University of South Bohemia in České Budějovice
Faculty of Science
Department of Molecular Biology

BACHELOR THESIS

Interaction of proteins *Methoprene tolerant* and *Taiman* – Preparation of constructs for expression in S2 cells

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ANNOTATION

The aim of this thesis was to create a set of constructs consisted of actin or Hsp70 promoter and complete coding DNA sequence (CDS) of *Met* (*Methoprene tolerant*) or *tai* (*taiman*) genes from linden bug (*Pyrrhocoris apterus*) tagged by green fluorescent protein, or red fluorescent protein. These constructs will be used to study interaction of these proteins *in vivo* (Schneider S2 cell line) in the future.

AFFIRMATION

I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section. 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, Date František Kitzberger

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I would like to thank to Mgr. Olga Bazalová for patient guidance and supervision during the lab work and thesis overall. I would also like to thank to Mgr. David Doležel PhD. for allowing me working on this interesting topic and also for the professional supervision. My next big thank goes to the Prof. RNDr. Libor Grubhoffer CSc. for never ending support of students of this curriculum.

My last thanks go to my family and friends for support and care.

ABSTRACT

Protein-protein interactions are essential for many biochemical processes. However, it is often difficult to study these interactions in complex environment of a particular organism. Therefore, artificial cell culture reporter assays were developed. Usually, simple system, such as yeast cells or *Drosophila* embryonic cells, is used as a host for expression of proteins from delivered plasmids. These engineered plasmids contain promoters allowing either constituent or inducible expression of heterologous protein. These proteins of interest are often tagged with either short epitope or fluorescent protein.

Usually, combination of several complementary experiments, relying on slightly modified constructs and different tags, are necessary to elucidate mechanism underpinning interactions of even small number of proteins. Construct preparation is a tedious, time and resources demanding task.

Therefore, I have explored reliability, effectiveness and applicability of Gateway cloning system for specific needs in the Laboratory of Molecular Chronobiology. I have verified sequence of original GFP and mRFP tagging clones. Using clonase reaction I have successfully created several new plasmids. My work indicates that, despite some difficulties with one construct set, Gateway cloning is efficient approach for building whole collection of reporters either for *Drosophila* or for yeast expressing systems.

AIMS OF THE WORK

My aim was testing effectiveness and applicability of Gateway cloning system on two genes, *Met* and *tai*. Specifically, my aim was to prepare clones containing eGFP or RFP tag upstream or downstream to the *Met* or *Tai* ORFs with either Actin5C or HSP70 promotor.

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1. List of Abbreviations

AMP	Ampicillin
CAM	Chloramphenicol
CK2	Casein Kinase 2
CRY	Cryptochrome
CWO	Clockwork Orange
DBT	Doubletime
GE	Gel Electrophoresis
GFP	Green Fluorescent Protein
JH	Juvenile Hormone
KAN	Kanamycin
LB	Luria-Bertani
MET	Methoprene tolerant
PCR	Polymerase chain reaction
PDP1ε	PAR Domain Protein 1ε
PER	Period
RFP	Red Fluorescent Protein
RPM	Rotations per minute
SGG	Shaggy
SOC medium	Super Optimal Broth with Catabolite repression
TAE buffer	Tris-acetate-EDTA
TAI	Taiman
TIM	Timeless
VRI	Vrille

2. INTRODUCTION

2.1 Circadian and photoperiodic clock

Circadian clock is a time measurement system in a body which is responsible for the time dependent activities and maintaining the periodicity. It evolved as a response to the periodical activities and oscillations which occur due to the periodical movements of the Earth in space (day and night cycle, lunar cycle). It results in the changing of the light intensity, temperature and humidity. This mechanism occurs in most of eukaryotes and also in some prokaryotes. It is endogenous but it can be entrained to environmental changes, so the organism can be kept in the constant light or constant darkness and still can maintain the periodicity of daily activities. It causes that various activities such as feeding, daily motion, but also egg hatching are triggered in the most favourable time. The molecular mechanisms of the circadian clock is well studied and quite well described in many species across the "tree of live". Since 1971, when Konopka and Benzer showed first clock mutant flies, circadian clocks were well studied in insect organisms *Drosophila melanogaster*, which became a model organisms. The insight to the molecular mechanisms of the *Drosophila* clock is well established, there are so-called clock genes involved in this machinery consisted of at least three feedback loops.

These clocks are controlled by a group of proteins.

CLOCK, which works as a transcription factor in *Drosophila* (Allada et al., 1998) and in mammals (King et al., 1997). This protein drives expression of proteins like PERIOD and TIMELESS. CYCLE, which is an interaction partner of the CLOCK and which forms heterodimer with it (Rutila et al., 1998). Tohether they work as a transcription factor for other proteins. CLOCKWORK ORANGE is a protein whose level oscillates in the cell with time. It works as a repressor of the CLK target genes (Kadener et al., 2007). Its absence in the organism results in decreased level of mRNA of *per*, *tim*, *vri* and *pdp1ε* genes. Subsequently it leads to loss of mRNA oscillations which suggest that CWO works mainly as transcriptional activator of these genes (Richier B et al., 2008). Timeless and Period that create 24 hours feedback loop repressing CLK-CYC mediated transcription. This is caused by the fact that increased concentration of PERIOD inhibits the transcription of *per* in the cell. When the PERIOD protein accumulates it dimerizes with TIMELESS. This dimer enters the cell where it inhibits its own activators CLK-CYC dimer (Ishida N et al., 1999).

The level of these proteins oscillates in a body/cell with 24 hour periodicity. When the transcription is inhibited, the levels of PER and TIM also decreases as the TIM is inhibited by light sensitive protein CRYPTOCHROME (CRY) (Ceriani MF et al., 1999) and therefore cannot complex PER which is then hyperphosphorylated and targeted for digestion by protein DOUBLETIME (DBT) (Kivimäe S et al., 2008; Grima et al., 2002; Ko et al., 2002). VRILLE is a protein that forms another feedback loop that is linked with oscillation of the CLK protein. Its transcription is activated by CLK-CYC heterodimer (Glossop et al., 1999).When this protein is exprimed, it enters the nucleus and it inhibits the transcription of the CLK by binding to its promotor. However in this loop works one more protein called PAR domain protein 1ε (PDP1ε). This protein was found to be the activator of the CLK expression (Cyran et al., 2003). This protein is exprimed later and then it is thought to bind to the promotor competitively with the VRI and activates the transcription of the CLK (Benito J et al., 2007). Genes of proteins that affect phosphorylation are *doubletime (dbt)* (Kloss et al., 1998; Price et al., 1998; Kivimae et al., 2008; Grima et al., 2002; Ko et al., 2002; Cyran et al., 2005), *casein kinase 2 (ck2)* (Price et al., 1998; Lin et al., 2002; Akten et al., 2003). The phosphorylation probably affects the timing of entrance of the TIM and PER into the nucleus (Cyran et al., 2005; Meyer et al., 2006). DBT interacts with PER and enters the nucleus with PER-TIM heterodimer (Kloss et al., 2001). When DBT is transported to the cell by PER it can phosphorylate CLK and inactivate it. When the PER is hyperphosporylated in the cytosol, it is degraded rapidly. (Grima et al., 2002; Ko et al., 2002) SHAGGY (SGG) is a kinase that phosphorylates TIM so it speeds up its binding to PER (Martinek et al., 2001) (see Fig 1).

