University of South Bohemia in České Budějovice

Faculty of Science

Department of Molecular Biology

BACHELOR THESIS

Interaction of proteins Clock and Cycle – Preparation of constructs for subsequent expression in S2 cells

Simona Fišerová

Supervisor: Mgr. David Doležel PhD

Co-Supervisor: Mgr. Olga Bazalová PhD

Institute of Entomology, The Czech Academy of Sciences

Laboratory of Molecular Chronobiology

České Budějovice 2017

This thesis should be cited as: Fišerová, S. 2017. Interaction of proteins Clock and Cycle – Preparation of constructs for subsequent expression in S2 cells. Faculty of Science. University of South Bohemia in České Budějovice.

Annotation

The aim of this thesis was to create constructs containing actin or Hsp70 promoter, complete coding DNA sequence of *Clock* and *cycle* genes from the linden bug (*Pyrrhocoris apterus*) and tag – green or red fluorescent protein. Such prepared constructs were designed to be used to study interactions of Clock and Cycle proteins *in vivo* in Schneider S2 cell line. In addition, effectiveness, reliability and applicability of the used method Gateway cloning was examined.

Sworn declaration

I hereby declare that I have worked on the submitted bachelor thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice 10/5/2017 Simona Fišerová

Acknowledgement

First of all, I would like to thank my thesis advisor Mgr. Olga Bazalová PhD for leading me through this project. I would also like to thank Mgr. David Doležel PhD for allowing me to work on such a topic. While supporting my curiosity, they both gave me a courage and motivation which was necessary part of the work.

Secondly, I would like to mention and thank Prof. RNDr. Libor Grubhoffer, CSc. and Univ.-Prof. Dr. Norbert Müller for the establishment and management of the Biological Chemistry program.

At last, I would like to thank and express my huge gratitude to my family and friends. Without their unfailing support and encouragement, not just while the process of writing my thesis but throughout my all years of study, this accomplishment would not have been possible. I do thank you.

Abbreviations

(alphabetical order)

AMP	AMPICILLIN
bHLH	BASIC HELIX-LOOP-HELIX DOMAIN
CAM	CHLORAMPHENICOL
CaM	CALMODULIN
CLK	CLOCK
CRY	CRYPTOCHROME
CWO	CLOCKWORK ORANGE
CYC	CYCLE
GFP	GREEN FLUORESCENT PROTEIN
HSP	HEAT SHOCK PROMOTER
KAN	KANAMYCIN SULPHATE
LB	LURIA-BERTANI LIQUID MEDIUM
MET	METHOPRENE TOLERANT
ORF	OPEN READING FRAME
pENTR	ENTRY CLONE
PER	PERIOD
RFP	RED FLUORESCENT PROTEIN
RPM	ROTATIONS PER MINUTE
SOC	SUPER OPTIMAL BROTH REPRESSION MEDIUM
TAE	TRIS-ACETATE-EDTA BUFFER
TAI	TAIMAN
TIM	TIMELESS
UNF	UNFULFILLED
VRI	VRILLE

Abstract

Protein-protein interactions are essential for biological processes although their study is challenging. It is generally difficult to study protein-protein interactions *in vivo* in an organism and particularly in non-model species. Therefore, a variety of artificial systems are used, such as yeast cells or various cell lines. However, interactions observed in these artificial systems may not reflect true interactions in the original organism and the comparison of various experimental expression systems is desirable. Because of that, a staggering number of expression plasmids needs to be prepared. These plasmids differ in promoters and protein tags, but contain identical open reading frame coding for the studied protein. The aim of this work was to test possibilities and a robustness of Gateway cloning system. Second goal of this thesis was to verify original plasmids containing either “eGFP” or “mRFP” tags combined with actin and heat shock promoters, respectively. Results of this thesis indicate Gateway cloning as a powerful and reliable cloning approach.

Abstrakt

Studium proteinových interakcí je často velmi složité, ale zároveň neodmyslitelné pro pochopení mechanismů většiny biologických dějů. Mnohdy je extrémně obtížné sledovat tyto interakce v živých organismech a u nemodelových organismů je to prakticky nemožné. Proto jsou hojně používány arteficiální systémy jako například kvasinky nebo různé druhy buněčných linií. Interakce v těchto systémech ale nemusí věrně odpovídat situaci v původním organismu, a proto je nutné porovnávat výsledky z různých systémů. Z tohoto důvodu je k expresi studovaných proteinů potřeba vytvořit velké množství plasmidů, které se liší například svými promotory, ale obsahují identický úsek kódující testovaný protein. Cílem této práce bylo otestovat možnosti efektivního překlonování čtecích rámců mezi různými plasmidy a to pomocí systému „Gateway cloning“. Zkoumané vlastnosti této metody byly efektivita, spolehlivost a aplikovatelnost. Druhým cílem bylo ověření originální sekvence plasmidů, obsahující fluorescenční proteiny „eGFP” a „mRFP“ řízených aktinovým nebo Hsp70 promotorem. Výsledky projektu naznačují, že způsob klonování, „Gateway cloning“ je účinným postupem při přípravě konstruktů.

Content

1. Introduction 8
  1.1 Motivation 8
  1.2 Circadian clock 8
    1.2.1 Clock and Cycle 10
  1.3 Gateway cloning 11
2. Aims of the study 12
3. Materials and Methods 13
  3.1 List of materials 13
    3.1.1 List of instruments 13
    3.1.2 Used programs 13
    3.1.3 List of primers 14
  3.2 Growth of *E. coli* cells with vectors 14
  3.3 Miniprep preparation 14
  3.4 Plasmid DNA isolation 15
  3.5 Preparation of sequencing samples 15
  3.6 LR recombination 15
  3.7 Transformation of *E. coli* cells 17
  3.8 Polymerase chain reaction 17
  3.9 Agarose gel electrophoresis 17
  3.10 Conservation of cells 18
4. Results 19
  4.1 Attachment sites verification 19
  4.2 Production of expression clones *in silico* 20
  4.3 Production of expression clones in *E. coli* cells 22
    4.3.1 LR recombination verification 22
    4.3.2 Production of constructs 23
5. Discussion 25
6. Conclusion 26
7. References 27

1. Introduction

1.1 Motivation

Many processes in living organisms depend on the proper protein-protein interaction and understanding these interactions is often the key step in elucidating a detailed molecular mechanism behind the particular biological process. The laboratory of chronobiology uses the linden bug (*Pyrrhocoris apterus*) as a model organism to decipher molecular basis of insect Circadian clock. The key feature of the Circadian clock is a precisely controlled interaction and a cellular localization of several proteins. Since it is extremely difficult to study these interactions directly in an insect, different heterologous expression systems are used instead. For some type of a quick test yeast two hybrid assays are used [1], sometimes insect cells might be used, and under specific circumstances even mammalian cell cultures are preferred. Importantly, each of above mentioned systems requires different regulatory sequences. Moreover, a various protein tags might be preferred according to the application: fluorescent proteins are excellent for *in vivo* monitoring but are relatively large (250 amino acids). Therefore, they might interfere with the structure of the protein or prevent an interaction with a protein partner. Studied proteins might be large which could further complicate sub-cloning between plasmids. Therefore, fast and reliable sub-cloning between various vectors is necessary for an efficient research. The goal of this bachelor thesis was to explore possibility of using Gateway cloning system and to test a few commercially available plasmids.

1.2 Circadian clock

Since there is a planet movement in space, there is also a periodical occurrence of natural events. Most important are surely the daily cycles, annual period and lastly the lunar cycle. Parameters like light/dark conditions, light intensity, temperature and humidity are affected. These changes are remarkably important for nearly all organisms. For instance, plants regulate photosynthesis according to the day and night cycle, fruit flies (*Drosophila melanogaster*) [2] need to ecdyse into adult during the morning before the dry part of the day starts, and many mammals need to avoid predators. Therefore, a reliable mechanism allowing prediction (anticipation) of these events is really beneficial. Indeed, many organisms evolved so called Circadian clock that "keeps ticking" with a period of approximately 24 hours even under constant conditions. [1]. The mechanism of the Circadian clock is very well understood at the molecular and genetic level in a few model organisms, such as the mouse [3], fruit fly [2] or variety of other insect species [4, 5, 6, 7].

