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Implementation of thiamine pyrophosphate (TPP) riboswitches as synthetic biosensors and regulatory tools in cyanobacteria

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Abstract

Implementation of thiamine pyrophosphate (TPP) riboswitches as synthetic biosensors and regulatory tools in cyanobacteria

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The natural occurrence of the non-mevalonate (also called MEP after the compound methyl-erythriol phosphate) pathway in the model cyanobacterium *Synechocystis* sp. PCC 6803 allows for biosynthesis of various high-value terpenoid compounds. An important co-factor of this pathway is thiamine pyrophosphate (TPP), coenzyme to the 1-deoxy-D-xylulose-5-phosphate synthase (DXS) reaction in the initial step of the MEP pathway. Concurrently, TPP biosynthesis derives partially from 1-deoxy-D-xylulose phosphate, the product of DXS. This makes TPP a potentially significant measure of MEP pathway activity, and thus terpenoid productivity. The implementation of a molecular biosensor for TPP could be a promising approach towards on-line assessment and feedback regulation of MEP pathway activity and this application is therefore investigated in this work.

Riboswitches have been suggested as versatile RNA-based tools for biotechnological applications in bacteria, including various cyanobacterial species. However, TPP-responsive riboswitches have not been addressed in cyanobacteria thus far. This project therefore aims at the evaluation and implementation of TPP-responsive riboswitches in *Synechocystis*, using a yellow fluorescent reporter protein as quantitative readout of translational regulation. Native putative OFF-switches from two cyanobacterial species are investigated along with one synthetic ON-switch, originally based on the native riboswitch from *E. coli*. The induction effects are assessed on both RNA and protein level for both TPP and its precursor thiamine. The synthetic riboswitch is found to be effective in *Synechocystis* and is further examined for its dynamic range. Several protocols for fluorescence and transcript level experiments are developed. Several continuation experiments are suggested, including further investigation of the cyanobacterial OFF-switches.

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Sammanfattning

Mänskligheten står inför ett problem av proportioner som den aldrig har sett förut. Den globala uppvärmningen hotar att utrota inte bara oss människor utan även alla andra levande varelser på jorden. Om vi ska lyckas vända trenden måste vi hitta ett sätt att sluta använda fossila resurser. Detta är dock praktiskt taget omöjligt om vi inte kan hitta alternativa lösningar, inte bara för bränslen utan även för de kemikalier vi nu producerar från fossila resurser. Terpenoider är ett utmärkt exempel på sådana kemikalier. Förutom som bränslen så kan vissa terpenoider användas för produktion av farmaceutiska produkter och mattillsatser.

Men hur kan vi tillverka dessa produkter utan att använda fossila resurser? Ett svar på detta kan finnas i syntetisk biologi, i möjligheten att få organismer att producera ämnen som de annars inte skulle tillverka alls eller i tillräcklig mängd. De flesta rekombinanta produktionsvärdar kräver dock att en källa till organiskt kol och energi såsom glukos tillsätts till tillväxtmediet. Detta innebär en stor produktionskostnad och kräver dessutom en area på vilken den glukosproducerande växten kan odlas. Cyanobakterier däremot är fotoautotrofer och kan därför odlas utan någon annan kol-eller energitillförsel än koldioxid och solljus. Men för att kunna använda cyanobakterier för detta så måste vi ha verktygen för att modifiera dem. Många sådana verktyg finns redan, såsom CRISPRi, promotorer och ribosomala bindningsställen. En annan typ av verktyg är riboswitches, korta RNA-bitar som ändrar konfiguration som respons på närvaron av en viss metabolit.

I den här studien undersöks flera riboswitches som reagerar på tiamine-pyrofosfat (TPP), en viktig kofaktor för ett av enzymerna som leder till terpenoidproduktion. I slutändan undersöks en riboswitch för sitt dynamiska intervall på både protein- och RNA-nivå.

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Abbreviations

cDNA	complementary deoxyribonucleic acid
CRISPRi	clustered regularly interspaced palindromic repeats - interference
dH_2O	deionized water
DMSO	dimethyl sulfoxide
DXP	1-deoxy-D-xylulose phosphate
DXR	1-deoxy-D-xylulose phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
EVC	empty vector control
GAP	glyceraldehyde-3-phosphate
LB	Luria Bertani broth
MEP	methylerythriol-phosphate
NADP	nicotinamide-adenine-dinucleotide-phosphate
NADPH	nicotidamide-adenine-dinucleotide-phosphate, reduced
NTC	non-template control
OD	optical density
PCC	Pasteur culture collection
PCR	polymerase chain reaction
q-RT-PCR	quantitative real-time polymerase chain reaction
RBS	ribosomal binding site
RT	reverse transcription
SD	Shine - Dalgarno
TPP	thiamine pyrophosphate
UTR	untranslated region
YFP	yellow fluorescent protein

Introduction

Humanity is facing a problem of proportions it has never seen before. Global warming threatens to exterminate not only humans but most other life on Earth as well. To turn the trend around we must stop using fossil resources. However, this will be practically impossible unless we can find alternate solutions, not only for fuels but also for other chemicals produced from fossil resources. An excellent example are terpenoids, some of which can be used in production of pharmaceuticals and food additives in addition to fuels (Englund 2016). As such they are compounds of great interest in this matter, but without fossil resources how can such compounds be made? One answer lies in synthetic biology, the possibility to genetically engineer organisms to produce compounds that they otherwise would not produce at all or in sufficient quantities. Most recombinant production hosts however require a source of organic carbon and energy such as glucose to be added to the growth medium. This imposes a production cost and means that a larger area is needed since one also must grow the sugar providing plant.

Cyanobacteria however, are photoautotrophic and thus do not need any source of organic carbon or energy other than carbon dioxide and sunlight. However, to be able to use cyanobacteria as recombinant production hosts there must be sufficient molecular tools. Many common tools for genetic engineering are available for cyanobacteria; CRISPRi (Yao *et al.* 2016), promoters and ribosomal binding sites (RBS: s) (Englund *et al.* 2016) and protein degradation tags (Huang *et al.* 2010) are only a few. However, more such tools are needed, particularly for regulatory applications to control the gene expression, in order to further develop the field. An example of such tools are riboswitches, short modulating RNA segments which fold differentially in response to the presence of a certain metabolite (Nomura & Yokobayashi 2007). In this work, several riboswitches responsive to thiamine pyrophosphate (TPP), an important metabolite in the pathway to isoprene production, are investigated at both protein and RNA level for their response to both TPP and its precursor thiamine. Finally, one riboswitch is selected and investigated for its dynamic range on both protein and RNA level, with some analysis pending.

1 Background

1.1 Cyanobacteria

Cyanobacteria are a diverse type of prokaryotes capable of growing in various environments, including the cryosphere (Quesada & Vincent 2012), freshwater (Scott & Marcarelli 2012), the oceans and hypersaline environments (Whitton 2012) and in symbiosis with other organisms (Adams *et al.* 2012). Due to their ability to survive with only sunlight, water and carbon dioxide (CO_2) as energy, electron and carbon sources respectively, they are currently being widely investigated as production hosts of many important compounds, including biofuels (Miao *et al.* 2017), (Gao *et al.* 2012), (Khetkorn *et al.* 2013), terpenoids (Lindberg *et al.* 2010) and pharmaceuticals (Prasanna *et al.* 2010).

As chassis for recombinant biofuel production, cyanobacteria are advantageous since they can grow phototrophically, compared to heterotrophs such as *Escherichia coli* or *Saccharomyces cerevisiae* which require an organic energy source as feedstock. However, the supply of CO_2 can be limiting (Oliver & Atsumi 2015) and must be considered in the case of large scale production. It has however been suggested to utilize the carbon dioxide in the flue gases from e.g. cement production, thereby both supplying the cyanobacteria with their carbon source and sequestering the carbon dioxide released in the production (Kumar *et al.* 2011). Compared to other prokaryotic production hosts cyanobacteria grow slowly (a doubling time of 8-12 hours was reported by Williams *et al.* 1988) but in comparison to plant hosts such as *Arabidopsis thaliana* (generation time of approximately 7-8 weeks, (Ochatt & Sangwan 2008)) the generation time is significantly shorter.

The cyanobacterial strain used in this work is *Synechocystis* PCC 6803, originally isolated from a freshwater lake in California, USA (Rippka *et al.* 1979), hereafter referred to as *Synechocystis*. It is unicellular and commonly used to study e.g. photosynthesis and cyanobacterial genetics in general. Additionally, it is relatively easy to manipulate genetically (e.g. by transformation or conjugation of a plasmid, with the possibility of homologous recombination for incorporation into the genome) and has a fully sequenced genome, making it a good host for engineered fuel biosynthesis (Englund 2016).

1.2 *Escherichia coli* DH5α

A common strategy when working with cyanobacteria, also applied in this work, is to assemble the desired constructs in *E. coli* and from there transfer the constructs, by transformation or conjugation, to the cyanobacterial host. In this work *E. coli* DH5α was used for cloning due to its good capacity for transformation and plasmid accumulation (Invitrogen Inc., Carlsbad, CA, USA).

1.3 Riboswitches

Riboswitches are *cis*-regulatory RNA elements which fold differentially upon binding of a small metabolite (Nomura & Yokobayashi 2007). They are situated in the RNA of the 5' untranslated region (5'UTR) and are comprised of two parts: an aptamer to which the metabolite binds and an expression platform which changes its conformation in response to the induction.

If the metabolite binding leads to the formation of a transcription termination stem-loop the riboswitch is referred to as a transcriptional OFF-switch. If it instead promotes an alternative folding such that the terminator cannot fold it is called a transcriptional ON-switch (Nudler & Mironov 2004). For a schematic illustration of what this might look like, see Figure 1.

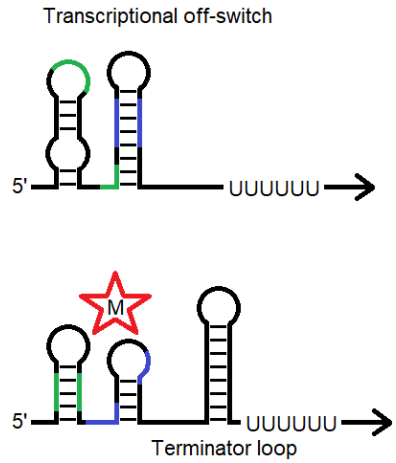

Figure 1. Schematic view of a transcriptional OFF-switch. M here denotes the metabolite inducing the riboswitches.

If the metabolite binding leads to the sequestration of the Shine-Dalgarno (SD) sequence, responsible for the ribosomal binding, the riboswitch is referred to as a translational OFF-switch. If it instead leads to the SD sequence being made accessible, the riboswitch is referred to as a translational ON-switch. (Nudler & Mironov 2004). For a schematic illustration of translational riboswitches see Figure 2.

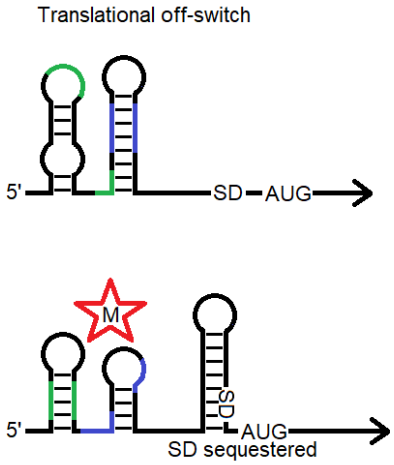

Figure 2. Schematic view of a translational OFF-switch. SD here refers to a Shine-Dalgarno sequence, responsible for ribosomal binding to the transcript. AUG here denotes the start codon of the translation. M denotes the metabolite responsible for the induction of the riboswitches.

As riboswitches are often responsible for the negative feedback control of their controlling metabolite, most of the previously found riboswitches are off switches, i.e. the transcription or translation of the controlled gene is decreased upon metabolite binding (Nomura & Yokobayashi 2007).

A great benefit of riboswitch control is that it is orthogonal to promoter control. Hence it is possible to construct AND, NAND or similar logical gates by having a riboswitch sensitive to one metabolite present upstream of a promoter sensitive to another metabolite (Morra *et al.* 2016). This means a great expansion of the available toolbox of any genetic engineer and it is thus of great interest to investigate such tools.

1.4 The non-mevalonate pathway and TPP

When producing terpenoids or similar chemicals in cyanobacteria, the non-mevalonate pathway (MEP pathway, see Figure 3) is often the starting point. And at the starting point of the MEP pathway is the enzyme DXS (1-deoxy-D-xylulose-5-phosphate synthase), converting pyruvate and glyceraldehyde-3-phosphate (GAP) to DXP (1-deoxy-D-xylulose-5-phosphate), the first substrate of the MEP pathway (Pattanaik & Lindberg 2015). From MEP there is then a long series of reactions ultimately leading to terpenoids. Thiamine pyrophosphate (TPP, see Figure 4) is a cofactor of DXS enzyme and is thus an interesting compound to investigate, see Figure 3.

1.5 TPP riboswitches in *Synechocystis*

In *Synechocystis*, TPP production is believed to be controlled by a negative feedback loop (Rodionov *et al.* 2002), where TPP inhibits its own production by binding to a putative translational riboswitch. This riboswitch, and TPP riboswitches in general, are interesting to study for two separate reasons: because they have a relevant function in the native system and because they can be used as regulatory elements in genetic circuits, e.g. as a biosensor. The biosensor function can then be used to make the system responsive to any changes in the cell leading to changed TPP concentrations.

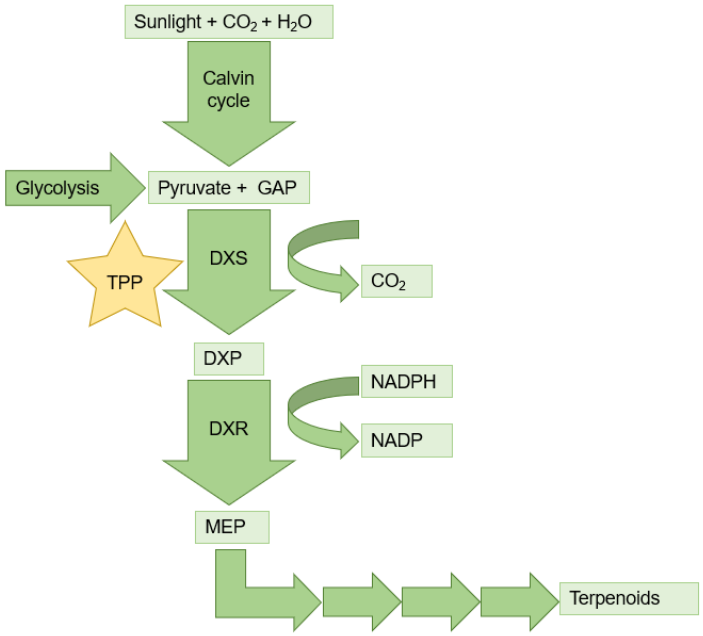

Figure 3. The non-mevalonate pathway (MEP) and the relevance of thiamine pyrophosphate (TPP). Abbreviations: GAP: glyceraldehyde-3-phosphate, DXS: 1-deoxy-d-xylulose-5-phosphate synthase, DXP:1 methylerythritol-4-phosphate, DXR: 1-deoxy-d-xylulose 5-phosphate reductoisomerase, MEP: methylerythritol-4-phosphate, NADP: Nicotinamide-adenine-dinucleotide phosphate, NADPH: NADP, reduced.

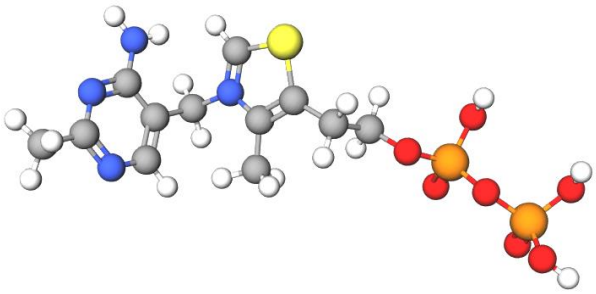

Figure 4. Thiamine pyrophosphate, TPP. Image generated with Molview, see http://molview.org/. Carbon molecules in grey, nitrogen in blue, Sulphur in yellow, oxygen in red, phosphorous in orange and hydrogen in white.

1.6 Constructs

This study investigates a total of nine constructs, summarized in Table 1. Each construct has the basic structure shown in Figure 5; a plasmid containing two different antibiotic resistance cassettes (against spectinomycin and kanamycin respectively) with a Biobrick prefix and suffix flanking the reporter gene site. The latter contains the TetR-repressible promoter PLO3 (in this study used as a constitutive promoter) from (Huang & Lindblad 2013) and the synthetic cisD50 (provided by the Ribonets project, ended in 2017), followed by a codon optimized CDS encoding the yellow fluorescent protein (YFP) *mVenus*.