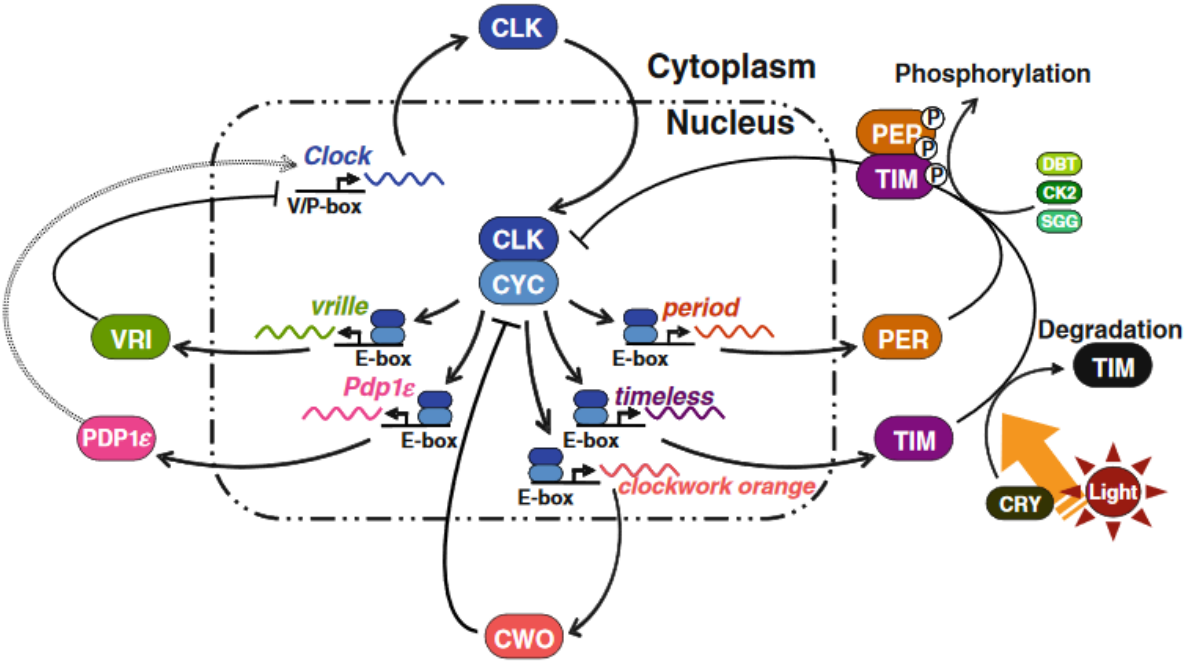

Figure 1: Graphical representation of the molecular interactions involved in the circadian rhythm in *Drosophila melanogaster* (Taken from Tomioka and Matsumoto, 2009)

Some organisms also need to adapt to seasonal changes during the year. They anticipate seasonal changes by measuring the changing ratio in night- or day-length (i.e. photoperiod).

To cope these seasonal variabilities animals either migrate to warmer habitats, or set their metabolisms to minimum and hibernate, or entry the diapause (Hahn and Delinger, 2011). This behaviour is driven by so called photoperiodic clocks. *Pyrrhocoris apterus* seems to be the perfect model organisms for studying photoperiodic clock and diapause, since it exhibit strong diapause response to the photoperiod (Socha, 1993). In contrast to circadian clock, mechanisms of photoperiodic clock still remains unclear, but an overlap with the circadian clock machinery is either suggested (Kostal, 2011; Saunders and Bertossa, 2011), or denied (Bradshaw and Holzapfel, 2010; Emerson et al., 2009a; b). There are some evidences suggesting genetic interaction between circadian clock factors and JH signalling playing the role in diapause of the linden bug (Bajgar at al., 2013a; b).

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2.2 Juvenile Hormones

Juvenile Hormones (JHs) are group of isoprenoid hormones which regulate many aspects of insect physiology e.g. reproduction and growth. JHs are responsible for the maintaining larval state in lot of insects. The concentration of JHs in haemolymph decreases over time of development, when it is depleted, metamorphosis is started and larva develops into an adult. This means that development of the insect is dependent on the hormone concentration (Wigglesworth, 1964). Later in life this hormone is necessary for reproduction, because it stimulates female vitelogenesis.

The structure of the JH in the *P. apterus* is not known, the structure of JH III is shown below (see Fig. 2). Involvement of JH in reproduction is suggested genetically and anatomically. When the synthesis of JH was stopped by the removal of the organ responsible for synthesis, the ovaries did not grow to proper size (Smykal et al., 2013).

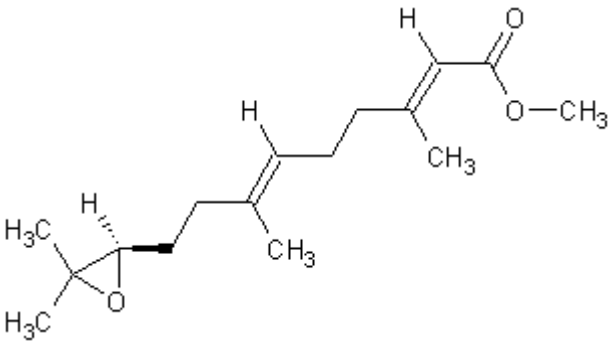

Figure 2: Structure of Juvenile Hormone III

The genetic evidence shows that *Methoprene tolerant* RNA interference results in the undeveloped and small ovaries which remain the same even after addition of synthetic analogues of JH. That proves that *Met* is receptor of JH in *P. apterus* (Bajgar et al., 2013a).

2.3 METHOPRENE TOLERANT (MET)

METHOPRENE TOLERANT is a bHLH-PAS protein which was found to be receptor for the Juvenile Hormone (Charles et al., 2011). It forms heterodimer with another bHLH-PAS protein CYCLE (CYC). This heterodimer mediates JHIII gene expression in insects. (SW Shin et al., 2012). If it is absent in the organism, it causes development defects in insects (Konopova and Jindra, 2007). It is involved in the reproduction in *P. apterus.* (Bajgar et al., 2013a). Two types of MET-like proteins can be found in *Drosophila* (MET and GCE) which makes research complicated due to the gene duplication.

2.4 TAIMAN (TAI)

TAIMAN is an interaction partner of METHOPRENE TOLERANT. It is a large protein (1260AA in *P. apterus*) which shoved binding to MET in yeast two hybrid assays (Li et al., 2011). Homologues of this protein can be found in various insects such as *Aedes aegypti* and *Tribolium castaneum*. In *P. apterus* it is involved in the reception of Juvenile Hormone in the fat tissue (Smykal et al., 2013).

2.5 Motivation of this study

Photoperiodic clock is a fascinating time-measuring machine with elusive molecular mechanism. Clearly, interaction of at least one dozen of proteins might contribute remarkably. While these candidates are known at protein sequence level, their mutual interaction is not well elucidated. Master thesis by Marion Sieber (Sieber M, 2013) indicates that yeast two-hybrid assay is a powerful screening approach for protein-protein interactions. However, since yeast assays are prone to artefact, further validations and parallel platforms are needed.

Since some of photoperiodic clock candidates are large proteins, cloning PCR products for corresponding open reading frames is a tedious task, often hampered by mutations introduced during the amplifications. Therefore it would be ideal to have a platform, where one successfully cloned ORF is be used for all different constructs. Such cloning is difficult due to the size of ORF, variability in promoters needed, and diversity of tags (different fluorescent proteins, luciferase, short tags) that can be attached either to N- or C- terminus. Optimally, one would need system allowing for an easy switch between yeast, *Drosophila* or bacterial expression vectors.

Therefore, I was exploring applicability of Gateway cloning system for specific needs in the Laboratory of Molecular Chronobiology. My aim was to prepare clones containing eGFP or RFP tag upstream or downstream to the *Met* or *Tag* ORFs with either Actin5C or HSP70 promotor.

2.6 Gateway cloning

Gateway Cloning technology is based on the site-specific recombination system used by phage l to integrate their DNA to *E. coli* chromosome. Both organisms have specific recombination att sites – attP in phage and attB in *E.coli*. The integration is catalysed by two enzymes, one in phage – Integrase, and one in *E.coli* Integration Host Factor. During

integration, attB and attP recombination sites are recombined and generate attL and attR sites (see Fig. 3).