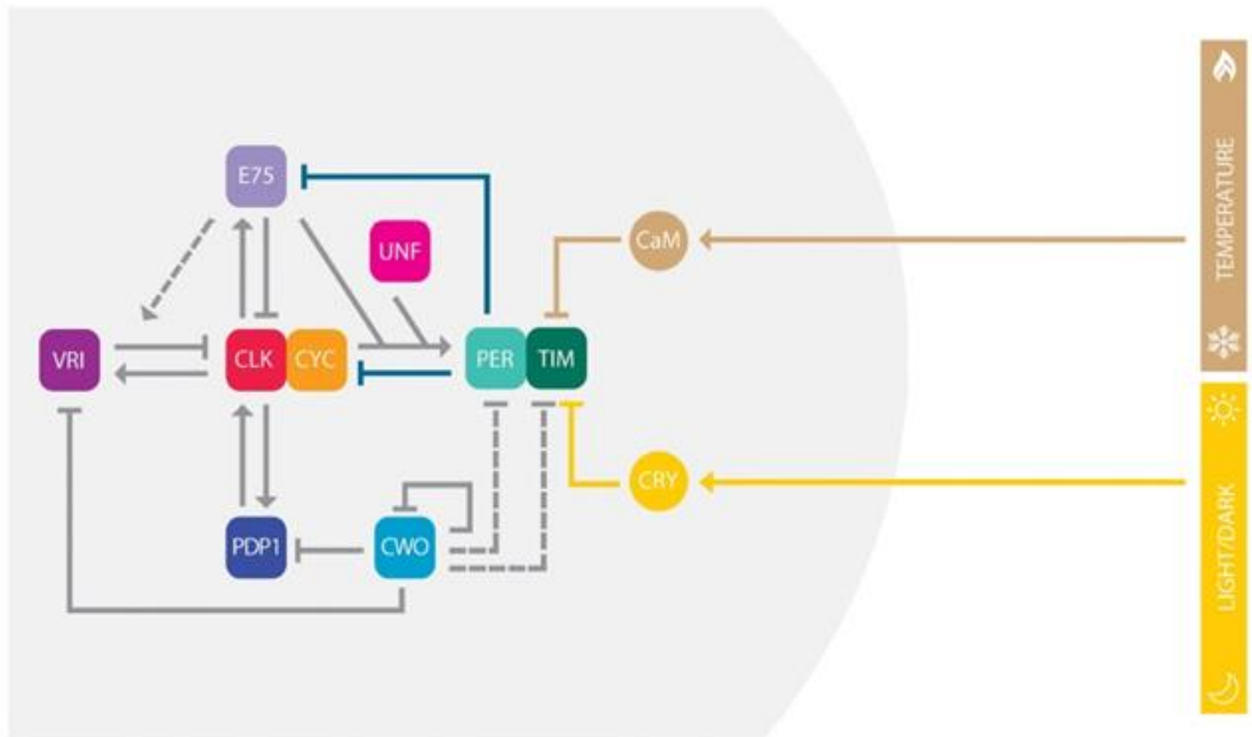

Figure 1: Circadian clock model (adapted from Rivas et al. [8]). For simplification, both genes and proteins are depicted using the same drawing symbols and capital letters [8]. Model is showing the regulatory interactions within *Drosophila* feedback loops. Positive regulation is represented by arrows and negative regulation is represented by lines. The main part of the scheme is CLK-CYC heterodimer placed in the center of the loops. Dark blue lines show protein-protein interactions, grey lines transcription regulation and yellow lines represent direction of the light. Brown color is used for the temperature pathway depiction [8]. Abbreviation CWO is used for the protein called CLOCKWORK ORANGE. Its level in the cell oscillates with time and it works as a repressor for the *Clock* (*Clk*) target genes [9]. Absence of *cwo* in the organism causes decrease of mRNA levels for other genes, namely *per, tim, vri* and *pdp1*. Following loss of mRNA levels and therefore oscillations loss implies that CWO also and primarily works as a transcriptional activator of mentioned genes [10]. Forenamed VRI, VRILLE, as a protein is a part of the loop connected to a CLK oscillation. Its transcription is activated by CLK-CYC heterodimer [11] and when VRI expresses, it enters the nucleus and by binding to CLK promoter it inhibits CLK´s transcription. Another part of this loop is PDP1 which was discovered to be a CLK´s expression activator [12]. The PDP1 develops the claim to be a competitive protein to the VRI considering binding CLK promoter and by doing so activating the CLK transcription [13]. A new feedback loop is formed by two nuclear receptors, *unfulfilled (unf)* and E75, and its molecular mechanism has not been clarified yet [8]. Nevertheless, it is known that both receptors are expressed in the circadian pacemaker and both play a role in the CLK-CYC mediated transcription of PER whereas not TIM [14,15]. Examination of E75 receptor shows that it takes a part in the *Clk* expression by repressing it and on the other hand supports VRI repression. Such a finding shows that either with or without VRI, E75 receptor modulate *Clk* regulation. Moreover, it was found that PER puts down activity of E75 and by this works as a de-repressor for CLK transcription [16]. Abbreviation CRY is used for the flavoprotein CRYPTOCHROME. CRY characterizes the molecular mechanism of the light/dark cycles. Change in the CRY conformation is caused by presence or absence of light. In its presence, the conformational change occurs in a way that it activates CRY which afterwards interacts with TIM [8]. TIM is also influenced by

temperature, concretely by CALMODULIN (CaM). CaM is triggered by Ca^{2+} ions and it afterwards degrades TIM using Small Optic Lobe (SOL) protease [17].

1.2.1 Clock and Cycle

Clock (Clk) affects both the persistence and also the period of the circadian rhythms. It works as a transcription factor in *Drosophila* [18] and its function is conserved in mammals as well [3]. *Clk* contains basic helix-loop-helix domain (bHLH), which is important for interaction with DNA. An interaction partner of CLK is cycle (CYC), another bHLH protein, together they form heterodimer [19] and they work as a transcription activator factor for other CLK genes.

First, CLK and CYC form heterodimer [19], which drives expression of many genes, including *period* (*per*) and *timeless* (*tim*). Subsequently, PER and TIM are translated, accumulate in the cytoplasm and at some point dimerize and the dimer is translocated to nucleus. In nucleus, these proteins interact with CLK and CYC heterodimer and inhibit their transcription. (In fact, *clockwork orange (cwo)* works as a transcriptional repressor which synergizes with PER and thereby PER inhibits its own transcription.) Consequently, no *per* and *tim* mRNA are transcribed. Therefore, no PER and TIM accumulate in the cytoplasm, do not enter the nucleus, and the CLK-CYC heterodimer is released from inhibition and the transcription starts again. This whole mechanism is called negative feedback loop [20, 21, 22, 23, 24].

The role of CLK and CYC in Circadian clock is well conserved across taxa. However, there are several interesting and important differences. For instance, in *Drosophila*, the activation domain is localized on CLK, whereas for mammals it is found to be on CYC, which is usually known as BMAL in mammals [1]. Additional factors influencing the Circadian clock and Circadian clock genes might play non-circadian role. In mosquitoes, physical interaction between CYC and Methoprene tolerant (MET) was observed [25]. MET contains bHLH and is recently identified receptor of Juvenile hormone in insect [26]. In the linden bug (*Pyrrhocoris apterus*) genetic experiments suggest possible interaction between MET and CLK or CYC [27].

As it was mentioned in an Introduction (Motivation, chapter 1.1) it is very difficult to study interaction of proteins in a living organism, *Pyrrhocoris apterus.* It would be interesting to know where proteins interact. If it is in cytoplasm or in nucleus. Also get to know further information about how they interact. Therefore, it is important to be able to study these

interactions in the laboratory conditions as in insect´s cell cultures or in yeast and it is needed to find genetic tools enabling an efficient comparison of obtained results.

1.3 Gateway cloning

Used method for this project was Gateway cloning which was thought to provide the efficient and suitable cloning in an artificial surroundings. Its principle lies in a working procedure without using restriction enzymes or ligation. Gateway Cloning Technology also ensures consistent and desired end results in a very short time. It operates with existing primers, vectors ready for cloning and included ligation reagents. Recombination efficiency is up to 95% and cloning time into expression vector takes about one hour when neglecting miniprep preparation [28].

