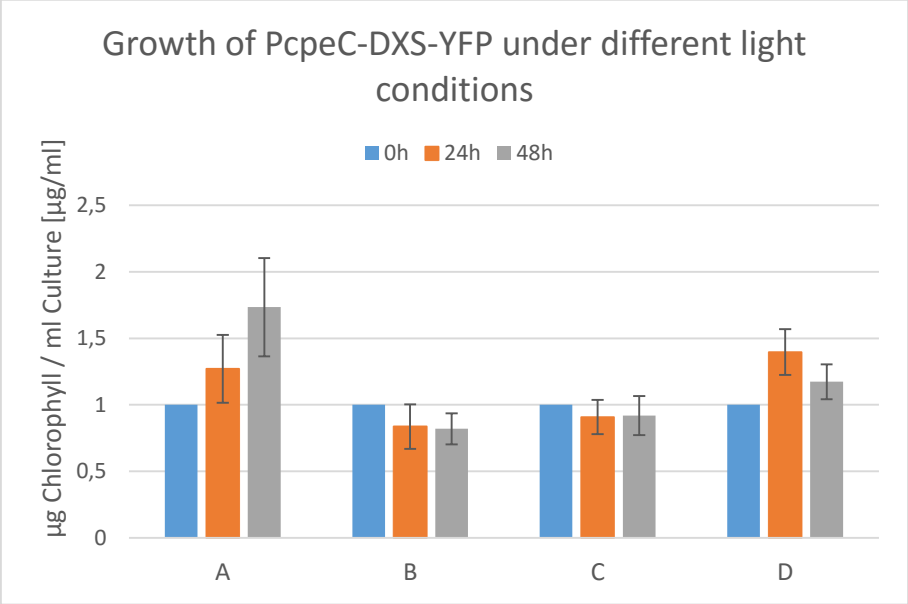

Figure 23: Growth of P*cpeC*-DXS-YFP. P*cpeC*-DXS-YFP in pSAW transformed into *Nostoc punctiforme* ATCC 29133was treated with under different µmol photons·m^{-2}·s^{-1} of green light (0/1.5/7.5/15) and the chlorophyll content was measured at 0h, 24h and 48h. The chlorophyll concentration at time point 0h was set to 1 µg·ml^{-1} culture.

5.5.3 GROWTH CURVE OF THE STRAIN P*cpeC*-FBP-YFP

The growth of P*cpeC*-FBP-YFP under red light (Figure 24A) shows a clear growth already after 24 h, which then increases to three times the amount of 0 h at 48 h. At low green light conditions (Figure 24B) the strain does not grow after 24 or 48 h. At 7.5 µmol photons·m^{-2}·s^{-1} green light (Figure 24C), the strain shows slightly increased chlorophyll content after 24 and 48 h, whereas at 15 µmol photons·m^{-2}·s^{-1} green light (Figure 24D) the chlorophyll a content does not increase after 24 h although it raised to 1.5-fold in the first 24 h.

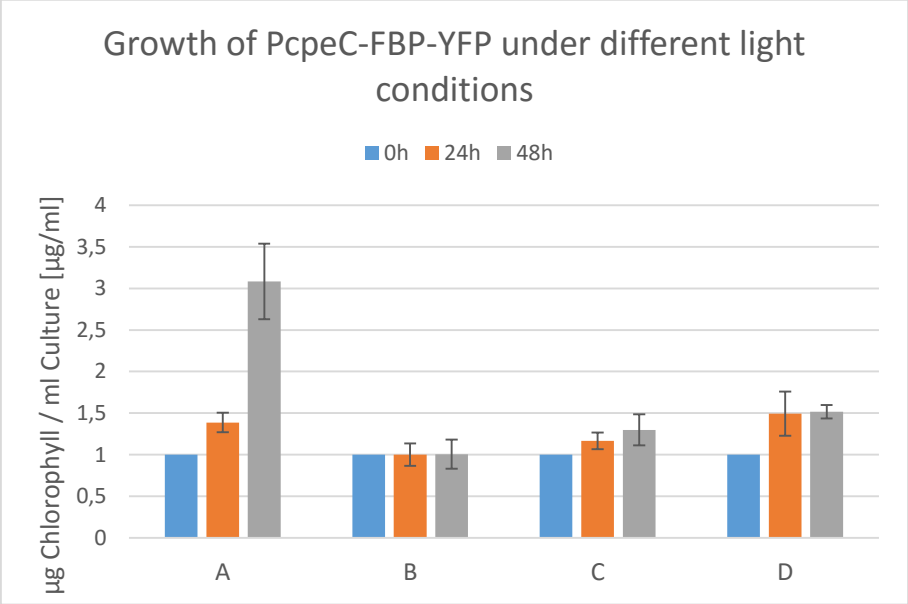

Figure 24: Growth of P*cpeC*-FBP-YFP. P*cpeC*-FBP-YFP in pSAW transformed into *Nostoc punctiforme* ATCC 29133was treated with under different µmol photons·m^{-2}·s^{-1} of green light (0/1.5/7.5/15) and the chlorophyll content was measured at 0h, 24h and 48h. The chlorophyll concentration at time point 0h was set to 1 µg·ml^{-1} culture.

5.5.4 COMPARISON OF GROWTH UNDER THE SAME LIGHT CONDITIONS

There are two different growth patterns of P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP. If the cultures are treated without green light and 15 µmol photons·m^{-2}·s^{-1}red light (Figure 25A), there is a significant growth during the 48 h of the analysis (Figure 25A). The chlorophyll a content of P*cpeC*-FBP-YFP is nearly double the amount as P*cpeC*-DXS-YFP. When the cultures are treated with 15 µmol photons·m^{-2}·s^{-1} green light (Figure 25B) they show nearly no growth between 24 and 48 h, although the measurements at 24 h would lead to the conclusion that there is slow growth of the strains.