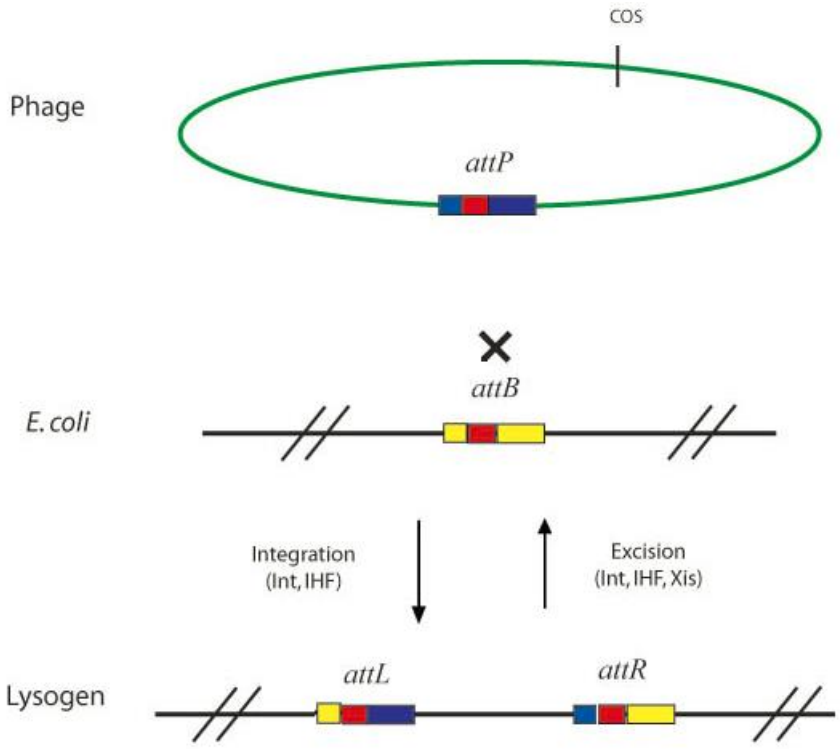

Figure 3: Phage l recombination in *E.coli*, attP stands for att site in Phage, attB stands for att site in Bacteria, attL and attR in lysogen stand for Left and Right att site (taken from http://tools.thermofisher.com/downloads/gateway-the-basics-seminar.html).

Gateway reactions are in vitro versions of integration and excision reactions which occur usually in the cells.

The goal of the GATEWAY reactions is to create an expression clone. This is usually done in two steps.

The first step is preparation of Entry Clone by cloning gene of interest into Entry Vector using BP recombination. In this step attB1 and attB2 sequences are added to 5´and 3´ends of the gene of interest. Then it is amplified by PCR using specific primers. The amplification products are mixed with Gateway Donor vector. During this reaction attP and attB sites are transformed to attL sites. It is catalysed by BP Clonase enzyme mix. (Katzen F et al., 2007) (see Fig. 4)

Another way to prepare Entry Clone is TOPO® cloning. TOPO vectors carry specific sequence 5´-(C/T)CCTT-3' on the linear ends and attL1 and attL2 sites on them. On the free 3´ ends of the sequence is attached topoisomerase enzyme. When PCR product is added, their 5´ends attach to the 3´ends and the attached topoisomerase links them together. (The technology behind TOPO® Cloning". Life Technologies. Retrieved 16 July 2014.) (see Fig. 4)

Next way to prepare the entry clone is restriction enzyme digestion and ligation. In this method the restriction enzyme digests the entry vector and another restriction enzyme (that creates compatible overhang) digests the gene of interest which is then mixed with the entry vector and the ligase is added to ligate the plasmid. (Life Sciences - Restriction Enzyme Digestion and DNA Modification) (see Fig. 4)

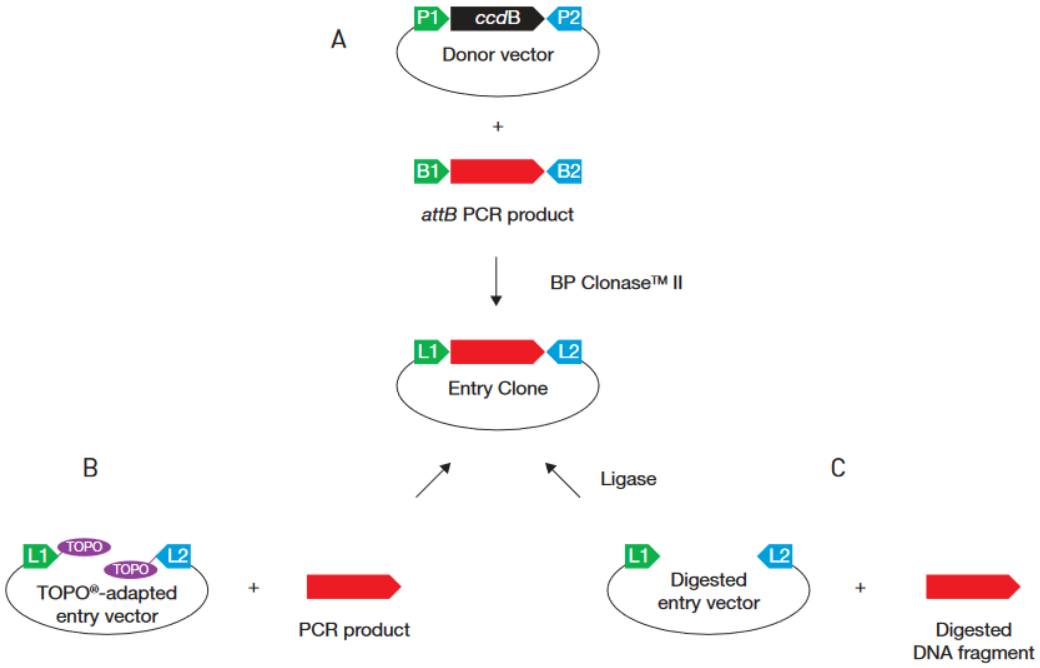

Figure 4: Three ways of preparation of entry clone A – BP cloning, B – TOPO® cloning, C – restriction enzyme and ligase cloning, red arrows represent the gene of interest (taken from Life Technologies—Gateway® cloning technology manual).

The second step involves subcloning the gene of interest from Entry Clone into destination vector using LR recombination and producing Expression Clone. The Entry Clone is then transferred to the Destination Vector – vector that contains attR recombination sites. In this

reaction the recombination between attL and attR sites occurs. This reaction is catalysed by LR Clonase enzyme mix and produces the Expression clone (see fig. 5).

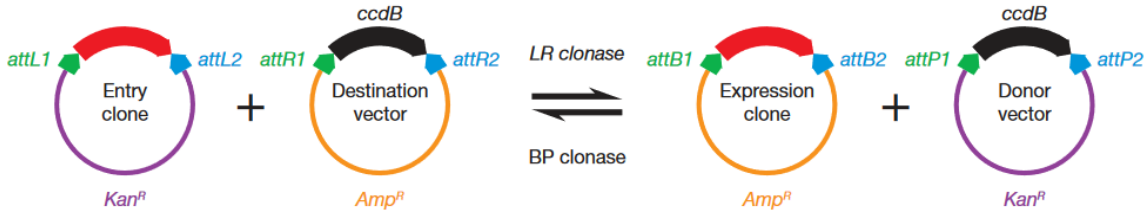

Figure 5: The four types of plasmids involved in the reactions, red arrow represents the gene of interest (taken from Life Technologies—Gateway® cloning technonolgy manual).

Products of this reaction are Expression clone and so called Donor Vector. The expression clone is under two forms of selection: antibiotic selection and negative selection by CcdB protein which is toxic *(*Katzen F et al., April 2007).