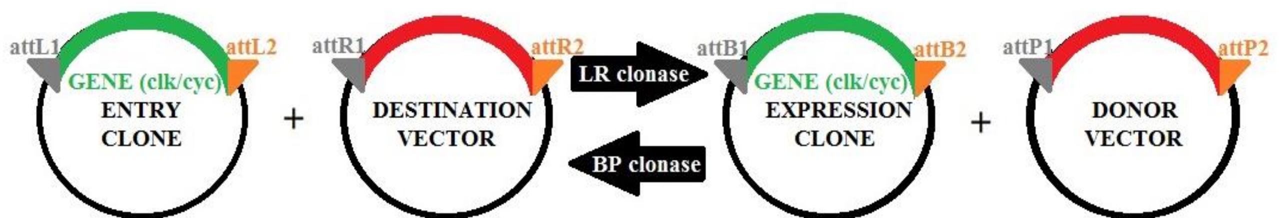

Figure 2: Gateway cloning principle scheme

Gateway cloning consists of three basic steps. Firstly, determination of the Entry clone. Secondly, mediation of the reaction using LR Clonase enzymes and lastly, selection of the Destination vector. The whole process starts in the Entry clone. It contains gene of interest bounded by attL sites. AttL sites are used to recombine with attR sequences to produce an expression clone. When the Entry clone is prepared, the gene of interest is shifted to a secondary plasmid called the Destination vector. Such reaction is realized using already mentioned LR Clonase mix. It contains protein machinery which is needed for cutting out the gene of interest from the Entry clone and including it into the Destination vector. Destination vector with an inserted part afterwards becomes an expression clone. Donor vector is also created during the process but is considered as a waste product. (If necessary, the whole reaction could be reversed with BP reaction, recombination between attB and attP sites, using BP Clonase enzyme mix.). Once the gene of interest is cloned into a Gateway vector, it could be put into as many Destination vectors as needed [28].

2. Aims of the study

The main aim of this study was to create constructs containing actin or Hsp70 promoter, complete coding DNA sequence of *Clock* and *cycle* genes from the linden bug (*Pyrrhocoris apterus*) and tag – green or red fluorescent protein. Such prepared constructs are intended to be used to study interactions of Clock and Cycle proteins *in vivo* in Schneider S2 cell line. Used way of cloning in this project was Gateway cloning. Its effectiveness, reliability and applicability was examined as another goal of the thesis.

3. Materials and Methods

3.1 List of materials

- SOC medium

  Used for the growth of bacteria after transformation. Prepared using tryptone, NaCl, yeast extract, water, KCl, $MgCl_2$ and glucose.
- LB-agar with AMP + CAM

  Used for selection of *E. coli* containing Destination vector. Prepared using LB-agar, water, AMP, CAM.
- LB-agar with AMP

  Used for selection of *E. coli* with expression clone. Prepared using LB-agar, water and AMP.
- LB-agar with KAN

  Used for selection of *E. coli* with Entry clone (also called pENTR). Prepared using LB-agar, KAN and water.
- LB-medium AMP or AMP and CAM or KAN

  Used for growth of *E. coli* cells. Prepared using LB-medium, water and AMP and/or CAM or KAN.
- TAE-buffer (10x)

  Used for gel electrophoresis. Prepared using Tris base, acetic acid and EDTA.

3.1.1 List of instruments

- Autocycler – Bioer, XP-Cycler
- Centrifuge – Hettich, Universal 320R
- Electrophoresis – Biokeystone, Liberty 120
- Heating block – SBH130D
- Incubator – Incucell
- NanoDrop – Nanodrop 2000
- Shaking incubator – Environmental Shaker-Incubator ES-20

3.1.2 Used programs

- Geneious 8.0.0 – sequence analysis and comparison, primer design, *in silico* recombination
- Alpha Imager – pictures from gel electrophoresis

3.1.3 List of primers

type	forward primers (5´ - 3´)	reverse primers (5´ - 3´)
Pa cyc	ACAGATTGCCGAGGAAGTGATT	GGGTCGGTAAGGAAATGTCACT
Pa Clk	ATGGTCTTCAAAATCTTCATCT	TTCAACCTTAATACTCCGATT
RFP	GCTGCGCGGCACCAACTTCC	GGACAGCTTCAAGTAGTCGG
GW5	ATCGAGGCCTGTCTAGAGAAGC	–
GW	–	GGGTGCCTAATGCGGCCGCC
GW GFPV	GGAGTACAACTACAACAGCC	CTTCGGGCATGGCGGACTTG

3.2 Growth of *E. coli* cells with vectors

LB-agar plates containing either AMP and CAM - for *E. coli* with Destination vector – or KAN – for *E. coli* with Entry clone – or AMP – for *E. coli* with expression clone – were prepared. AMP and CAM antibiotics were used for Destination vectors since they contain AMP and CAM resistance, KAN was used for Entry clones as they contain KAN resistance and AMP was used for the expression clone because it contains AMP resistance only.

Plate preparation was done using 16 g LB-agar powder (broth), which were dissolved in 400 ml distilled water and autoclaved due to sterilization for 40 min at 120°C. Solution was afterwards tempered to 50 – 60°C and needed antibiotics were added – 800 µL were taken from 50 mg/ml AMP stock solution, 100 µL were taken from 25 mg/ml CAM stock solution and 400 µL were taken from 50 mg/ml KAN. Such prepared solution was put onto Petri dish and let to cool down to solidify. *E. coli* cells (DH5α, TOP10 or usually used XL1) containing plasmids were inoculated on plates and let grow overnight in an incubation apparatus at 37°C. The next day, single colonies were chosen and transferred to a new agar plate containing particular antibiotics. Plates were subsequently put to the incubator once again.

3.3 Miniprep preparation

LB medium containing either AMP and CAM or AMP or KAN separately was prepared (depending on the vector). From such medium, 3 ml were transferred to a plastic test tube and a particular colony of bacteria was added via scratch of the colony using a plastic tip. The solution with a tip, meaning the colony, was left to shake at 37°C for 16 – 20 hours with 180 rpm.

3.4 Plasmid DNA isolation

After shaking, the solution was transferred into 1.5 ml Eppendorf tube and centrifuged for 2 minutes at 4°C with 8000 rpm. The supernatant was discarded while the pellet was kept. High Pure Plasmid Isolation Kit (Roche) was used for the DNA isolation process. After following all steps written in the manual, the concentration of the DNA was measured using NanoDrop (Thermo Fisher Scientific). Plasmid DNA was isolated from cells containing Destination vectors as well as Entry clones (provided by the supervisor) and sent for the sequencing – always done via Seqme s.r.o. – due to correctness check of recombination sites, att sites. After the LR recombination, plasmid DNA from *E. coli* containing produced expression clones (verification done by PCR) was isolated and sent for sequencing, positive colonies were kept as the result.

3.5 Preparation of sequencing samples

Samples contained 500 ng of plasmid DNA, 25 pmol of primer and water which was filled up to 10 μL. Samples were then sent for the sequencing. Resulting sequences were compared to the original sequences using Geneious program. Bacteria containing correct plasmids were kept. Plasmid DNA was used further as a Destination vector in a LR recombination.

3.6 LR recombination

LR recombination was performed after verification of correct recombination sites of destination plasmids and Entry clones, which was done by sequencing. For such recombination, agar plates containing AMP were prepared. 150 ng of Entry vector were mixed with 150 ng of Destination vector (with either GFP or RFP) and solution was filled up with TAE buffer – pH 8.0 – up to 8 μL. Afterwards, 2 μL of the LR clonase II (Invitrogen) were added. The solution was incubated for 1 hour at the room temperature and then 1 μL of proteinase K (2 μg/μL) was added. Solution with proteinase K addition was incubated for 10 minutes at 37°C. The final mix was heated up to 75° C for 10 minutes. Consequently, the transformation of the mixture into *E. coli* cells was performed.