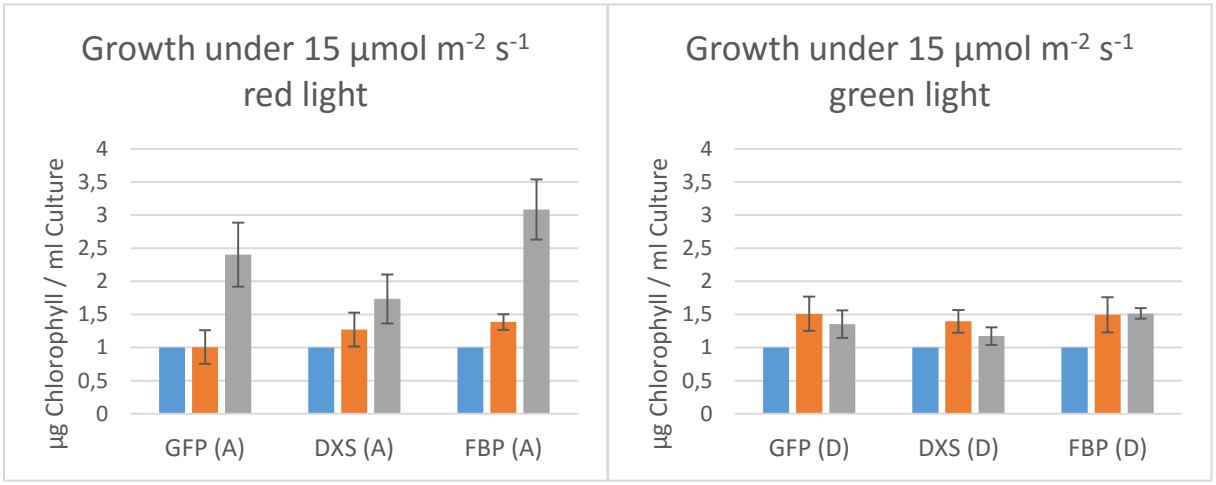

Figure 25: Growth of Nostoc punctiforme ATCC 29133 with three different plasmids under different light conditions. The diagram shows the growth patterns of P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP under red light (A) and under green light (B) after 0 h (blue), 24 h (red) and 48 h (green).

5.6 Confocal microscopy

To observe the uniform expression of the genes in *N. punctiforme* the fluorescence from the three different constructs, P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP, were observed under the confocal microscope for 48 h. To have comparable results, the fluorescence from one specific filament was observed over the time of the experiment. All three strains showed growth on the agarose plates, which led to breakage of the filament of P*cpeC*-GFP and P*cpeC*-FBP-YFP.

5.6.1 CONFOCAL MICROSCOPY OF P*CPEC*-GFP OVER 48H

The construct P*cpeC*-GFP shows a uniform fluorescence signal throughout the whole filament (Figure 26). A steady increase of the fluorescence signal was clearly visible for the whole 48 h.

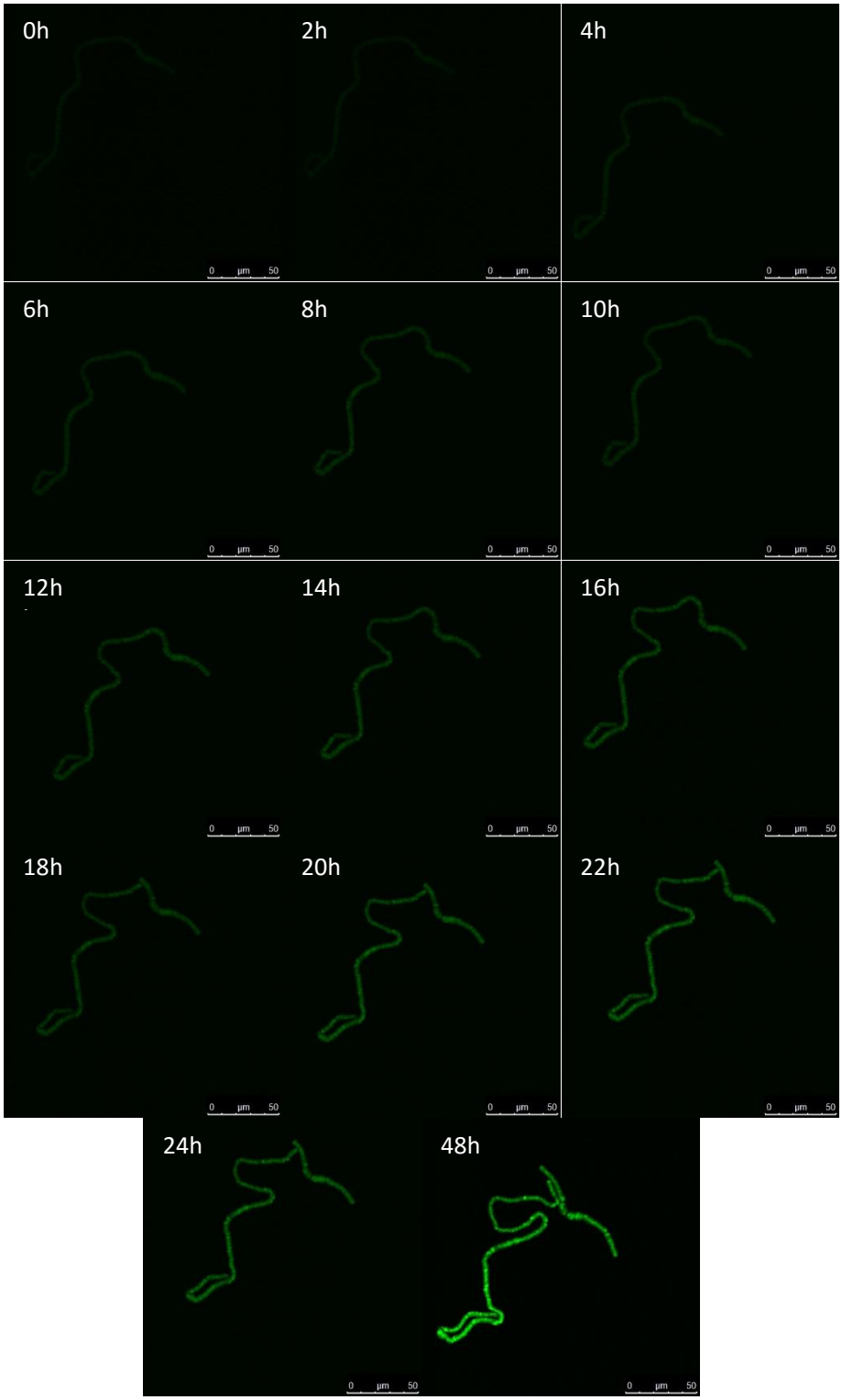

Figure 26: Investigation of fluorescence increase under confocal microscope of P*cpeC*-GFP in *Nostoc punctiforme* ATCC 29133. The protein expression of P*cpeC*-GFP in pAW in *N. punctiforme* ATCC 29133 over 48 h is shown by the fluorescence increase under the confocal microscope. For illustration the same filament was analyzed every two h for 24 h and an additional picture after 48 h.

