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MASTER THESIS

Protein-protein interaction of photoperiodic clock factors in *Pyrrhocoris apterus*

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ANNOTATION

In this work the interaction of genes supposedly involved in the circadian and/or the photoperiodic clock of *Pyrrhocoris apterus* was studied using Yeast 2 Hybrid assays.

I hereby declare under oath that I did the research work for this thesis by myself. I also declare that this thesis was written solely by me. Any additional sources and help from others are cited correctly.

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AIM OF THE PROJECT

The main goal of this study was to test direct interactions between several circadian clock proteins and juvenile hormone (JH) receptors of the Linden bug, *Pyrrhocoris apterus*. Particularly this means cloning coding regions of the relevant genes and preparing corresponding “bait” and “prey” constructs, which were then used in yeast two-hybrid assays to identify possible interacting partners. The interactions of certain JH putative receptors were compared in the absence and presence of a JH-mimicking compound, respectively. The results of this study should help us propose a model for JH reception in *P. apterus*.

CONTENTS

ABSTRACT

Evidence from several insect models suggests a connection between circadian clocks and diapause output as well as photoperiodic clocks, but the actual mechanism of this connection is still unknown. This is why our laboratory would like to shed some light on the molecular mechanisms of these systems. Due to its robust, photoperiodic clock controlled diapause, *Pyrrhocoris apterus* was the model organism of choice. In this project we tried to screen for protein-protein interactions among the following photoperiodic clock factors: CLOCK, CYCLE, METHOPRENE TOLERANT, TAIMAN, CLOCKWORK ORANGE, PAR DOMAIN PROTEIN 1, VRILLE and KAYAK. Additionally we also measured the influence of the JH III mimic methoprene on potential interactions.

This way, we were able to confirm the direct protein-protein interactions known from other species (CLK-CYC, MET-TAI, MET-CYC) also in *P. apterus*. We also saw that methoprene influences MET-TAI and MET-CYC interactions in the same way as previously described. Additionally we discovered that VRI is able to form homodimers and that TGO can bind to both, CLK and CYC. Moreover we found that PDP1_ISO1 seems to bind to almost any given partner, while PDP1_ISO2 does not. Future experiments have been planned to clarify the reason for this behavior.

In addition to the results above, which we received from the yeast two-hybrid essays, this study also provided us with pENTR clones for many important *P. apterus* genes. These plasmids can be used to produce vectors for other experiments (e.g. immunoprecipitation) by LR-recombination, saving time and effort in the future. Moreover we found that the second isoform of the *Clock* gene (*Clk_iso2*) is expressed in *P. apterus* gut tissue and we can conduct further experiments to elucidate its function.

INTRODUCTION

Circadian and photoperiodic clocks

Due to the movement of our planet in space, everything in nature occurs periodically. The 24 h cycle of each day, the oscillation of the tides (~12.4 h), the lunar cycle of about a month (~29.4 days) and the annual period are the most important examples. These oscillations influence many parameters such as light intensity, temperature and humidity. To cope with these changes, most organisms have evolved an internal time measurement system called the circadian clock. Circadian oscillations occur in most eukaryotes and some prokaryotes. They are endogenous, but can usually be entrained to environmental variables. This means that organisms kept in constant light (LL) or darkness (DD) can maintain a daily activity rhythm close to 24h without any external signals.

The four properties required for definition of a biological clock are endogeneity, a natural period close to but not exactly the environmental cycle, accuracy in a wide variability of conditions (such as different temperatures) and the ability for entrainment. This way, individual organisms as well as whole populations are provided with the possibility to perform various tasks at the most favorable points in time. Not only activities which recur on a daily basis such as general locomotion or feeding are clock controlled, but also 'once-in-a-lifetime' events such as egg hatching or molting are timed by such a mechanism.

Organisms also have to synchronize life events properly with the seasons during the year. The right decisions have to be made in advance, often at the end of summer. Temperature and humidity can differ among years and hence these factors are not reliable for predicting seasonal changes. The precise information is the ratio of day- to night-length, the so called photoperiod. The photoperiodic clock is the time-measuring device, which evolved in various organisms to orchestrate many changes necessary for optimal adaption to seasonal variations. In insects, the most common adaptation is diapause, a state with remarkably reduced development, metabolism and activity, when nutrients are targeted for energy storage and low temperature survival. Diapause can be scheduled to various developmental stages. In case of adult diapause, which we observe for instance in *P. apterus*, the typical hallmark is reproductive arrest.

A lot of research has been done on the molecular mechanisms underlying the phenomenon of circadian clock systems. The model organism in which circadian rhythms are best understood right now is *Drosophila melanogaster*. In this fly, the molecular clock is built up by 3 connected feedback loops, leading to an oscillation with a period of about 24 h. The formation of a heterodimer between CLOCK (CLK) and CYCLE (CYC) is central and activates the transcription of many other genes by binding to their E-box elements. The products of some of these genes feed back negatively on the production of CLK and the formation of the heterodimer. The influence of light leads to the degradation of TIMELESS (TIM), therefore allowing the system to be entrained. Figure 1 below illustrates these findings.

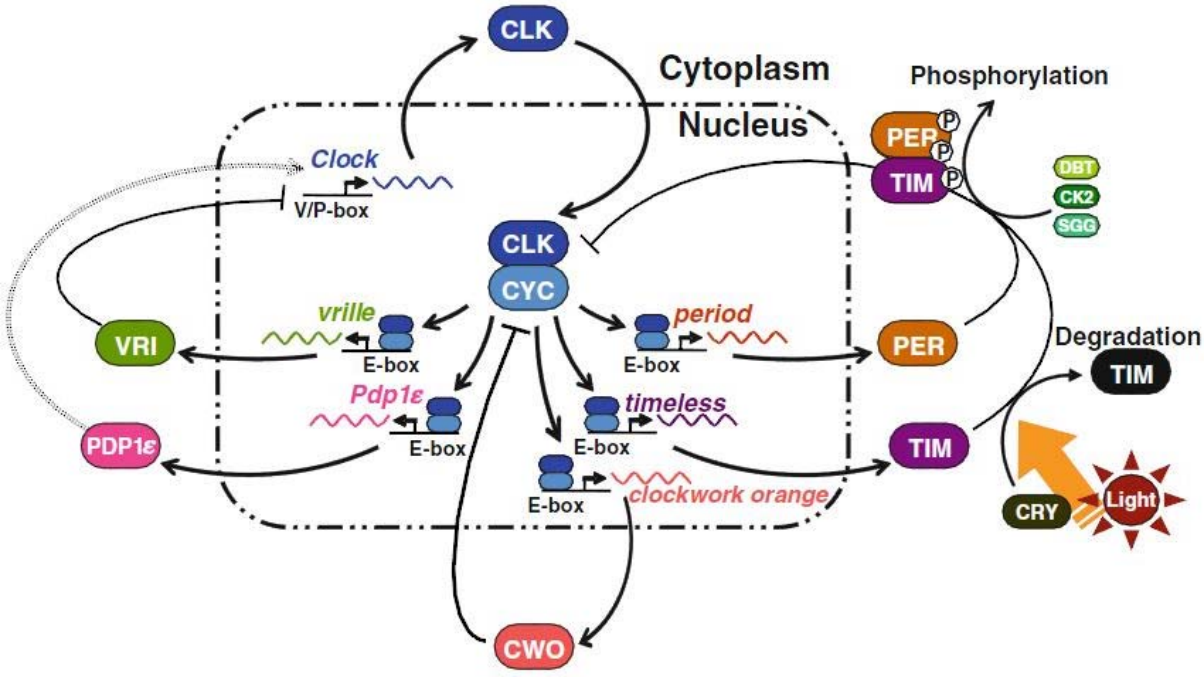

Figure 1: Graphical representation of the molecular interactions involved in the circadian rhythm of the fruit fly *Drosophila melanogaster*. (Taken from Tomioka & Matsumoto, 2009)

The photoperiodic clock is much less understood than the circadian one, but an overlap between these two time-measuring devices is either suggested (Kostal, 2011; Saunders & Bertossa, 2011) or denied (Bradshaw & Holzapfel, 2010; Emerson et al, 2009a, b). Since *Drosophila melanogaster* does not show a robust seasonal rhythm, a different model organism needs to be used for investigation of the photoperiodic clock. The firebug *Pyrrhocoris apterus* served as an excellent model for insect endocrinology in the last fifty years (Socha, 1993). Since it has a robust photoperiod-dependent response it is the model of diapause research in our laboratory. It exhibits a tightly regulated adult reproductive diapause in response to short days (SD, 12h light, 12h dark), while the bugs are reproductively active in long days (LD, 18h light, 6h dark).

As mentioned before, the actual mechanism of the photoperiodic clock has not yet been elucidated. The fact that knocking down some circadian genes in the bean bug, *Riptortus pedestris* by RNA interference (RNAi) leads to a change in diapause response (Ikeno et al, 2011a, b; Ikeno et al, 2010) highly supports the assumption that circadian factors are involved in the photoperiodic clock mechanism or diapause execution. Recent data from our laboratory suggest genetic interaction between circadian clock factors and JH signaling (Bajgar et al, 2013). However, the actual mechanistic model of molecular architecture remains elusive. Importantly, remarkable differences exist between mammalian and *Drosophila* circadian clock proteins and their interactions. Interestingly, *P. apterus* genome contains factors known both in *Drosophila* and mammals, providing an attractive model to elucidate evolutionary aspects of circadian clock development. Therefore we decided to screen for possible interactions between several candidate proteins.

Genes and Proteins of Interest

Most proteins we chose to investigate in this project are either known to be involved in the circadian clock of insects (*D. melanogaster*) and mammals or they have shown to be relevant in the diapause signaling and reproduction of *P. apterus*. These proteins are generally transcription factors and belong to three protein families: (1) basic helix-loop-helix (bHLH) proteins with Per-Arnt-Sim homology domain (bHLH-PAS), (2) bHLH with Orange domain (bHLH-O) and (3) proteins with a basic leucine zipper (bZIP).

bHLH-PAS

This structure is typical for transcription factors and many members of this family are known to bind specifically to gene promoter regions. They do this mainly in heterodimeric forms, but also homodimers can be formed and the interaction might be dependent on a specific ligand (Charles et al, 2011). The N-terminus contains a basic region that binds DNA, followed by a HLH motif and PAS domain, which are mediating protein-protein interactions. The polymerase interaction domains are localized in C-terminal regions of some of these proteins. For my study, I have focused on following bHLH-PAS proteins: CLOCK (CLK), CYCLE (CYC), TANGO (TGO), METHOPRENE TOLERANT (MET) and TAIMAN (TAI). A descriptive model of the bHLH-PAS structure is shown in figure 2 below.