Finally, all nine constructs are investigated with an AAV degradation tag (Andersen *et al.* 1998) inserted between the end of the *mVenus* gene and the Biobrick suffix, see Figure 5. This tag reduces the lifetime of the fluorescent protein and thereby increases the temporal sensitivity. This results in the nine constructs shown in Figure 6, each in the general shape shown in Figure 5 with the "Riboswitch" representing one of the riboswitches in Table 1. Each AAV-tagged construct was investigated in both *E. coli* and *Synechocystis* hosts and are referred to as the *E. coli* and *Synechocystis* strains respectively. Three versions of the basic plasmid was also investigated: without the riboswitch (named cisD50_mVenus_AAV), without either riboswitch or the AAV-tag (named cisD50_mVenus) and finally an empty vector control with a chloramphenicol resistance cassette instead of the mVenus gene (named EVC). The sequences for these are shown in Appendix A.

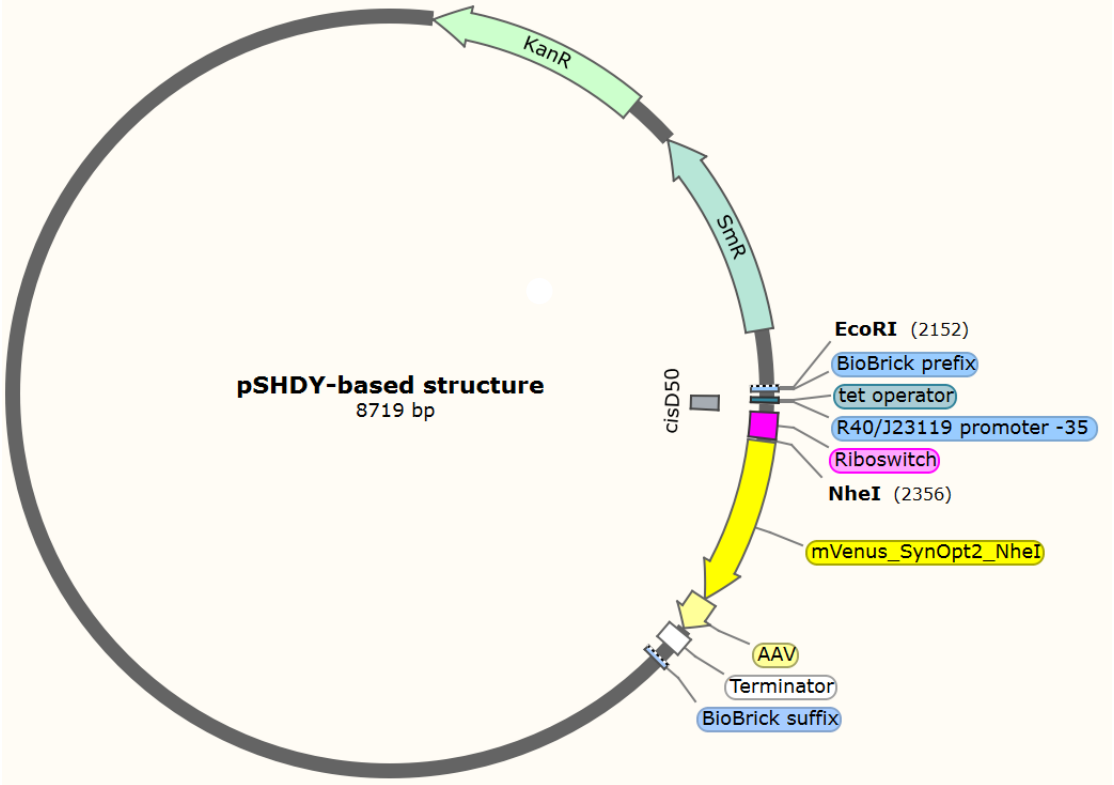

Figure 5. General structure for the AAV-tagged riboswitch constructs. *KanR* stands for kanamycin resistance gene, SmR for spectinomycin resistance gene. EcoRI and NheI are the used restriction sites. *mVenus* is the used fluorescence gene. The pink block "Riboswitch" is where the different riboswitches will be situated. The AAV-tag is situated just after the *mVenus* gene, shown in yellow. Image generated with Snapgene, see http://www.snapgene.com/

1.6.1 The *mVenus* reporter

In synthetic devices such as those designed in this work it is common to use fluorescence as a reporter system. The activity of the device can then be quantified by the *in vivo* fluorescence. Depending on the genetic circuit and experimental design this can give information regarding expression pattern of riboswitches, promoters, other genetic elements, or even other genes in the genetic circuit. In this study the fluorescent protein *mVenus* was used, a variant of YFP developed by Nagai *et al.* (2002). The emission of *mVenus* peaks at around 545 nm which is useful especially in cyanobacteria since they have pigments which absorb light at many other wavelengths (see Figure 17 under Results for an example), thereby obscuring signals from e.g. green fluorescent protein, which has its emission maximum around 508 nm (Inouye & Tsuji 1994).

1.6.2 Riboswitches investigated in this work

Three translational riboswitches have been investigated in this work; two native cyanobacterial OFF-switches from *Synechocystis* (construct *thiC*6803-) and *Nostoc* PCC 7120 (construct *thiC*7120-) respectively, and one synthetic ON-switch based on a native switch from *E. coli* (construct Seq8-), designed by Nomura & Yokobayashi (2007). Each of these three riboswitches have also been investigated in an extended version where five codons from the natively following gene (from *Synechocystis*, *Nostoc* or *E. coli*) are added to the 3'-end of the riboswitch (denoted with a + instead of the – seen above). This was done to investigate whether these codons affected the expression or inducibility of the riboswitches. Additionally,

three control constructs (constructs *ThiM*2mut2, Theo and Theo-RBS) are used. Construct *ThiM*2mut2 is designed by Nomura & Yokobayashi (2007) and is a form of construct Seq8 which is mutated to be constitutively on. Constructs Theo and Theo-RBS are theophylline ON-switches found by Ma *et al.* (2014), where construct Theo-RBS has been deprived of its RBS and is thus theoretically translationally silent (always OFF). The sequences for the riboswitch parts (gBlocks ordered and inserted into the plasmid) are shown in Appendix B.

Table 1. Summary of the nine riboswitches used in this study. *Synechocystis* denotes *Synechocystis* PCC 6803, *Nostoc* denotes *Nostoc* PCC 7120. Each riboswitch was inserted into the plasmid cisD50_*mVenus*_AAV containing the fluorescence gene *mVenus*. Each riboswitch corresponds to one *E. coli* and one *Synechocystis* strain. These strains were then denoted with the riboswitch name, e.g. for riboswitch *thiC*6803- the strain is riboswitch strain *thiC*6803-.

Riboswitch name	Origin	Description	Interesting for
*thiC*6803-	*Synechocystis*	Inducible by TPP, off, shorter version	Investigated
*thiC*6803+	*Synechocystis*	Inducible by TPP, off, longer version	Investigated
*thiC*7120-	*Nostoc*	Inducible by TPP, off, shorter version	Investigated
*thiC*7120+	*Nostoc*	Inducible by TPP, off, longer version	Investigated
*ThiM*2mut2	Synthetic, *E. coli*	Constitutively on	Positive Control
Seq8-	Synthetic, *E. coli*	Inducible by TPP, on, shorter version	Investigated
Seq8+	Synthetic, *E. coli*	Inducible by TPP, on, longer version	Investigated
Theo	*E. coli*	Inducible by theophylline, on	Induction, negative Control
Theo-RBS	*E. coli*	Inducible by theophylline, RBS inactivated	Induction, negative Control

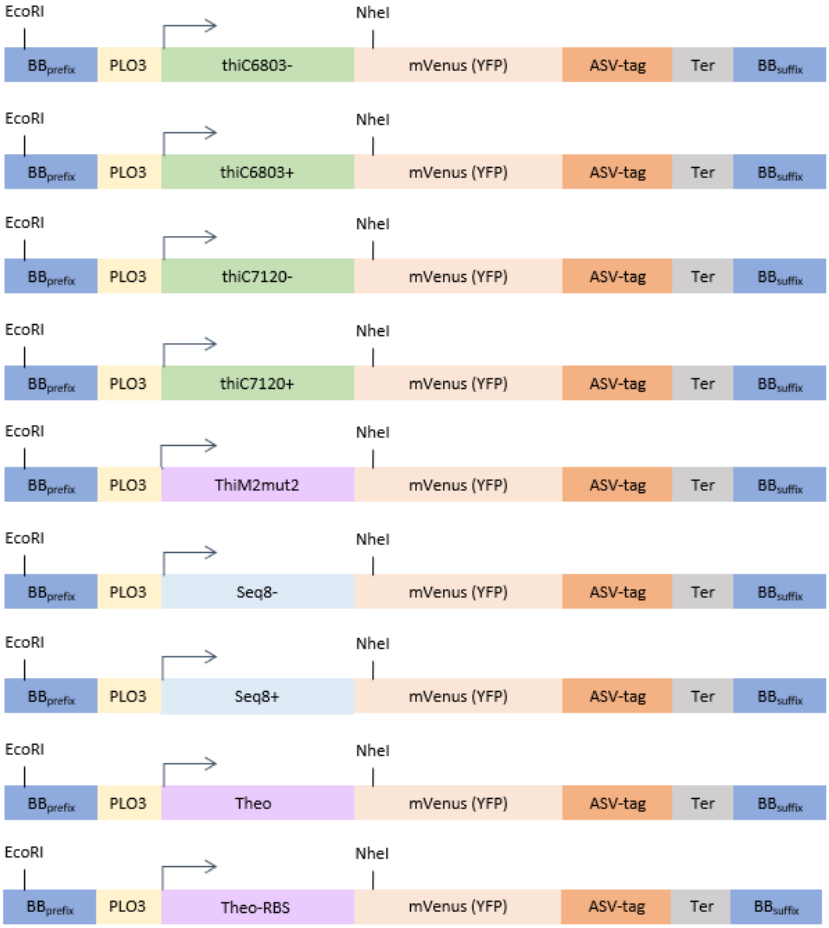

Figure 6. Summary of the AAV-tagged constructs. Strains *thiC*6803-, *thiC*6803+, *thiC*7120- and *thiC*7120+ all have cyanobacterial OFF-switches, strains with a + contain the first five codons from the following gene, from *Synechocystis* PCC 6803 or *Nostoc* PCC 7120 respectively. Strain *ThiM*2mut2 is a constitutively ON-turned version of the *E. coli* OFF-switch. Strains Seq8- and Seq8+ are synthetic ON-switches based on that from *E. coli*, again the + signifies that Seq8+ contains the five extra codons of the *E. coli* gene. Theo and Theo-RBS are theophylline ON-switches used as controls, with Theo-RBS having an inactivated RBS.

Table 2. Three basic plasmids are used as controls in the experiments, each with its corresponding *E. coli* and *Synechocystis* basic strains. cisD50_*mVenus*_AAV is the basic plasmid into which each of the riboswitches are inserted. EVC stands for empty vector control and contains a chloramphenicol resistance cassette instead of the *mVenus* gene. cisD50_*mVenus* is the same plasmid as cisD50_*mVenus*_AAV but without the AAV-tag.

Basic plasmid	AAV-tag?	*mVenus* or Chloramphenicol?	Investigated or control?
cisD50_*mVenus*_AAV	Yes	*mVenus*	Investigated
EVC	No	Chloramphenicol resistance	Control, negative
cisD50_*mVenus*	No	*mVenus*	Control, positive

1.7 Quantitative Real-Time Polymerase Chain Reaction

One method used extensively in this work is quantitative real-time polymerase chain reaction or q-RT-PCR. In this report all such methods will be denoted with qPCR since the RT is a bit ambiguous and may in some cases mean reverse transcription. The method is based on the common PCR method where two short DNA-segments called primers are used to amplify a certain DNA or cDNA (complementary DNA generated by reverse transcription from RNA) section flanked by the two primers. Today, this is performed in a machine which cycles certain temperatures appropriate for the primer binding, the section elongation and the separation of the strands.

qPCR is performed in the same way, except that a fluorescent intercalating dye which binds to the DNA is added and the fluorescence is measured continuously during the amplification. Since the dye only fluoresces when bound to the DNA, this gives a continuous measure of the amount of DNA in the sample. The cycle where the amplification turns from exponential to logarithmic is known as the cycle of quantification and is, as the name implies, used to quantify the amount of DNA originally added to the reaction. This usually includes using at least one control gene and one control sample, however in this work only control genes were used due to time constraints.

2 Materials

2.1 Genetic materials

All gBlocks® Gene Fragments, hereafter referred to as gBlocks, containing each of the riboswitches in Table 1 were ordered from Integrated DNA Technologies (IDT).

The basic plasmids in Table 2 were supplied in the form of frozen glycerol stocks from co-supervisor Dennis Dienst.

Synechocystis wild type culture was supplied from Rui Miao in the form of frozen DMSO stocks.

3 Methods

3.1 Sterilization

3.1.1 Autoclaving

All liquid media, non-filter pipette tips, E-flasks, beakers, 1-2 ml reaction tubes and PCR tubes were sterilized by autoclaving.

3.1.2 Sterile filtering

To make solutions sterile without autoclaving (either to save time or to prevent heat degradation, e.g. for stock solution 4 for BG11), they were sterile filtered using 13 mm Acrodisc® Syringe Filters, 0.2 µM Supor Membrane from Pall Life Science, excepting those containing DMSO which were filtered using Acrodisc® DMSO safe Syringe Filter from Pall corporation.

3.1.3 Presterilized materials

Some materials were purchased in presterilized form: filter pipette tips, qPCR plates, Experion chips, 6-well and 96-well plates.

3.2 Theophylline, thiamine and TPP stock solutions

Stock solutions of 100 mM were prepared of theophylline, thiamine and TPP where the first was dissolved in 100 % DMSO and the latter two were dissolved in dH_2O. Each solution was sterile filtered into a fresh 15 ml Falcon tube.

3.3 Growth media and agar plates

3.3.1 BG11 medium and BG11 agar plates

BG11 medium was prepared as described as below:

Stock solutions 1-6 were prepared as described in Table 3 and transferred to 50 ml falcon tubes, stored at -20 °C until needed, after that at 4 °C.

Table 3. BG11 recipe. Lists substances and amounts to add to make stock solutions for BG11 medium.

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

Stock solutions of $NaNO_3$: 150 g/L and 1 M TES, titrated to pH 8.0 with KOH were also prepared. A few hundred ml deionized water was added to a 1 L bottle.

1 mL of each of stock solutions 1, 2, 3, 5 and 6 were added. Stock solution 4 was added after autoclaving to prevent precipitation of the iron. 10 ml each of the $NaNO_3$ and TES-KOH solutions was added, and the bottle filled to 1 L with deionized water.

The bottle was autoclaved. Stock solution 4 was sterile filtered and 1 ml added just prior to first use. The bottle was stored in room temperature and opened only in a sterile hood to prevent contamination.

The same protocol with half the volume of deionized water was used to prepare 2x BG11 medium to be used for BG11 agar plates. Unless specified otherwise all 1x BG11 medium contained 25 µg/ml spectinomycin and 50 µg/ml kanamycin, added just before use.

3.3.2 BG11 agar plates

6 g agar powder of Type A, plant cell culture tested from Sigma® Life Sciences was mixed with 200 ml deionized water and autoclaved. When needed it was microwaved and mixed with an equal volume 2x BG11 medium. The mixture was allowed to cool until possible to touch with a bare hand and the appropriate antibiotics were added. Unless otherwise specified, the plates contained 50 µg/ml kanamycin and 25 µg/ml spectinomycin. The solution was poured in petri dishes, approximately 40 ml per plate, yielding an average of 10 plates. Plates were usually prepared fresh, and otherwise stored in 4 °C.

3.3.3 Luria Bertani broth medium

LB (Luria Bertani broth) medium was prepared from 8 g LB Broth powder from Sigma Life Sciences mixed with 400 ml water and autoclaved. All LB medium contained 25 ng/ul spectinomycin and 50 ng/ul kanamycin added just before use.

3.3.4 LB agar plates

LB agar was prepared directly as 1 % agar powder in 1x LB solution and autoclaved. The solution was heated in microwave until the gel was melted and allowed to cool until possible to touch with a bare hand. Kanamycin was added to 50 ng/µl and spectinomycin to 25 ng/µl and the solution poured in petri dishes, approximately 20 ml per plate, yielding about 20 plates. Plates were stored in 4 °C. Agar used for *E. coli* was for bacteriology, from VWR.