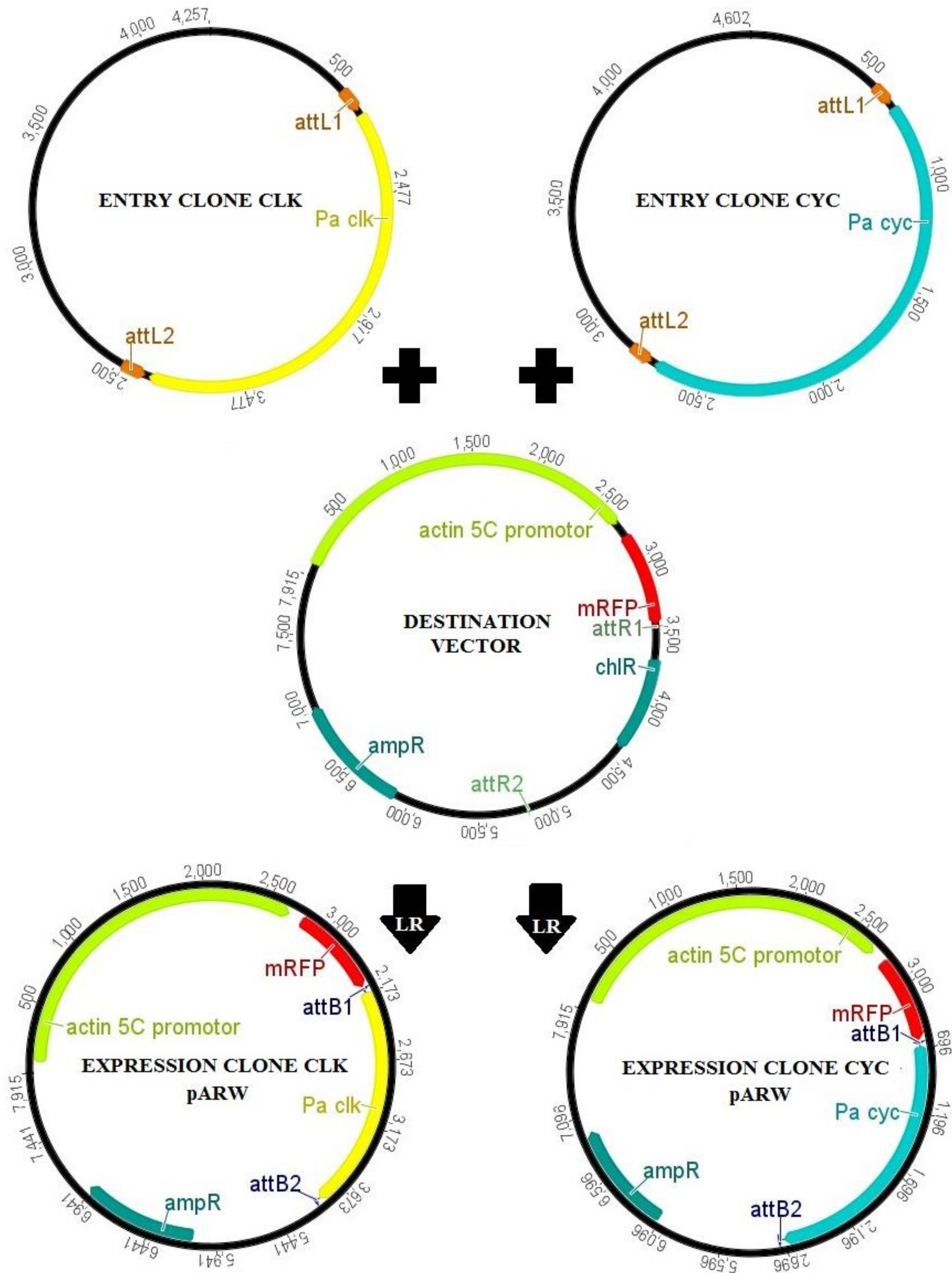

Figure 3: LR recombination example scheme [29] shows an Entry clone either containing *Clk* (left) or *cyc* (right) mixed with a Destination vector. Entry clone and Destination vector contain att sites (attL1 and attL2 in Entry, attR1 and attR2 in Destination vector). After LR recombination, expression clone is created, sites are marked with attB1 and attB2 holding the exchanged part in between – *Clk* denoted using yellow and *cyc* denoted using blue color. Exchanged parts are tagged with a protein, in this case mRFP (red fluorescent protein) downstream the gene of interest. Destination vector and expression clones contain actin promoter and ampR (ampicillin resistance gene). Both LR reactions proceed separately for *Clk* and *cyc*. For the schematic purpose was Donor vector neglected when considering as a waste product (Gateway cloning, chapter 1.3).

3.7 Transformation of *E. coli* cells

E. coli cells were taken out from -80°C and put on ice to melt. 100 µL XL1 cells were used for each reaction separately. Afterwards, 2 µL of the recombination mix were added with a careful circular motion and the solution was put on ice for 20 minutes. Next the heat shock for 45 seconds at 42°C was used for an introduction of a new plasmid to *E. coli* cells. Cooling on ice again for 1 – 2 minutes was needed after the heat shock. Subsequently, 250 µL of the SOC medium were added and the solution was incubated in the preheated shaker for 50 – 60 minutes at 37°C. After incubation, 150 µL of the solution were put on the agar plate with AMP and it was let to grow overnight at 37°C. Grown colonies were transferred to a new agar plate containing AMP and analyzed using PCR, afterwards the plasmid DNA was isolated and sent for sequencing. Using Geneious program obtained sequences were compared to the sequences made *in silico*. Bacterial cells including valid plasmids were conserved.

3.8 Polymerase chain reaction

PCR or polymerase chain reaction is a method when the DNA strand is multiplied in a fast an efficient way. The method is based on a nucleic acid replication, when DNA parts have to be bounded on 3´ and 5´ends by primers. Synthesis of new strands is done using thermostable DNA polymerase in thermocycler (XP cycler, Bioer). This method was used as a proof of presence of *Pa Clk* or *Pa cyc* sequence in the Destination vector (after the recombination). Agarose gel electrophoresis was used as the PCR result verification.

Firstly, PCR mix had to be prepared. It was prepared using 2X PPP Master mix, Top Bio (150 mM Tris-HCl, 40 mM $(NH_4)_2SO_4$, 0.02 % Tween 20, 5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U/mL Taq DNA polymerase, color additives and stabilizators), forward and reverse primers (400 nM) and water. For the PCR test tubes, 7 µL of the PCR mix were used for each, inoculum from bacteria cells was used as a DNA template. Test tubes were placed into a thermocycler.
Following procedure was used: 94 °C for 2 minutes as an initialization, 35 cycles of 94 °C – 30 s, 56 °C – 30 s, 72 °C – 60 s and afterwards a final elongation at 72 °C for 10 min. Agarose gel electrophoresis came next.

3.9 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate mixture of RNA and DNA in an agarose gel matrix. Firstly, 1% gel was prepared using 0.65 g of agarose (SeaKem® LE Agarose, Lonza) and 65 mL of 1x TAE buffer. The mixture was afterwards heated up in the microwave. The

gel stain (3 μL, GelRed, Biotium) was then added to the cooled down mixture. All the solution was poured to the electrophoresis stand, comb was inserted to form wells and the gel was let to solidify. The PCR product was put into wells using pipettes, 7 μL into each. Marker was also put to the proper well. Apparatus (Liberty 120 high speed gel system, Biokeystone) was filled with 1x TAE buffer according to the manufacturer´s instruction. Thereafter, the picture of the gel was taken using Alpha Imager mini. If the size of the DNA fragment was correct, minipreps were prepared, DNA plasmid isolation performed in the next step and send for verification via sequencing.

3.10 Conservation of cells

Following procedure was conservation of cells. From the LB medium containing AMP, 3 mL were taken and put into a plastic test tube. The cells were introduced via scratch with a micropipette tip and put into the solution. Cells were let to grow for 8 – 12 hours and afterwards mixed with 80% glycerol (sterilized by autoclaving) in a 1 : 1 ratio. Such mix could be kept in the freezer at -80°C.

4. Results

4.1 Attachment sites verification

First, the correctness of the attachment recombination sites (att sites) in the sequence in both Destination and Entry vector were checked. The alignment of obtained sequences and Destination vector are used as an illustration (Fig. 4). The same procedure was done with the Entry vector (data not shown).

COMPLETE LINEARIZED PLASMID SEQUENCE WITH HIGHLIGHTED ATT SITES

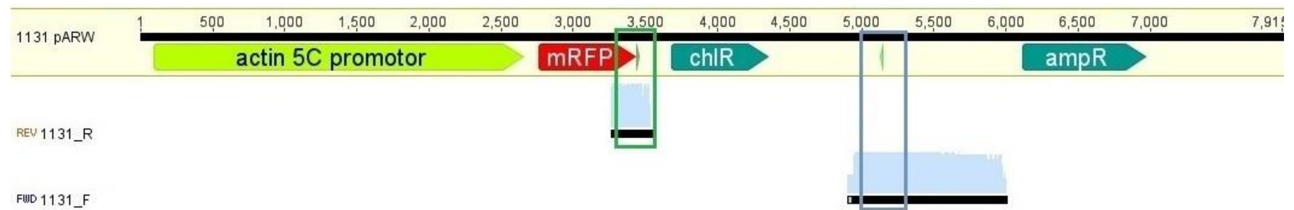

Figure 4: Sequencing of the Destination vector: Sequencing results 1131_R and 1131_F aligned to the Destination vector sequence (1131 pARW) in Geneious program [29]. Important features of the Destination vector are highlighted - actin 5C promoter, mRFP gene, CAM (chlR) resistance gene, AMP resistance gene and att recombination sites: attR1 green line in the green box, attR2 green line in the blue box. Black background in an alignment means similar sequences**.**