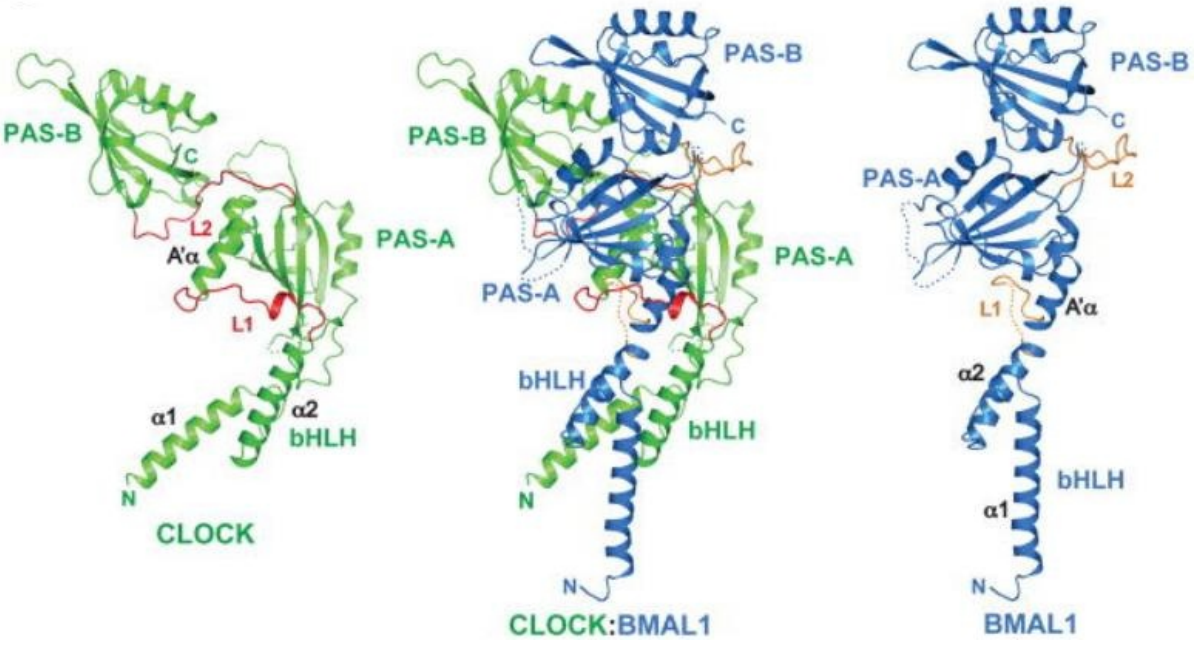

Figure 2: 3D models of bHLH-PAS proteins. Shown are the crystal structures of mammalian CLOCK and BMAL1 proteins and how they dimerize (Taken from Huang et al, 2012).

CLOCK (CLK) is a transcription factor known in both, *Drosophila* (Allada et al, 1998) and mammals (King et al, 1997). This protein drives expression of classical circadian genes such as *period* and *timeless*. In *P. apterus Clk* is more similar to the gene known in mammals than to the *Drosophila* one. Detailed analysis revealed two different splicing isoforms (*Clk_iso1* and *Clk_iso2*) *in silico*, which differ by a 42 AA deletion in the C-terminal part of the second isoform.

CLK interacts with another bHLH PAS protein **CYCLE** (**CYC**) (Rutila et al, 1998). Interestingly, in *Drosophila*, the activation domain is localized on CLK, while in mammals it is found on its dimerizing partner CYC, which is in mammals named BMAL. Recently, the interaction of mammalian CLK and BMAL was resolved crystallographically (Huang et al, 2012). The CYC protein in *P. apterus* resembles more the mammalian BMAL than the *Drosophila* CYC, which means that it contains the activation domain.

TANGO (TGO) is the phylogenetically closest relative to the BMAL/CYC protein, which has not been identified as clock factor so far (Shin et al, 2012). It is an orthologue of the mammalian ARNT protein and can form heterodimers with other bHLH-PAS proteins (Sonnenfeld et al, 1997). Therefore we have included this protein into our dataset as negative control, although possible interactions with some of the tested factors cannot be excluded.

METHOPRENE TOLERANT (MET) is a protein recently identified as a receptor of Juvenile Hormone (JH, see below for details) (Charles et al, 2011). Its depletion results in developmental defects in various insects (Konopova & Jindra, 2007) including *P. apterus* (Konopova et al, 2011) and its involvement in reproduction was recently identified also in *P. apterus* (Bajgar et al, 2013; Smykal et al, in press). There are two types of MET-like proteins in *Drosophila* (MET and GCE). This gene duplication makes research in *Drosophila* complicated.

An interacting partner of MET is **TAIMAN (TAI)**. It is a large (1260 AA in *P. apterus*) protein that was shown to bind MET in yeast two hybrid assays (Li et al, 2011). Analogues called SRC, FISC or TAIMAN exist in various insects such as *Aedes aegypti* and *Tribolium castaneum*. In *P. apterus* it is involved in JH reception in the fat body tissue (Smykal et al, in press).

bHLH-O

Proteins of this structural family typically bind DNA in an E-Box region. A bHLH domain is combined with an orange domain, which responsible for this binding interaction. For this study only one protein with this structure was selected: CLOCKWORK ORANGE (CWO).

CLOCKWORK ORANGE (CWO) is known to be involved in the circadian clock of *Drosophila,* where it acts as a transcriptional repressor of CLK-mediated genes (Kadener et al, 2007; Matsumoto et al, 2007; Lim et al, 2007). In mammals two homologs, DEC1 and DEC2, exist, which supposedly have a similar feedback function (Honma et al, 2002; Kadener et al, 2007). The *P. apterus* homolog is phylogenetically closer related to the insect type than to the mammalian one. In our *de novo* genome assembly (Provaznik et al, unpublished) we were able to identify four different possible coding versions (*cwo_iso1*, *cwo_iso2*, *cwo_iso3* and *cwo_iso4*) of this gene generated by alternative splicing. Figure 3 below shows the differences between them.

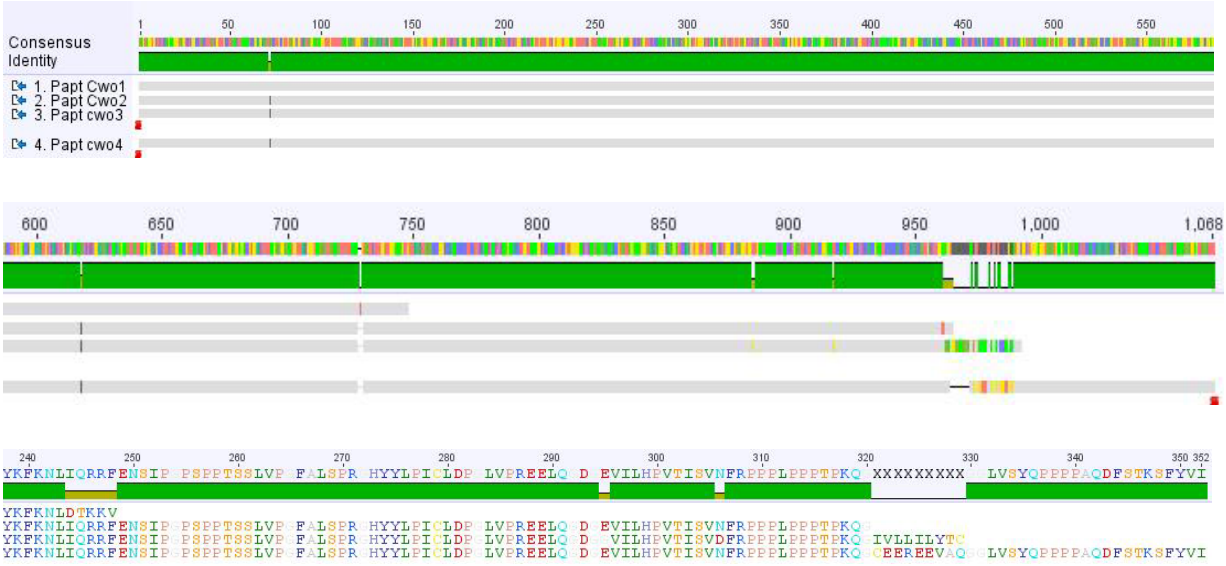

Figure 3: Alignment of the four different predicted coding versions of *cwo* found in *P. apterus*. The differences lie mainly in the 3'-end of the gene. The detailed view shows the alignment of the theoretical C-terminal parts of the CWO protein versions.

bZIP

This structure is also very common for DNA binding transcription factors. The DNA binding regions contain basic amino acids and are responsible for sequence specificity, whereas the leucine zipper holds the two dimerizing partners together. A model of this structure can be seen in figure 4 below. In this study, the following bZIP proteins were investigated: two different isoforms of PAR DOMAIN PROTEIN 1 (PDP1_iso1, PDP1_iso2), VRILLE (VRI) and KAYAK (KAY).

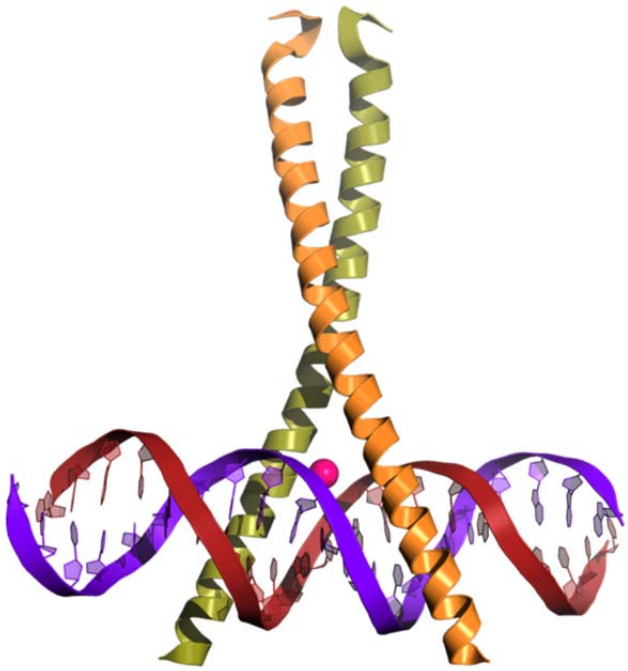

Figure 4: Example of the structure of a bZIP protein domain, when binding to a DNA double helix.

In *Drosophila* **PAR DOMAIN PROTEIN 1 ε (PDP1 ε)** expression is activated by the CLK-CYC-heterodimer and activates *Clk* transcription in a delayed manner, forming one of the feedback loops of the circadian clock (Cyran, 2003). In *P. apterus* we found two isoforms (*Pdp1_iso1* and *Pdp1_iso2*), which differ in the N-terminal part of the protein. *Pdp1_iso1* expression in the gut of the bug is JH and CLK dependent, while the expression of *Pdp1_iso2* seems to require neither JH nor CLK (Bajgar et al, 2013; Bajgar et al, unpublished). In *Drosophila* there are two additional bZip proteins interacting with PDP1: VRILLE (VRI) and KAYAK (KAY).

The second constituent of the above mentioned feedback loop is **VRILLE (VRI)**. It competes with PDP1 for binding to the *Clk* promoter. It acts about 3-6 h prior to PDP1, but repressing the transcription of *Clk* rather than activating it (Cyran, 2003). In *P. apterus* we found a conserved homolog, but so far we have not identified its function.