3.3.5 Agar plates with inducer

Agar plates containing 1 mM theophylline or thiamine were prepared as for regular LB agar plates, with the theophylline or thiamine added from 100 mM stocks at the same time as the antibiotics. *E. coli* cells were streaked on three different plates from the same original colony: plates containing theophylline, thiamine or no inducer (regular LB agar plate) and grown overnight in 37 °C.

3.4 Cell growth

3.4.1 *Synechocystis* wildtype

Synechocystis PCC 6803 wildtype strain was grown and maintained (backdiluted every three weeks or so) in 25 ml antibiotic-free BG11 medium in a 100 ml E-flask at 30 ° under approximately 50 µE with constant shaking (120 rpm). Contamination control was done on antibiotic-free BG11 plates with 1 % glucose overnight at 30 °C before conjugation.

3.4.2 *Synechocystis*

Synechocystis cells were grown on BG11 agar plates. After conjugation, six colonies for each strain were restreaked on fresh BG11 agar plates using pipette tips after around 7 days and then transferred with sterile plastic loops to 100 ml Erlenmeyer flasks containing 25 ml BG11 medium. All *Synechocystis* cells were grown at 30 °C under approximately 50 µE light with constant shaking (120 rpm) for liquid cultures. Plates were placed in a plastic box containing a small Erlenmeyer flask of water, and the plates switched daily to provide for equal light exposure.

3.4.3 *E. coli*

E. coli cells were grown on LB agar plates. 10 colonies for each strain were restreaked after one night at 37 °C and then transferred with pipette tips to 15 ml growth tubes containing 5 ml LB liquid medium and grown overnight in 37 °C.

3.4.4 Cryo cultures

Cryo cultures were made as soon as possible after the respective transformation or conjugation for *E. coli* and *Synechocystis* respectively. Cryo cultures for *E. coli* were prepared from overnight culture in 15 % glycerol and stored at -80 °C. Cryo cultures for *Synechocystis* were prepared from liquid cultures in 7 % DMSO.

3.4.5 Spectrophotometric measurements of optical density and whole cell scans

The optical densities (ODs) of *E. coli* cultures were measured at 600 nm and those of *Synechocystis* cultures were measured at 750 nm. This was done using a Cary UV-Visible Spectrophotometer (Varian) and polystyrol/polystyrene cuvettes (Sarstedt). Blanking was performed with LB medium and BG11 medium, if possible from the same bottle as the one used when inoculating the cultures. The same spectrophotometer was used for scanning the absorbance spectra of the cultures, then at wavelengths between 400 nm and 750 nm, only performed for the *Synechocystis* cultures.

3.4.6 Plate reader measurements

A Chameleon Multilabel detection platform plate reader from Hidex was used to measure both *E. coli* and *Synechocystis* cultures. OD was measured at 600 and 750 nm respectively and the fluorescence measured with excitation at 485 nm and emission at 535 nm. 150 µl was added to each well of a black-walled 96-well plate. A few wells were always filled with blank medium, LB for *E. coli* and BG11 for *Synechocystis* measurement.

3.5 Generation of Riboswitch-reporter constructs

3.5.1 Chemically competent cells

Competent cells of DH5α *E. coli* were prepared according to the protocol "Preparation of chemical competent *E. coli* cells (w/ calcium chloride)" co-authored with Dennis Dienst, to be found on the DOI number: dx.doi.org/10.17504/protocols.io.qe4dtgw

3.5.2 Plasmid extraction

Plasmid extraction from *E. coli* was performed with the GeneJET Plasmid Miniprep Kit from Thermo Scientific, according to the manufacturer's instructions.

3.5.3 DNA purification and concentration

DNA was purified and concentrated using DNA clean & concentrator™ from Zymoresearch, Nordic Biolabs.

3.5.4 Digestion

Digestion was performed using FastDigest EcoRI and NheI restriction enzymes from Thermo Scientific, according to the manufacturer's instructions.

3.5.5 Agarose gels and gel purifications

Agarose gels were made with 1 % agarose for shorter fragments such as PCR amplified gBlocks and 0.8 % agarose for longer fragments such as plasmid backbones. Gels were made with 1x TAE buffer diluted from 50x stock prepared according to the recipe in Table 4. 0.1 % 10 mM thiazole orange was added as a DNA visualization dye. GeneRuler 1 kb and 100 bp DNA ladder from Thermo Scientific were used as references depending on the length of the expected fragment. To each sample 10x green FastDigest buffer was added from Thermo Scientific as a loading dye.

Table 4. 50 x TAE buffer recipe. The amounts noted are those required for a 1 L 50x stock solution.

Substance	Amount for 1 L 50 x solution
Tris-base	242 g
Acetate (100 % acetic acid)	57.1 ml
0.5 M Sodium EDTA	100 ml
dH_2O	Up to 1 L

For gel purifications, the appropriate gel segments were cut out and purified using Zymoclean Gel DNA Recovery Kit from Zymo Research, Nordic Biolabs, according to the manufacturer's instructions, excepting that 600 µl agarose dissolving buffer was used in all cases and the gel slices melted in 42 °C for approximately 20 minutes.

3.5.6 Ligation

Ligation was performed using the Quick ligase kit from New England Biolabs Inc.™, according to the manufacturer's instructions.

3.5.7 PCR

3.5.7.1 Preparatory PCR

Preparatory PCRs were run using Phusion High Fidelity DNA Polymerase from Thermo Scientific according to the manufacturer's instructions using 10 mM primer solutions.

3.5.7.2 Colony PCR

For colony PCRs of *E. coli*, five of the ten restreaked colonies were selected. For *Synechocystis* colony PCRs, three colonies out of six restreaked were investigated. In both cases, some colony was scraped up with a pipette tip and dissolved in 10 µl of deionized water. 1 µl of this was then used in a PCR using DreamTaq polymerase with Green DreamTaq Buffer from Thermo Scientific according to the manufacturer's instructions. In some cases, Taq DNA Polymerase Thermo Scientific was used instead with the same protocol using 10 mM primer solutions.

3.5.8 Transformation

Transformation was performed using Chemically competent DH5α *E. coli* cells as described in the protocol "Transformation of *E. coli* cells of strain DH5α", to be found on the DOI: dx.doi.org/10.17504/protocols.io.mqqc5vw

3.5.9 Sequencing

Sequencing was performed by the overnight TubeSeq service of Eurofins Genomics from plasmid miniprep for *E. coli* and gel purified colony PCR product for *Synechocystis*.

3.6 Conjugation

Conjugation was performed according to the protocol "Triparental mating of *Synechocystis* (Conjugation)" by Anna Behle, to be found on the DOI: dx.doi.org/10.17504/protocols.io.ftpbnmn

One modification was done: After spreading the cyanobacteria on the HATF filter and incubation for 48 h, the filter was transferred to a fresh BG11 agar plate containing antibiotics.

3.7 RNA extraction and handling

Throughout all RNA extraction, purification, quantitation etc., nuclease-free, molecular biology grade water from Thermo Scientific was used.

3.7.1 RNA extraction

RNA extraction was performed according to Pinto *et al.* (2009). Cell lysis was performed with a Precellys 24 lysis & homogenization from Bertin Technologies and 0.2 g acid-washed glass beads added to each tube.

3.7.2 RNA quantitation and quality control

RNA quantitation and quality control were achieved by capillary gel electrophoresis using the Experion Automated Electrophoresis System from Bio-Rad according to manufacturer's instructions. Due to a shortage of the accompanying RNA ladder, the RiboRuler High Range RNA ladder from Thermo Scientific was used. An initial estimation of the RNA concentration was also gained with a NanoDrop 2000 Spectrophotometer from Thermo Scientific.

3.7.3 DNase I digestion

Extracted and quantified RNA was treated with DNase I from Thermo Scientific or Ambion according to the manufacturer's protocol. The DNase was removed either by use of the kit accompanying the RapidOut DNA Removal kit, according to the manufacturer's instructions or with phenol chloroform extraction as described below.

3.7.4 Phenol-chloroform extraction

To each 50 µl DNase I digestion reaction, 150 µl nuclease-free H_2O was added followed by 100 µl Phenol (acid pH) and 100 µl Chloroform. The tubes were centrifuged at 4 °C for 10 minutes at 12700 g for 10 minutes and then supernatant transferred to a fresh tube. 600 µl sterile filtered 30:1 ethanol: sodium acetate (3M) was added and the RNA was precipitated at -20 °C over night. 1 µl RNA grade glycogen from Thermo Scientific was added and the RNA allowed to precipitate once again over night. The samples were centrifuged at 4 °C at high speed for 30 minutes and the supernatant removed. The samples were washed with 300 µl 70 % sterile filtered ethanol three times with 10 minutes centrifugation at 4 °C and high speed in between. All ethanol was carefully removed, and the pellet resuspended in 11 µl nuclease-free water.

3.7.5 Primer efficiency

Primer efficiency of the primer pairs for *rnpB*, *mVenus* and *kanR* was estimated by running a qPCR using DNA from the genome of *Synechocystis* as a template for the first primer pair, and purified basic plasmid cisD50_mVenus for the latter two. This was used to calculate the cQ and the primer efficiency of each primer pair.

3.7.6 Reverse transcription

Total DNase I-treated RNA was reverse transcribed into the corresponding cDNA using iScript RT Supermix for RT qPCR from Bio-Rad according to the manufacturer's instructions.

3.7.7 qPCR

Quantitative PCR (qPCR) was conducted in white-welled 96-well plates using SYBR Green Master mix from Quanta bio Perfecta™ according to the manufacturer's instructions. In all experiments the RNA levels were normalized after the *rnpB* primer pair (primers 7 and 8, see Table 5). This was done using the software accompanying the qPCR machine (CFX Connect™ Real-Time System from Bio-Rad), by calculating the ΔcQ where the cQ is the cycle at which the PCR amplification reaches its logarithmic phase. It is also possible to calculate the ΔΔcQ, also comparing to a control well on the same plate but this was not done due to lack of control sample.

Table 5. Primers used in this work. Included is a description of the primer sites, whether they are forward or reverse primers and their sequence. *KanR* is the kanamycin resistance cassette, rnpB is a reference gene in the *Synechocystis* genome, *mVenus* is the reporter fluorescence gene and *thiC* is the native *Synechocystis* gene following the native riboswitch.

Primer no.	Description	Forward or reverse	Sequence
1	Biobrick prefix primer	Forward	ccaggaattcgcggccgcttctagag
2	Biobrick suffix primer	Reverse	gctcctgcagcggccgctactagta
3	For the gene *slr* in the genome, used to detect residual DNA	Forward	gcatcctaggactaaacccattgcccccctc
4	For the gene *slr* in the genome, used to detect residual DNA	Reverse	gatccacttccgctaccactaacccc actccttagccc
5	*kanR*	Forward	attgtatgggaagcccgatg
6	*kanR*	Reverse	attccgtcagccagtttagtc
7	*rnpB*	Forward	agaggtactggctcggtaaa
8	*rnpB*	Reverse	tcaagcggttccaccaatc
9	*mVenus*	Forward	tagtcacgaccctcggttat
10	*mVenus*	Reverse	ccgttcttgaacatatccctct
11	*thiC*	Forward	ggtggtgacttagacgtgattc
12	*thiC*	Reverse	ctctccagggcttggtaaatg

3.8 Plate reader experiments

3.8.1 Evaporation and growth evaluation in 6-well plates

In order to determine both the approximate evaporation from each well and the approximate growth of the culture over one day, a short measurement was performed. Each culture used in *Synechocystis* experiment 3 (strain *thiC*6803+ in three biological replicates and all three basic strains) was used to inoculate one well in a 6-well plate to an $OD_{initial}$ of 0.4. The 6-well plate was then sealed with plaster tape. The following day the OD and volume of each well was measured and averaged to assess the average OD_{final} and remaining volume V_r over all wells. The average OD was used to calculate the growth factor $F_g = OD_{initial}/OD_{final}$.

These values were used to calculate the Volume to keep = $V_{keep} = F_g * V_{desired}$, and from that the volume to remove = $V_{remove} = 4\ ml - V_{keep}$ and the volume to replenish = $V_{replenish} = V_{desired} - V_{keep}$.

3.8.2 *Synechocystis* experiments in *Synechocystis*

Three different *Synechocystis* experiments were executed for the *Synechocystis* riboswitch strains with AAV-tags and the three basic strains as controls in some cases. The first experiment was carried out similarly to the third *E. coli* experiment, to get a first glance into the functionality of the riboswitches in *Synechocystis*. The second *Synechocystis* experiment was performed in E-flasks using both thiamine and TPP in an effort to investigate whether TPP is more efficient than thiamine in inducing the riboswitches. Finally, the third experiment used either thiamine or TPP in order to discern whether the effect seen in experiment 2 was due to thiamine or TPP. It also investigated the dynamic range of both thiamine and TPP between 0 and 1 mM.

3.8.2.1 Synechocystis experiment 1

Synechocystis experiment 1 was carried out in 6-well plates over three days. Before the first day inoculations of three biological replicates of each cyanobacterial riboswitch strain were made in both 6-well plates (4 ml/well) and allowed to grow to $OD_{750\ nm}$ around 1.

On the first day, the OD of each 6-well was measured and the values used to inoculate three new wells per biological replicate to $OD_{750\ nm}$ = 0.2 in 4 ml BG11 (leading to a total of 18 6-well plates). One additional ml of $OD_{750\ nm}$ = 0.2 was prepared separately of each culture and used to measure the 0-day fluorescence values in technical quadruplicates in the plate reader. Each six-well was then induced with either 1 mM theophylline, 1 mM thiamine or nothing (referred to as the LB control). The plates were sealed with plaster tape to reduce the evaporation.

On the second day, 1 ml samples were taken for OD measurement and an additional 0.5 ml taken and with the help of the OD measurements diluted to $OD_{750\ nm}$ = 0.2 before measuring the fluorescence in the plate reader. The plates were resealed with the same plaster tape. This procedure was repeated on the third day and the cultures discarded.

3.8.2.2 Synechocystis experiment 2

The second fluorescence measurement was performed in 100 ml E-flasks which were inoculated in 20 ml BG11 medium, with each of the riboswitch strains as well as the basic strains cisD50_*mVenus*_AAV and EVC (not cisD50_*mVenus*, this was excluded to reduce the workload), each in two technical replicates. All cultures were set to grow over three days and then backdiluted to OD = 0.2 in fresh E-flasks. The cultures were allowed to grow over night and two types of samples were taken. First, 10 ml was taken from one replicate (replicate 1) of each strain and immediately used for RNA extraction as described. 10 ml were also removed from each of the other replicates (replicate 2 for each strain) and discarded. Second, the remaining culture was backdiluted with another 10 ml of BG11, to an approximate OD of 0.2 (not measured) and induced with both TPP and thiamine, each to a concentration of 1 mM. This was repeated on two more days (complete with fresh TPP and thiamine to a concentration of 1 mM each in the replenishing medium).

3.8.2.3 Synechocystis experiment 3

The third and final *Synechocystis* experiment included only one of the riboswitch strains, strain Seq8+, in three biological replicates as well as three controls, the basic *Synechocystis* strains. Each of these was used to inoculate 10 separate 6-wells to 4 ml of OD = 0.4. 1 ml with the same OD was also prepared separately and used to measure the fluorescence in the plate reader. Each of the ten replicates of each strain was then induced with either TPP or thiamine to one of the concentrations 0, 50, 250, 500 or 1000 µM using stock solutions of 100 mM. The 6-well plates were sealed with plaster tape to reduce evaporation and allowed to grow until the next day. Simultaneously, the first biological replicate of strain Seq8+, along with the basic strain cisD50_*mVenus*_AAV, were inoculated to OD = 0.4 in two replicates in E-flasks. Each replicate was induced with 1 mM either thiamine or TPP and allowed to grow until the next day.

The following day 10 ml samples were taken from the E-flasks and used for RNA extraction as described. The BG11 and thiamine or TPP was replenished as before. For the 6-well plates the calculated evaporation volume V_e and growth factor F_g were used to calculate the volume to take out and replenish as described in the "Evaporation and growth evaluation in 6-well plates", see above, using $V_{desired}$ = 5 ml. The calculated volumes were removed and replenished with fresh BG11 medium, leading to an approximate backdilution to OD = 0.4. 1 ml samples were taken from each well and used for *Synechocystis* experiment. The appropriate concentrations of thiamine or TPP were replenished in proportions corresponding to the replenished volume. This was repeated again on the third day.

4 Results

4.1 Cloning

Below follow the results of the cloning procedure with example results shown in Appendix C.

4.1.1 Basic plasmid preparation

E. coli cells already containing the basic plasmid cisD50_mVenus_AAV were grown overnight and the plasmid was extracted. The plasmid was digested with EcoRI and NheI, and a fraction of approximately 8400 bp, the expected length, was isolated by gel purification, see Appendix C, Figure 28 for an example gel.