5.6.2 CONFOCAL MICROSCOPY OF P*CPEC*-DXS-YFP OVER 48H

The construct P*cpeC*-DXS-YFP shows a uniform fluorescence signal throughout the whole filament (Figure 27). A steady increase of the fluorescence signal was clearly visible for the whole 48 h.

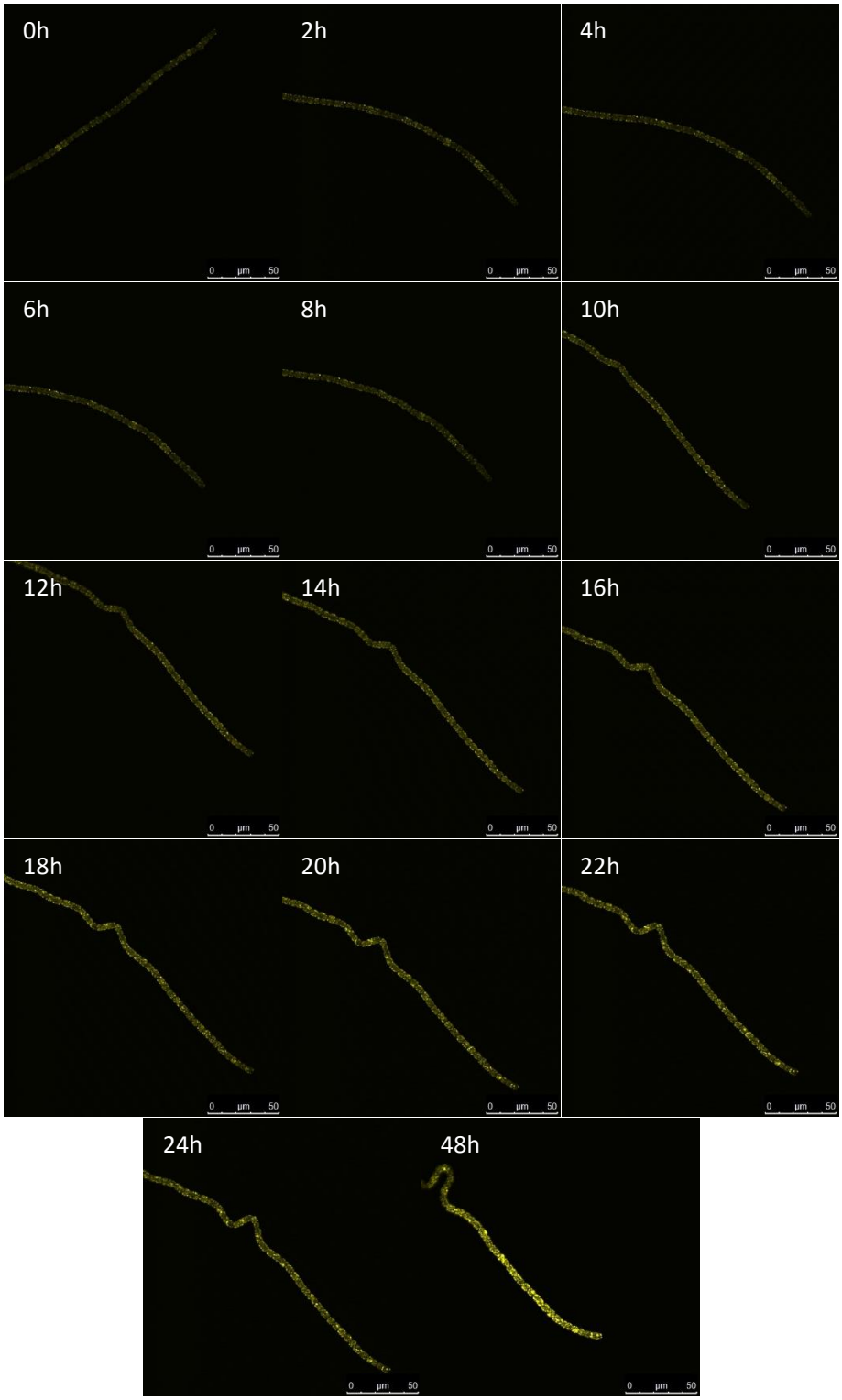

Figure27: Investigation of fluorescence increase under confocal microscope of P*cpeC*-DXS-YFP in *Nostoc punctiforme* ATCC 29133. The protein expression of P*cpeC*-DXS-YFP in pSAW in *N. punctiforme* ATCC 29133 over 48 h is shown by the fluorescence increase under the confocal microscope. For illustration the same filament was analyzed for every two h for 24 h and an additional picture after 48 h.

5.6.3 CONFOCAL MICROSCOPY OF P*CPEC*-FBP-YFP OVER 48H

The construct P*cpeC*-FBP-YFP shows a uniform fluorescence signal throughout the whole filament (Figure 28). A steady increase of the fluorescence signal was clearly visible for the whole 48 h.