KAYAK (KAY) is a transcription factor known from development of both insects and mammals, where it is more often called FOS. In *Drosophila* KAY can bind to VRI and inhibit the activation of *Clk* transcription. Additionally it can also repress the activity of the CLK protein. “The double role of KAY in the two transcriptional loops controlling *Drosophila* circadian behavior brings precision and stability to their oscillations.” (Ling et al, 2012) The *kay* gene we found in *P. apterus* is highly conserved, but its function has yet to be identified.

Previous experiments have shown that an RNAi knockdown of *Clk*, *cyc*, *Met* and *tai* in *P. apterus* leads to similar phenotypes in terms of diapause response (Bajgar et al, 2013). These results show the overlap between circadian (*Clk*, *cyc*) machinery and JH signaling (*tai*). Weather this link is established via direct protein-protein interaction or indirectly will be tested in this study. Additionally we are interested in different factors influencing the interactions between proteins such as environmental conditions or the presence of cofactors. The most interesting molecules for us are Juvenile hormones, because of their important role in diapause signaling.

Juvenile Hormones

Juvenile hormones (JHs) are a group of isoprenoid hormones regulating many aspects of insect physiology such as growth and reproduction. The presence of JHs is essential for maintaining larval status in many Insect species and its depletion, either natural or achieved experimentally, leads to metamorphosis. The concentration of JHs in the haemolymph of the developing insect decreases over time and therefore allows the animal to develop into an adult. This means that the developmental stage of the insect is tightly controlled by the hormone level (Wigglesworth, 1964). Later in Insect life, JH is necessary for reproduction by stimulating female vitelogenesis.

As the exact chemical structure of JH is unknown in *P. apterus,* the structure of JH III which is known in most insect species is shown in figure 5 below. However the involvement of JH in reproduction in this insect is suggested both anatomically and genetically. Microsurgical removal of the *Corpus Allatum* (CA), a neurohemal organ necessary for JH synthesis, results in small ovaries. This absence of endogenous JH can be rescued by providing a mimetic compound, methoprene (Smykal et al, in press).

In *P. apterus*, JH absence is the hallmark of reproductive diapause, making it an important factor when investigating photoperiodic rhythms (Denlinger et al, 2012). Genetic evidence indicates that *Methoprene tolerant* (*Met*) RNA interference results in small undeveloped ovaries and importantly these *Met* depleted females retain small ovaries even after addition of synthetic JH analogs, confirming that MET is a JH receptor in *P. apterus* (Bajgar et al, 2013). As it affects some protein-protein interaction in a dose dependent manner (Li et al, 2010; Shin et al, 2012) we also wanted to investigate its influence on possible interactions.

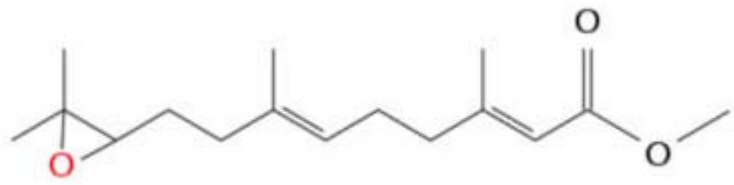

Figure 5: Chemical structure of JH III as it has been identified in Lepidoptera (IUPAC: methyl (2*E*,6*E*,10*R*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate) © User: Jp33 / Wikimedia Commons / CC-BY-SA-3.0

For laboratory experiments JH can be replaced by analogues and mimicking compounds, such as methoprene. Its very similar chemical structure is shown in figure 6 below. As it acts as a growth regulator in insects, while having no toxic effects on humans, it is widely used as a pesticide. In *P. apterus* it has shown to break the photoperiodically induced diapause and induce ovarian growth (Bajgar et al, 2013). Methoprene has shown to effectively mimic JH III as cofactor in the interaction between mosquito MET and TAI (Li et al, 2010), while it did not have this effect on the interaction between MET and CYC (Shin et al, 2012). Since the biological activity of methoprene on *P. apterus* can be specifically eliminated by RNAi depletion of *Met* (Bajgar et al, 2013) or *tai* (Smykal et al, in press), we expected that methoprene will also have an impact on interactions of these proteins when expressed in yeast cells.

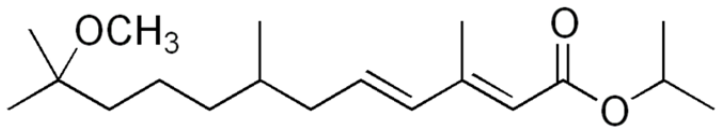

Figure 6: Chemical structure of methoprene (IUPAC: 1-methylethyl (*E,E*)-11- methoxy-3,7,11-trimethyl- 2,4-dodecadienoate)

Given the fact we have many candidate proteins and we wanted to test all combinations, both in the presence and the absence of methoprene, we needed an efficient high throughput technique. This technique also had to be useful for comparing different splice variants under physiological conditions. Doing this at the cost of precision and inability to directly measure the biological relevance is acceptable, because we can later check the interactions we find using different approaches. These are the reasons why Yeast Two-Hybrid (Y2H) screening seemed a perfect choice.

Yeast Two-Hybrid Screening

Yeast two-hybrid (Y2H) screening is a molecular biology technique, which allows monitoring protein-protein interactions in vivo (Fields & Song, 1989). For this study the ProQuest Two-Hybrid system was purchased as a kit (Invitrogen Life Technologies, 2005). To study, weather two proteins of interest interact or not, yeast cells are cotransformed with two plasmid constructs called prey and bait. The bait plasmid contains a domain (GAL 4 DBD) prior to the gene of interest, which allows binding of the protein construct to the promoter regions of UAS GAL4, whereas the prey plasmid the GAL4 activation domain (GAL4 AD). Figure 7 below shows a map of the two plasmid constructs.

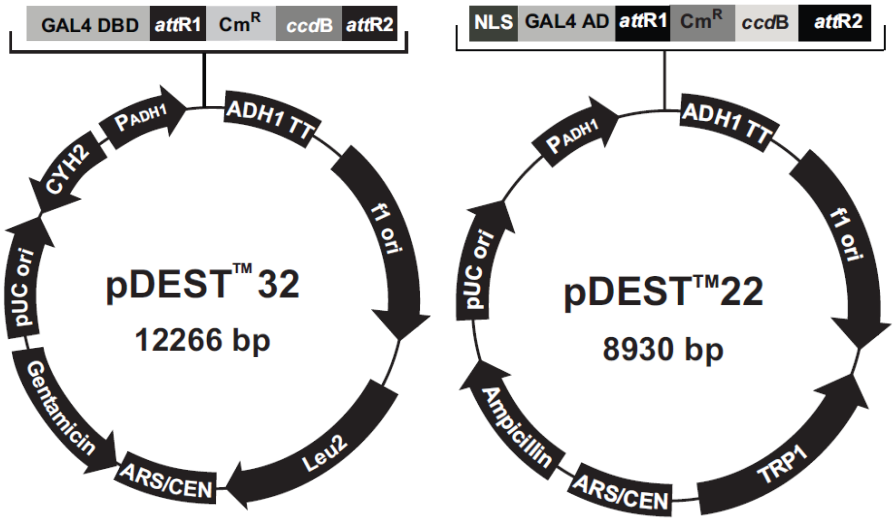

Figure 7: Empty bait (pDEST 32) and prey (pDEST 22) plasmids. Genes of interest are cloned between attR sites by LR-recombination reaction. Both plasmids contain a marker for selection in both, bacteria (antibiotic resistance to Gentamicin or Ampicillin) and yeasts (auxotrophies for leucine or tryptophane). (Adapted from Invitrogen Life Technologies, 2005)

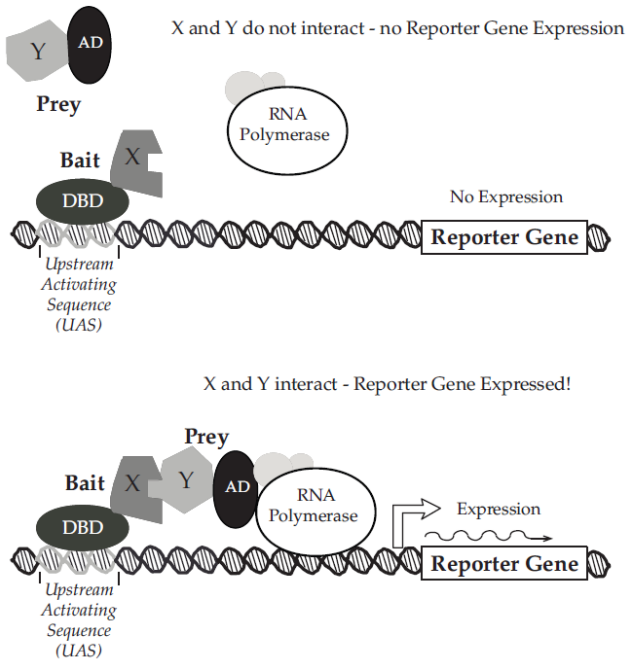

Figure 8: Scheme of reporter gene expression upon bait and prey interaction in Y2H screening. (Taken from Invitrogen Life Technologies, 2005)

If both plasmids are present in a yeast cell, it can selectively grow on medium lacking both, leucine and tryptophane (SC-Leu-Trp). Additionally an interaction of the protein products of the two genes of interest leads to the activation of the reporter gene(s) like shown in figure 8 above. In the MaV203 yeast strain we used, 3 reporter genes have a GAL 4 binding site within their promoter region: LacZ, HIS3 and URA3. This means an interaction leads to the expression of LacZ and the colonies can grow in medium lacking histidine and/or uracil.

As shown in figure 9 on the next page, we used four different selective media to discriminate between strong (++), weak (+) and no (-) interaction. First, an X-Gal assay leads to a dark (++) or very light (+) blue color of the colonies in case of an interaction, while the rest of the yeasts (-) stay white to slightly yellow. Medium lacking uracil supports growth of all colonies expressing URA3 (++ and +). Since MaV203 expresses a basal level of HIS3, which can be inhibited by 3-Amino-1,2,4-Triazole (3AT) SC-Leu-Trp-His+3AT plates can be used to discriminate strong and weak interactions. For this method to work reliably, the concentration of 3AT has to be slightly higher than the concentration required to inhibit the growth of the self-activation controls (yeasts transformed with one plasmid containing a gene and an empty one).Finally medium containing 5-fluoroorotic acid (5FOA) was used to selectively inhibit the growth of yeasts expressing URA3 (++ and +).