4.1.2 gBlock preparation

gBlocks of all nine riboswitches were amplified by PCR and fractions of around 250 bp, the expected length, were isolated by gel purification, see Appendix C, Figure 29 for an example gel. After purification, samples were digested with EcoRI and NheI, purified and concentrated.

4.1.3 Ligation and transformation

The digested basic plasmid was ligated together with each of the digested gBlocks, yielding the final constructs described in Figure 6, and the resulting ligation mixture was used in a transformation of competent *E. coli* DH5α cells. The colonies were restreaked and grown in LB medium overnight and the plasmids purified.

4.1.4 Colony PCR and sequencing

Colony PCR analysis with primers 16 and 17 (see Table 5) was performed for each of the first five restreaked colonies if present, yielding fragments of around 1100 bp, visualized in an agarose gel. This was done in several batches in order to reduce workload, but one example gel is shown in Appendix C, Figure 30. The identified colonies were checked by sequencing. Eventually one clone each from all riboswitch strains was confirmed this way.

4.2 Conjugation

The chosen *E. coli* colonies were used for conjugation of *Synechocystis* cells. The resulting colonies were restreaked on BG11 agar plates and then grown in E-flasks. A colony PCR using primers 1 and 2 was run from the restreaks, and the resulting 1100 bp fragments, when present, were isolated by gel purification (see gel in Appendix C, Figure 31) and sent for sequencing. Again, some of the fragments appear to be somewhat shorter than the expected 1100 bp but were still assumed to be correct. Results were positive for all clones except clone one of construct 9, where no match was found. Constructs 6 and 7 were not sent for sequencing as they did not have any positive bands on the gel, and thus the sequence identities of these clones are unknown.

4.3 *In vivo* fluorescence of *E. coli* reporter strains

In order to pre-test which clones, and which riboswitches were active, three different types of *E. coli* fluorescence measurements were performed.

4.3.1 Inducer-supplemented agar plates

The first *E. coli* fluorescence measurements investigated LB agar plates containing 1 mM theophylline or thiamine respectively. Transformed *E. coli* cells (of all riboswitch strains) were streaked on the plates and viewed under ultraviolet light, with some example results shown in Figure 7. The difference between either strains or inductions or even colonies is difficult to discern, but some fluorescence is visible for all constructs, including those not shown in Figure 7.

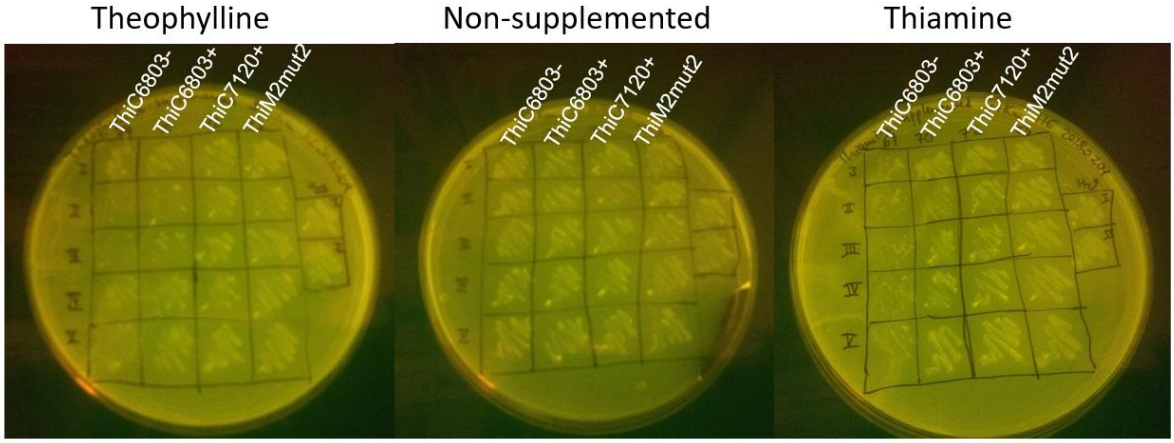

Figure 7. Example of inducer supplemented LB agar plates under UV blue light. See Table 1 for further descriptions of the strains.

Similar results were obtained for the other strains. These results indicated too small differences between both strains and inductions. One theory was that the background fluorescence of the LB agar plate obscured the signal.

4.3.2 Liquid cultures

Each clone was then inoculated in liquid culture, cells spun down and the supernatant removed. This showed a clear difference discernible between strains, see Figure 8. Strains *thiC*6803+, *thiC*7120+ and possibly Seq8+ had a higher basic fluorescence level, as did the basic strain cisD50_*mVenus*_AAV. However, there was a concern that different cell densities of the different strains might affect the signal and so this was not done with inductions.

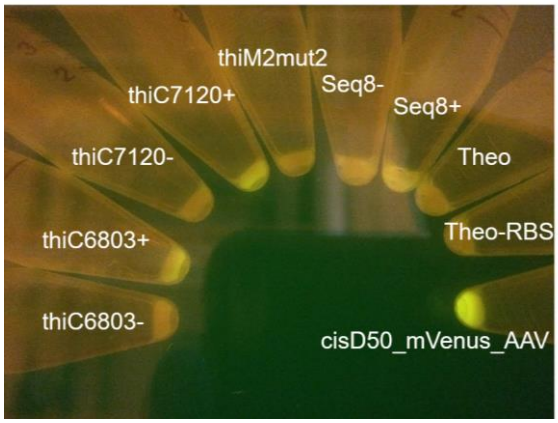

Figure 8. *E. coli* cell pellets under UV/blue light illumination. See Table 1 for further descriptions of the riboswitch strains. See Table 2 for further description of cisD50_*mVenus*_AAV.

4.3.3 Plate reader

To correct for both the background fluorescence of the LB medium and the cell density normalization, the same experiment was performed in the plate reader with cells spun down and resuspended in NaCl solution, see Figure 9. It was performed with all three different inductions in all nine riboswitch strains and for the basic strain cisD50_*mVenus*_AAV. The same differences between strains were observable though no significant difference between inductions could be seen. At least a slight increase can be noted in riboswitch strain Seq8+ in response to thiamine, but it is not quite significant, and the level goes down again by the next day. There is also an increase visible for strain *thiC*7120+, but this is deemed to be an artefact since this is an OFF-switch and the level goes down again by the next day.

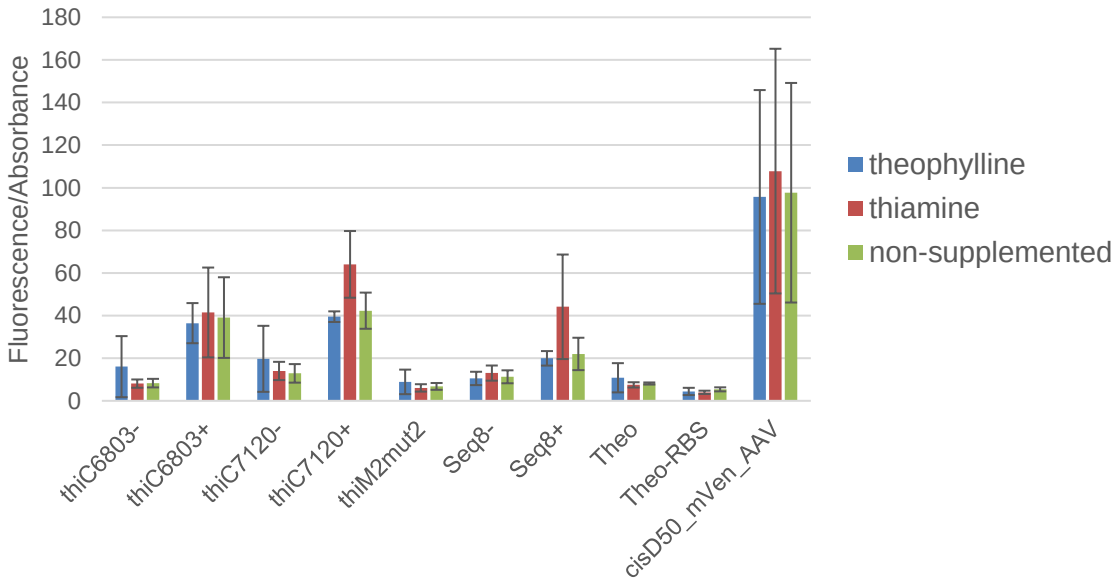

Figure 9. Plate reader experiment with *E. coli* strains. Cells spun down and resuspended in NaCl. See Table 1 for further descriptions of the riboswitch strains. See Table 2 for further descriptions of the basic strain cisD50_*mVenus*_AAV.

4.4 Pre-experiments *Synechocystis*

Some pre-experiments were performed for the basic cyanobacterial strains (cisD50_*mVenus*_AAV, EVC and cisD50_*mVenus*, see Table 2). This was done for the purpose of establishing protocols for plate reader measurements, RNA extraction and qPCR, as well as generating reference data to which the data of the riboswitch strains could later be compared.

4.4.1 Growth curve and basic fluorescence measurement

A growth curve was established, see Figure 10, over three days for each of the three basic strains. The cultures were grown in E-flasks without any induction, with the purpose to establish the range of linear growth for the cells. It seems that the growth is roughly linear (lowest R^2-value is 0.9927) in the range $OD_{750\ nm}$ = 0.2 to 1.2, for all three basic strains.

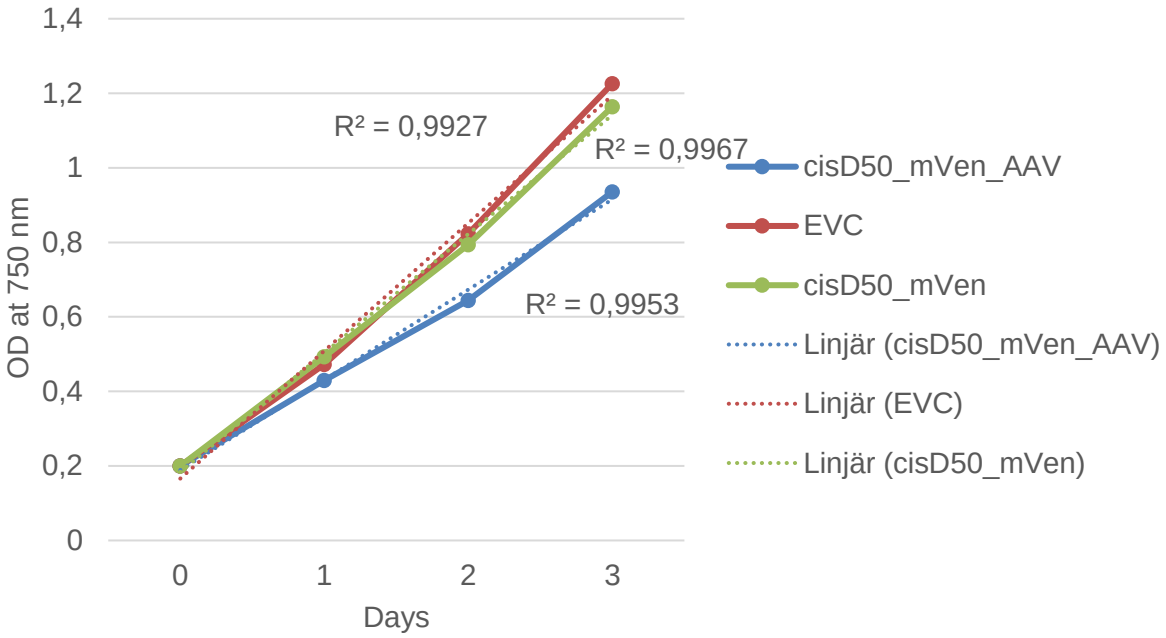

Figure 10. Growth curves of the basic strains. For further description of the basic strains, see Table 2.

The fluorescence shown in Figure 11 was measured both in order to verify the behaviour of the strains and to establish the plate reader protocol. The fluorescence increased in the case of basic strains cisD50_*mVenus*_AAV and cisD50_*mVenus* but remained low for the EVC strain. This, as well as the higher general fluorescence for cisD50_*mVenus*, is expected since the EVC strain lacks the gene for the fluorescent protein and cisD50_*mVenus* has the fluorescence gene and lacks the degradation tag. This is interpreted as meaning that the basic strains are behaving as expected and that the plate reader protocol is successful.

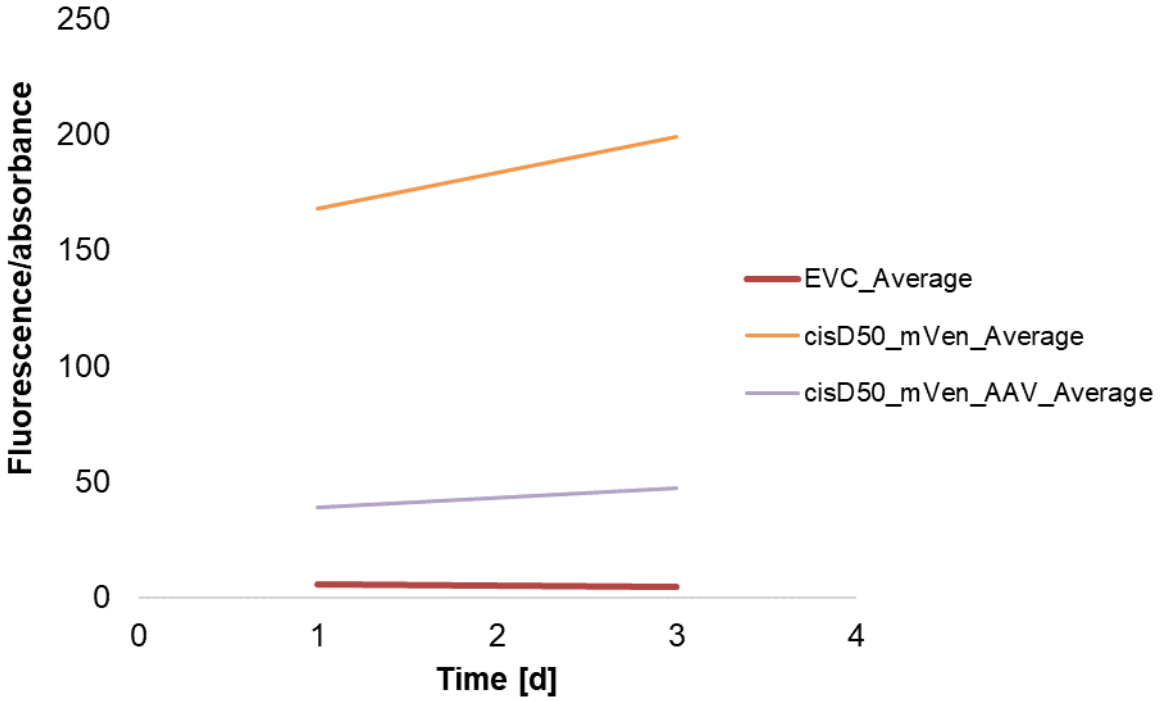

Figure 11. Fluorescence measurement of all basic cyanobacterial strains. The average of four technical replicates is shown for each strain. The culture was grown for three days but only measured on days 1 and 3. See Table 2 for further descriptions of the basic strains.

4.4.2 RNA measurements

To establish the fluorescence measurement, RNA preparation and qPCR protocols and to monitor the transcript levels of the basic strains in order to compare to those of later experiments with the riboswitch constructs, total RNA was extracted from each of the three basic strains.

To assess and compare the accuracy of the NanoDrop and Experion methods of RNA quantification, the extracted RNA from the basic strains was quantified both with NanoDrop (only nondiluted) and in a dilution series in Experion, giving the results in Figure 12. From this it seems that the two methods are roughly equal in quantification, although cisD50_*mVenus* diverges between the two methods. However, given the R^2 value and the data points of lower dilution factor, it is believed that the RNA concentration is actually somewhere in between what is found by the two methods. Thus, it was assumed that the NanoDrop and Experion were equally trustworthy for quantitation.