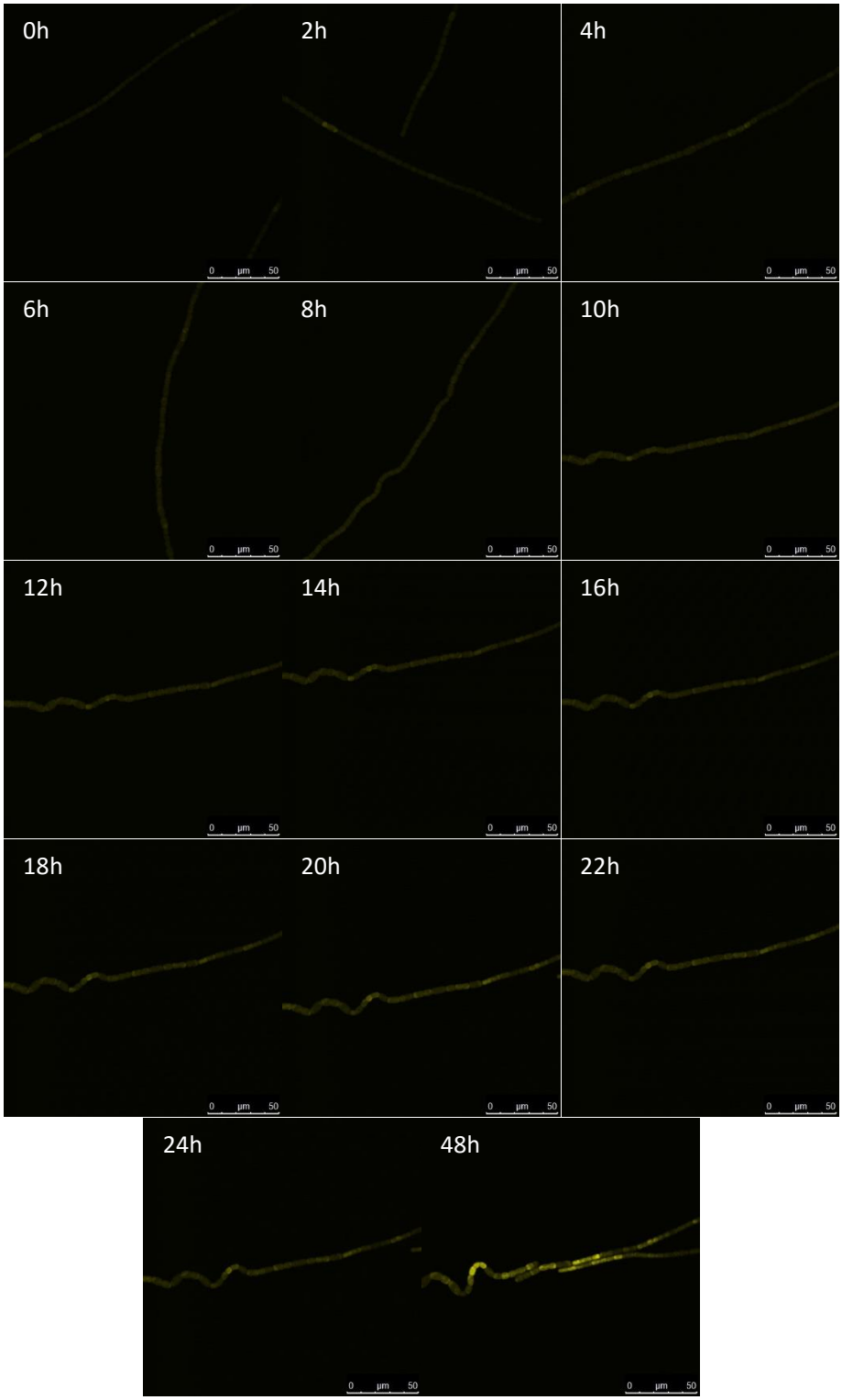

Figure 28: Investigation of fluorescence increase under confocal microscope of P*cpeC*-FBP-YFP in *Nostoc punctiforme* ATCC 29133. The protein expression of P*cpeC*-FBP-YFP in pSAW in *N. punctiforme* ATCC 29133 over 48 h is shown by the fluorescence increase under the confocal microscope. For illustration the same filament was analyzed for every two h for 24 h and an additional picture after 48 h.

6 DISCUSSION

6.1 Selected Cyanobacteria

The reason why *N. puntiforme* ATCC 29133 and not a well-studied filamentous cyanobacterium like *Anabaena* sp. PCC 7120 was chosen for this project is the increased potential of *N. punctiforme* as a H_2 producing platform [31]. H_2 which is produced during nitrogen fixation in heterocystous cyanobacteria is reused by the cell due to reoxidation of H_2 by an uptake hydrogenase. The reducing power can then be used for N_2 fixation by the nitrogenase complex [31].

In *N. punctiforme* there is just one known uptake hydrogenase (HupSL), which could be knocked out and lead to molecular hydrogen production [31]. Furthermore, there are attempts to insert a heterologous hydrogenase of *Chlamydomonas reinhardtii* (HydA1) into the heterocysts of *N. punctiforme* to potentially increase the net hydrogen production [32].

6.2 Expression level of different genes under P*cpeC* in *Nostoc punctiforme*

To answer the question if the expression level of a gene of interest under the promoter of *cpeC* depends on the gene which is expressed, it is visible that there is a significant difference between the fluorescent signals of the YFP-fusion protein with the DXS-gene and the FBP-gene (Figure 19). The ratio 'fluorescence signal of P*cpeC*-DXS-YFP to fluorescence signal of P*cpeC*-FBP-YFP' varies from 2.5 (at 1.5 µmol photons·m^{-2}·s^{-1}) to 3.9 (at 15 µmol photons·m^{-2}·s^{-1}) after 48 h. This is a surprising result taking into account that the DXS-gene (1920 bp) is nearly double the length of the FBP-gene (1035 bp). Usually we would assume that with the same amount of mRNA a longer gene would lead to lower protein expression due to higher probability of the ribosome to drop off and thereby stop the translation [33]. Beside the possibility of a different mRNA structure, which would lead to different binding probabilities for the ribosomes, there is also the possibility of translation difference because of antisense RNA [34] or different codon bias [35].

Both genes were taken from *Synechocystis* sp. PCC 6803 and are therefore based on the codon bias of another organism. Although they are both cyanobacteria the codon usage shows that there are differences (Figure 21).