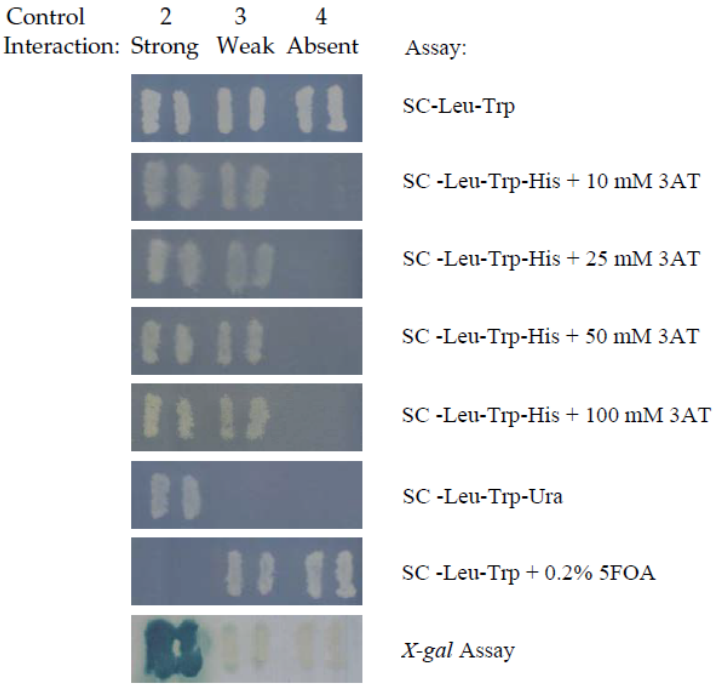

Figure 9: Different media are used to distinguish strong, weak and no interaction between prey and bait plasmids. Once the proper 3 AT concentration is determined on the self-activation controls, the 5 plates containing 3 AT can be reduced to one. (Taken from Invitrogen Life Technologies, 2005)

Yeast 2 Hybrid Screening using the ProQuest Two-Hybrid system is a rather cheap and easy way to monitor protein-protein interactions in vivo. It also allows for the screening of novel protein libraries on a large scale. Moreover, the use of mutated genes can give insight on the protein sites required for identified binding interactions. Although all the conditions are physiological, the assay does not allow for a conclusion about the biological relevance of a detected interaction.

As the ProQuest Two-Hybrid system uses the Gateway (Invitrogen Life Technologies, 2003) system, further test can easily be done using the constructs that have been prepared for Y2H. Subcloning to different expression vectors can be achieved easily and with only a very low risk of introducing mistakes in the sequence. An additional reason why Y2H was chosen for this study is the fact that it has shown to work well for similar purposes (Li et al, 2010; Shin et al, 2012).

MATERIALS

Table 1: Alphabetical list of chemicals used. DMF stands for dimethylformamide.

Name	Ingredients	Concentration or amount	Notes
AA powder mix	Adenine sulfate	3 g	
	Alanine	3 g	
	Arginine	3 g	
	Aspartic acid	3 g	
	Asparagine	3 g	
	Cysteine	3 g	
	Glutamic acid	3 g	
	Glutamine	3 g	
	Glycine	3 g	
	Isoleucine	3 g	
	Lysine	3 g	
	Methionine	3 g	
	Phenylalanine	3 g	
	Proline	3 g	
	Serine	3 g	
	Threonine	3 g	
	Tyrosine	3 g	
	Valine	3 g	
Glucose solution	Glucose	40%	Autoclave
Histidine solution	Histidine.HCl	100 mM	Filter sterilize
Leucine solution	Leucine	100 mM	Filter sterilize
Lithium Acetate solution (10x)	$LiAc.2H_2O$	1 M	Filter sterilize
1x LiAc / 0.5x TE	10x LiAc	1 ml	Filter sterilize
	10x TE	0.5 ml	
	Sterile water	8.5 ml	
1x LiAc / 40% PEG / 0.5x TE	10x LiAc	1 ml	Filter sterilize
	10x TE	0.5 ml	
	PEG-3350	4 g	
	Sterile water	Up to 10 ml	
TE-buffer (10x)	Tris.HCl	100 mM	pH = 7.5 Autoclave
	EDTA	10 mM	
Tryptophane solution	Tryptophane	40 mM	Filter sterilize
Uracil solution	Uracil	20 mM	Filter sterilize
X-gal stock solution	X-gal	100 mg/ml	In DMF
X-gal working solution	X-gal stock	100 µl	Sufficient for 1 membrane
	2-mercaptoethanol	60 µl	
	Z buffer	10 ml	
Z buffer	$Na_2HPO_4.7H_2O$	3.22 g	pH = 7 Filter sterilize
	$NaH_2PO_4.H_2O$	1.1 g	
	KCl	150 mg	
	$MgSO_4.7H_2O$	49.2 mg	
	Sterile water	Up to 200 ml	

Table 2: List of media used for growth of yeasts and bacteria

Name	Use	Ingredients	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+K	Selection of *E. coli* with pENTR	LB-agar	16 g	Autoclave
		Water	400 ml	
		Kanamycin	400 µl	50mg/ml stock
LB-Agar+G	Selection of *E. coli* with bait	LB-agar	16g	Autoclave
		Water	400 ml	
		Gentamycin	80 µl	50mg/ml stock
LB-Agar+AMP	Selection of E. coli with prey	LB-agar	16 g	Autoclave
		Water	400 ml	
		Ampicilin	800 µl	50mg/ml stock
LB-Medium	Growth of *E. coli*	LB-medium	5 g	Selection with K, G or AMP as above
		Water	200 ml	
YPAD-Medium	Growth of yeasts before transformation	Bacto-yeast extract	4 g	pH = 6 Autoclave
		Bacto-peptone	8 g	
		Glucose	8 g	
		Adenine.SO_4	40 mg	
		Water	400 ml	
YPAD-Agar	Growth of yeasts	YPAD	400 ml	pH = 6 Autoclave
		Agar	8 g	
SC-Agar	Selection of transformed yeasts	Yeast nitrogen base (no AA)	6.7 g	pH = 5.9 Autoclave
		AA powder mix	1.35 g	
		Water	500 ml	
		Agar	10 g	Autoclave separately and combine after
		Water	225 ml	
		Glucose	25 ml	40% stock
		Auxotrophy solutions	8 ml	Of each one, the yeast does NOT have an aux-otrophy for
5FOA-plates	Selection for -	2x SC-Leu-Trp	252.8 ml	2x final concentration of ingredients as above Autoclave
		5FOA powder	200 mg	pH = 4.5
		Agar	8 g	Autoclave separately
		Water	180 ml	
3AT-plates	Selection for ++ and +	SC-Leu-Trp -His	400 ml	Already combined with agar
		3AT powder	336 mg	10 mM plates

Table 3: List of purchased materials and kits

Name	Use	Supplier
1 Kb Plus DNA ladder	All DNA gels	Invitrogen
High Pure Plasmid Isolation Kit	Isolation of plasmids from *E. coli*	Roche
Hybond-N Nylon membrane	Growing yeasts for X-gal essays	Amersham Life Science
iQ™ SYBR® Green Supermix	qPCR	Bio-Rad
Longlife™ Zymolase®	Plasmid isolation from yeasts	G-Biosciences
pENTR™ Directional TOPO® Cloning Kit	Production of entry clones	Invitrogen
*Pfx*50™ DNA Polymerase	PCR	Invitrogen
PPP mastermix	PCR of colonies	Top-Bio
ProQuest™ Two-Hybid System Kit	Y2H essay	Invitrogen
Qiaex® II Gel Extraction Kit	Purification of PCR products	Qiagen
SuperScript® III Reverse Transcriptase	cDNA production from RNA	Invitrogen
SuperTag™ Plus Polymerase	PCR	Invitrogen
One Shot® Top 10 chemically competent *E. coli*	Transformation of *E. coli*	Invitrogen
TRIzol® Reagent	RNA isolation from tissue	Ambion
TURBO™ DNase	DNA removal from RNA	Ambion
Hard-Shell® 96-well plate	qPCR	Bio-Rad

Table 4: List of used software

Software	Use	Copyright holder
Geneious 6.1.2	Sequence analysis and comparison	Biomatters Limited
Oligo Analyzer 3.1	Primer design	Integrated DNA Technologies, Inc

Table 5: List of primers used for PCR, qPCR and sequencing. All primers were ordered from generi biotech. Samples were sent for Sanger sequencing to SEQme s.r.o. HK stands for housekeeping.

METHODS

Isolation of mRNA from P. apterus tissue

2 individuals of LD and SD firebug females were dissected at different time points (ZT 0, 6, 12, 18, 24). Gut and fat body tissue samples from each animal were frozen in liquid nitrogen. The tissue samples were homogenized and treated according to the RNA isolation procedure described in the protocol provided with the TRIzol reagent. For qPCR samples the routine DNase treatment was performed according to the TURBO DNase manual. The DNA-free RNA was then diluted to 1.5 µg per tube and reverse transcribed to cDNA following the instructions in the protocol provided with the SuperScript III reverse transcriptase. For the use as a PCR template the cDNA from all time points was combined and diluted 5x.

Amplification of desired genes by PCR

The primers shown in table 5 above were designed to amplify the full length ORF of all predicted genes and isoforms using the Oligo Analyzer. In case of *tai* a primer leading to a truncated version (1711 bp) was designed as well. For the forward primers a CACC overhang was included on the 5' end, to allow for cloning into the pENTR vector later on. The genes of interest were amplified by PCR using the primers described in table 5 above. A 20 µl PCR mixture contained: 1 µl template, 14.52 µl water, 2 µl buffer (10x), 1.6 µl dNTPs (2,5 mM each), 0.08 µl polymerase and 0.4 µl of each primer (20 µM). For most genes the standard PCR program shown in table 6 below was used. For some, adaptations of annealing temperature and/or elongation time had to be made. Afterwards the products were separated by gel electrophoresis on a 1 % agarose gel. Subsequently the proper bands were cut out and the PCR products were isolated from the gel using the Qiaex II kit.

Table 6: Standard PCR program.

Step	Temperature	Time	
Initial denaturation	94° C	120 s	
Denaturation	94° C	30 s	30x
Annealing	56° C	30 s	
Elongation	72° C	100 s	
Final elongation	72° C	600 s	
Cooling	14° C	overnight	

Search for alternative splicing variants by qPCR

Since we wanted to confirm the presence of the second splicing variant of the *Clock* gene we had predicted in silico, selective primers for both variants were used for qPCR. For each reaction, 2.52 µl water were combined with 6 µl qPCR Supermix, 0.24 µl of each primer (20 µM) and 3 µl template in one of the wells of a 96 well-plate. The different primers and templates used can be seen in figure 10 below.