3.MATERIALS

Table 1: List of used materials

Name	**Use**	**Ingredients**	**Concentration or amount**	**Notes**
S.O.C-medium	Growth of bacteria after transformation	Tryptone	20 g	Dissolve
		NaCl	0.5 g	
		Yeast extract	5 g	
		Water	950 mL	
		KCl	2.5 mM	pH = 7, V = 1L, Autoclave
		$MgCl_2$	5 mL	2 M sterile stock
		Glucose	20 mL	1 M sterile stock
LB-agar + AMP + CAM	Selection of *E.coli* containing destination vector	LB-agar	16 g	Autoclaved
		Water	400 mL	
		Ampicillin	800 µL	Stock 50 mg/mL
		Chloramphenicol	800 µL	Stock 50 mg/mL
LB-agar + KAN	Selection of *E.coli* with pENTR (entry clone)	LB-agar	16 g	Autoclaved,
		Water	400 mL	
		Kanamycin	400 µL	Stock 50 mg/mL
LB-medium AMP or AMP+CAM, or KAN	*E.coli* cells growth	LB-Medium	5 g	Autoclave
		water	200 mL	
		Ampicillin	400 µL	Stock 50 mg/µL
		Chloramphenicol	400 µL	Stock 50 mg/µL
		Kanamycin	200 µL	Stock 50 mg/µL
LB-agar + AMP	Selection of *E.coli* with expression clone	LB-agar	16 g	Autoclaved
		Water	400 mL	
		Ampicillin	800 µL	Stock 50 mg/mL
TAE-buffer(10x)	For gel elctrophoresis	Tris	400 mM	pH = 8.3
		Acetic Acid	200 mM	Autoclave
		EDTA	10 mM	

Table 2: List of purchased kit and materials

Name	**Use**	**Supplier**
1 Kb Plus DNA ladder	All DNA gels	Invitrogen
PPP mastermix	PCR	Top-Bio
Gel red	Staining of gels for electrophoresis	Biotium
High Pure Plasmid Isolation Kit	Isolation of plasmid DNA from *E.coli*	Roche
One Shot ® Top 10 chemically competent *E.coli*	Transformation of *E.coli*	Invitrogen
XL 1 *E.coli*	Transformation of *E.coli*	
DH5α *E.coli* cells containing destination vectors	obtaining destination vectors	Carnegie Institution for Science

Table 3: List of instruments and used software

Name	**Use/ Type**	**Copyright holder/ manufacturer**
Geneious 8.0.0	Sequence analysis, comparison, primer design, in silico recombination	Biomatters Limited
Alpha Imager	Pictures after Gel elctrophoresis	Protein Simple
Autocycler	XP-Cycler	Bioer
Centrifuge	Universal 320R	Hettich Zentrifugen
Electrophoresis	Liberty 120	Biokeystone
Heating block	SBH130D	Stuart
Incubator	Incucell	BMT
Nanodrop	Nanodrop 2000	Thermo scientific
Shaking incubator	Environmental Shaker-Incubator ES-20	Biosan

Table 4: List of primers used for PCR and sequencing (Generi Biotech Company). Samples were sent for Sanger sequencing to SEQme s.r.o. The size of produced sequence is shown for PCR primers only (Papt tai F1, Papt tai R1, Papt met F1, Papt met R1). , the other primers were used for sequencing.

Type	**Sequence**	**Size of produced sequence**
GW GFPV F	GGAGTACAACTACAACAGCC	-
GW GFPV R	CAAGTCCGCCATGCCCGAAG	-
RFPF	GCTGCGCGGCACCAACTTCC	-
RFPR	GGACAGCTTCAAGTAGTCGG	-
Papt tai F1	ACACGGGAAGCAGCATAAAC	348 bp
Papt tai R1	AGAGGCGAGAACGCATAAGA	348 bp
Papt met F1	ATGTTGGATGGAACGATTGT	644 bp
Papt met R1	GGAAGGACGGCTGTATCG	644 bp

4. METHODS

4.1 Growth of E.coli cells with vectors

LB-agar plates containing ampicillin and chloramphenicol (for *E.coli* with destination vector), or containing kanamycin sulphate (*E.coli* with entry clone), or containing ampicillin (*E.coli* with expression clone) were prepared in a way that 16g LB-agar powder (Broth) was dissolved in 400ml of distilled water and autoclaved to sterilize (120 °C, 40 min). The solution was then tempered to 50-60 °C and antibiotics were added (see tab.1). The solution was poured on sterile Petri dishes and let cool down to solidify. *E.coli* cells (DH5α, TOP10, or XL1) containing plasmids were inoculated on the plates and let grow overnight (37 °C). Next day single colonies were selected and transferred on another agar plate containing ATB and were grow overnight (37 °C).

4.2 Miniprep preparation and plasmid DNA isolation

LB medium containing either ampicillin and chloramphenicol or ampicillin or kanamycin was prepared (see tab.1 for concentration of antibiotics). Ampicillin and chloramphenicol antibiotics were used the destination vectors because they contain ampicillin and chloramphenicol resistance, kanamycin was used for entry clones as they contain kanamycin resistance and ampicillin was used for the expression clone because it contain only ampicillin resistance. 3 ml of the solution was transferred to a plastic test tube and the specific colony of bacteria was added via scratch of the colony with the plastic tip. The solution with the colony was shaken in the 37 °C at 180 RPM for 16-20 hours. After 16-20 hours the solution was transferred to 1.5ml Eppendorf tube and centrifuged at 8000 RPM for 2 minutes, 4 °C. Pellet was kept and the supernatant was discarded. For DNA isolation "High pure plasmid DNA isolation kit" (Roche) was used. The steps are written in the "High pure plasmid DNA isolation kit" manual. The concentration of the DNA was measured via Nanodrop (Thermo Scientific). Plasmid DNA of destination vectors and entry clones were isolated and sent for sequencing (Seqme s.r.o.) to check correctness of recombination sites (att sites). From *E.coli* cells containing plasmids of expression clones (verified by colony PCR – see subchapter 4.6 Polymerase Chain Reaction) minipreps were prepared, plasmid DNA was isolated and then also sent for sequencing.

4.3 Preparation of samples for sequencing

The sample for the sequencing was prepared in a way that it contains 500 ng of plasmid DNA, 25 pmol of primer and the rest was filled with water up to 10 µL. The sample was then sent for sequencing to company Seqme.s.r.o..

Resulting sequences were then compared to the original sequence with use of GENEIOUS program. Bacteria containing correct plasmids were kept and plasmid DNA was used for the next step as a destination vector in LR recombination.

4.4 LR recombination

After validation of correct recombination sites of destination plasmids and entry clones by sequencing, the LR recombination was performed. First of all, agar plates containing ampicillin were prepared. 150 ng of entry vector was mixed with 150 ng of destination vector (with eGFP or RFP) and solution was filled with TE buffer, pH8.0 up to the 8 µL. Then 2 µL of the LR clonase II enzyme (Invitrogen) was added. Then the solution was incubated for 1 hour at the room temperature. After this 1µL of proteinase K (2 µg/µL) was added and then the solution was incubated for 10 minutes at 37 °C. The mix was heated to 75 °C for 10 minutes. The mixture was then directly transformed into *E.coli* cells. (See fig. 6)