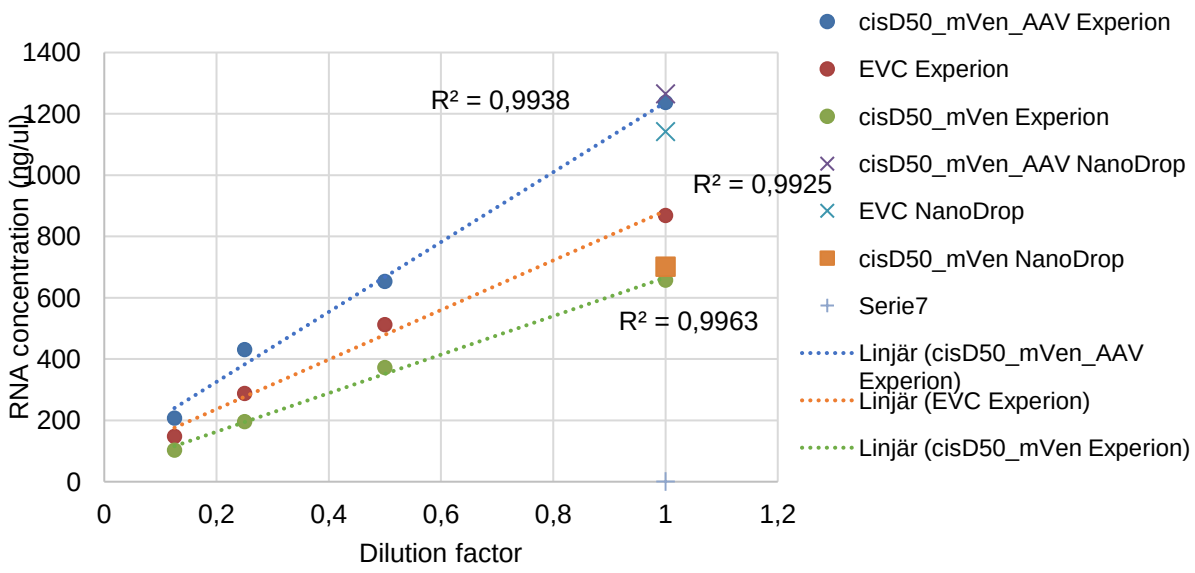

Figure 12. Comparison between NanoDrop and Experion measurements. EVC stands for empty vector control. cisD50_mVen_AAV is the basic plasmid later containing the riboswitches, and cisD50_mVen is the same plasmid without the AAV-tag.

For qualitative analysis however, the Experion is vastly preferable. The same Experion run as used for the quantitation also gave a qualitative result showing the expected peaks for total RNA in *Synechocystis*, see Figure 13 for the generated *in silico* gel image and Figure 14 for one example chromatogram. These show the expected RNA bands, corresponding to the ribosomal RNA in cyanobacteria, which is interpreted as good RNA quality. These chromatograms set the basis for the expected chromatograms from later RNA extractions.

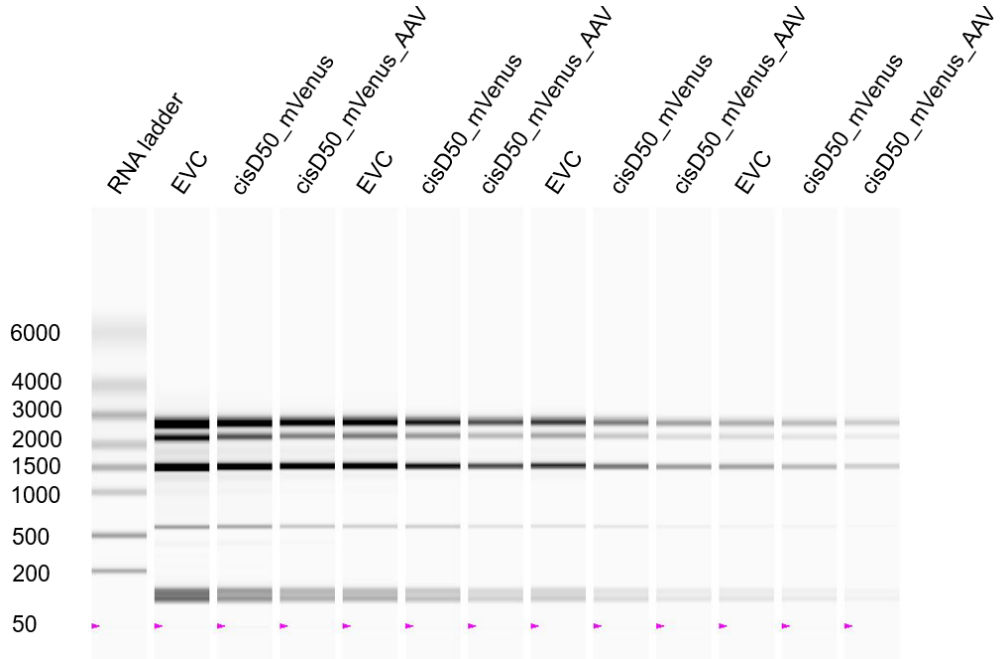

Figure 13. Experion dilution series of the basic strains. Dilution factors are decreasing from left to right: 1, 0.5, 0.25, 0.0625, with all three strains in each dilution as shown. See Table 2 for further descriptions of the basic strains.

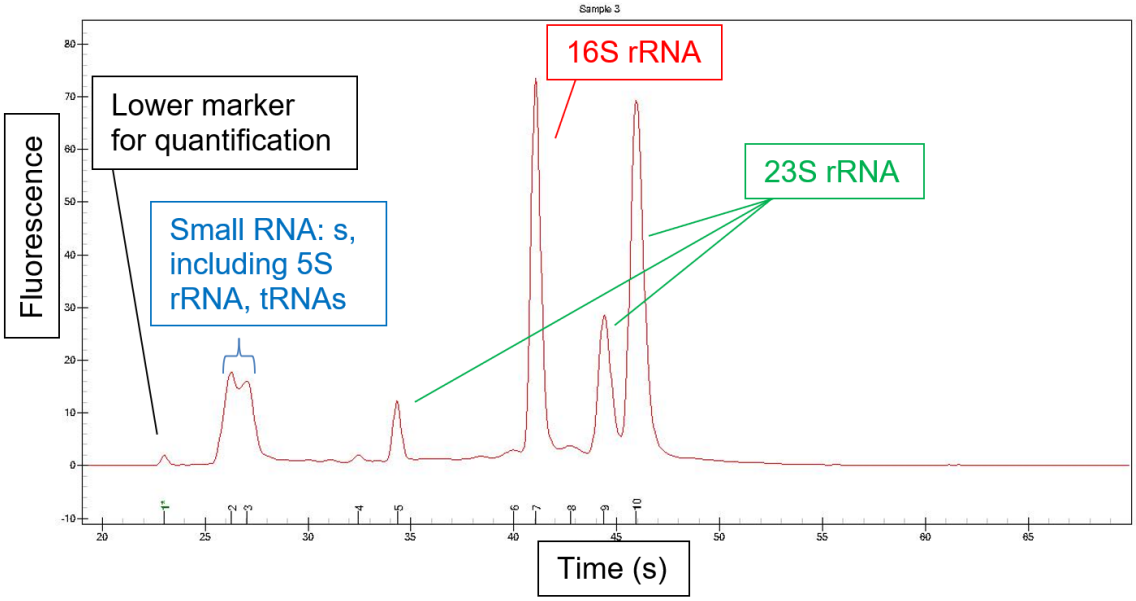

Figure 14. Chromatogram for cisD50_*mVenus*_AAV, dilution factor 1. See Table 2 for further description of cisD50_*mVenus*_AAV.

To remove any contaminating DNA before the reverse transcription, the RNA was treated with DNase I. It was then used in a PCR with accompanying agarose gel, see Figure 15. Some untreated RNA from each strain were used as controls, as well as a DNA sample from one of the basic strains.

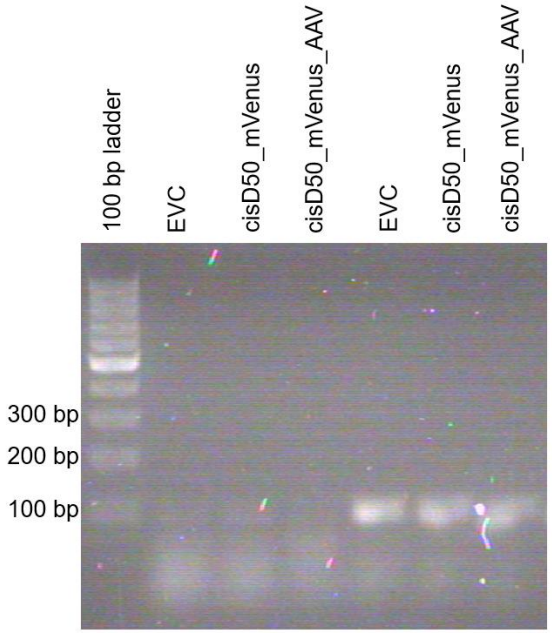

Figure 15. DNase treatment sufficiency test. A PCR was run using primers for a gene from the genome of *Synechocystis* to screen for remaining contaminating DNA. For further description of the basic plasmids, see Table 2.

As can be seen in Figure 15, the DNA degradation was only partially successful, with some very faint bands at 250 bp remaining after DNase digestion. This is however deemed to be little enough that it should hopefully not interfere with the qPCR.

4.4.3 Primer Efficiency test

In order to find out how well the primers planned for the qPCR worked, a primer efficiency test was performed for the primers 5 through 10 for the genes *mVenus*, kanamycin cassette (both on the basic plasmid cisD50_mVenus) as well as *rnpB* from the genome, with basic plasmid cisD50_mVenus and genomic *Synechocystis* DNA as the respective templates, see Table 6. The idea behind this was to achieve a more accurate quantification of the transcript by adjusting for the fact that the efficiency is usually not 100 %.

This would later be used for real-time PCR of DNA from *mVenus* and the kanamycin cassette (*kanR*), both on the basic plasmid, as well as from *rnpB*, which is encoded on the *Synechocystis* genome. cisD50_mVenus plasmid and genomic *Synechocystis* DNA were used as the respective templates, see table 6. However, the primer efficiency for primers 11 and 12, for the gene *thiC*, were not originally planned and so no primer efficiency test was performed. Thus, the standard primer efficiency of 100 % was used for these primers.

Table 6. Templates and found primer efficiencies for the three primer pairs *kanR* (for the kanamycin resistance gene), mVen (for *mVenus*) and *rnpB* (in the *Synechocystis* genome). For further description of the basic plasmid cisD50_mVenus, see Table 2.

Primer numbers /template gene	Template	Primer efficiency (%)
5 and 6/*kanR*	cisD50_mVenus	96.33
9 and 10/*mVenus*	cisD50_mVenus	93.86
7 and 8/*rnpB*	*Synechocystis* 6803 genomic DNA	107.34

4.4.4 PCR for the basic strains

The DNase-treated samples were reverse transcribed into cDNA and used in a qPCR, with calculated transcript levels shown in Figure 16. This was done in order to get an idea of whether the *mVenus* expression was affected by the differences between the basic strains on the transcript level.

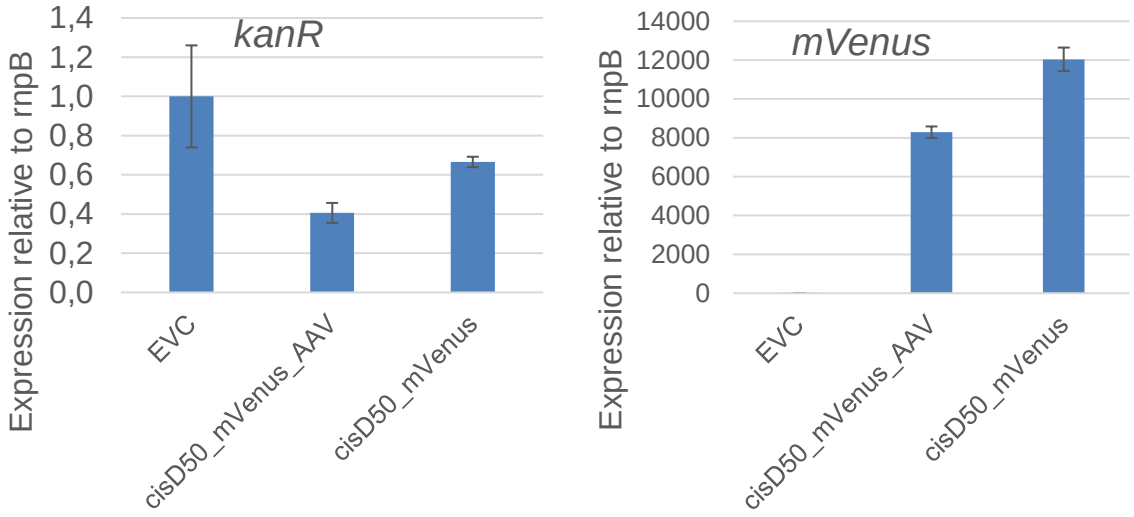

Figure 16. Evaluation of basic strains on the transcript level. Kan: used kanamycin resistance gene primers. mVen: used *mVenus* gene primers. See Table 2 for further descriptions of the basic strains.

The qPCR results in Figure 16 suggest that the basic strain cisD50_*mVenus*_AAV gives lower transcript levels of both mVenus and *kanR* when compared to cisD50_*mVenus*. The cisD50_mVenus_AAV level appear to be about ¾ of the cisD50_mVenus levels in both cases. Since the difference is roughly the same this is interpreted as a difference in plasmid expression, the cisD50_mVenus producing more of the plasmid than cisD50_mVenus_AAV. The empty vector control behaves as expected, with *kanR* values in the same range as the other genes (albeit somewhat higher) and mVen values in much lower quantities.

4.5 Assessment of TPP-riboswitch function in *Synechocystis*

Once the AAV-tagged constructs had been conjugated into *Synechocystis*, the resulting strains were investigated on a number of different levels; growth rate, fluorescence and RNA. The absorbance spectra between 400 and 750 nm of the liquid cultures were compared to those of the basic strains. Three different fluorescence experiments were performed, in order to assess the expression levels, and the two latter were complemented with RNA analysis.

4.5.1 Absorbance spectra

Absorbance spectra of each of the basic plasmid- containing *Synechocystis* cultures were taken as a background and compared to absorbance spectra of each of the constructs 1-9 to estimate any differences in pigmentation due to the riboswitches. The spectra are shown in Figure 17 and Figure 18 respectively. They appear similar in general shape, with peaks in the same general positions and of the same approximate dimensions. This is interpreted as meaning that no significant pigment changes have occurred due to the riboswitches.

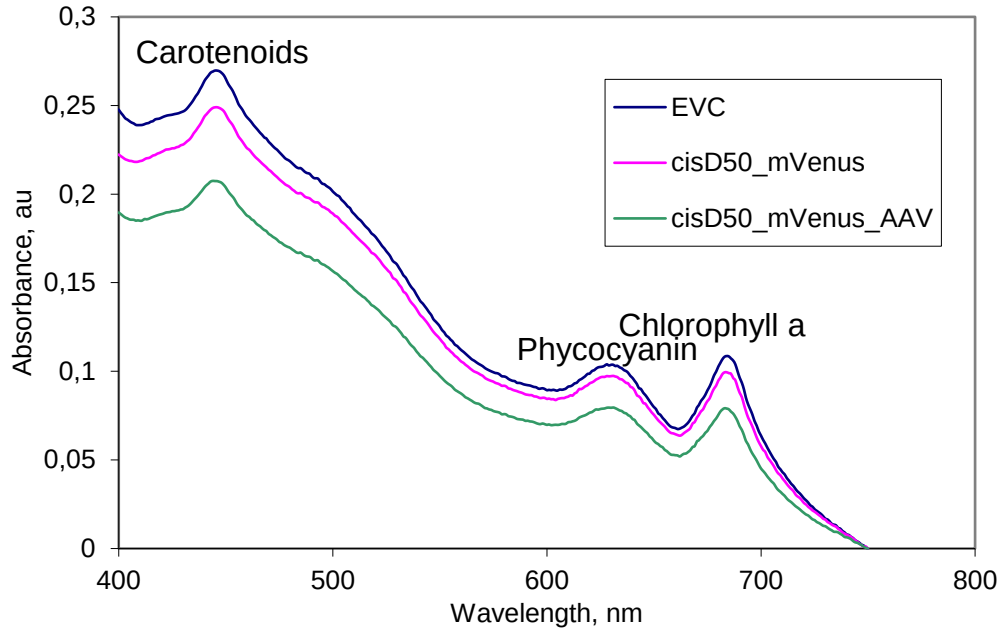

Figure 17. Whole cell scan of the basic plasmids. Noted are the major pigments and their respective peaks. For further description of the basic plasmids, see Table 2.