	1	2	3	4	5	6	7	8	9	10	11	12
	1	1	1	2	2	2	3	3	3	4	4	4
A	LD_ZT0	LD_ZT0	LD_ZT0	LD_ZT6	LD_ZT6	LD_ZT6	LD_ZT12	LD_ZT12	LD_ZT12	LD_ZT18	LD_ZT18	LD_ZT18
	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1
	5	5	5	6	6	6	7	7	7	8	8	8
B	LD_ZT24	LD_ZT24	LD_ZT24	LD_ZT0	LD_ZT0	LD_ZT0	LD_ZT6	LD_ZT6	LD_ZT6	LD_ZT12	LD_ZT12	LD_ZT12
	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2
	9	9	9	10	10	10	11	11	11	12	12	12
C	LD_ZT18	LD_ZT18	LD_ZT18	LD_ZT24	LD_ZT24	LD_ZT24	LD_ZT0	LD_ZT0	LD_ZT0	LD_ZT6	LD_ZT6	LD_ZT6
	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	HK	HK	HK	HK	HK	HK
	13	13	13	14	14	14	15	15	15	16	16	16
D	LD_ZT12	LD_ZT12	LD_ZT12	LD_ZT18	LD_ZT18	LD_ZT18	LD_ZT24	LD_ZT24	LD_ZT24	SD_ZT0	SD_ZT0	SD_ZT0
	HK	HK	HK	HK	HK	HK	HK	HK	HK	Clk_iso1	Clk_iso1	Clk_iso1
	17	17	17	18	18	18	19	19	19	20	20	20
E	SD_ZT6	SD_ZT6	SD_ZT6	SD_ZT12	SD_ZT12	SD_ZT12	SD_ZT18	SD_ZT18	SD_ZT18	SD_ZT24	SD_ZT24	SD_ZT24
	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1
	21	21	21	22	22	22	23	23	23	24	24	24
F	SD_ZT0	SD_ZT0	SD_ZT0	SD_ZT6	SD_ZT6	SD_ZT6	SD_ZT12	SD_ZT12	SD_ZT12	SD_ZT18	SD_ZT18	SD_ZT18
	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2	Clk_iso2
	25	25	25	26	26	26	27	27	27	28	28	28
G	SD_ZT24	SD_ZT24	SD_ZT24	SD_ZT0	SD_ZT0	SD_ZT0	SD_ZT6	SD_ZT6	SD_ZT6	SD_ZT12	SD_ZT12	SD_ZT12
	Clk_iso2	Clk_iso2	Clk_iso2	HK	HK	HK	HK	HK	HK	HK	HK	HK
	29	29	29	30	30	30	REF	REF	REF	NC	NC	NC
H	SD_ZT18	SD_ZT18	SD_ZT18	SD_ZT24	SD_ZT24	SD_ZT24	LD2_ZT0	LD2_ZT1	LD2_ZT2	NONE	NONE	NONE
	HK	HK	HK	HK	HK	HK	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso1	Clk_iso2	HK

Figure 10: Primers (third row) and templates (second row) used for qPCR. Each reaction was done 3 x. HK means housekeeping, for this product ribosomal protein (rp49) primers were used. The level of this mRNA should be constant throughout the day and the values can therefore be used to normalize for differences in the sample which occurred during the isolation procedure. NC stands for negative control. Reference (REF) means that cDNA from the second sample set was used to be able to set the numbers from the 2 individual plates in relation and compare them.

The plate was sealed, centrifuged and the reaction was run in the C1000^{TM} Thermal Cycler (Bio-Rad). The temperature program used can be seen in figure 11 on the next page. The experiment was run twice, using the gut samples from the second bug that was sampled at each time point. For data analysis the ΔΔCt-method (Livak & Schmittgen, 2001) was used.

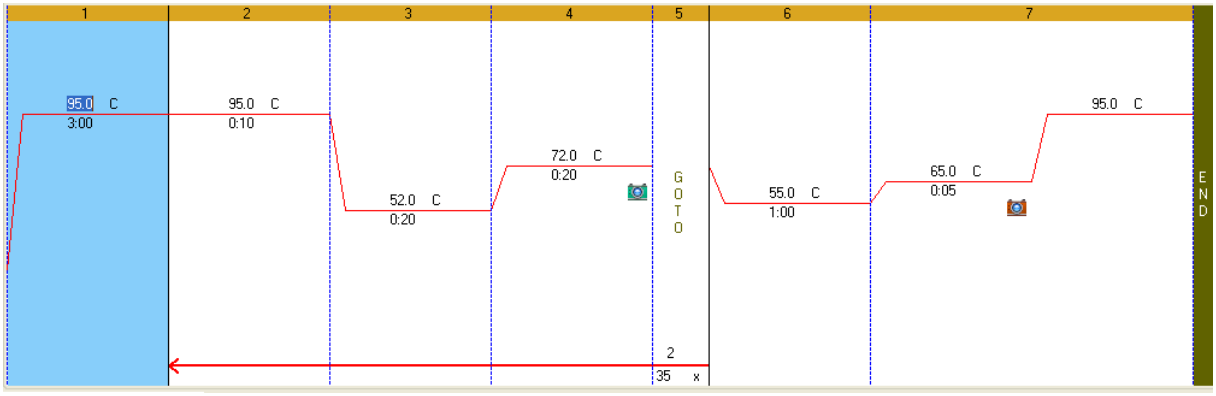

Figure 11: Temperature program used for qPCR. Camera symbols depict measurement timepoints.

Production of entry clones

The PCR product was cloned into the pENTR vector (Invitrogen Life Technologies, 2012) according to the pENTR manual, p. 12. The incubation time was extended to 30 minutes, before the product was used for transformation of Top 10 chemically competent cells according to the pENTR manual, p. 14. Also during this procedure a long incubation time of 25 minutes was chosen. After overnight growth on selective plates (LB+K), the colonies were checked by PCR for insert size (7 µl PCR mixture contained: 3.5 µl PPP mastermix, 3.22 µl H_2O, 0.14 µl of M13 F and 0.14 µl of M13 R primer (20 µM)). Promising ones were cultivated in liquid medium (LB+K) overnight and the plasmids isolated using the High Pure Plasmid Isolation Kit.

Production of prey and bait plasmids by LR-recombination

The plasmids obtained from the entry clones were sent for Sanger sequencing to SEQme. The results were aligned with the predicted CDS sequences using Geneious. Only vectors that did not have mistakes, influencing binding domains on the protein level were further used. The LR-recombination reaction was performed according to the procedure in the ProQuest manual, p. 22. Transformation of Top 10 chemically competent cells was repeated as before and the cells were grown on selective plates (LB+AMP and LB+G respectively) overnight. The colonies were checked again by PCR, grown in liquid medium (LB+AMP/LB+G) and isolated plasmids were sequenced again to ensure no mistakes had happened during the LR-reaction.

Production of self-activation controls

MaV203 yeast cells were transformed with empty pDEST32 + prey plasmid (Control 7) or bait plasmid + empty pDEST22 (Control 6) according to the ProQuest manual, p. 28. The transformed yeasts were grown on SC-Leu-Trp plates for two days. Afterwards they were streaked in patters on SC-Leu-Trp plates and grown overnight. The next day the plates were replica plated on SC-Leu-Trp-His+3AT (0, 10, 25, 50 and 100 mM 3AT) plates. They were replica cleaned right away and a second time the day after. Two days later the minimum inhibiting 3AT concentration was determined for each control.

Yeast Two-Hybrid assays

For each interaction, MaV203 cells were transformed with bait + prey plasmids according to the ProQuest manual, p. 28. The cells were grown on SC-Leu-Trp plates for two days and then streaked on SC-Leu-Trp plates in the pattern shown in figure 12 on the next page. After overnight growth, the colonies were replica plated on the four different selective plates required: YPAD plates with a membrane on top, SC-Leu-Trp-Ura, SC-Leu-Trp-His+3AT and SC-Leu-Trp+5FOA. The 3AT and the 5FOA plates were replica cleaned immediately. After one day, an X-gal essay was performed on the membranes according to the manual, p. 32. The remaining plates were replica cleaned and grown for two more days before all the results were evaluated.

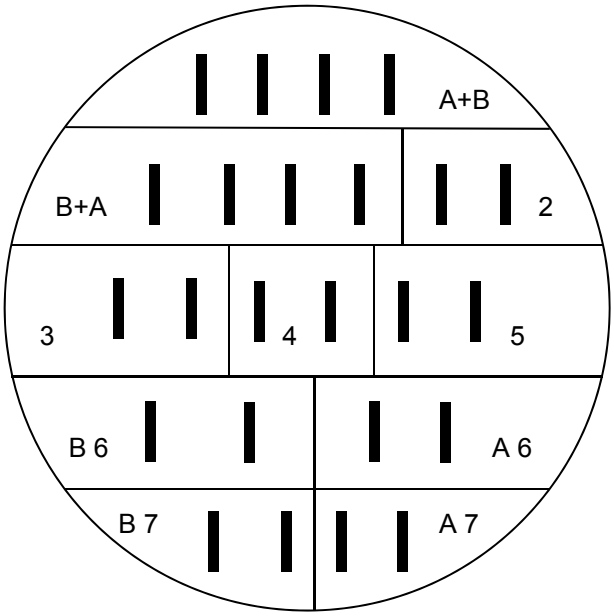

Figure 12: For all Y2H assays the colonies were streaked in this pattern. A+B: Gene A in pDEST 32 + Gene B in pDEST 22; B+A: Gene B in pDEST 32 + Gene A in pDEST 22; 2: Krev1 + RalGDS-wt, strong positive interaction control; 3: Krev1 + RalGDS-m1, weak positive interaction control; 4: Krev1 + RalGDS-m2, negative interaction control; 5: pDEST 32 + pDEST 22, negative activation control; A/B 6: Gene A/B in pDEST 32 + pDEST 22, self activation control 6; A/B 7: pDEST 32 + Gene A/B in pDEST 22, self activation control 7. For all interactions described later Gene A is always the one mentioned first e.g. *Clock* and *cycle* means that *Clk* is gene A and *cyc* gene B. “Interaction yest cells” summarizes A+B and B+A. Note also that photos from X-gal essays are taken from top, this means the pattern stays the same, while the ones from other plates are taken from the bottom, leading to the inversion of the pattern around a vertical axis.

Analysis of interaction colonies

If we observed controversies in the results of the Y2H essays, the genetic composition of the interaction colonies was further analyzed. For this purpose the yeasts were grown in liquid YPAD overnight and the plasmids isolated according to the ProQuest manual, p. 48 ff. To produce higher amounts of plasmids, they were used to transform XL-1 chemically competent *E. Coli* according to the pENTR manual, p. 14. The bacteria were grown on both, LB+G and LB+AMP agar overnight. Then the colonies were screened for the proper inserts by PCR as described above. For each interaction a colony containing only the bait but not the prey and one containing only the prey but not the bait plasmid were selected and grown in liquid media overnight. After isolation of the plasmid DNA with the High Pure Plasmid Isolation Kit the plasmids were sent for Sanger sequencing to SEQme. The results were analyzed with Geneious.

RESULTS AND DISCUSSION

Search for alternative splicing variants by qPCR

This experiment showed, that both predicted isoforms are expressed in the gut tissue of *P. apterus*. In figure 13 below, the results for LD females are shown. Very similar ones were obtained for SD females as well as for a second set of samples.