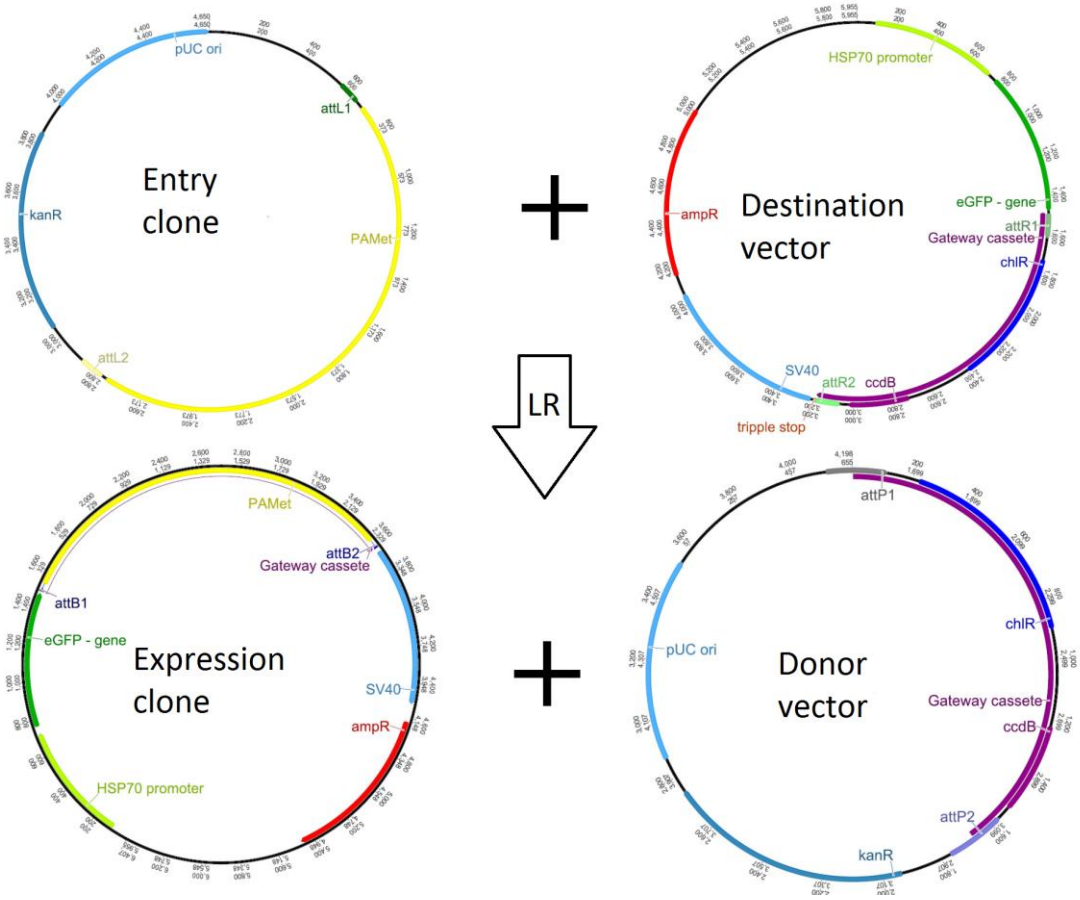

Figure 6: Example of the LR recombination: pENTR vector (entry clone) is mixed with destination vector, after LR recombination Expression clone and Donor vector are created; pUC ori – origin of replication; SV40 – origin of replication; PAMet – *Methoprene tolerant* gene CDS from *P. apterus*; kanR – Kanamycin Resistance gene; HSP70 promotor – Heat shock protein 70 promotor; eGFP – gene – green fluorescent protein gene; chlR – Chloramphenicol resistance gene; ccdB – gene of toxic protein that kills cell when exprimed; triple stop – stopping sequence; ampR – Ampicillin resistance gene; attL, attR, attP, attB – recombination sites; Gateway cassette – sequence between attR recombination sites on destination vector that exchanges with sequence between attL sites on Entry clone

4.5 Transformation of immunocompetent E.coli *cells*

The *E.coli* cells were removed from the -80 °C and were kept on the ice until the solution thawed. 25 µL Top 10, or 100 µL XL1 cells was used for 1 reaction. Then 2 µL of the recombination mix was added with careful slow circular motion during releasing. This solution was kept on the ice for other 20 minutes. Then the heat shock of 42 °C for 45 sec was used to introduce new plasmid to the *E.coli* cells. After the heat shock the mix was cooled on ice for 1-2 minutes. Then 250 µL of the SOC medium was added and the solution was incubated in preheated shaker to 37 °C for 50-60 minutes. Then 150 µL of the solution was placed on the agar plate containing ampicillin and let to grow overnight in 37 °C. Grown

colonies were then criss-crossed to a new agar plate containing ampicillin and were analysed by PCR reaction. Then the plasmid DNA was isolated and sent for sequencing. Obtained sequences were compared to the sequences made by in silico cloning (programme Geneious). Bacteria cells containing proper plasmids were conserved.

4.6 Polymerase chain reaction

Polymerase chain reaction or PCR is method of fast and easy multiplication of part of DNA strand. This method is based on replication of nucleic acids. Parts of DNA must be bounded on the 3´ and 5´ ends by primers. The new strands are synthesized with help of thermostable DNA polymerase in thermocycler (XP cycler, Bioer), which is able to rapidly increase or decrease temperature.

We used this method to prove that bacteria cells after recombination contain either Pa *met* or Pa *tai*. The result of PCR reaction was then checked by the agarose gel electrophoresis.

The PCR mix was prepared. It consisted of 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, colour, additives and stabilizators), forward and reverse primers (400 nM) and water.7 µL of this mix was put to PCR test tubes and an inoculum from bacteria cells was used as a DNA template. The test tubes were placed into the thermocycler, used procedure was: 94 °C – 2min. (initialization), 35 cycles of: 94 °C – 30 s, 56 °C – 30 s, 72 °C – 60 s final elongation (72 °C – 10 min). Agarose gel electrophoresis was then performed.

4.7 Agarose gel electrophoresis

This method is widely used in Biochemistry, molecular biology and genetics to separate the mixture of RNA or DNA in agarose gel matrix.

We used this method to check presence of the Pa *met* or Pa *tai* genes after the PCR reaction (see Tab. 4 for the sizes of multiplied sequences).

At first the 1 % gel was prepared in a way that 0.65 g of agarose (SeaKem® LE Agarose, Lonza), was mixed with 65 mL of 1xTAE buffer. The mixture was then heated in the microwave and the temperature decreased to 70 °C. Into the mix was then added the gel stain (3 µL, GelRed, Biotium) and it was poured to the electrophoresis stand, the comb was then inserted to form wells and the gel was let to cool down. The product of PCR was then pipetted into each well and into one well was pipetted 7 µL of marker. Machine (Liberty 120

high speed gel system, Biokeystone) was then filled with 1xTAE buffer on edges next to the positive and negative electrodes and with distilled water over the gel. The lid was then closed and the gel electrophoresed at constant voltage of 120 V for 2 min. Then the voltage was increased to 220 V. After approximately 20 minutes the device was turned off and the picture of the gel was taken (Alpha Imager mini, Protein simple). If the size of DNA fragment was accurate, in the next step minipreps were prepared and plasmid DNA was isolated.

4.8 Conservation of cells

3 mL of LB medium containing Ampicillin were pipetted to the plastic test tube and the cells were introduced via scratch of the colony with micropipette tip and put in it to the solution. The cells were then let grow for 8-12 hours and then mixed with 80 % glycerol (sterilized by autoclaving) in 1:1 ratio. This mixture was then kept in the freezer at -80 °C.

5. RESULTS

5.1 PCR and agarose gel electrophoresis

As a control of the result of the LR recombination we performed colony PCR with specific primers for *Met* or *Tai* genes and the agarose gel electrophoresis. The product of the PCR was put on the gel, which was then electrophoresed and picture of the gel was taken. Positive colonies were identified according to their size (*met* – 644 kbp; *tai* 348kbp) and some of them were then sent for sequencing (see Fig. 7 – 8).

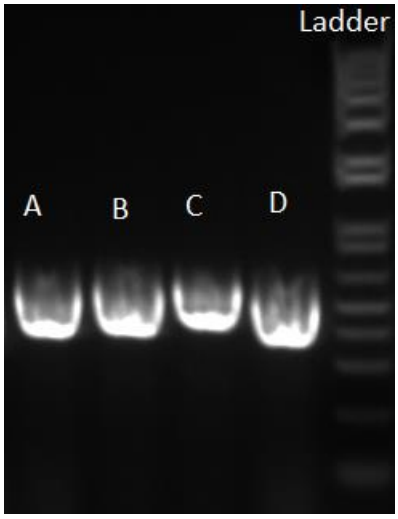

Figure 7: PCR on Bacteria colonies after LR recombination. Plasmids should containing whole *Tai* ORF and RFP tag. For sequencing sent only A and D samples (their size was correct around 348 kbp). C could not be used the size was not appropriate. Ladder – 1kb plus DNA ladder (Invitrogen).