It seems that the sequence of *dxs* of *Synechocystis* sp PCC 6803 is more compatible with the codon bias of *N. punctiforme*, because most of the codons reach a value around 1, which indicates a perfect relative amount of this codon for *N. punctiforme*. However, the codons of *fbp* of *Synechocystis* sp PC 6803 show a different distribution, where it is more likely that a codon is used more often compared to the codon bias of *N. punctiforme* (frequency > 1). A frequency above 1 indicates a codon which is rarely used in the normal codon bias of *N. punctiforme* compared to the rate of the codon in the gene *fbp* of *Synechocystis* sp PC 6803. The usage of the rare codons could be part of the explanation for the lower expression level of the FBP-YFP-protein. Through the low accessibility of the right tRNA for the high frequent codons, the translation is slowed down [35]. Whereas the comparison of codons which were used in these two genes and usually do not appear in *N. punctiforme* show that in *dxs* there are more not occurring codons, which should compensate the effect explained above.

Therefore, for future studies it is better to codon optimize the sequence of a heterologous gene to ensure that there is no difference on expression level because of translation speed [35].

To ensure that the difference in fluorescence is due to different ribosome binding probabilities it is also necessary to do an analysis of the mRNA level from each construct. With semi-quantitative RT-PCR or RT-qPCR the mRNA concentrations can be compared between the two different strains. This analysis was started within this project, but did not lead to reasonable results until the end of the project. Furthermore, it is necessary to investigate the P*cpeC* activity of a higher number of genes, to ensure that this effect is not due to the effect of the protein, encoded by the tested genes, on the metabolism of the cell which may directs the metabolic flux into a different direction as shown by Englund *et al.* (2016) for the overexpression of *dxs* in *Synechocystis* sp. PCC 6803 [36].

The fluorescence signals of the three different strains tested during this master thesis show similar expression patterns. The expression of P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP

can be completely repressed under 15 µmol photons·m^{-2}·s^{-1} of red light in all three cases (Figure 19). This is shown by the fact that the intensity of the fluorescence signal does not change significantly after 24 and 48 h. The different starting fluorescence signals are because of different delay time in white light during culture preparation for the experiment.

The expression pattern under 7.5 and 15 µmol photons·m^{-2}·s^{-1} green light differ from the pattern under 1.5 µmol photons·m^{-2}·s^{-1}. The fluorescence increase between 24 and 48 h is bigger than between 0 and 24 h and therefore not linear any more over the whole 48 h. This increase could be explained by the assumption that the adaption of cyanobacteria to the new green light conditions takes some time after the phycobilisomes have been adapted to the 15 µmol photons·m^{-2}·s^{-1} red light during the cultivation. In the presence of green light, the cells have to change their phycobilisome composition to a high part of PE. The adaption is a complex process, where DNA has to be transcribed first before they are translated into proteins and correctly folded and integrated into the phycobilisomes. Until this adaption is reached not the whole energy of the green light can be used and the metabolism is slowed down. Bittencourt-Oliveira *et al.* (2012) already showed that metabolism of cyanobacteria is dependent on the light intensity [37].

Another reason can be that not only the cell needs time to adapt, but also the initiation of the transcription of DNA and the translation of mRNA is a time consuming step.

To compare the expression levels of P*cpeC*-GFP with the other two constructs with YFP a quantitative comparison of fluorescence signals of GFP and YFP was performed. There was no literature found on the excitation and emission probability of the two different fluorescence proteins. Although YFP is derived from GFP and the difference in the amino acid sequence is small, there would be some uncertainties in the assumption that they both would lead to the same fluorescence intensity. It would be necessary to produce two strains, one with GFP and one with YFP, which produce the same amount of fluorescence protein. With the comparison of these fluorescence signals it would be possible to calculate a ratio of the fluorescence signals of GFP and YFP. The P*cpeC*-GFP strain is more relevant for the second question, if the expression level can be regulated by different light intensities.

6.3 Regulation of P*cpeC* by different light intensities

The results of the fluorescence signal of P*cpeC*-GFP, P*cpeC*-DXS-YPF and P*cpeC*-FBP-YFP (Figure 20) show clearly that it is possible to regulate the expression level with different intensities of green light. In all cases, after 24 h and 48 h, it is clearly visible that there is an increase in fluorescence signal for all three strains at higher light intensities compared to lower light intensities, which indicates a higher protein amount expressed under higher intensity of green light.

The flattening of the increase in fluorescence signals at higher green light intensities after 24 h could be explained by the time of the adaption of the phycobilisomes to the light conditions. My results indicate that the adaption time, until the cells are completely adapted to the 15 µmol photons·m^{-2}·s^{-1} green light, takes a relative big part of the induction time of 24 h. One possible explanation of the low fluorescence signal at 15 µmol photons·m^{-2}·s^{-1} green light could be that the cells cannot use the complete energy of the light from the beginning, because their phycobilisomes are not able to harvest the complete energy delivered by light. In contrast the energy of the 1.5 µmol photons·m^{-2}·s^{-1} green light is nearly completely absorbed over the whole 24 h. The time point until all of the light is absorbed µmol photons·m^{-2}·s^{-1} green light can be absorbed should be reached earlier at 1.5 µmol photons·m^{-2}·s^{-1}. This would include that at 48 h the adaption period's part of the 48 h is half of the part at 24 h and so the cells are longer able to use all of the energy of the 15 µmol photons·m^{-2}·s^{-1} green light. Therefore, the three strains show a more linear behavior after 48 h than after 24 h.

Additionally, the protein expression might also be a limiting factor, because it not only takes the cell time until it is able to use all the energy of the light, but it also take some time before the expression is on its maximum level. If the fluorescence signals at 24 and 48 h are compared it is visible that the fluorescence signals at 48 h is about 5 times higher than the signal at 24 h which indicates that there is a long period where the expression is not on its maximum level. Out of these results it is not possible to say if the promoter reached its maximum expression level after 48 h. To answer this question, further measurements at higher light intensities are needed. With the result of higher light intensities, it would also be possible to identify the

limitations of the induction of P*cpeC* by green light. At higher light intensities, the degradation of chlorophyll also needs to be analyzed [38].

Nonetheless with the results at 48 h it is visible that it is possible to regulate the protein expression under P*cpeC* with different green light intensities.