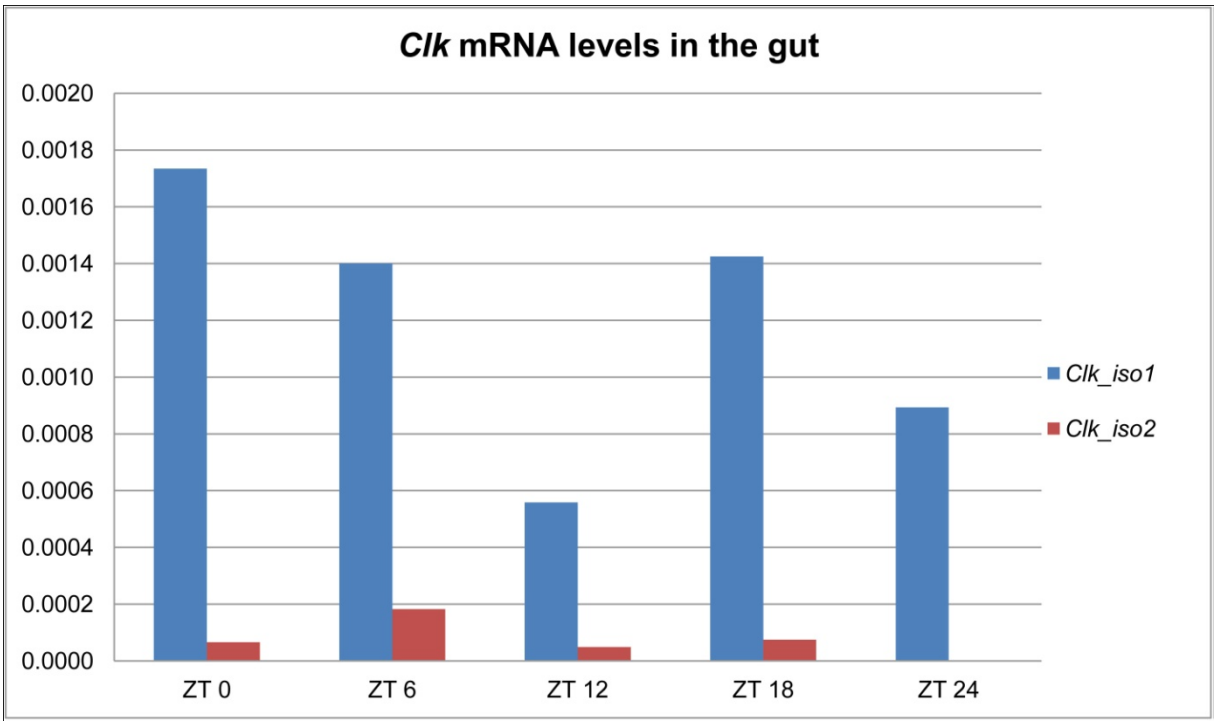

Figure 13: Comparison of mRNA levels of the two different *Clk* isoforms in *P. apterus* gut tissue from LD females.

Unfortunately we were not able to amplify the *Clk_iso2* isoform by PCR. Also using cDNA from those time points, where *Clk_iso2* expression is highest as template did not improve the situation. This might be due to the fact that the concentration of *Clk_iso2* mRNA in the tissue is approximately 10 to 20x lower than the one of *Clk_iso1*. Therefore we could not explore a possible biological relevance of this predicted isoform further. Given above mentioned difficulties to amplify *Clk_iso2*, an alternative approach to obtain it might be employed. The easiest one would be PCR-generated deletion using *Clk_iso1* clone as template.

Production of entry clones

For most genes the standard PCR program, shown in table 6, yielded sufficient results. Entry clones for the following genes were successfully produced: *Clk*, *cyc*, *Met*, *vri*, *tgo*, *Pdp1_iso1*, *Pdp1_iso2*, *tai_short*, *kay*, *cwo_iso2* and *cwo_iso4*. Unfortunately we were not able to amplify *cwo_iso1* and for *cwo_iso3* as well as the full length *tai* we have not obtained a proper entry clone so far. In case of *cwo_iso1* we are not sure if this splicing variant is expressed in the bug and when. In order to find out, we could do a qPCR experiment like we did for *Clk_iso2*. The other two genes did yield a PCR product, but none of the colonies we received after transformation had the proper insert. For both genes the transformation efficiency was very low. In case of *tai* this is very likely due to its size. This means we would have to optimize the conditions of the cloning reaction for larger PCR products, but there was not enough time to do this during this project. Therefore we decided to work with the truncated version (see figure 14) which contains all domains necessary for interaction with MET, as shown in case of TAI from mosquito (Li et al, 2011) and beetle (Charles et al, 2011).

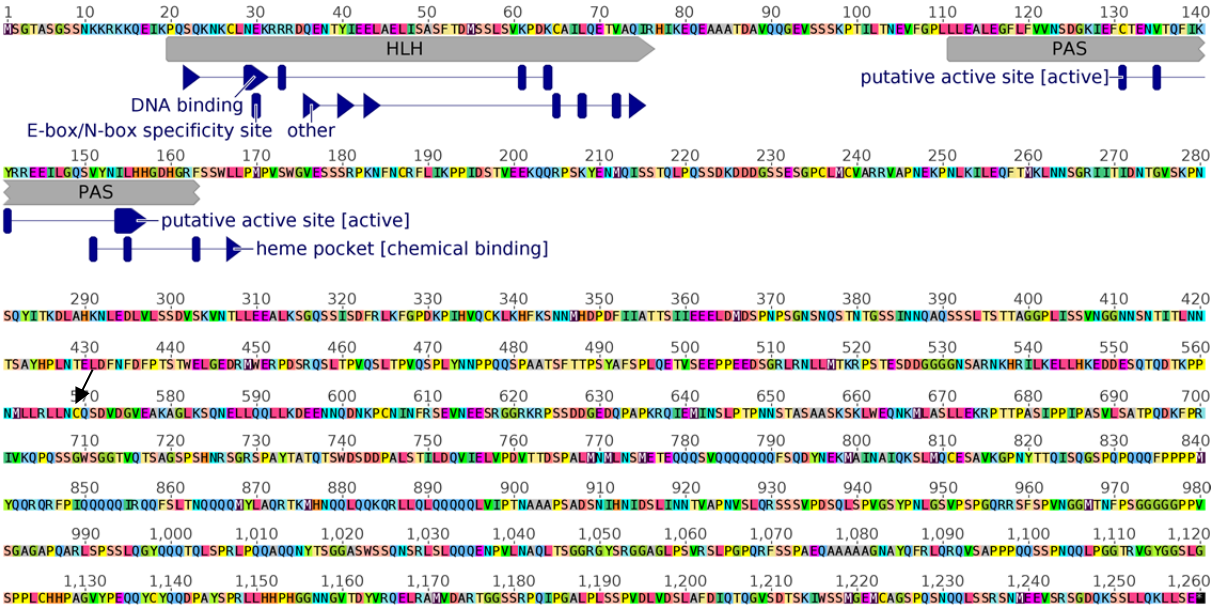

Figure 14: Map of the *P. apterus* TAI protein showing the domains and the truncation site indicated by an arrow. The last triplet of the truncated gene was altered to be a stop codon.

Production of prey and bait plasmids by LR-recombination

The LR-recombination reaction worked well on all entry clones and no mistakes were discovered in the sequencing results. This shows the advantage of the Gateway system and opens up a wide variety of future experiments. This method could for example be used to subclone all our genes of interest to vectors containing a V5 or MYC-tag and use the protein products for immunoprecipitation experiments with commercially available antibodies. Another possibility is to use the LR-recombination for production of insect cell expression vectors. The two interacting partners can directly activate the respective promoter linked to a reporter gene, (such as the *period* promoter driving expression of luciferase, see Kobelkova et al, 2010) in the context of a more realistic insect cell environment. This essay could be used to further investigate the biological relevance of the interactions found in this preliminary screening.

Production of self-activation controls

The results of colony growth on different concentrations of 3AT in the media are summarized in table 7 below. If a control is resistant to 3AT, this means the construct is problematic for Y2H, because a weak interaction cannot be distinguished from the background. We have not used these constructs for further experiments. In case of the *tgo*, control 6 can even self-activate strongly, giving a blue color in an X-gal essay (see figure 15). This means *tgo* cannot be used as bait at all.

Table 7: 3AT sensitivity of self-activation controls

Gene	Control	3AT Sensitivity
Clk	6	25mM
	7	25mM
cyc	6	50 mM
	7	25 mM
Met	6	10 mM
	7	10 mM
Pdp1_iso1	6	10 mM
	7	10 mM
Pdp1_iso2	6	10 mM
	7	10 mM
vri	6	10 mM
	7	10 mM
tgo	6	resistant
	7	25mM
tai_short	6	10 mM
	7	10 mM
kay	6	10 mM
	7	10 mM
cwo_iso2	6	50 mM
	7	25 mM
cwo_iso4	6	resistant
	7	25 mM

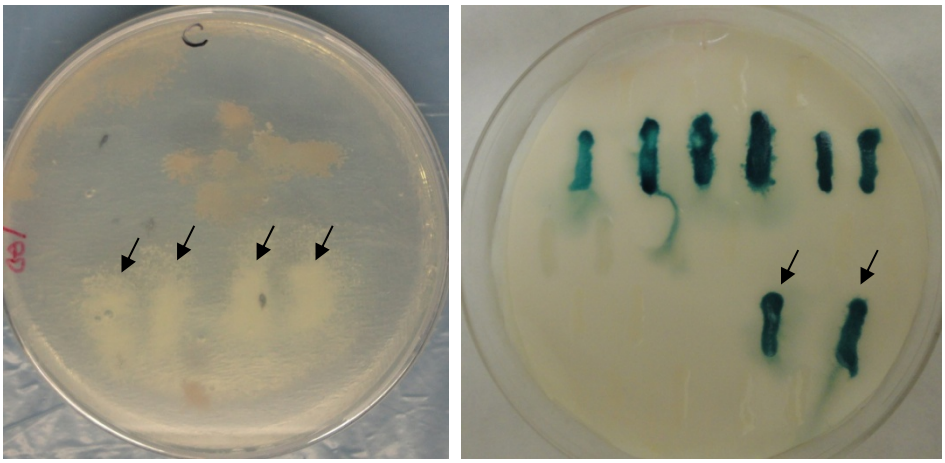

Figure 15: *tgo* control 6 is 3AT (100 mM) resistant (left) and self activates in Y2H e.g. lead to a strong blue color in X-gal essays (right). Arrows indicate *tgo* control 6 colonies in both plates.

Yeast Two-Hybrid assays

If not denoted otherwise, all pictures presented in this part follow the pattern described in figure 12 before. Generally, the interaction of each possible combination of bait and prey can be tested. Since the time to do this would exceed this study, I focused on those interactions, which were more likely to give positive results before randomly trying many others as well. The most important interactions are:

Clock and *cycle*

Since the formation of the CKL-CYC heterodimer lies in the center of the circadian clock in *D. melanogaster*, we expected to find the same in *P. apterus*. In fact, this is suggested by our results. Figure 16 below shows the plates of one essay, both with and without methoprene. The ones from the repeat look essentially the same. The problem is that in both essays some of the colonies showed stronger interactions then others. Nevertheless, we can definitely conclude that there is a weak interaction between *P. apterus* CLK and CYC, which is not influenced by the presence of methoprene.