AttR1 DETAIL

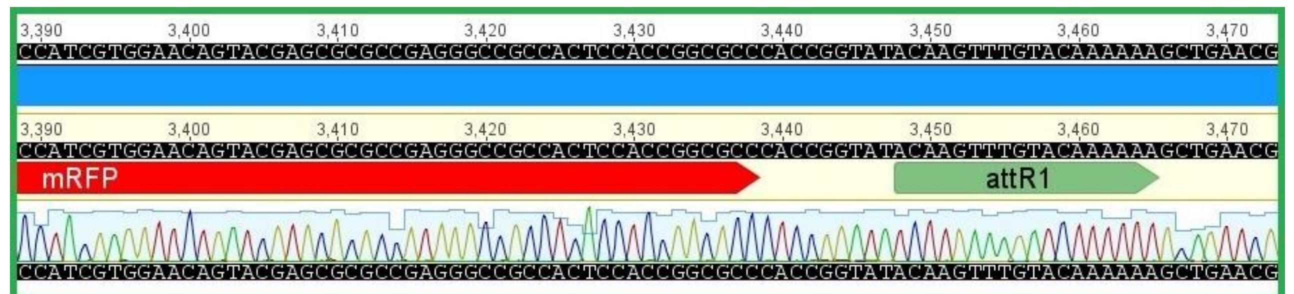

Figure 4.1: Area showing a verified attR1 site. Green box from the **Fig. 4** – zoomed using program Geneious [29].

AttR2 DETAIL

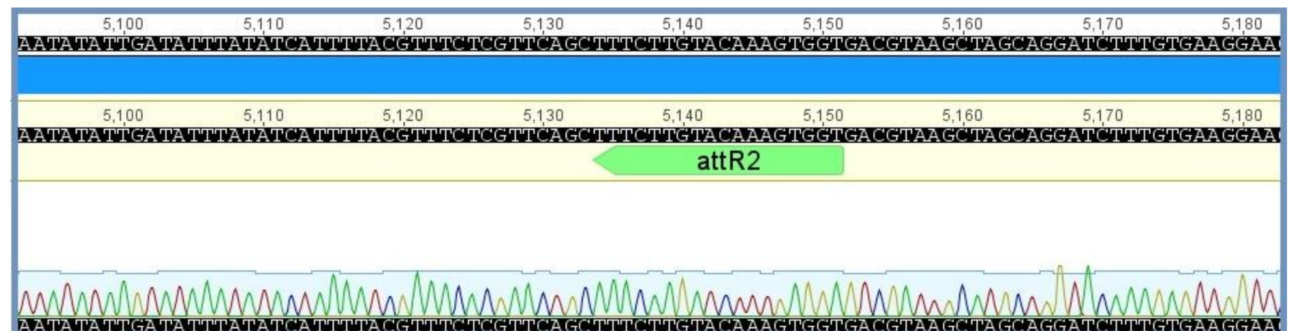

Figure 4.2: Area showing a verified attR2 site. Blue box from the **Fig. 4** – zoomed using program Geneious [29].

4.2 Production of expression clones *in silico*

Using Geneious program, LR recombination was first performed *in silico*. Sequences of *in silico* created expression vectors (Fig. 5 – 8) were linearized and then used as the reference in an alignment of the *in vitro* produced expression vectors (Fig. 10 – 17).

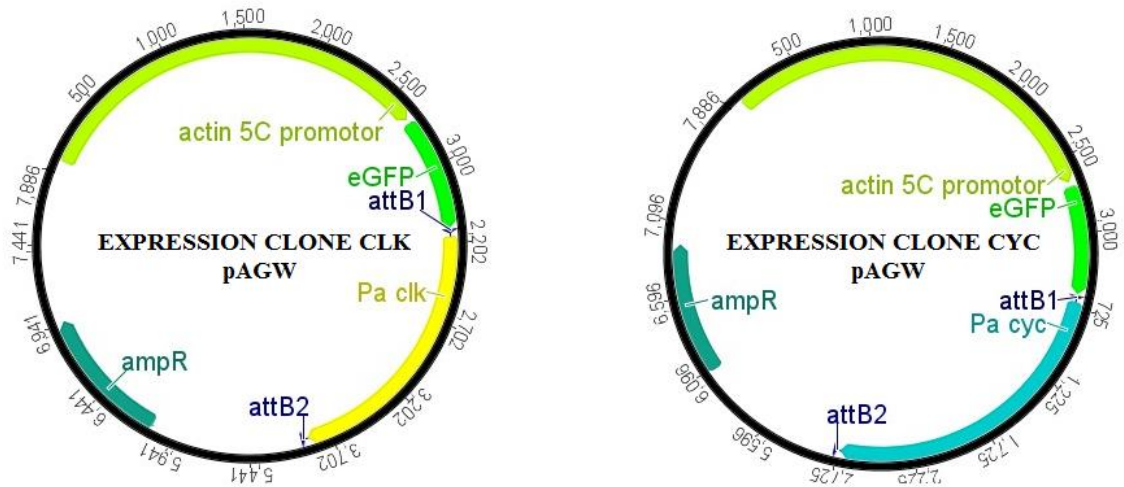

Figure 5: *In silico* produced expression clones after LR recombination of pAGW vector (as donor) and pENTR vector containing *Pa Clk* (left) or *Pa cyc* (right) [29]. Expression clones contain actin 5C promoter, eGFP gene downstream the gene of interest, AMP resistance gene and attB1 and attB2 recombination sites. Expression clone on the left contains *Pa Clk* ORF denoted using yellow color. Expression clone on the right contains *Pa cyc* ORF denoted using blue color.

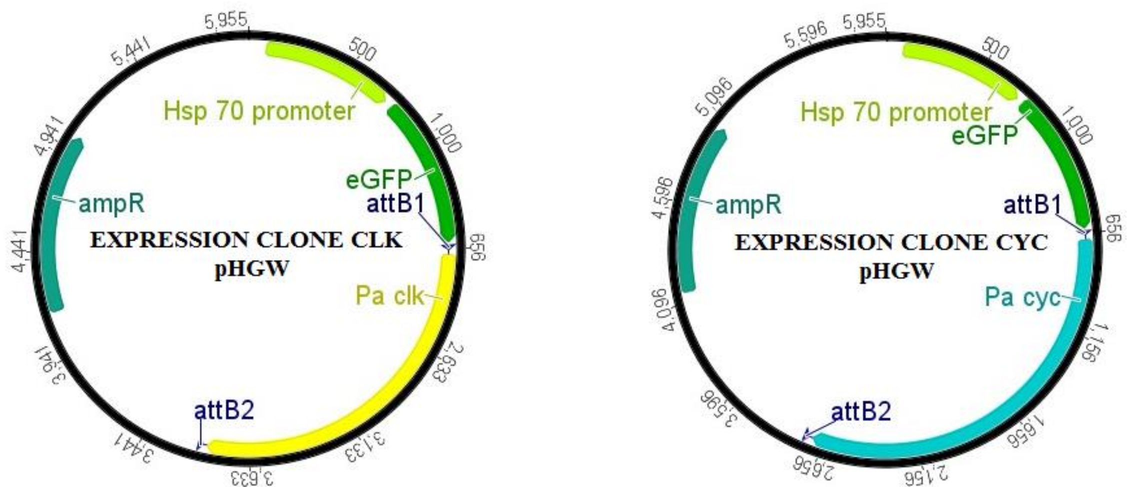

Figure 6: *In silico* produced expression clones after LR recombination of pHGW vector (as donor) and pENTR vector containing *Pa Clk* (left) or *Pa cyc* (right) [29]. Expression clones contain Hsp 70 promoter, eGFP gene downstream the gene of interest, AMP resistance gene and attB1 and attB2 recombination sites. Expression clone on the left contains *Pa Clk* ORF denoted using yellow color. Expression clone on the right contains *Pa cyc* ORF denoted using blue color.