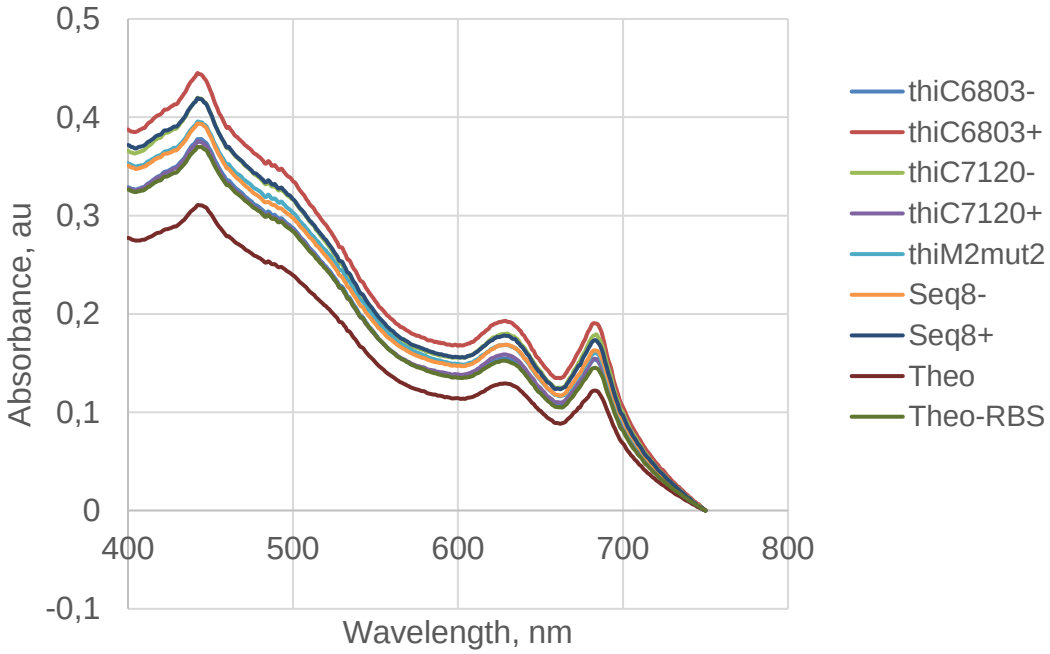

Figure 18. Whole cell scan of all riboswitch strains. For further description of the riboswitch strains, see Table 1.

4.5.2 *Synechocystis* experiment 1

Similar to the plate reader experiment on *E. coli*, *Synechocystis* experiment 1 investigated thiamine, theophylline and no induction on each of the riboswitch strains as well as the basic strains, giving the results in Figure 19. It was noticed that the theophylline-induced cultures had formed clumps or islands of cells whereas the remaining solution was clear, see Figure 20. This indicated that the cells were either dead or in a severely stressed state, either of which would compromise the experiment. Additionally, no difference other than that otherwise noted between the strains was visible. Both theophylline strains Theo and Theo-RBS seem unresponsive to theophylline as well as thiamine, further indicating that this experimental setup was not successful for the theophylline case.

As for the thiamine induction, strains *thiC*6803+ and *thiC*7120+ did not give significantly higher expression than strain *thiC*7120-, as noted for the *E. coli* experiment. Strain *ThiM*2mut2, which should be constitutively ON shows a fluorescence only slightly higher than that of the EVC, which does not encode *mVenus*. Strains Seq8- and Seq8+ show significantly higher expression. This shows a difference in expression in *E. coli* and *Synechocystis* on the strain level since the Seq8 strains were lower in expression in *E. coli*. However, the differences between inductions were not reliable for either and the levels seem to decrease over time for all constructs, including control strains. It is possible that thiamine is not taken up by the cells and/or converted into TPP, which could explain the lack of a clear effect.

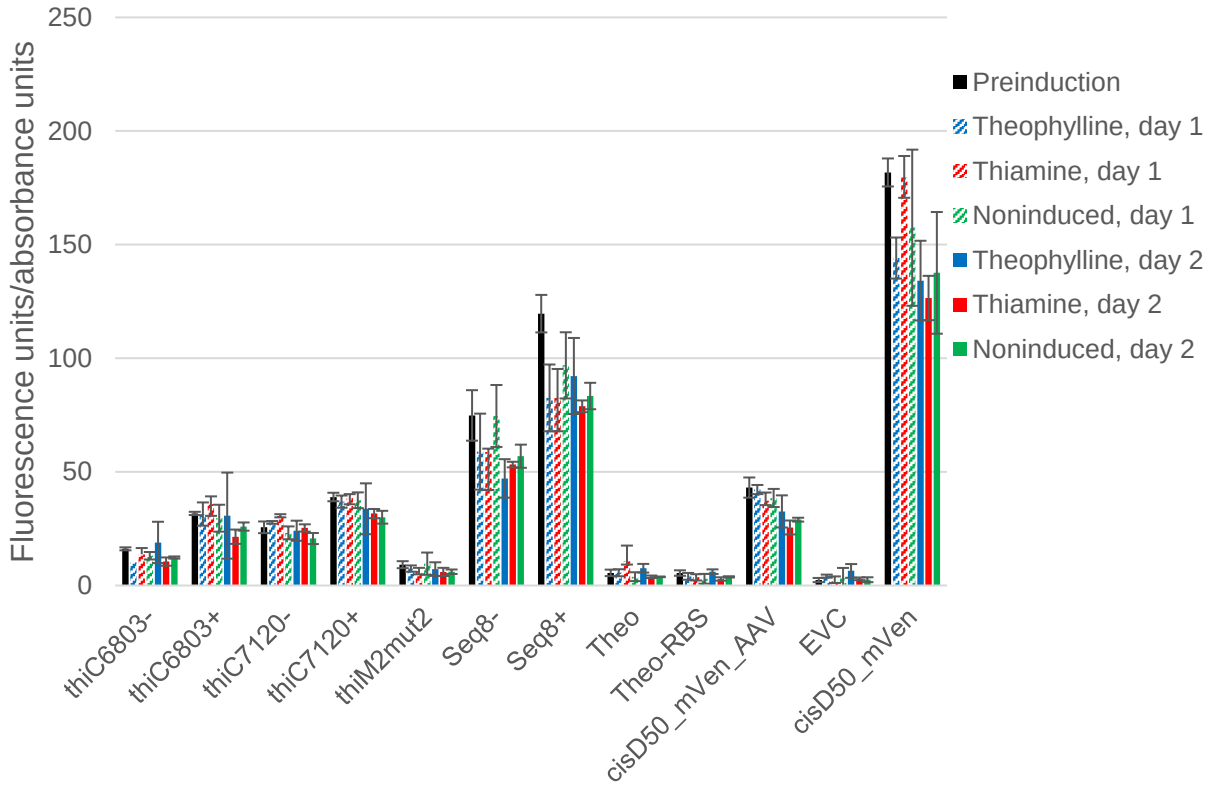

Figure 19. Results of *Synechocystis* experiment 1. Cultures were induced with 1 mM theophylline or thiamine, or no induction. Samples were taken prior to induction as well as at 24 h and 48 h after induction. Shows average of three biological replicates of each of the *Synechocystis* riboswitch strains as well as all three basic strains. For further description of the riboswitch strains, see Table 1. For further description of the basic strains, see Table 2.

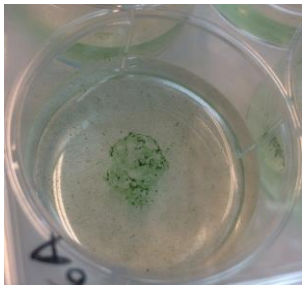

Figure 20. One well containing theophylline-induced *Synechocystis* culture.

4.5.3 *Synechocystis* experiment 2

Due to the poor growth and fluorescence levels seen for the theophylline induction in the *Synechocystis* experiment 1, these inducers were not investigated in fluorescence experiments 2 or 3. In order to be able to sample reasonable volumes for RNA extraction (in this work 10 ml culture was sampled from each strain), *Synechocystis* experiment 2 was conducted in E-flasks. Due to the doubts about thiamine being taken up and/or converted to TPP, both substances were added to the medium in a co-induction. The plan was to do an additional experiment (*Synechocystis* experiment 3) later to differentiate between their effects, if any were observable. In addition, each culture was diluted back 2 times every day, also compensating for the volume removed for RNA extraction.

The fluorescence results are shown in Figure 21. The same basic level of each strain is seen in *Synechocystis* experiment 1, but strain Seq8+ shows a clear increased fluorescence after induction, with its shorter version strain Seq8- also seeming to increase after induction. For this reason, strain Seq8+ was chosen for further experiments. The basic strains both display the expected behaviour. *cis*D50_*mVenus*_AAV stayed at an intermediate level between strain Seq8+ and the EVC, and the EVC did not fluoresce significantly. Strains *ThiM*2mut2, Theo and Theo-RBS do not display significant fluorescence and *thiC*6803- is only marginally stronger. Strains *thiC*6803+ and *thiC*7120- are slightly under the fluorescence level showed by cisD50_*mVenus*_AAV, with no significant change over time. Strain *thiC*7120+ on the other hand shows an increased fluorescence over time despite being believed to be an OFF-switch. This could be interesting to investigate in the future but is not examined further in this report.

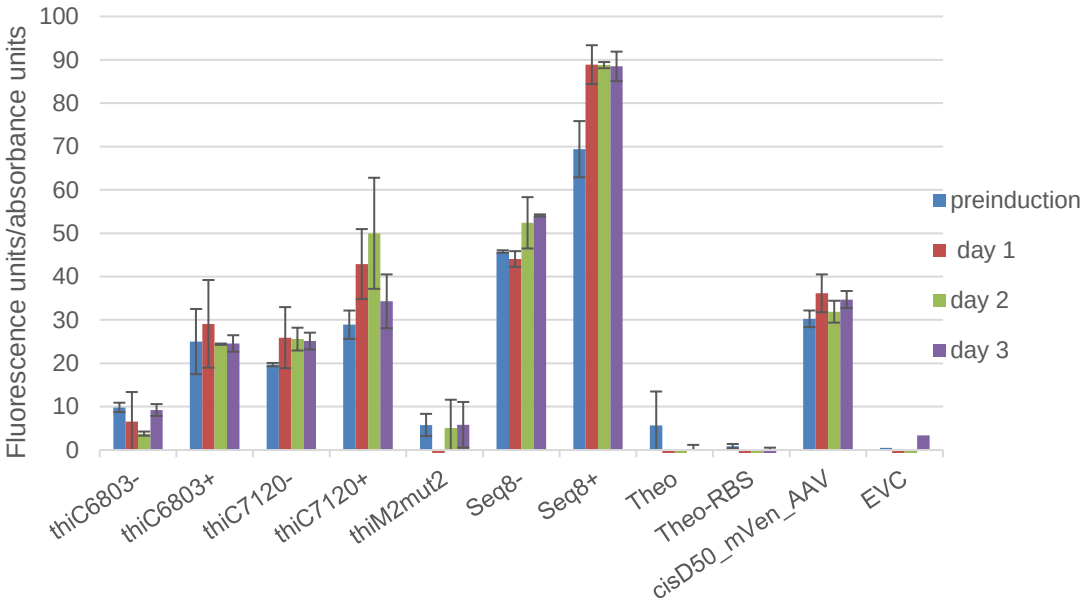

Figure 21. *Synechocystis* experiment 2. Co-induction with 1 mM TPP and 1 mM thiamine. For further description of the riboswitch strains, see Table 1. For further description of the basic strains, see Table 2.

To examine the results of *Synechocystis* experiment 2 on the transcript level, the extracted RNA was inspected for quality and quantity using Experion, with gel pictures found in Figure 22 and Figure 23. In order to reduce workload, only the samples taken on day 0 and day 1 were investigated. The RNA was treated with DNase I and the enzyme removed either with phenol-chloroform extraction or with the RapidOut DNA removal's accompanying kit. A PCR and gel electrophoresis to verify DNA removal is pending. Both Appendix C, Figure 22 and Appendix C, Figure 23 show that the RNA extracted in *Synechocystis* experiment 2 is of good quality with varying concentrations, possibly with the exception of strain Seq8- from day 1, where only the marker band is visible. In Figure 22 the marker band is absent and thus the quantification is off, and in Figure 23 the RNA ladder is degraded and thus the samples were only quantified with NanoDrop.

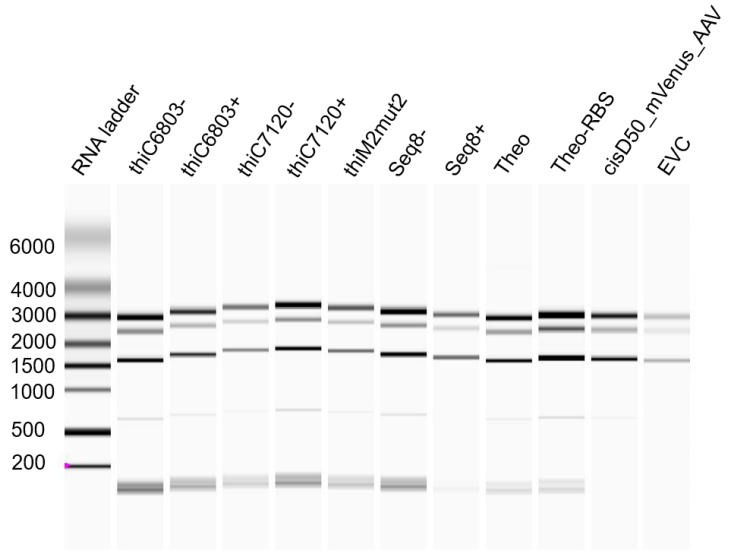

Figure 22. *In silico* gels of the RNA extracted in *Synechocystis* experiment 2, produced by Experion. Samples from day 0. For further description of riboswitch strains, see Table 1. For further description of basic strains, see Table 2.

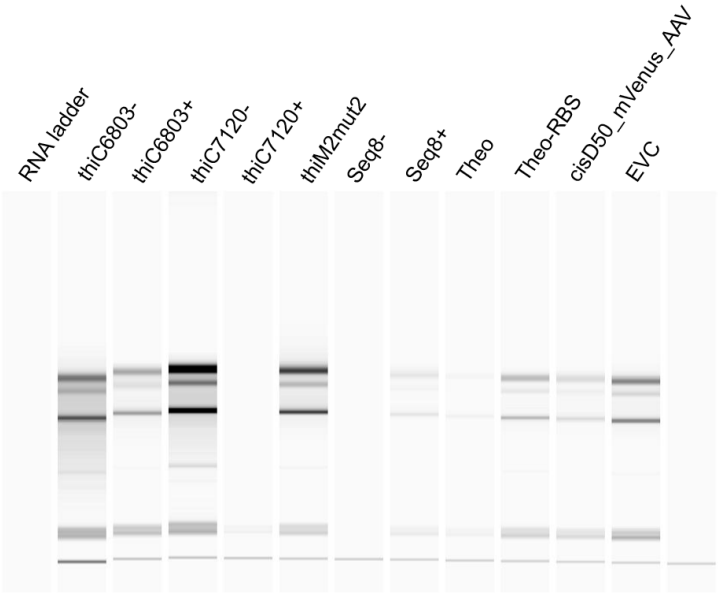

Figure 23. *In silico* gels of the RNA extracted in *Synechocystis* experiment 2, produced by Experion. Samples from day 1. Ladder was added to the appropriate well but was either not loaded or degraded. For further description of the riboswitch strains, see Table 1. For further description of the basic strains, see Table 2.

To study the effects of the induction on transcript level, the RNA was reverse transcribed into cDNA and run in a qPCR (see Figure 24). The qPCR data is shown from *Synechocystis* experiment 2 for all strains except strain Theo-RBS and the basic strains. These were on a separate qPCR plate, as were the -RT controls, and these plates mostly gave no results because the *rnpB* (used as an internal control) wells gave too low signals and thus relative quantification was impossible. Therefore, these results are pending and will be carried out after this project is formally concluded. The transcription levels for *mVenus* (see Figure 24), in riboswitch strains *thiC*6803-, *thiC*6803+ and *thiC*7120- indicated significant decreases by day 1, with the most remarkable difference seen for strain *thiC*7120.