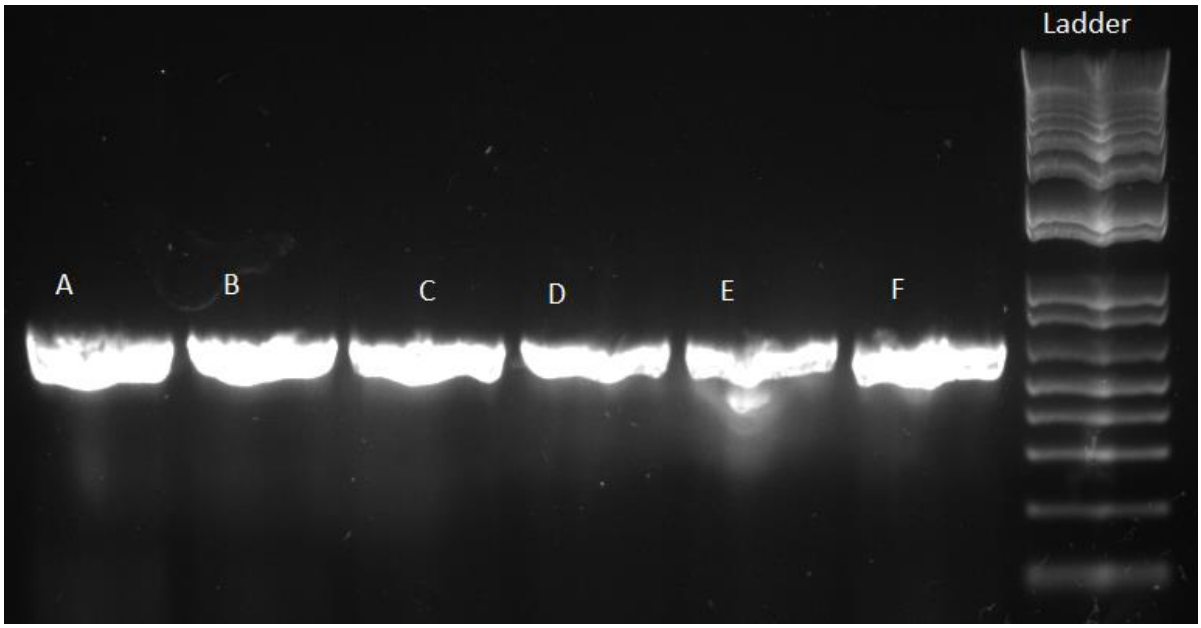

Figure 8: PCR on bacteria colonies after LR recombination. Plasmids should contain whole *Methoprene tolerant* gene ORF and eGFP gene, For sequencing sent only clones A and D because they had correct size (around 644 kbp), Ladder – 1kb plus DNA ladder (Invitrogen).

5.2 LR recombination

5.2.1. Sequencing of entry clones and destination vectors.

First of all sequences (correct recombination sites) of destination vectors and entry clones were checked using Geneious program (Fig. 9).

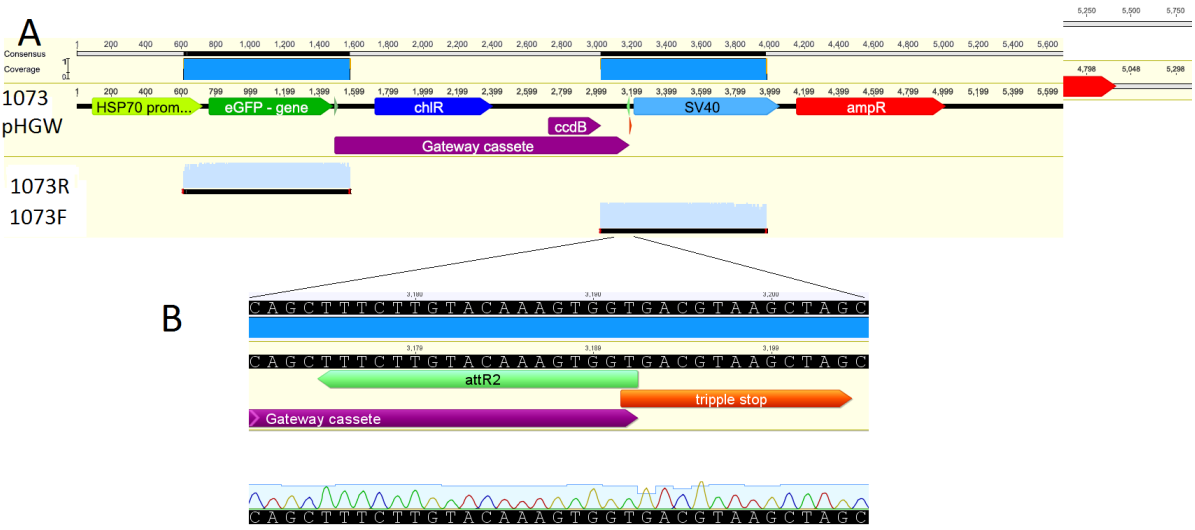

Figure 9: (A) Sequence of the destination vector that contains HSP70 promotor, eGFP gene, Chloramphenicol resistance, ccdB, SV40 – origin of replication, Ampicillin resistance and attR recombination sites – light green triangles. 1073 pHGW - the reference sequence, 1073F and 1073R - the compared sequences. Identic sequences are coloured in black, the red colour on compared sequences represents erased ends. (B) Zoomed area showing the correct attR2 site.

5.2.2. Production of expression clones

Next the Genious program was used to perform *in silico* LR recombination. Sequences of *in silico* expression vectors (see Fig. 10 – 13) were assembled to *in vitro* produced expression vectors (see Fig. 14 – 20) and bacteria colonies containing correct expression vectors (the *Pa Met* ORF or *Pa tai* ORF was recombined to vectors containing Tags) were maintained for future use (not a part of this thesis). We have constructed 3 Tag/*Met* ORF, 2 *Met* ORF/Tag; 2 Tag/*tai* ORF expression vectors (see Fig. 14 – 20).

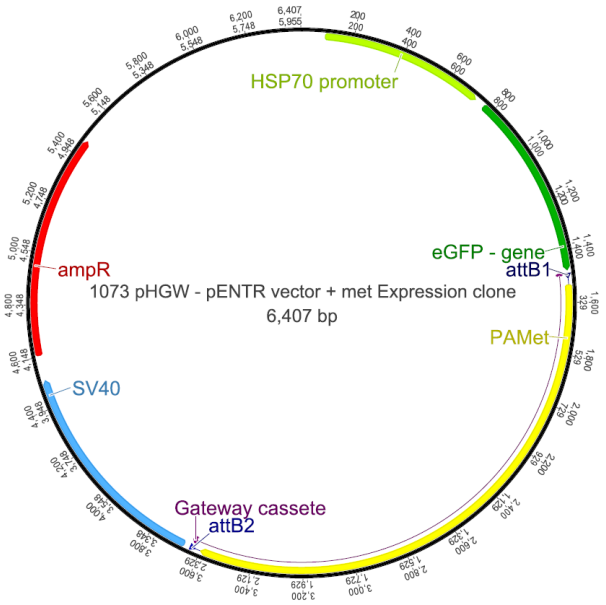

Figure 10: *In silico* produced expression clone containing eGFP and *Met* ORF; ampR – Ampicillin resistance gene, HSP70 – Heat shock protein 70 promotor; eGFP gene – green fluorescence protein gene ; PAMet – *Methoprene tolerant* ORF from *P. apterus*; SV40 – origin of replication, attB1 and 2 –recombination sites; Gateway cassette – original sequence replaced by *Met* ORF.