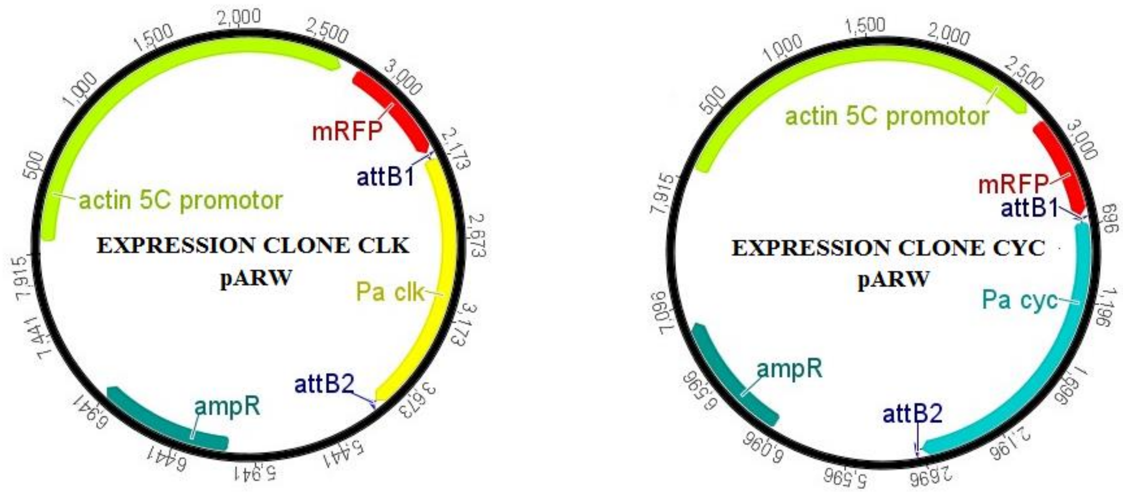

Figure 7: *In silico* produced expression clones after LR recombination of pARW vector (as donor) and pENTR vector containing *Pa Clk* (left) or *Pa cyc* (right) [29]. Expression clones contain actin 5C promoter, mRFP gene downstream the gene of interest, AMP resistance gene and attB1 and attB2 recombination sites each. Expression clone on the left contains *Pa Clk* ORF denoted using yellow color. Expression clone on the right contains *Pa cyc* ORF denoted using blue color.

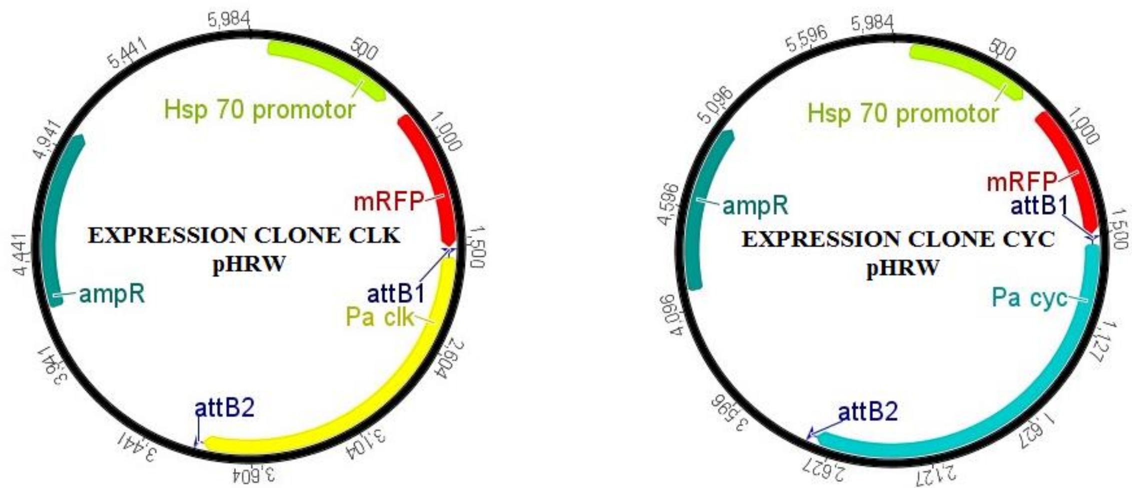

Figure 8: *In silico* produced expression clones after LR recombination of pHRW vector (as donor) and pENTR vector containing *Pa Clk* (left) or *Pa cyc* (right) [29]. Expression clones contain Hsp 70 promoter, mRFP gene downstream the gene of interest, AMP resistance gene and attB1 and attB2 recombination sites each. Expression clone on the left contains *Pa Clk* ORF denoted using yellow color. Expression clone on the right contains *Pa cyc* ORF denoted using blue color.

4.3 Production of expression clones in *E. coli* cells

4.3.1 LR recombination verification

As a check-up of the LR recombination results, PCR (Polymerase chain reaction, chapter 3.8) on colonies with specific primers for *Pa Clk* or *Pa cyc* was performed together with agarose gel electrophoresis. PCR product was put on the gel which was subsequently electrophoresed and the picture of the gel was taken. Positive colonies containing desired input were identified according to the appearance or absence of the band on the gel, using 1 kb plus DNA Ladder (Invitrogen). Plasmid DNA from positive colonies was isolated and verified by sequencing.

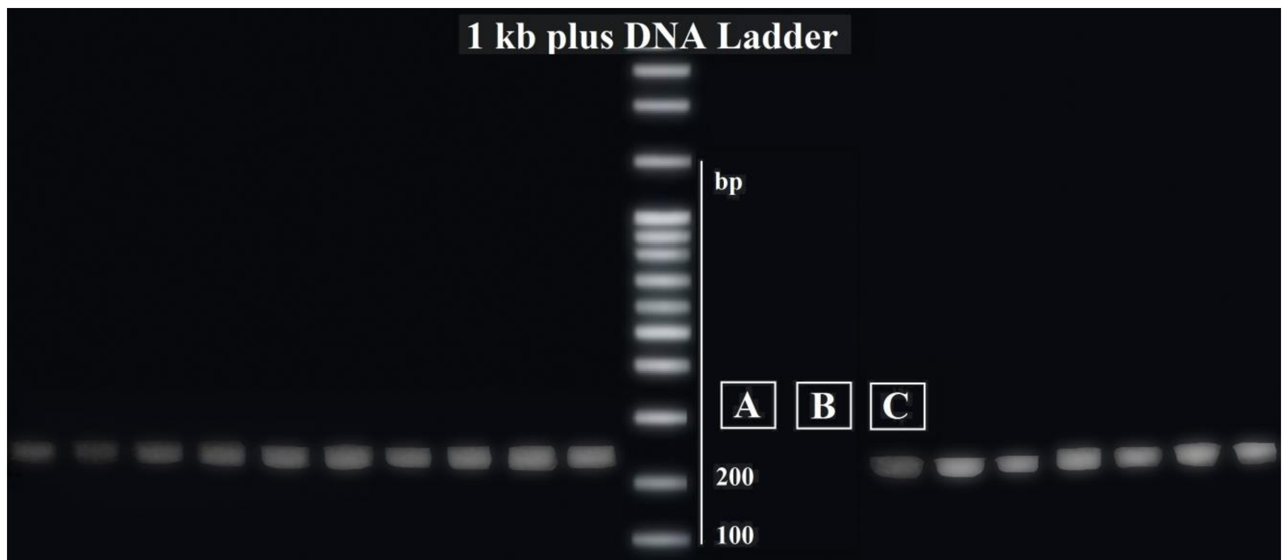

Figure 9: An example of the agarose gel electrophoresis after the PCR on the bacteria colonies. Illustrative sample of the colony marked with a letter C represents positive proceed of the LR recombination together with all colonies samples that appear. Missing bands marked with A and B show negative LR recombination meaning that the LR recombination in those two particular bacteria colonies did not proceed.

4.3.2 Production of constructs

The obtained sequences were aligned to the linearized *in silico* expression vectors taken as the reference. All pictures of constructs (Fig. 10 – 17) contain promoter, actin or Hsp70, fluorescent protein, eGFP or mRFP, AMP resistance gene and *Pa Clk* or *Pa cyc* ORFs.