6.4 Growth under different Light conditions

There are two different growth patterns of P*cpeC*-GFP, P*cpeC*-DXS-YFP and P*cpeC*-FBP-YFP. If the cultures are treated without green light and only red light, which was used during cultivation before the experiment, there is a significant growth during the 48 h's period of the analysis visible (Figure 25; A) although there is not always an obvious growth after 24 h. When the cultures are treated with different intensities of green light (Figure 25; B), there is no significant difference between 24 and 48 h. The difference between 0 h and 24 h might be due to the unequal distribution of the biomass within the media, because of agglomerations of the cells. Before the dilution to 1 µg chlorophyll a·ml^{-1} of culture, the chlorophyll of a 20 ml culture was measured three times, and always some agglomerations of cells was seen. With the determined values of the 20 ml culture, the 5 ml cultures for the fluorescence measurement were prepared by diluting the culture to a chlorophyll a content of 1 µg·ml^{-1}. These cultures were then homogenized with a syringe and a sterile needle to get a good cell homogenization for the 24 h and the 48 h measurements.

The growth patterns could be explained by the composition of the phycobilisomes. In the presence of red light, the phycobilisomes are already adapted to the light conditions due to the cultivation under red light. The cells are able to absorb the red light (620 nm) due to their high PC content in the phycobilisomes and therefore these cell could keep the same growth rate as from the cultivation phase.

It is also visible that the P*cpeC*-FBP construct shows higher growth than the P*cpeC*-DXS construct under repressing conditions, although there should be no protein expressed and therefore, no effect visible. The difference in growth might be due to small protein amounts which are expressed during the preparation phase of the cultures, although the amount of expressed protein should be low, because of the short preparation time.

6.5 Growth on agar plates (Confocal microscopy)

The confocal pictures, which were taken of the same filament of one of the three *N. punctiforme* strains for 48 h, show that there is a homogenous expression throughout the whole filament. Some filaments seem to show more fluorescence than others, but this is reasoned by the growth of the filaments as it is not possible to get the whole filament into one focal plane. After 48 h, the filaments on agarose plates show a significant growth under green light. This is in contrast to the observation of the strains under green light in liquid culture. There are at least two possible explanations for this. On the one hand chlorophyll is light sensitive and therefore, it might be degraded under higher green light intensities, which would lead to conclusion that the chlorophyll amount is not a good strategy to determine biomass at higher light intensities.

On the other hand, *N. punctiforme* is known to survive under completely dark heterotrophic conditions by converting sucrose, glucose or fructose. This leads to the assumption that *N. punctiforme* maybe is also able to convert galactose out of the agarose of the plates and use it for growth.

7 CONCLUSION

With the results discussed above it is possible to partly answer the two questions (1) is the expression level of a gene under the light inducible P*cpeC* in *N. punctiforme* ATCC 29133 dependent on the expressed gene and (2) is the expression level is regulated by different light intensities.

It was possible to express different genes under P*cpeC* in *N. punctiforme*. All three strains containing the different PcpeC – GOI – YFP constructs show clear light regulation properties on different light conditions, although it seems that the protein expression needs time to reach its maximum level. Despite the different expression levels of the different constructs, it is not clear if these expression levels derive from different secondary structures of the mRNA and therefore, from the ribosome binding capability. To ensure that the difference seen is due to different translation rates and not because of a difference in transcript levels the mRNA amounts need to be compared to a reference gene (for example 16S rRNA). To check the mRNA amount it is necessary to transform the already available constructs in *E. coli*, made in this project, into *N. punctiforme* and compare the mRNA levels of these five constructs to find out if there are any differences.

It is also necessary to find out the effect of the five different enzymes on the cells by overexpressing them in *N. punctiforme*.

In another approach, the promoter strength should be analyzed. This could be achieved by transforming the constructs of this thesis under another, well known promoter into *N. punctiforme* and comparing the fluorescence signal with the results of this thesis.

The second question about the possibility to regulate the promoter by different light intensities is answered clearly. It is possible to regulate the gene expression of P*cpeC* by different green light intensities.

I conclude that P*cpeC* therefore can become a useful tool for controlled gene expression in further approaches (for example biofuel production) in *N. puntiforme* due to its ability to repress protein expression completely under red light and its ability to regulate protein

expression by different green light intensities. Especially for toxic substrates the long time it takes, until the expression rate is on its maximum level, could be an additional advantage.

8 APPENDIX

8.1 BG_{11}

Table 3:BG_{11}

Stock	Components	1000x (g/L)
Stock 1	K_2HPO_4	40
Stock 2	$MgSO_4 + 7\ H_2O$	75
Stock 3	$CaCl_2 + 2\ H_2O$	36
	Citric Acid	6.0
Stock 4	Ferric ammonium citrate	6.0
	EDTA disodium salt	1.0
Stock 5	Na_2CO_3	20
Stock 6	H_3BO_3	2.86
	$MnCl_2 + 2\ H_2O$	1.81
	$ZnSO_4 + 2\ H_2O$	0.222
	Na_2MoO_4	0.395
	$CuSO_4 + 5\ H_2O$	0.0790
	$Co\ (NO_3)_2 + 5\ H_2O$	0.0494

8.2 Sequences

P*cpeC*

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CTGCGGACTCCCATTAAGAAAATATTGCAAAAAGTAACAATTTCTAGTTTTCGTTGCTTCTTTCCGATTT
CTTTACCATTTCCTGAGCGATTTCCTTACTTTTACTGTGTAATTTTCTATTCTATGTAGGTTTTTAATAAC
CTCCTTTAGACGTTCTAAAAGAACTCTATGTTTAGCCAGGGGATTTTTAGAAA

P*rbcL*

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slr1192

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GACTTTGCTGTGCGCCATGACATTAAACCCGTGGTGGAACAATTTAGCTTTGATCAGATCAACGAGGC
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dxs

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fbp

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hydA1

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CATCCGAAGATGGACGCGGCGGAATTACTTTGCGCGTCGCTGTGGCCAACGGCTTAGGTAATGCTAA
GAAATTGATCACCAAGATGCAAGCGGGTGAAGCCAAATACGATTTCGTCGAGATCATGGCATGTCCT
GCCGGTTGTGTTGGTGGTGGTGGTCAACCCCGAAGTACTGATAAAGCGATCACGCAAAAACGCCAAG
CAGCGTTATACAATTTAGACGAAAAATCTACGTTGCGTCGCAGTCATGAGAATCCCTCTATCCGAGAA
TTATATGACACTTATTTAGGAGAACCCTTAGGTCATAAAGCGCATGAACTCTTACATACTCATTACGTC
GGT

8.3 Primers

Table 4: Primers.