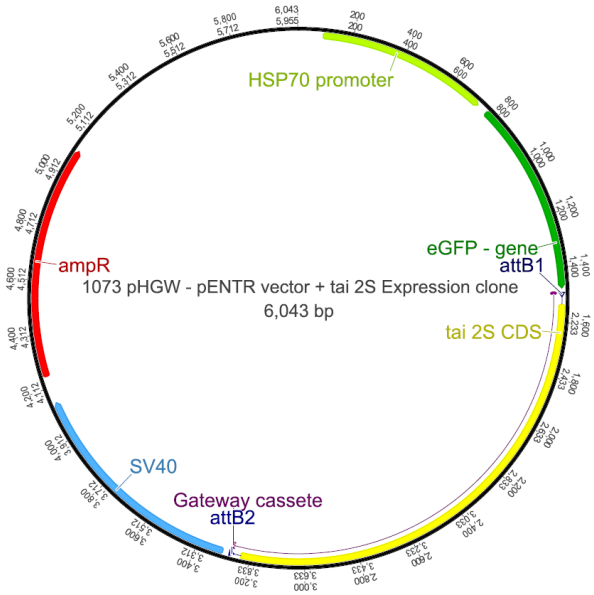

Figure 11: – *In silico* produced expression clone containing eGFP and *Tai* ORF; HSP70 promotor – Heat shock protein 70 promotor; eGFP gene – green fluorescence protein gene; Tai 2S CDS – *Pa Taiman* ORF, SV40 – origin of replication, ampR – Ampicillin resistance gene, attB1 and 2 –recombination sites, Gateway cassette – the original sequence replaced by *Pa Tai* ORF.

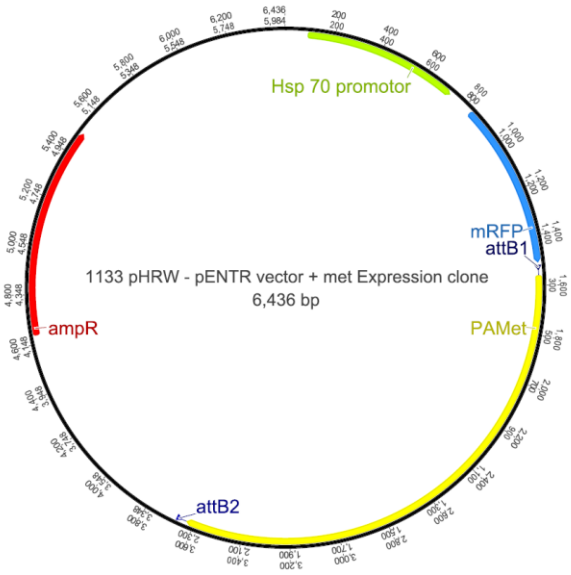

Figure 12: *In silico* produced Expression clone containing RFP and *Met* ORF; HSP70 promotor – Heat shock protein 70 promotor; mRFP – red fluorescent protein gene; PAMet – *Methoprene tolerant* ORF; ampR – Ampicillin resistance; attB – att recombination sites

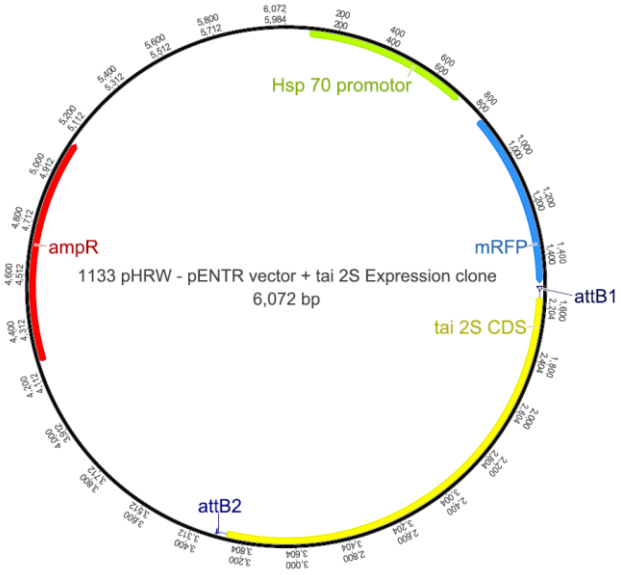

Figure 13: *In silico* produced Expression clone containing RFP and *Tai* ORF; HSP70 promotor – Heat shock protein 70 promotor; mRFP – red fluorescent protein gene; Tai 2S CDS – *Taiman* ORF; ampR – Ampicillin resistance; attB – att recombination sites

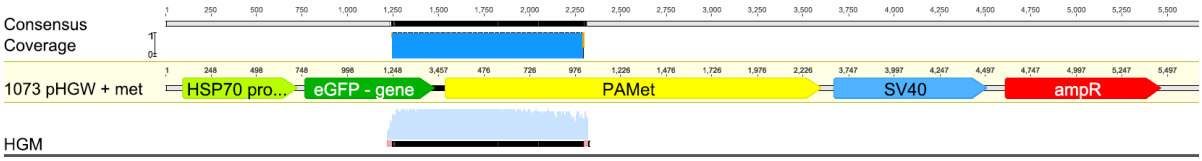

Figure 14: Comparison of obtained sequence result (HGM) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa met* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful. ; HSP70 promotor – Heat shock protein 70 promotor; eGFP gene – green fluorescent protein gene; PAMet – *Pa Methoprene tolerant* ORF; ampR – Ampicillin resistance gene; 1073 pHGW + met - reference sequence; HGM – obtained sequence (compared sequence) –, trim annotated ends (not used for alignments) in pink; HGM - *Pa met* CDS was recombined to destination vector with Hsp70 promotor and eGFP tag downstream the *Met* ORF.

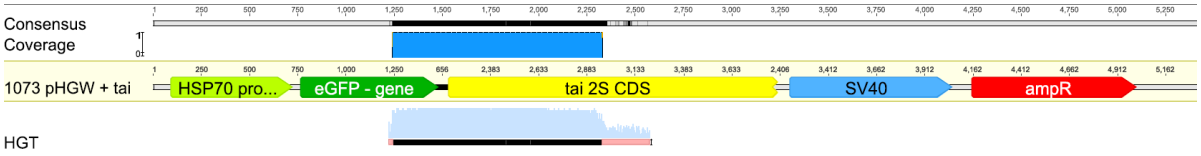

Figure 15: Comparison of obtained sequence result (HGT) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa Tai* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful.; HSP70 promotor – Heat shock protein 70 promotor; eGFP gene – eGFP gene; Tai 2S CDS – *Taiman* ORF; SV40 – origin of replication; ampR – Ampicillin resistance; 1073 pHGW + tai - reference sequence; HGT – obtained sequence (compared sequence) – black colour means identical sequence, pink colour are annotated (trim annotated) ends – not used for alignments; HGT - *Pa Tai* CDS was recombined to destination vector with Hsp70 promotor and eGFP tag downstream the *Tai* ORF.

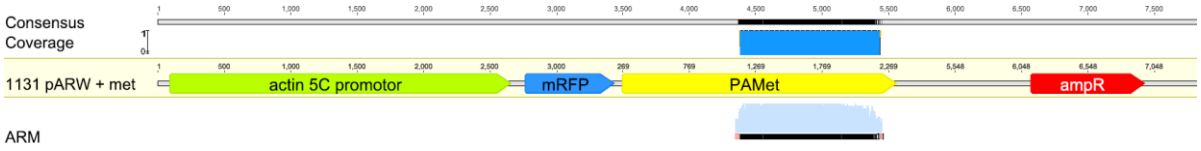

Figure 16: Comparison of obtained sequence result (ARM) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa met* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful. Actin 5C promotor – Actin 5C promotor; mRFP – red fluorescent protein gene; PAMet – *Methoprene tolerant* ORF; ampR – Ampicillin resistance; 1131 pARW + met - reference sequence; ARM – obtained sequence (compared sequence) – black colour means identical sequence, pink colour are annotated (trim annotated) ends – not used for alignments; ARM - *Pa met* CDS was recombined to destination vector with Actin 5C promotor and RFP tag downstream the *Met* ORF.