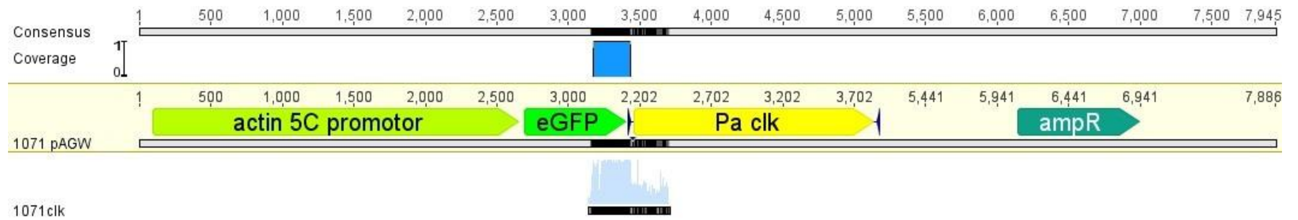

Figure 10: Obtained sequence 1071 clk was aligned to *Pa Clk in silico* produced expression clone 1071 pAGW + clk [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

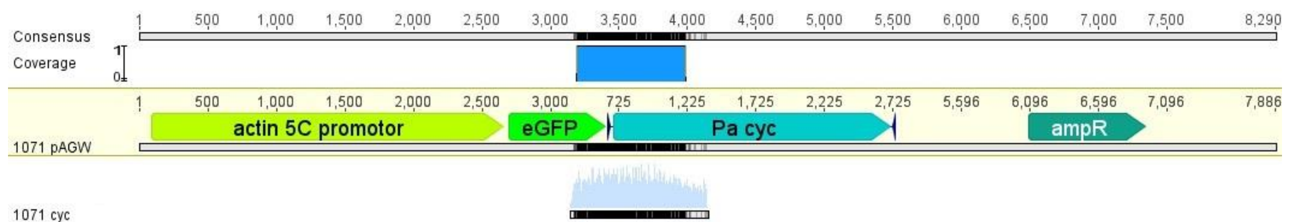

Figure 11: Obtained sequence 1071 cyc was aligned to *Pa cyc in silico* produced expression clone 1071 pAGW + cyc [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

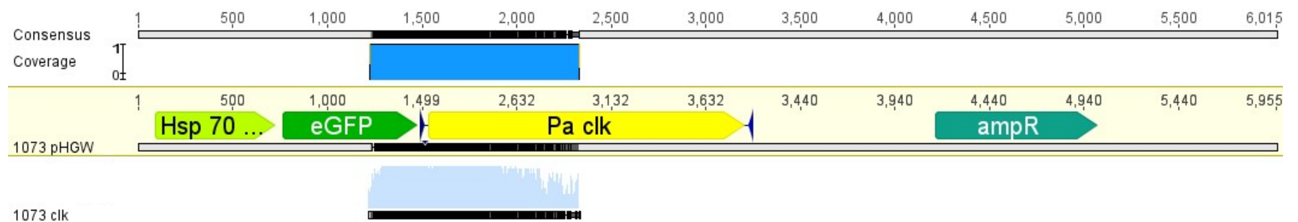

Figure 12: Obtained sequence 1073 clk was aligned to *Pa Clk in silico* produced expression clone 1073 pHGW + clk [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

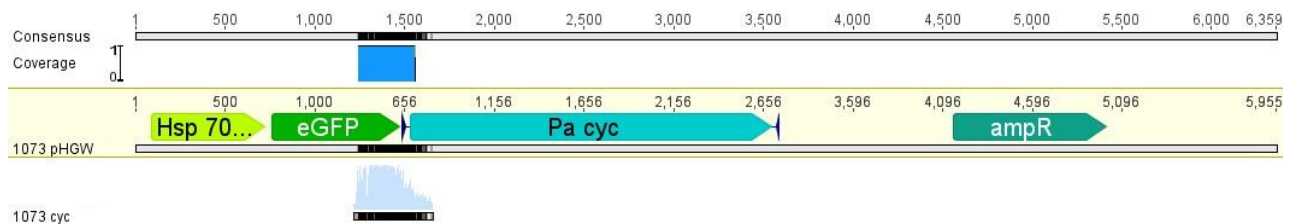

Figure 13: Obtained sequence 1073 cyc was aligned to *Pa cyc in silico* produced expression clone 1073 pHGW + cyc [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

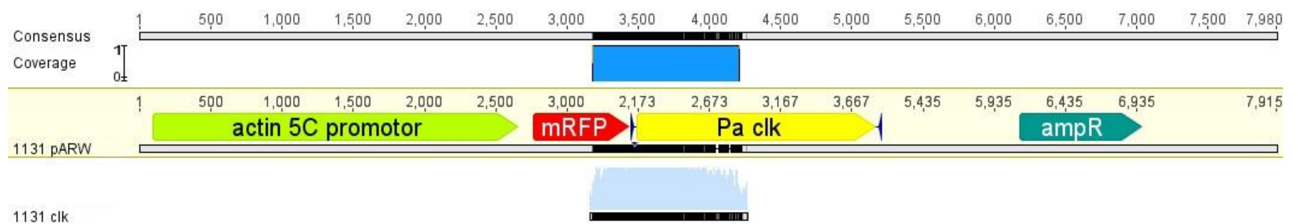

Figure 14: Obtained sequence 1131 clk was aligned to *Pa Clk in silico* produced expression clone 1131 pARW + clk. [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

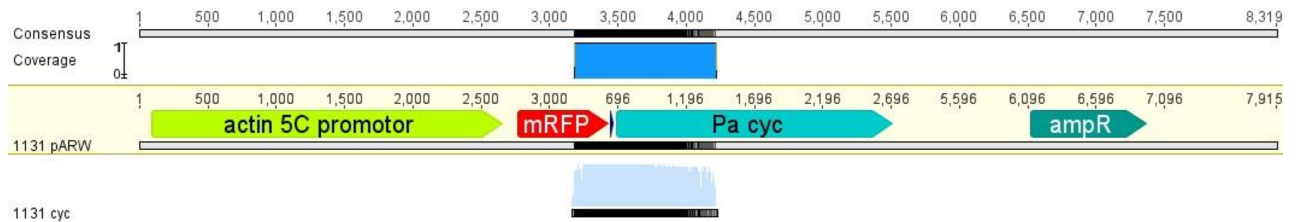

Figure 15: Obtained sequence 1131 cyc was aligned to *Pa cyc in silico* produced expression clone 1131 pARW + cyc [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

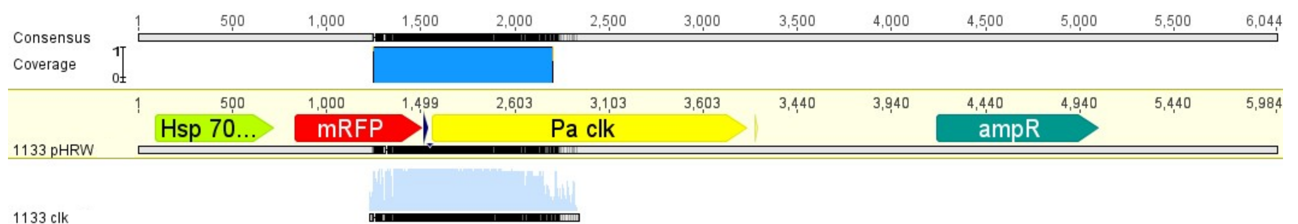

Figure 16: Obtained sequence 1133 clk was aligned to *Pa Clk in silico* produced expression clone 1133 pHRW + clk [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

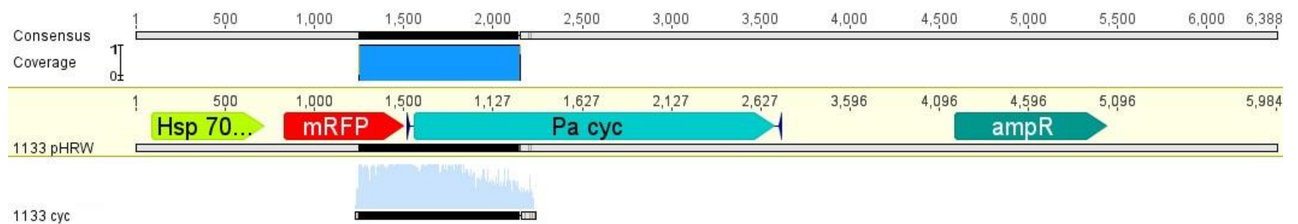

Figure 17: Obtained sequence 1133 cyc was aligned to *Pa cyc in silico* produced expression clone 1133 pHRW + cyc [29]. 100 % identity is shown in black color, which means that LR recombination was successful.

5. Discussion

The grand goal of this study was to construct various plasmids by using Gateway cloning strategy. Constructs were created in a way to contain promoter, actin or Hsp70, followed by the complete coding DNA sequence of *Clock* and *cycle* genes from the linden bug (*Pyrrhocoris apterus*) and tag, green or red fluorescent protein. Such prepared constructs are intended to be used to study interactions of Clock and Cycle proteins *in vivo* in Schneider S2 cell line. In addition, effectiveness, reliability and applicability of the used method Gateway cloning was examined.