Name of Primer	Sequence (5' to 3')	Primer length (+Restriction site) (no. of nt.)	Melting point [°C]
P*cpeC*_fwd (Sal1)	GTCGACGTACATCATCTTCAACTAAGAGAATTCG	28(+6)	63
P*cpeC*_rev (Xho1)	CTCGAGTTTCTAAAAATCCCCTGGC	19(+6)	60
P*cpeC*_rev (OL_SLR1192)	GGCTTTAATCATTTTCTAAAAATCCCCTGGC	19(+12)	60
SLR1192_fwd (OL_P*cpeC*)	GATTTTTAGAAAATGATTAAAGCCTACGCTGC	20(+12)	61
SLR1192_rev (Xho1_Linke)	CTCGAGACTTCCGCTACCCTAATTTTTACTATGGCTGAGCACTAC	27(+18)	61
P*cpeC*_rev (OL_DXS)	TGATGTGCATTTTCTAAAAATCCCCTGGC	19 (+10)	60
DXS_fwd (OL_P*cpeC*)	TTTTTAGAAAATGCACATCAGCGAACTGAC	20(+10)	64
DXS_rev (Xho1_Link)	CTCGAGACTTCCGCTACCCTAACTAACTCCAGGAGCGACAAC	24(+18)	64
P*cpeC*_rev (OL_FBP)	CTTTGCCCATTTTCTAAAAATCCCCTGGC	19(+10)	60
FBP_fwd (OL_P*cpeC*)	TTTTTAGAAAATGGGCAAAGGTGAAAAAAAC	21(+10)	64
FBP_rev (Xho1_Link)	CTCGAGACTTCCGCTACCTTAATGCAGTTGGATTACTTTGGG	24(+18)	65
P*cpeC*_rev (OL_HydA1)	GAGCAGCCATTTTCTAAAAATCCCCTGGC	19(+10)	60
Hyda1_fwd (OL_P*cpeC*)	TTTTTAGAAAATGGCTGCTCCTGCTGCTG	19	69
Hyda1_rev (Xho1_Link)	CTCGAGACTTCCGCTACCACCGACGTAATGAGTATGTAAGAGTTCATG	30	67
P*rbcL*_fwd (Sal1)	GTCGACGTGCGATTTTTTATCTTTACGC	22(+6)	61
P*rbcL*_rev (Xho1)	CTCGAGATTTTATCCTTCCCTGAAATCAC	23(+6)	61
P*rbcL*_rev (OL_SLR1192)	GGCTTTAATCATATTTTATCCTTCCCTGAAATCAC	23(+12)	61
SLR1192_fwd (OL_P*rbcL*)	GAAGGATAAAATATGATTAAAGCCTACGCTGC	20(+12)	61
P*rbcL*_rev (OL_DXS)	GCTGATGTGCATATTTTATCCTTCCCTGAAATCAC	23(+12)	61

DXS_fwd (OL_P*rbcL*)	GAAGGATAAAATATGCACATCAG CGAACTGAC	20(+12)	64
P*rbcL*_rev (OL_FBP)	CTTTGCCCATATTTTATCCTTCCCT GAAATCAC	23(+10)	61
FBP_fwd (OL_P*rbcL*)	AGGATAAAATATGGGCAAAGGT GAAAAAAAC	21(+10)	64
P*rbcL*_rev (OL_HydA1)	GAGCAGCCATATTTTATCCTTCCC TGAAATCAC	23(+10)	61
HydA1_fwd (OL_P*rbcL*)	AGGATAAAATATGGCTGCTCCTG CTGCTG	19(+10)	69

9 REFERENCE

[1] John H. Mercer. West Antarctic ice sheet and CO_2 greenhouse effect: a threat of disaster. Nature (Vol 271). 1978. pp. 321-325

[2] Brian Speer. Introduction to the Cyanobacteria. Online source. http://www.ucmp.berkeley.edu/bacteria/cyanointro.html . Date: 21.06.2017

[3] Ximing Cai, Xiao Zhang, Dingbao Wang. Land Availability for Biofuel Production. Environmental Science & Technology (Vol 45). 2011. pp. 334-339

[4] United Nations, Department of Economic and Social Affairs. World population projected to reach 9.7 billion by 2050. 29.07.2015. Online source. http://www.un.org/en/development/desa/news/population/2015-report.html Date: 21.06.2017

[5] Michael Lüders. Wer den Wind sät; Was westliche Politik im Orient anrichtet (23rd Edition). 2015. C.H.Beck. Munich.

[6] Daniel C. Ducat, Jeffrey C. Way, Pamela A. Silver. Engineering cyanobacteria to generate high-value products. Trends Biotechnology (Vol 29). 2011. pp. 95-103

[7] Nicole E. Nozzi, John W. K. Oliver, Shota Atsumi. Cyanobacteria as a Platform for Biofuel Production. Frontiers in Bioengineering and Biotechnology (Vol 1/2013). 2013. Published online

[8] David L. Nelson, Michael M. Cox. Lehninger Principles of Biochemistry (4th Edition). 2005. Palgrave Macmillan. Chapter 1: The foundations of biochemistry. pp. 1-44

[9] Nyok-Sean Lau, Minami Matsui, Amirul Al-Ashraf Abdullah. Cyanobacteria: Photoautotrophic Microbial Factories for the Sustainable Synthesis of Industrial Products. BioMed Research International Vol 2015 (2015). 9 pages.