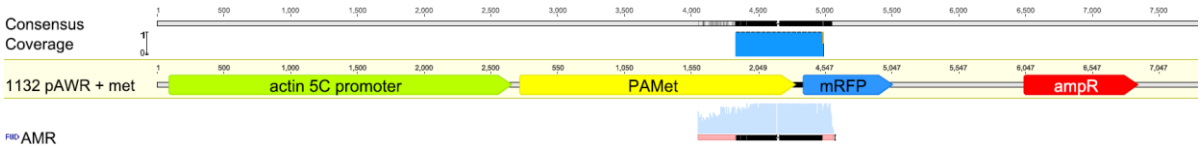

Figure 17: Comparison of obtained sequence result (AMR) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa met* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful. Actin 5C promotor – Actin 5C promotor; mRFP – red fluorescent protein gene; PAMet – *Methoprene tolerant* ORF; ampR – Ampicillin resistance; 1132 pAWR + met reference sequence; AMR – obtained sequence (compared sequence) – black colour means identical sequence, pink colour are annotated (trim annotated) ends – not used for alignments; AMR - *Pa met* CDS was recombined to destination vector with Actin 5C promotor and RFP tag upstream the *Met* ORF.

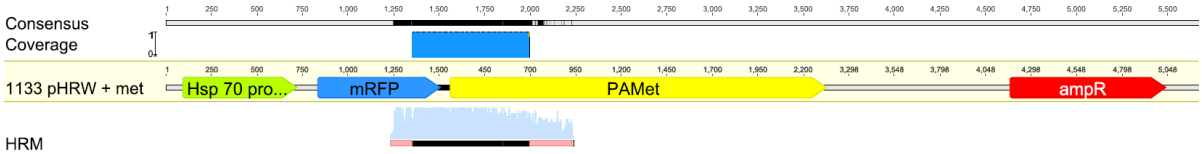

Figure 18: Comparison of obtained sequence result (HRM) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa met* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful. HSP 70 promotor – Heat shock protein 70 promotor; mRFP – red fluorescent protein gene; PAMet – *Methoprene tolerant* ORF; ampR – Ampicillin resistance; 1133 pHRW + met - reference sequence; HRM – obtained sequence (compared sequence) – black colour means identical sequence, pink colour are annotated (trim annotated) ends – not used for alignments; HRM - *Pa Met* CDS was recombined to destination vector with Hsp70 promotor and eGFP tag downstream the *Met* ORF.

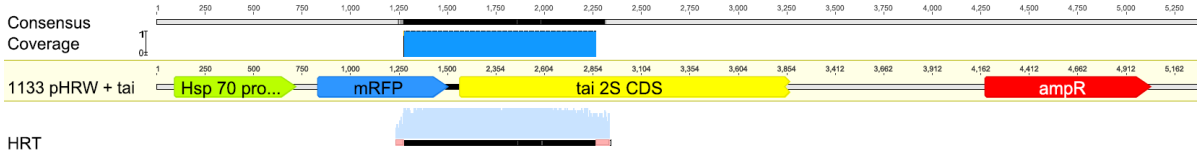

Figure 19: Comparison of obtained sequence result (HRT) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa Tai* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful. HSP 70 promotor – Heat shock protein 70 promotor; mRFP – red fluorescent protein gene; Tai 2S CDS – *Taiman* ORF; ampR – Ampicillin resistance; 1133 pHRW + tai - reference sequence; HRT – obtained sequence (compared sequence) – black colour means identical sequence, pink colour are annotated (trim annotated) ends – not used for alignments; HRT - *Pa Tai* CDS was recombined to destination vector with Hsp70 promotor and eGFP tag downstream the *Tai* ORF.

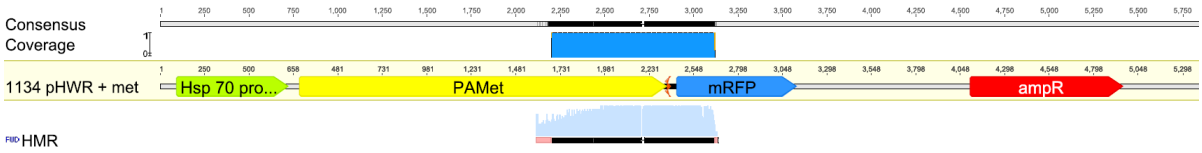

Figure 20: Comparison of obtained sequence result (HMR) to *in silico* produced expression clone. Obtained sequence was aligned to *Pa met* in *in silico* produced expression clone with 100 % identity (black colour) meaning LR recombination was successful.; HSP 70 promotor – Heat shock protein 70 promotor; mRFP – red fluorescent protein gene; PAMet – *Methoprene tolerant* ORF; ampR – Ampicillin resistance; 1134 pHWR + met - reference sequence; HMR – obtained sequence (compared sequence) – black colour means identical sequence, pink colour are annotated (trim annotated) ends – not used for alignments; HMR - *Pa Met* CDS was recombined to destination vector with Hsp70 promotor and eGFP tag upstream the *Met* ORF.

6. DISCUSSION

My aim was to test applicability and efficiency of GATEway cloning strategy, and prepare clones containing either *met* ORF or *tai* ORF, labelled with either eGFP or RFP upstream of downstream from the ORF in a constructs containing either Actin5C promotor or HSP70 promotor.

We wanted to prepare plasmids containing HSP70 promotor or Actin5C promotor because we did not know which of these would suit better for the expression. Most likely, each promotor is better for a specific purpose. The constitutive one is suitable for constant expression level, while the heat shock promotor allows the specific timing of protein expression. When co-transfected, cells constitutively expressing protein "A" may be heat shock to trigger expression of a protein "B". Then, impact of B on localization may easily be explored. Similar experiments benefit from many controls, for example: protein B is stably expressed first and then "A" expression is induced by a heat shock. In a third experimental set up both proteins are expressed after heat shock, so the age of protein is well-defined and possible temporal changes in localization might be explored.

The combination of various fluorescent proteins, distinguishable by their emission wavelength (colour), allows to visualize subcellular localization and colocalization of both proteins.

During my training I have prepared 4 clones that can be directly used for further work. These clones contained HSP70 promotor, *Met* or *Tai* ORF with the eGFP or RFP tag downstream the ORF. However 3 more clones were prepared 1 clone contained *Met* ORF with RFP tag upstream the ORF with HSP70 promotor, 1 contained *Met* ORF with RFP tag upstream the ORF with Actin5C promotor and last one contained RFP tag downstream the *Met* ORF with Actin5C promotor. The rest of plasmids did not recombine properly even though we repeated the recombination many times. The reason for that could be that the LR recombination did not work properly, maybe because the low recombination efficiency of the LR enzyme mix, which might be lowered by freeze-thawing the enzyme. We did not try to repeat the recombination with the fresh enzyme which might solve this problem, because the lack of time. For the further experiments was prepared one set of 4 clones because they contain the same promotor and the fluorescent tag is downstream the ORF.

7. CONCLUSION

My work indicates that Gateway cloning in general, and available gateway plasmids in particular, provide suitable platform for experiments addressing circadian proteins of *Pyrrhocoris apterus*. I produced 4 expression clones which could be used directly for future works. The further work with these clones would be transfection of the plasmid DNA to the *Drosophilla* S2 cell lines. One of these clones contained eGFP/ *Met* ORF, one contained RFP/ *Met* ORF, one eGFP/ *Tai* ORF and one contained RFP/ *Tai* ORF, all of them contained HSP70 promotor. We also produced 1 more clone which contained *Met* ORF/ RFP and HSP70 promotor, the further experiments would have needed preparation of other 3 clones with the fluorescent protein tags upstream the ORFs and HSP70 promotor.

Taken together, this approach is effective for creating plasmids expressing proteins with various tags under distinct promoters. The pallet of currently available tags and promoters seems to be suitable for experimental needs of our laboratory, and first constructs were successfully created during my tests.

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