Different promoters are used even in the same cell line, such as S2 cells, if a diverse expression control is needed. For instance, actin promoter is constitutively expressed, which is often sufficient for major biochemical experiments where protein is expressed to certain level and its properties are tested. For example, Chiu et al. used constitutive actin promoter to explore phosphorylation pattern on PER protein [30]. However, sometime more detailed temporal control of expression is needed. Indeed, experiment aiming at PER-TIM interaction required well-defined expression from the heat shock promoter, allowing to determine dynamics of cytoplasmic/nuclear localization of PER-TIM dimers [31]. Nevertheless, no matter specific qualities of both promoters separately, both showed promising results together with the usage of fluorescent proteins used for visualization of *Clock* and *cycle* genes. Such observation is supported by results of another thesis in which were constructs created using other genes namely *Met* and *taiman* (*tai*), which were also tagged by red or green fluorescent protein and also contained promoters [32].

An important aspect of all cloning is reliability. Circadian proteins are often large, for instance TIM is approximately 1400 amino acids long and PER contains around 1200 amino acids. Because of that, precise sub-cloning between various plasmids is needed, ideally avoiding PCR amplification which might introduce errors. Here-tested Gateway cloning requires only verification of region right next to att sites, but further laborious verifications of open reading frames are not necessary. Therefore, Gateway Cloning Technology [28] seems to be indeed suitable and efficient approach supported not only by obtained results but also by conclusion from preparation of constructs containing *Met* and *tai* [32]. All of produced expression clones could be directly of use for the future experiments.

6. Conclusion

Produced expression clones consisting of actin or Hsp70 promoter, fluorescent protein eGFP or mRFP and *Pa Clock* or *Pa cycle* ORF could be directly used for the future experiments. The future work would contain transferring the *Drosophila S2* cell lines with the plasmid DNA.

The available evidence seems to point out that the used procedure is suitable and efficient approach when building reporters. Effectiveness, reliability and applicability of the method was examined and supported by obtained results. Constructs were successfully created and seem to be sufficient for our laboratory needs.

7. References

[1] Sieber, M. 2013. Protein-protein interaction of photoperiodic clock factors in *Pyrrhocoris apterus*. Faculty of Science. University of South Bohemia in České Budějovice.

[2] Konopka, R.J., Benzer, S. 1971. Clock mutants of *Drosophila melanogaster*. Proc.Nat.Acad.Sci. USA 68, 2112-6.

[3] King, D.P., Zhao, Y.L., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W., Takahashi, J.S. 1997. Positional cloning of the mouse circadian Clock gene. *Cell* **89**: 641-653.

[4] Levine, J.D., Sauman, I., Imbalzano, M., Reppert, S.M., Jackson, F.R. 1995. Period protein from the giant silkmoth *Antheraea pernyi* functions as a circadian clock element in *Drosophila melanogaster*. Neuron 15, 147-57.

[5] Šauman, I., Tsai, T., Roca, A.L., Reppert, S.M. 1996. Period protein is necessary for circadian control of egg hatching behavior in the silkmoth *Antheraea pernyi*. Neuron 17, 901-9.

[6] Rubin, E.B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H.M., Bloch, G. 2006. Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis malifera*) and shed new light on the molecular evolution of the circadian clock. Genome Res. 2006 Nov;16(11):1352-65. Epub 2006 Oct 25.

[7] Tomioka, K., Matsumoto, A. 2010. A comparative view of insect circadian clock systems. Cell Mol Life Sci. May;67(9):1397-406.

[8] Rivas, G.B., Bauzer, L.G., Meireles-Filho, A.C. 2016. "The Environment is Everything That Isn't Me": Molecular Mechanisms and Evolutionary Dynamics of Insect Clocks in Variable Surroundings. Front Physiol. 2016 Jan 12;6:400. doi: 10.3389/fphys.2015.00400. eCollection 2015.

[9] Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M. 2007. Clockwork Orange is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. Genes Dev. 21(13): 1675--1686

[10] Richier, B., Michard-Vanhée, C., Lamouroux, A., Papin, C., Rouyer, F. 2008. The clockwork orange *Drosophila* protein functions as both an activator and a repressor of clock gene expression. J Biol Rhythms. 23(2):103-16.

[11] Glossop, N.R., Lyons, L.C., Hardin, P.E. 1999. Interlocked feedback loops within the *Drosophila* circadian oscillator. Science 286:766

[12] Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R.J, Hardin, P.E., Young, M.W., Storti, R.V., Blau, J. 2003. *vrille*, *Pdp1* and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112:329–341

[13] Benito, J., Zheng, H., Hardin, P.E. 2007. PDP1e functions downstream of the circadian oscillator to mediate behavioral rhythms. J Neurosci 27:2539–2547

[14] Beuchle, D., Jaumouillé, E., Nagoshi, E. 2012. The nuclear receptor unfulfilled is required for free-running clocks in Drosophila pacemaker neurons. Curr. Biol. 22, 1221 - 1227.

[15] Jaumouillé, E., Machado Almeida, P., Stähli, P., Koch, R., Nagoshi, E. 2015. Transcriptional regulation via nuclear receptor crosstalk required for the *Drosophila* circadian clock. Curr. Biol. 25, 1502–1508.

[16] Kumar, S., Chen, D., Jang, C., Nall, A., Zheng, X., Sehgal, A. 2014. An ecdysone-responsive nuclear receptor regulates circadian rhythms in *Drosophila*. Nat. Commun. 5:5697.

[17] Tataroglu, O., Zhao, X., Busza, A., Ling, J., O'Neill, J. S., Emery, P. 2015. Calcium and SOL protease mediate temperature resetting of circadian clocks. Cell 19, 1214–1224.

[18] Allada, R., White, N.E., So, W.V., Hal,l J.C., Rosbash, M. 1998. A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**: 791-804.

[19] Rutila, J.E., Suri, V., Le M., So, W.V., Rosbash, M., Hall, J.C. 1998. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* **93**: 805-814.

[20] Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M. 2007 *Clockwork orange* is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. Genes Dev 21:1675–1686

[21] Lim, C., Chung, B.Y., Pitman, J.L., McGill, J.J., Pradhan, S., Lee, J., Keegan, K.P., Choe, J., Allada, R. 2007. *Clockwork orange* encodes a transcriptional repressor important for circadian-clock amplitude in Drosophila. Curr Biol 17:1082–1089

[22] Matsumoto, A., Ukai-Tadenuma, M., Yamada, R.G., Houl, J., Uno, K.D., Kasukawa, T., Dauwalder, B., Itoh, T.Q., Takahashi, K., Ueda, R., Hardin, P.E., Tanimura, T., Ueda, H.R. 2007. A functional genomics strategy reveals *clockwork orange* as a transcriptional regulator. Genes Dev. 2007 Jul 1;21(13):1687-700. Epub 2007 Jun 19.

[23] Richier, B., Michard-Vanhe´e, C., Lamouroux, A., Papin, C., Rouyer, F. 2008. The *clockwork orange Drosophila* protein functions as both an activator and a repressor of clock gene expression. J Biol Rhythms 23:103–116

[24] Zhou, J., Yu. W., Hardin, P.E. 2016. CLOCKWORK ORANGE Enhances PERIOD Mediated Rhythms in Transcriptional Repression by Antagonizing E-box Binding by CLOCK-CYCLE. PLoS Genet. 2016 Nov; 12(11): e1006430.

[25] Shin, S.W., Zou, Z., Saha, T.T., Raikhel, A.S. 2012. bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. P Natl Acad Sci USA *109*, 16576-16581.

[26] Konopova, B., Jindra, M. 2007. Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle Triboliumcastaneum. *Proc Natl Acad Sci USA* **104**: 10488-10493

[27] Bajgar, A., Jindra, M., Dolezel, D. 2013. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc Natl Acad Sci U S A *110*, 4416-4421.

[28] Gateway Recombination Cloning Technology | Thermo Fisher Scientific. [online]. Available: https://www.thermofisher.com/cz/en/home/life-science/cloning/gateway-cloning/gateway-technology.html.

[29] Geneious version 8.1 created by Biomatters. Available from http://www.geneious.com.

[30] Chiu, J.C., Ko, H.W., Edery, I. 2011. NEMO/NLK Phosphorylates PERIOD to Initiate a Time-Delay Phosphorylation Circuit that Sets Circadian Clock Speed. Cell *145*, 357-370.

[31] Meyer, P., Saez, L., Young, M.W. 2006. PER-TIM interactions in living Drosophila cells: An interval timer for the circadian clock. Science *311*, 226-229.

[32] Kitzberger, F. 2016. Interaction of proteins Methoprene tolerant and Taiman – Preparation of constructs for expression in S2 cells. Faculty of Science. University of South Bohemia in České Budějovice.