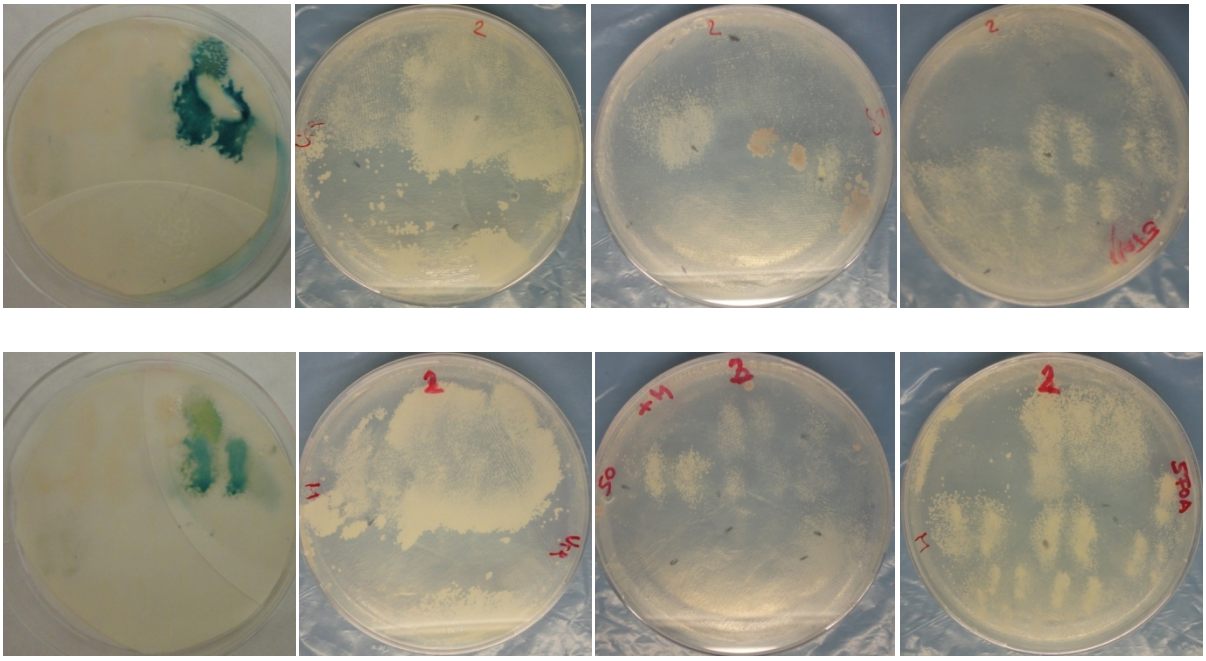

Figure 16: Top, from left to right: X-gal essay, SC-Leu-Trp-Ura, SC-Leu-Trp-His+3AT (50 mM), SC-Leu-Trp+5FOA, all without methoprene. Bottom: all with 1 µM methoprene. Slight blue color of some interaction cells could be observed in the X-gal essay. Innteraction yeast cells grow strongly on SC-Leu-Trp-Ura, weakly on SC-Leu-Trp-His+3AT and not at all on SC-Leu-Trp+5FOA. The only difference between the plates containing methoprene and the ones without is that 5FOA does not suppress the growth of all yeast colonies on the methoprene plates.

In order to find out what causes the slightly different responses e.g. in the X-gal essay, we further analyzed the genetic makeup of the interaction colonies. Unfortunately only the colonies containing the *Clk* plasmids grew, which were completely identical in all tested cases. Therefore the different responses to various media had to be caused by mutations in the *cyc* plasmids, which unfortunately didn't grow in bacteria cells. This means that we are, unfortunately, unable to tell how strong the interaction between *Clk* and *cyc* actually is. Further tests have to be done on the matter.

Methoprene tolerant and *cycle*

This interaction has been discovered by Shin et al. (2012) in the mosquito *Aedes aegypti*. They show that the formation of the MET-CYC heterodimer is dependent on the concentration of JH III. Unfortunately methoprene had not been a suitable JH analogue in this study. Since the exact structure of the JH(s) in *P. apterus* is not known, we nevertheless tried to reproduce these results, using methoprene as JH analog. The results shown in figure 17 on the next page proof, that also in *P. apterus* methoprene is not able to induce a MET-CYC heterodimer formation, because growth does not increase upon addition of methoprene. Nevertheless the results suggest that a weak interaction occurs between these proteins, because the cells grow strongly on SC-Leu-Trp-Ura and weakly on SC-Leu-Trp-His+3AT.

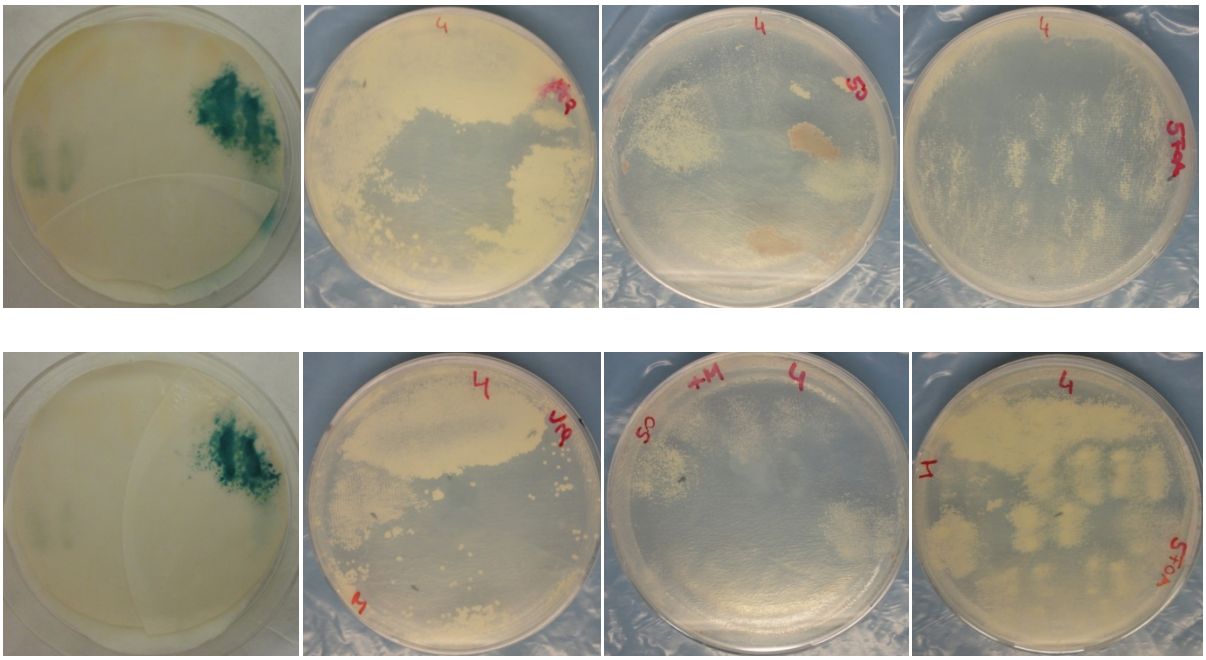

Figure 17: Arrangement of plates as described before. No blue color of the interaction cells could be observed in the X-gal essay. Strong growth of the *Met* + *cyc* interaction yeast cells occurs on SC-Leu-Trp-Ura. Weak growth can be seen on SC-Leu-Trp-His+3AT. On SC-Leu-Trp+5FOA growth of *Met* + *cyc* is reduced in the absence but not in the presence of methoprene, while *cyc* + *Met* colonies grow in both cases.

Homodimer of *Methoprene tolerant*

This interaction has previously been used as negative control (Shin et al, 2012). We can confirm that also in *P. apterus* no MET-MET homodimer is formed in the presence or absence of methoprene. This result is illustrated in figure 18 below.

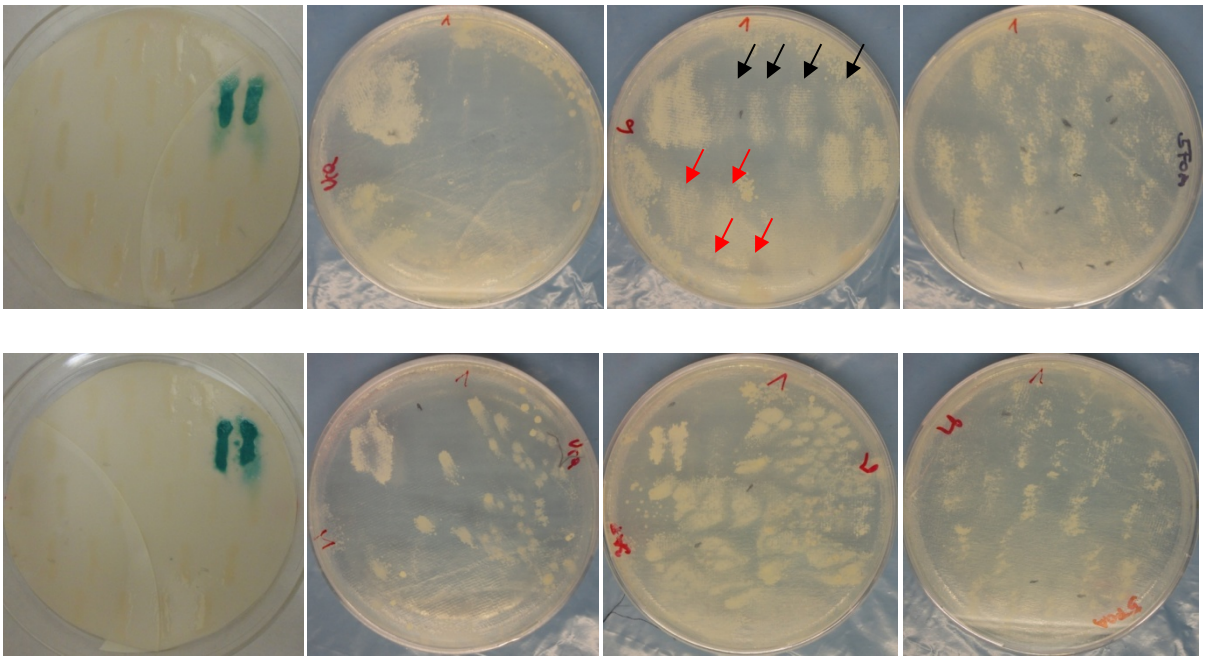

Figure 18: Arrangement of plates as described before. No evidence of an interaction can be seen. The top 4 colonies are *Met* + *Met*, the lower ones *cyc* + *cyc*, which also does not form homodimers. The growth that can be observed on the SC-Leu-Trp-His+3AT plate (black arrows) is due to the fact that 10 mM 3AT is not sufficient to suppress the background of cyc. Therefore also the controls 6 and 7 grow slightly (red arrows).

Methoprene tolerant and *taiman*

In *Aedes aegypti* MET and TAI form heterodimers in a JH dependent manner (Li et al, 2011). Furthermore the same study showed that methoprene is a suitable JH analogue for this interaction to occur. We can now confirm both of these results also in *P. apterus*. The increase of the methoprene concentration from 1 µM to 5µM increased the strength of the interaction enough, to lead to a blue color in the respective X-gal essay. Using the lower concentration of 1 µM, the interaction can only be seen due to the growth on selective plates. Figure 19 on the next page summarizes the findings.