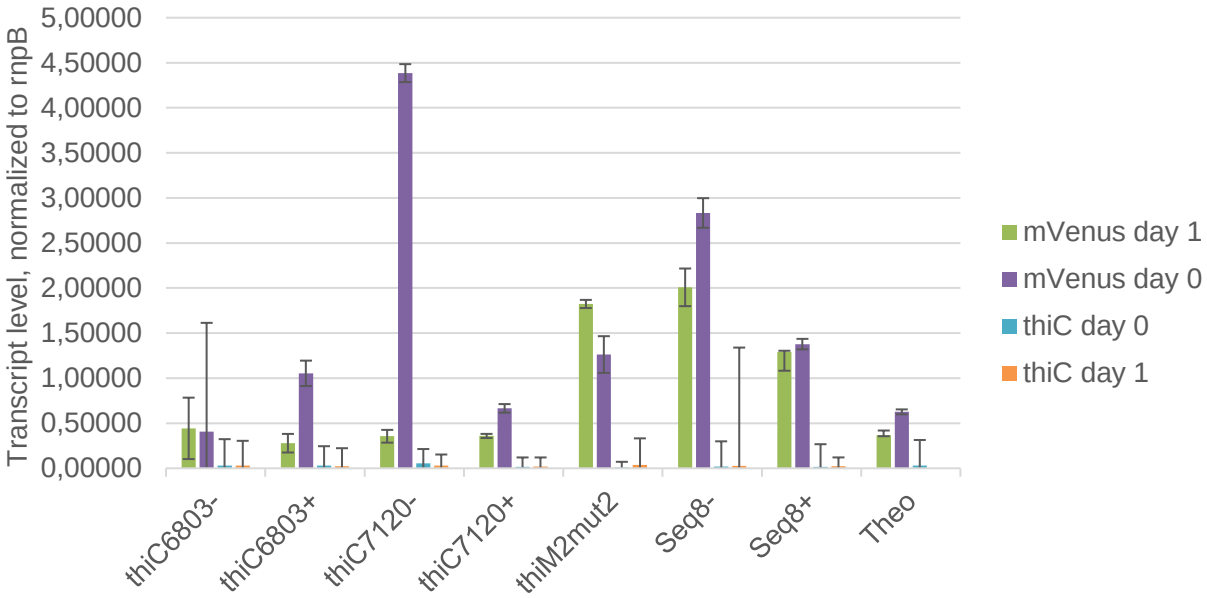

Figure 24. qPCR quantification data from *Synechocystis* experiment 2, *mVenus* and *thiC*. For further description of riboswitch strains, see Table 1.

The transcript levels of the *thiC* gene, also shown in Figure 24, were measured in order to get an idea of whether the thiamine and TPP affect the riboswitch before the *thiC* gene in the *Synechocystis* and whether this may be the true cause of any of the results for the *mVenus* gene. However, the variations in the different strains have such varying reactions to the induction that it is difficult to tell. Therefore, until more data is acquired these results will be interpreted as meaning that the *thiC* levels are not affected by the treatment per se but rather represent other variation.

4.5.4 *Synechocystis* experiment 3

After finding that Seq8+ responded to the induction with both thiamine and TPP it was decided to investigate if this response was more dependent on one or the other. In order to reduce workload only Seq8+ was investigated, with the basic strains as controls. Strain Seq8+ was investigated in biological triplicates while only one replicate was investigated for the basic strains. In order to determine the dynamic range of the riboswitch a dilution series of between 0 and 1 mM was investigated for both TPP and thiamine.

The fluorescence results are shown in Figure 25 and Figure 26. The fluorescence for EVC was too low in this case to be visible in the figure. The level of fluorescence for cisD50_*mVenus* is much higher than for all the other strains, as expected for an AAV-free strain. As for cisD50_*mVenus*_AAV and Seq8+, they were both intermediate with Seq8+ being higher than cisD50_*mVenus*_AAV. These patterns stayed true across all concentrations, for both strains and over all days. The relation between thiamine and TPP induced cultures varies over both days and concentrations and are thus believed to be more due to natural variation than to any difference in uptake or effect. Thus, it is believed that the effects seen in *Synechocystis* experiment 2 were due to the roughly equal effects of TPP and thiamine additions.

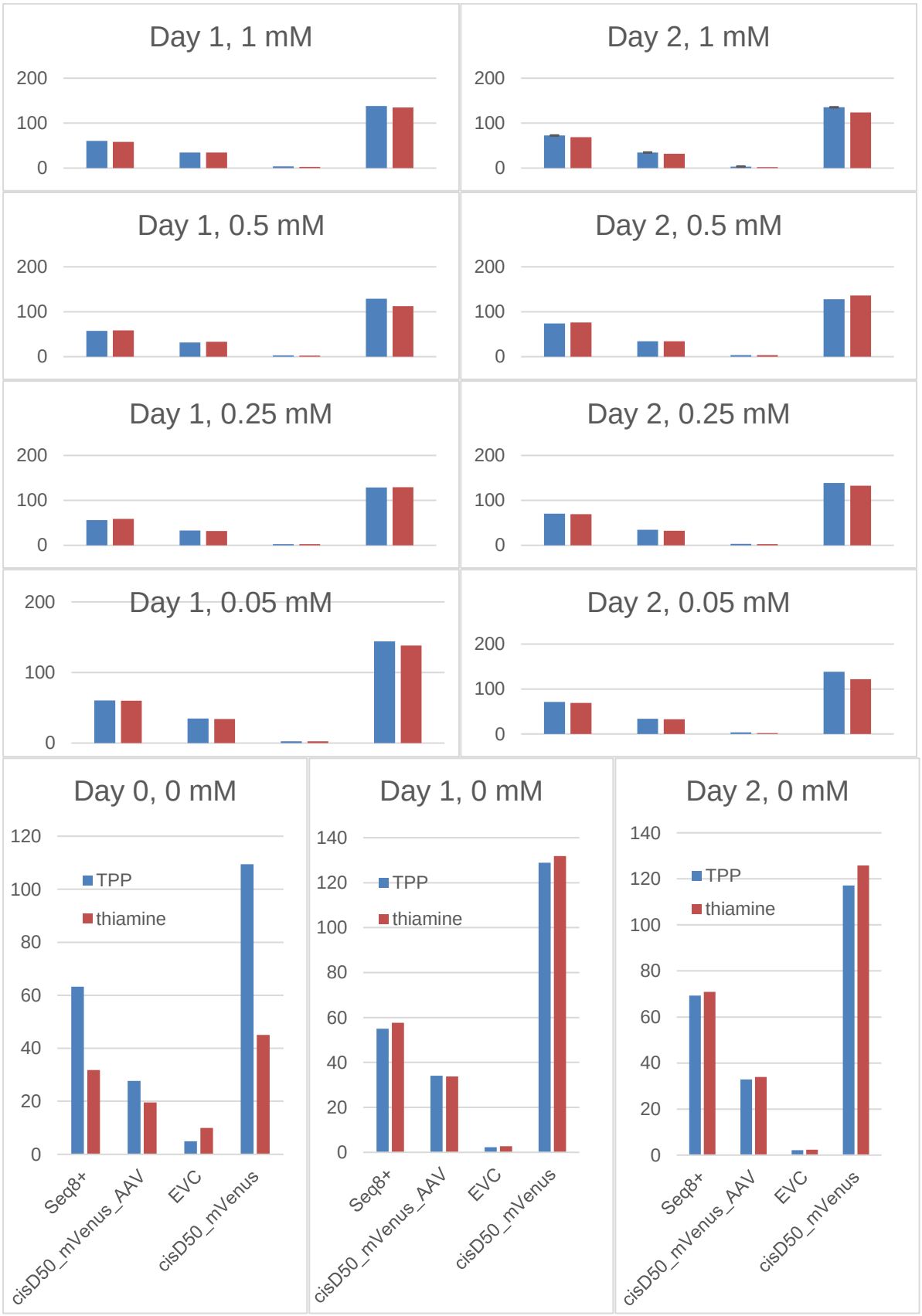

Figure 25. Fluorescence measured in *Synechocystis* experiment 3. The graphs for day 1 show the notation also used in the other graphs. On the Y-axis is the fluorescence divided by the absorbance. Further description of Seq8+ can be found in Table 1. Further descriptions of the three basic strains can be found in Table 2.

As for the variation over concentrations, this is difficult to determine in Figure 25 and thus a separate graph for only Seq8+ is shown in Figure 26. Here it seems as if the level increases from 0 to 0.05 mM and plateau beyond. For comparison the basic strain 1 is included, which has a corresponding but smaller increase. This would suggest that 0.05 mM TPP is sufficient to reach saturation, and that the dynamic range for Seq8+ is between 0 and 0.05 mM of TPP. However, this difference seems to disappear again at 0.25 and so this might be natural variation. As such, this experiment should be repeated before any definite conclusions can be made.

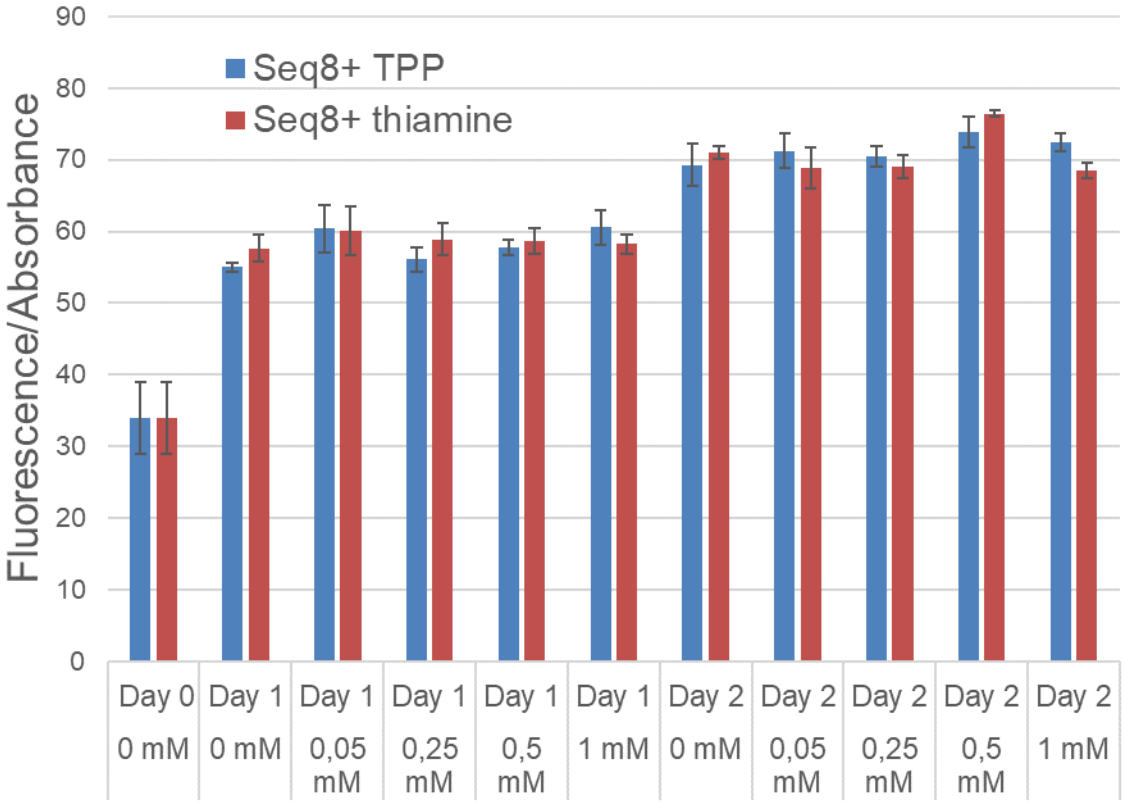

Figure 26. Fluorescence measured for TPP induced cultures in *Synechocystis* experiment 3. Shown are the Seq8+ measurements for all three days and all five concentrations, for induction with thiamine and TPP. Further description of Seq8+ can be found in Table 1. Further descriptions of the three basic strains can be found in Table 2.

In parallel to the fluorescence experiments described above, the transcript response to TPP and thiamine was investigated from cultures in E-flasks at either 0 or 1 mM TPP or thiamine respectively. RNA was extracted each day and tested as described.

The results of the qPCR can be found in Figure 27. The thiamine values were in many cases impossible to calculate since *rnpB* signals failed. The values after addition of thiamine are thus disregarded in this work since the high values observed here may simply be due to division by a very small, almost zero, signal for *rnpB*. This should however be re-examined at a later time to see whether the transcript level is indeed affected. All the TPP values were significant and appeared similar between the strains as in *Synechocystis* experiment 2. This suggests that riboswitch Seq8+ is not affected on the transcript level by thiamine or TPP.

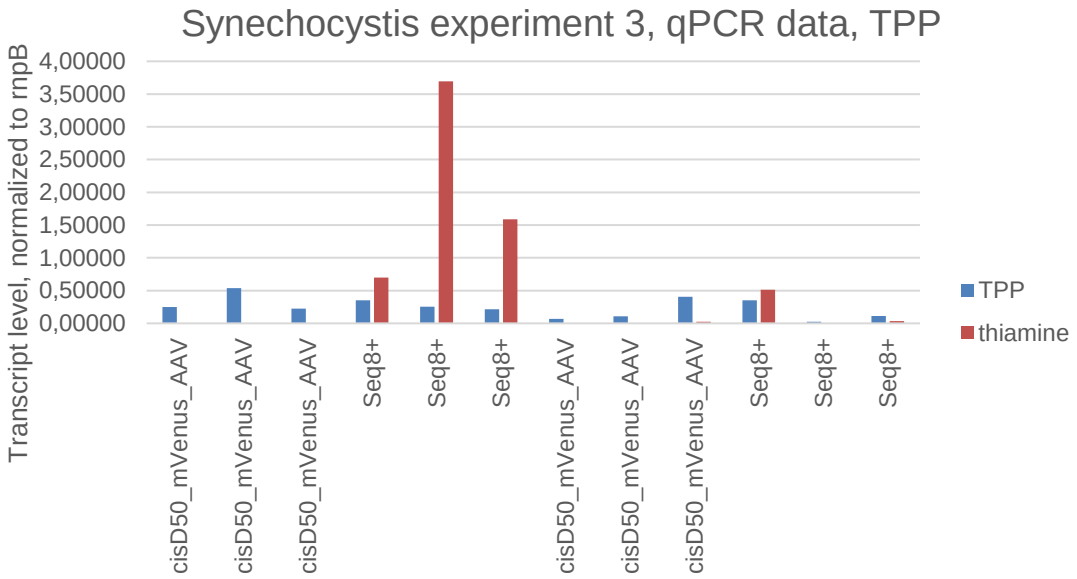

Figure 27. qPCR quantification data from *Synechocystis* experiment 3. Further description of Seq8+ can be found in Table 1. Further descriptions of cisD50-*mVenus* can be found in Table 2.

5 Discussion

This project aimed at implementing and evaluating nine TPP-responsive translational riboswitches in *Synechocystis* PCC 6803 as well as developing protocols for the relevant experiments. This was intended to extend the toolbox available for modification of cyanobacterial production systems for future industrial applications and make it possible to create more complex genetic circuits by combining riboswitches with promoters.

5.1 *E. coli* effects

The same basic fluorescence levels were observed for both *E. coli* and *Synechocystis* cultures when investigated in the plate reader (See Figure 9 and Figure 19). This suggests that the riboswitches investigated mainly in *Synechocystis* may also be interesting to investigate in *E. coli*. This is not so surprising for the synthetic switches Seq8- and Seq8+ since both originate from *E. coli* (Nomura & Yokobayashi 2007). However, the cyanobacterial riboswitches (thiC6803-, thiC6803+, thiC7120- and thiC7120+) might be interesting to investigate further in *E. coli* since they also behave similarly in the two host organisms.

5.2 Transcript level effects

The transcript levels for the basic strains, seen in Figure 16, indicate that the cisD50_mVenus_AAV strain is lower in plasmid copy number when compared with cisD50_mVenus. This is based on the fact that both *kanR* and mVenus levels for cisD50_mVenus_AAV are about ¾ of the values observed for cisD50_mVenus, and the ratio is about the same for both templates. Since no effect is expected for the *kanR* gene, and the two genes are on the same plasmid, in one replicate each, this is interpreted as a difference in plasmid copy numbers rather than in accumulation of the mVenus transcript. This could however simply be due to a difference in the conditions when the RNA was extracted, for instance a slight temperature, culture density or time difference between the cultures.

This lack of a difference between the *mVenus* RNA levels of the basic strains cisD50_*mVenus*_AAV and cisD50_*mVenus* (Figure 16) indicates that the AAV-degradation tag has no significant effect on mRNA accumulation. This is not overly surprising since the AAV-tag does not affect translation, merely causes the finished protein to be broken down, (Andersen *et al.* 1998). Further investigations however, with more biological replicates (here only technical replicates were used), would be needed to clarify this. The difference in plasmid copy numbers between cisD50_mVenus_AAV and cisD50_mVenus is believed to be due to some slight variation in the growth or extraction conditions. Further experiments should preferably be done to confirm this, since it is still possible that the difference is due to some effect of the AAV-tag, and this would be valuable information for many experiments.