[10] Elias Englund, Johan Andersen-Ranberg, Rui Miao, Björn Hamberger, Pia Lindberg. Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide. ACS Synthetic Biology (Vol 4).2015. pp. 1270-1278

[11] Kathrin Schütz, Thomas Happe, Olga Troshina, Peter Lindblad, Elsa Leitäo, Paulo Oliveira, Paula Tamagnini. Cyanobacterial H_2 production – a comparative analysis. Planta (Vol 218). 2004. pp. 350-359

[12] Donald Voet, Judith G. Voet. Biochemistry (4th Edition). 2011. Wiley-VCH. Chapter 24: Photosynthesis. pp 901-937

[13] Preksha Bhan. Structure of Cyanobacteria (Blue Green Algae). Online article. http://www.biologydiscussion.com/cytology/structure-of-cyanobacteria-blue-green-algae/36793. Date: 21.6.2017

[14] Robert MacColl. Cyanobacterial Phycobilisomes. Journal of structural biology (Vol 124). 1998. pp. 311-334

[15] Yuu Hirose, Rei Narikawa, Mitsunori Katayama, and Masahiko Ikeuchi. Cyanobacteriochrome CcaS regulates phycoerythrin accumulation in *Nostoc punctiforme*, a group II chromatic adapter. Proceedings of the National Academy of Sciences (Vol 107, No 19). 2010. pp. 8854-8859.

[16] Donald A. Bryant, Gerard Guglielmi, Nicole Tandeau de Marsac, Anne-Marie Castets, Germaine Cohen-Bazire. The structure of cyanobacterial phycobilisomes: a model. Archives of Microbiology (Vol 123). 1979. pp. 113-127

[17] Wikipedia. Allophycocyanin. Online source.
https://de.wikipedia.org/wiki/Allophycocyanin . Date: 14.07.17

[18] Wikipedia. Phycocyanin. Online source.
https://de.wikipedia.org/wiki/Phycocyanin . Date: 14.07.17

[19] Wikipedia. Phycoerythrocyanin. Online source.
https://en.wikipedia.org/wiki/Phycoerythrocyanin . Date: 14.07.17

[20] Wikipedia. Phycoerythrin. Online source.
https://de.wikipedia.org/wiki/Phycoerythrin . Date: 14.07.17

[21] David L. Nelson, Michael M. Cox. Lehninger Principles of Biochemistry (4th Edition). 2005. Palgrave Macmillan. Chapter 28: Regulation of gene expression. pp.1081-1119.

[22] Donald Voet, Judith G. Voet. Biochemistry. 4th Edition.2011. Wiley-VCH. Chapter 5: Nucleic Acids, Gene Expression and Recombinant DNA Technology. pp. 82-104.

[23] Chemically-regulated promoters. Online source
http://www.bios.net/daisy/promoters/271/272.html Date: 21.06.2017.

[24] Liangjiang Wang, Susan R. Wessler. Role of mRNA Secondary Structure in Translational Repression of the Maize Transcriptional Activator Lc. Plant Physiology (Vol 125). 2001. pp. 1380-1387

[25] Vivek K. Mutalik, Joao C. Guimaraes, Guillaume Cambray, Colin Lam, Marc Juul Christoffersen, Quynh-Anh Mai, Andrew B. Tran, Morgan Paull, Jay D. Keasling, Adam P. Arkin, Drew Endy. Precise and reliable gene expression via standard transcription and translation initiation elements. Nature methods 10. 2013. pp. 354-360

[26] Hsin-Ho Huang, Daniel Camsund, Peter Lindblad, Thorsten Heidorn. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Research (Vol 38). 2010. pp. 2577-2593

[27] JGI Genome Portal: *Nostoc punctiforme* ATCC 29133. Online source http://genome.jgi.doe.gov/nospu/nospu.home.html. Date: 17.6.2017.

[28] pSCR119. Online source http://www.uniprot.org/taxonomy/282192. Date: 17.06.2017

[29] Marcel Llavero Pasquina. Engineered light controlled cell development for enhanced hydrogen production in *Nostoc punctiforme* ATCC 29133. Master thesis, Uppsala University. 2016. pp.39

[30] Codon Usage *Nostoc punctiforme*. Online source. http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=63737&aa=4&style=N Date: 17.06.2017.

[31] Patricia Raleiras, Namita Khanna, Helder Miranda, Livia S. Mescaros, Henning Krassen, Felix Ho, Natalia Battchikova, Eva-Mari Aro, Ann Manuson, Peter Lindblad, Stenbjörn Styring. Turning around the electron flow in an uptake hydrogenase. EPR spectroscopy and in vivo activity of a designed mutant in HupSL from *Nostoc punctiforme*. Energy & Environmental Science (Issue 2). 2016. pp. 581-594

[32] Thorsten Heidorn, Peter Lindblad. Heterologous expression of an algal hydrogenase in a heterocystous cyanobacterium. 16th World Hydrogen Energy Conference. 2006.

[33] John R. Menninger. Peptidyl transfer RNA dissociates during protein synthesis from ribosomes of *Escherichia coli*. The Journal of Biological Chemistry (Vol 251). 1976. pp. 3392-3398

[34] Pamela J. Green, Ophry Pines, Masayori Inouye. The role of antisense RNA in gene regulation. Annual Review of Biochemistry (Volu 55). 1986. pp. 569-597

[35] Claes Gustafsson, Sridhar Govindarajan, Jeremy Minshull. Codon bias and heterologous protein expression. Trends Biotechnology (Vol 22). 2004. pp. 346-353

[36] Elias Englund, Pia Lindberg . Effect of Expression of MEP Pathway Enzymes on Production of Isoprene in *Escherichia coli* and *Synechocystis* sp. PCC 6803. Manuscript (2016)

[37] Bittencourt-Oliveira MC, Buch B, Hereman TC, Arruda-Neto JD, Moura AN, Zocchi SS. Effects of light intensity and temperature on *Cylindrospermopsis raciborskii* (Cyanobacteria) with straight and coiled trichomes: growth rate and morphology. Brazilian Journal of Biology (Vol 72). 2012. pp 343-351

[38] Brian Moss. Studies on the degradation of chlorophyll a and carotenoids in freshwaters. New Phytologist (Vol 67). 1968. pp. 49-59