The transcript levels for *Synechocystis* experiment 2 (see Figure 24) indicate that despite the fluorescence readout for strains *thiC*6803+, *thiC*7120- and *thiC*7120+ being unaffected by the induction, the transcript level is not. Note that what is shown is not the transcription of the gene but the accumulated level of transcript present in the cell, i.e. a snapshot of the RNA levels at the moment when the sample is taken. This means that not only the transcription initiation frequency, but also the degradation rate of the transcript factor in the observed transcript level. While the transcript is undergoing translation, as on day 0 for the OFF-

switches, the ribosome usually protects it from degradation by RNases (Dreyfus 2009). When the switch is turned off by the presence of TPP however, the mRNA becomes unprotected, and the decay rates are accelerated. This might mean that the riboswitches do work but that the readout is too low to be visible on the fluorescence level. The effect may of course also be due to natural variation in the strain; since only one replicate is used for RNA extraction it is difficult to tell.

It is difficult to draw any conclusions regarding the difference in induction capacity between thiamine and TPP from the qPCR data of *Synechocystis* experiment 3 since so many of the thiamine values are missing. However, the TPP values seem to verify the results seen in *Synechocystis* experiment 2 in that the mVenus transcript levels are roughly equal over time and between cisD50_mVenus_AAV and Seq8+. This in turn indicates that riboswitch Seq8+ is "truly" translational with no significant effect on the transcripts. Hence, it is inferred that the transcript levels for strain Seq8+ are unaffected by the induction with TPP even though the fluorescence levels are increased. For the other strains, only strain Seq8- show some indication of being affected on the fluorescence level. Strains *thiC*6803+, *thiC*7120- and *thiC*7120+ however are clearly affected on the transcript level and may possibly be affected on the fluorescence level albeit in a manner too small to distinguish in these experiments.

Some additional experiments might clarify the results found here. Strains *thiC*6803+, *thiC*7120- and *thiC*7120+ should be investigated on the protein level in some other manner such as with a Western blot using antibodies against the *mVenus* gene to see if the fluorescence readout is simply too low to be seen. Strains Seq8- and Seq8+ could be investigated further on both transcript and fluorescence level to determine the dynamic range more exactly and see the difference between them more clearly.

5.3 Comparing the present results to the relevant literature

Strain *ThiM*2mut2, designed by Nomura & Yokobayashi (2007), is supposedly a mutated OFF-switch, meaning that it is constitutively on. This leads us to expect no difference upon addition of either thiamine or TPP, and this is indeed observed in this work. However, in this case no significant expression is seen at all, under any conditions, suggesting that it does not react to thiamine or TPP and moreover does not support significant downstream translation. This means that the riboswitch leads to a much lower level than what is found without this riboswitch. It could still be called constitutive but only if there is actually expression, which is not evident here. Further experiments should be used to investigate this, both in *E. coli* and in *Synechocystis*. The slight decrease of transcript level over time for *mVenus* (Figure 24), may be natural variation.

The theophylline switch, here referred to as "Theo", designed by Ma *et al.* (2014) is an ON-switch, investigated here as a control due to its reported high induction levels at 1 mM theophylline. However, in *Synechocystis* experiment 1, we do not observe any readout at all, hardly surprising given the appearance of the culture seen in Figure 20. This effect could either be due to the theophylline concentration being too high for the strain to handle, or to the DMSO in which it is dissolved. However, the setup used here, with a 100 mM stock solution of theophylline dissolved in 100 % DMSO, is no different from that used by Ma *et al.* (2014). The only differences are the strain used, in their case WHSyn, and that their stock solution was at 200 mM. In the work by Ma *et al.* (2014) the inducer-free control cultures are instead supplied with the corresponding volume of pure DMSO. This was not done here since the

main purpose of the experiments was to investigate the thiamine effect, (thiamine was not dissolved in DMSO). However, if one would like to differentiate between the effects of theophylline and DMSO one might do a dilution series of theophylline in DMSO, starting from 0 mM and up to the concentration used here, 1 mM, and as a control have one of the concentrations diluted in water or directly added to the medium. This was deemed too time consuming for this work but might be interesting for the future.

It might also be noted that the effect shown in Figure 20 is not seen in the *E. coli* experiment in Figure 9, meaning that this could be a specific *Synechocystis* effect, possibly even specific to our strain.

5.4 Experimental procedure findings

The failure to see clear differences in fluorescence on agar plates may be in part due to background fluorescence of LB medium (Lafferty & Dycaico 2004) and in part to the differences in cell density of the restreaks (more cells in the same place may give a stronger signal). Once the supernatant was removed, by spinning the cells down and either simply pouring it off and observing the pellet or resuspending the pellet and measuring the fluorescence in the plate reader, the differences between strains were clearly discernible. This argues for the theory of LB fluorescence. On the other hand, the fact that the results are clearer in the plate reader may speak for the theory of different cell density since the fluorescence is there normalized by the absorbance, i.e. the cell density. However, this might also be due to the increased number of replicates giving a more accurate picture. Most likely both background fluorescence and cell density affect the result, meaning that the plate reader is the best option.

Another explanation for this is simply that the AAV-tag is too destabilizing for our experiments, particularly those with the riboswitch in focus, the Seq8+. A degradation tag is supposed to aid in assessing the dynamics of the system in a downregulation scenario: *mVenus* protein will be degraded shortly after being formed, so that the signal in time (or in our case decrease in fluorescence signal) represents current levels. This is most helpful in the case of OFF-switches where the desired signal rapidly disappears when translation is turned off. Rapid degradation is however a trade-off between measuring current levels and signal strength. Thus, it would be beneficial to use the AAV-tag for the cyanobacterial OFF-switches, but not for the synthetic ON-switches where proper kinetics would be observable in much higher signals.

Figure 19 also shows a decrease in fluorescence over time for all strains. However, this might be the result of the experimental design. Since the cultures were grown in batch form, the OD of the cultures increases, both because of growth but also because of significant evaporation. This causes a decrease in light availability in the cultures which might also affect the result. Additionally, the sampling further reduces the volume which might also affect the light availability as the path through the culture to the cells at the bottom is shorter. Finally, the lack of response to thiamine could reflect a failure in conversion into TPP after uptake by the cells. However, this is unlikely since roughly equal fluorescence levels were found for thiamine and TPP in *Synechocystis* experiment 3, see Figure 25.

5.5 Conclusions

In conclusion, riboswitch Seq8+ seem to be effective in *Synechocystis*, as assessed on the protein level. Riboswitches *thiC*6803+, *thiC*7120- and *thiC*7120+ seem to respond on an RNA level, with riboswitch *thiC*7120- being especially clear, and may in fact be effective also on the protein level, albeit not scored significantly in the experiments carried out here. There are further experiments to be executed to investigate this and to validate the results achieved with riboswitch Seq8+.

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Appendix A: Plasmid sequences

Below are the plasmid sequences as designed, with the region around the riboswitch being sequence confirmed at the *E. coli* level.

Basic plasmid cisD50_mVenus_AAV

ggtaccgaggtcgatgtcgactaacgaccctgccctgaaccgacgacaagctgacgaccgggtctccgcaagtggcacttttcgggg
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Basic plasmid EVC (Empty vector control)

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gggctgaatgatcgaccgagacaggccctgcggggctgcacacgcgccccccacccttcgggtaggggggaaaggccgctaaagcg
gctaaaagcgctccagcgtatttctgcggggtttggtgtggggtttagcgggctttgcccgcctttcccccctgccgcgcagcggtgggg
cggtgtgtagcctagcgcagcgaatagaccagctatccggcctctggccgggcatattgggcaagggcagcagcgccccacaagg
gcgctgataaccgcgcctagtggattattcttagataatcatggatggatttttccaacaccccgccagcccccgcccctgctgggtttgc
aggtttgggggcgtgacagttattgcaggggttcgtgacagttattgcaggggggcgtgacagttattgcaggggttcgtgacagttagt
acgggagtgacggg

Appendix B: gBlock sequences

Below are the gBlocks used in this work.

gBlock 1

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccacttgccatagctaggggtgtctagaaagctaggctgagaaaaaccccttagaacctgagactgggtaataccagcggag
ggaagctcaccattcgaggaaatttgtatggctagcaaaggagaggaactactagtagcggccgctgcaggagc

gBlock 2

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccacttgccatagctaggggtgtctagaaagctaggctgagaaaaaccccttagaacctgagactgggtaataccagcggag
ggaagctcaccattcgaggaaatttgtatgagaactgcttgggctagcaaaggagaggaactactagtagcggccgctgcaggagc

gBlock 3

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactcatccatgctaggggtgcttgcactaacaggctgagattacacccttaacacctgagactgggtaataccagcgaag
ggaagctgtttattgagggaatttcatatggctagcaaaggagaggaactactagtagcggccgctgcaggagc

gBlock 4

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactcatccatgctaggggtgcttgcactaacaggctgagattacacccttaacacctgagactgggtaataccagcgaag
ggaagctgtttattgagggaatttcatatgcggacagaatgggctagcaaaggagaggaactactagtagcggccgctgcaggagc

gBlock 5

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactaattgtgagcggataacaattcaattcaaccaaacgactcggggtgcccttctgcgtgaaggctgagaaatacccgt
atcacctgatgaggataatgccagcgtagggaagctattacaagatcatcaggagaaattaactatgggatcgcaccatcacgctagca
aaggagaggaactactagtagcggccgctgcaggagc

gBlock 6

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactaattgtgagcggataacaattcaattcaaccaaacgactcggggtgcccttctgcgtgaaggctgagaaatacccgt
atcacctgatctggataatgccagcgtagggaagctattacaagaccatcaggagaaattaactatgggatcgcaccatcacgctagca
aaggagaggaactactagtagcggccgctgcaggagc

gBlock 7

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactaattgtgagcggataacaattcaattcaaccaaacgactcggggtgcccttctgcgtgaaggctgagaaatacccgt
atcacctgatgaggataatgccagcgtagggaagctattacaagatcatcaggagaaattaactatggctagcaaaggagaggaacta
ctagtagcggccgctgcaggagc

gBlock 8

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactcggtacctgataagataggggtgataccagcatcgtcttgatgcccttggcagcaccaagggacaacaagatggct
agcaaaggagaggaactactagtagcggccgctgcaggagc

gBlock 9

Ccaggaattcgcggccgcttctagaggtacgcaattggcatgcgcattccctatcagtgatagagattgacatccctatcagtgatagat
ataatggccactcggtacctgataagataggggtgataccagcatcgtcttgatgcccttggcagcacctcatcacaacaagatggcta
gcaaaggagaggaactactagtagcggccgctgcaggagc

Appendix C: Additional results

Here follow example quality controls which were performed during this work.

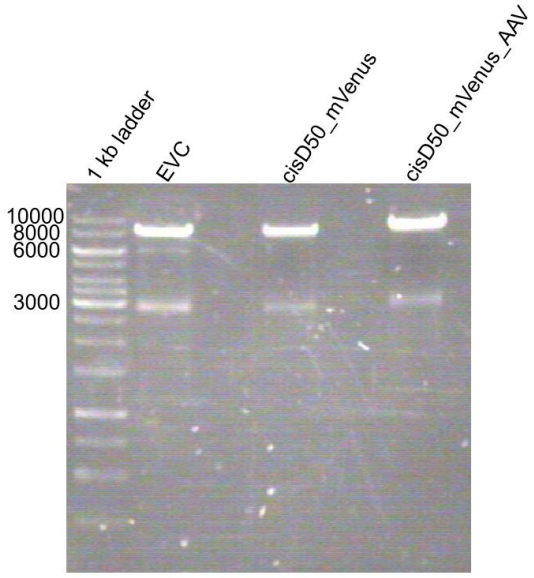

Figure 28. Digested basic plasmids. From this gel the fragments from basic plasmids cisD50_*mVenus* and EVC were excised and gel purified. Expected lengths are around 8400 bp. For further description of the basic plasmids, see Table 2.

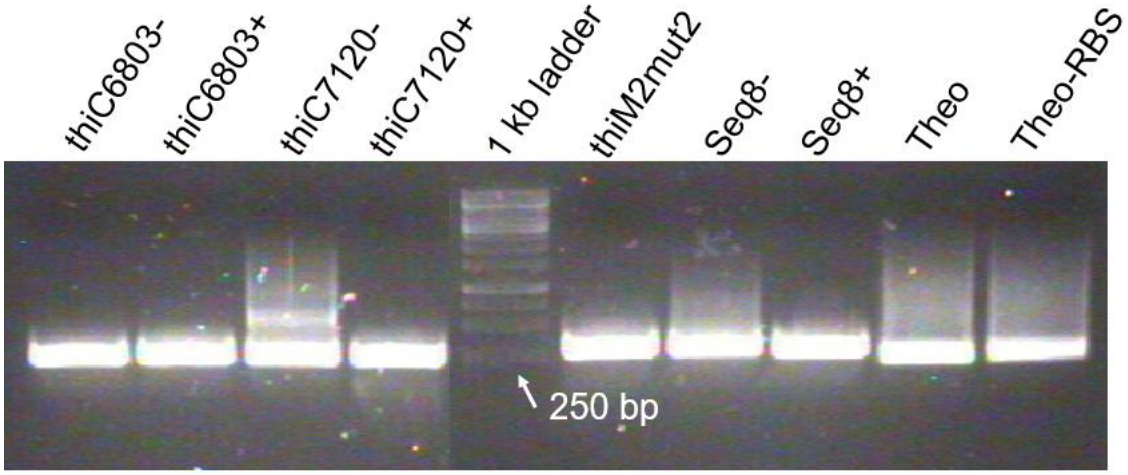

Figure 29. PCR products of gBlock amplification. Expected lengths are around 250 bp. The lower bands were excised and gel purified. For further description of the gBlocks, see Table 1.

Here, most wells contain a smear trailing behind in the gel, and gBlock *thiC*7120- also has an unknown band of around 500 bp clearly visible. Thus, the bands were excised with care not to carry any excess gel over to the purification tube.

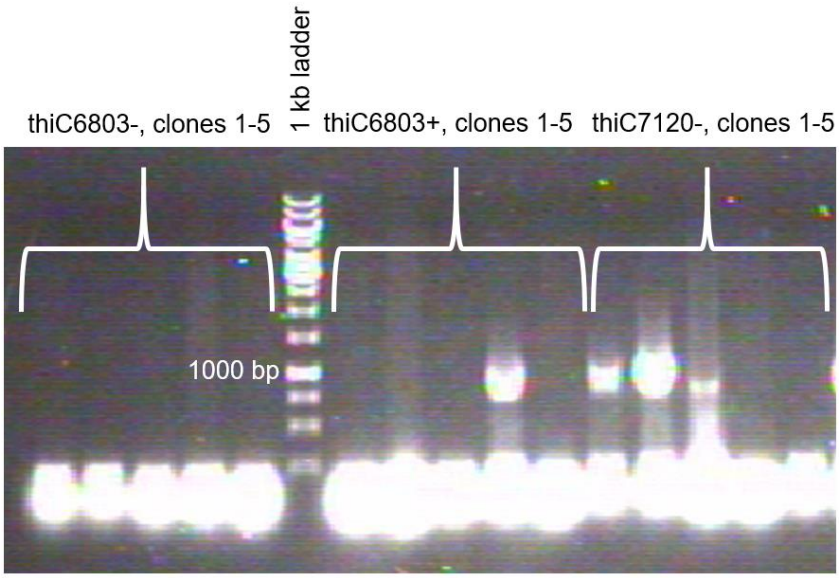

Figure 30. Example of a colony PCR agarose gel. Shown are the first five strains in *E. coli*. For further description of the strains, see Table 1.

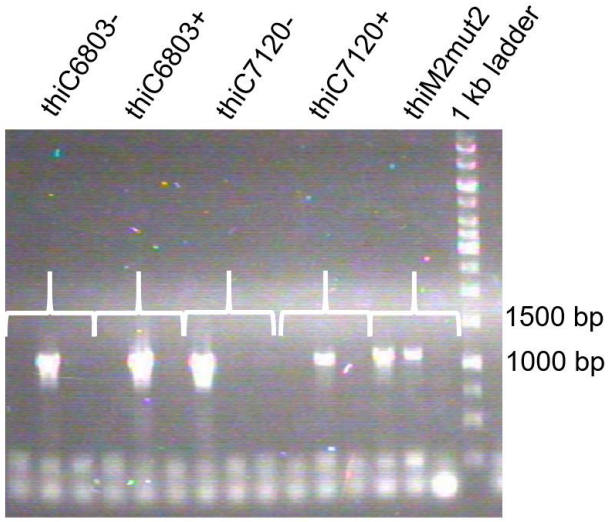

Figure 31. Colony PCR of *Synechocystis* clones. The rightmost lane shows a 1 kb ladder. Expected length is around 1200 bp. For further description of the strains, see Table 1.

In both colony PCRs some fragments appear to be slightly shorter than the expected 1100 bp but were still assumed to be of the correct length. From this gel it was assumed that colonies *thiC*6803+: 4, *thiC*7120-: 1, 2, 3 and *thiC*7120+: 1, 2 are correct, even though they appear to be somewhat shorter than the expected.