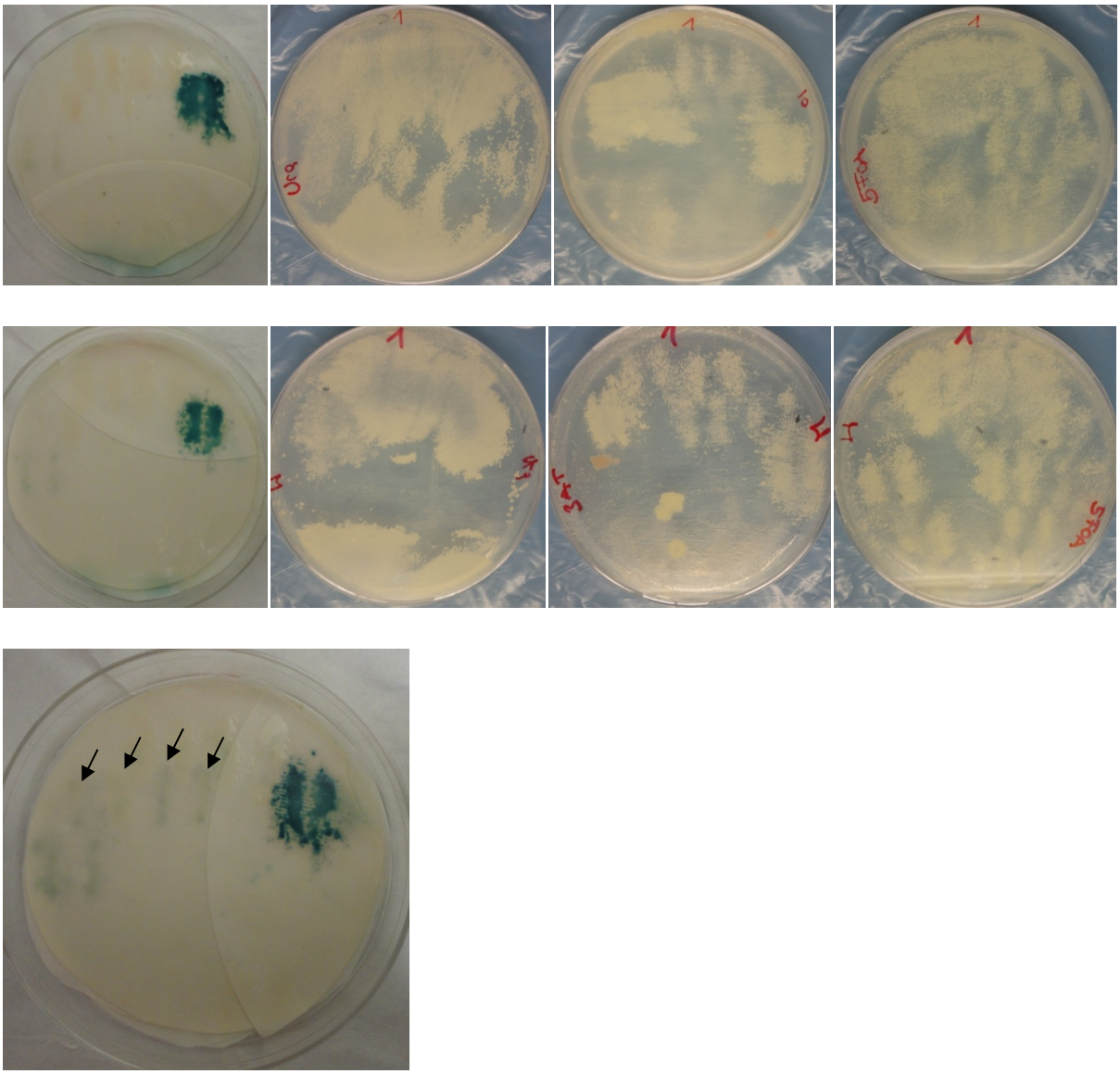

Figure 19: Top: plates without methoprene. Middle: plates with methoprene (1 µM). Bottom: X-gal essay with 5x increased concentration of methoprene (5 µM) in the growth medium. A weak interaction can be seen both with and without methoprene, but it clearly gets stronger with increasing methoprene concentration, because only then it is strong enough to trigger a response in the X-gal essay (black arrows).

Interestingly in this essay the level of interaction of MET and TAI in the absence of methoprene is still higher than in the one in the MET and TGO interaction used as control. Since the yeast did not grow on SC-Leu-Trp-Ura plates without methoprene in the other 3 conducted essays and only once on SC-Leu-Trp-His+3AT, this is could be an artifact of the one shown above. Maybe the interaction strength is so weak, that it can only be detected in rare cases.

Also in the mentioned SC-Leu-Trp-His+3AT plate, only 2 out of 4 colonies grew. To figure out the reason, we further analyzed their genetic makeup and found out, that the non-interacting ones had a mutation in the *tai* gene (see figure 20). This means that although we took special care to avoid mutations by sequencing the plasmids twice during the procedure, it is still possible that they occur within the yeast cells, adulterating the results. As it would be too time and cost intensive to do this analysis on all interaction colonies, we have to trust that the sample of 4 colonies on each plate is large enough to have at least one colony without mutations.

Figure 20: Alignment of the predicted *P. apterus* TAI protein and the translated sequencing results. The colonies showing no interaction (neg) have versions of the gene where a deletion caused a frameshift which led to a premature stop codon. The colonies showing an interaction (pos) have the proper version of the gene.

Since we could only use a short form of the *tai* gene, we also learned from this experiment, that the parts of the protein necessary for the dimer formation are most likely the domains lying in the N-terminal part of the protein. Another interesting fact is that this interaction works in both ways. It seems slightly stronger when TAI is bait and MET is prey (*tai* + *Met*), but it is still measurable the other way around (*Met* + *tai*), although this did not lead to a response in the X-gal essay (5 µM methoprene). In the future we can try to further increase the methoprene concentration to see if this interaction also gets strong enough to turn the colonies blue at some point.

Homodimer of *vrille*

Surprisingly we found the formation of a VRI-VRI homodimer in this screening. This interaction is a bit stronger than the weak interaction control and completely independent from the presence of methoprene. The results are shown in figure 21 below.

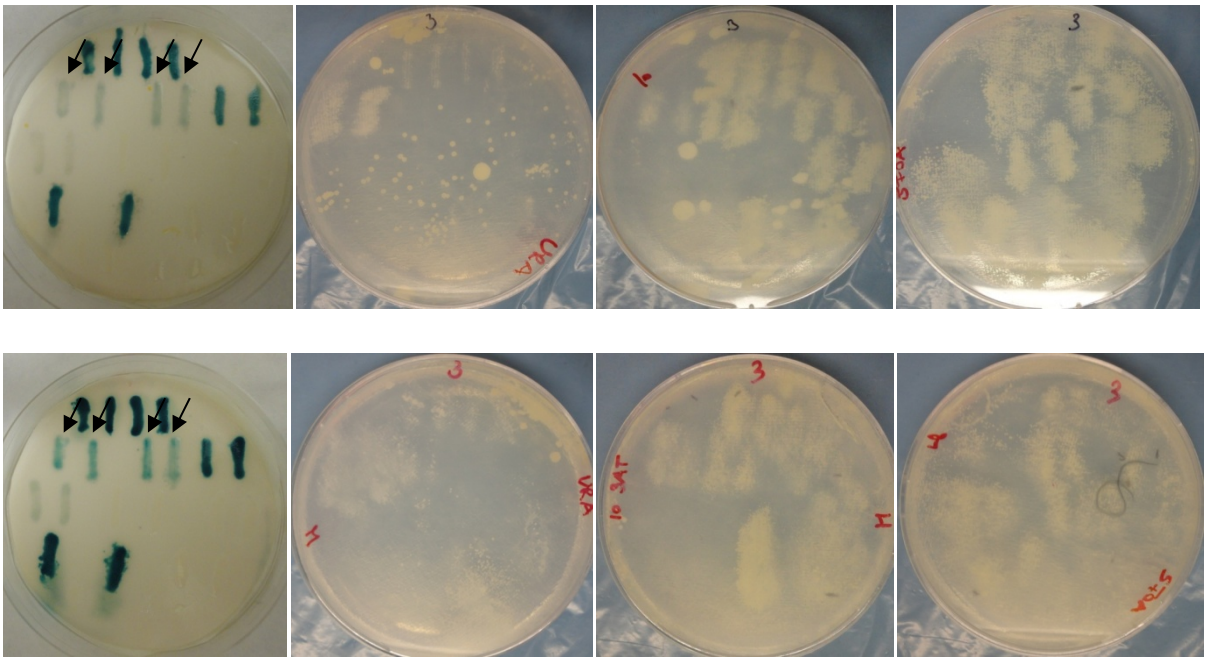

Figure 21: Arrangement of plates as described before. The top 4 interaction colonies are *tgo* + *tgo*. Since *tgo* control 6 self-activates, there is no possibility to judge if an interaction occurs or not. The lower interaction colonies are *vri* + *vri*. On the X-gal essay the blue color looks slightly stronger then the weak interaction control (black arrows).

tango as negative control

Once we found out that *tgo* control 6 self-activates and *tgo* therefore cannot be used as bait, we still tried to use the prey plasmid as negative control. While this worked well with *Met*, *tai* and *kay*, where we didn't see any interaction, it failed for *Clk* and *cyc* (see figure 22).

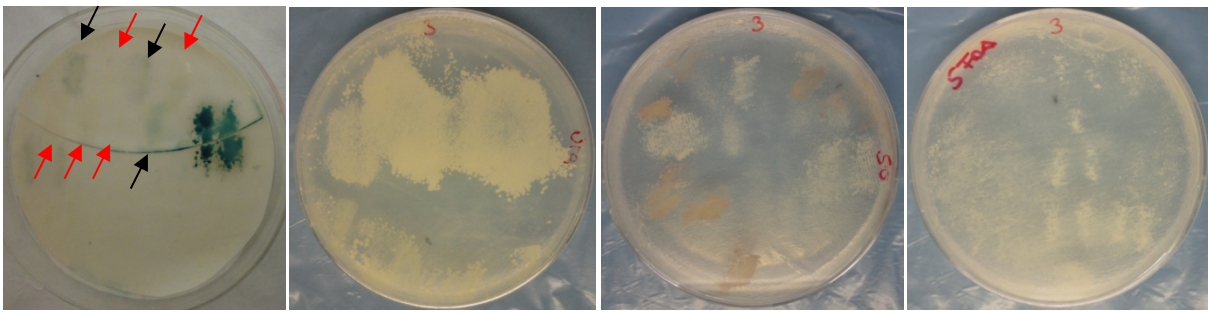

Figure 22: The interaction colonies on top are *Clk* + *tgo*, the ones in the second row are *cyc* + *tgo*. In both cases, some of the colonies show an interaction (black arrows), while others don't (red arrows).

Since the result was not consistent for the 4 different colonies patched on the plate, we further analyzed the genetic makeup of the colonies. This revealed that in case of *Clk* + *tgo*, the interacting colonies had the proper inserts in the pDEST vectors, while for the non-interacting ones the *tgo* gene did not amplify in PCR at all. For *cyc* + *tgo* we found a 28 AA deletion in the CYC protein of the non-interacting colonies. This suggests that in both cases a weak interaction takes place. We also learned that for the *cyc* + *tgo* interaction other parts of the CYC protein than the bHLH and PAS domains are important, because the deletion lies in a part which is highly variable among species and has not been yet been annotated as a specific type of domain (see figure 23).

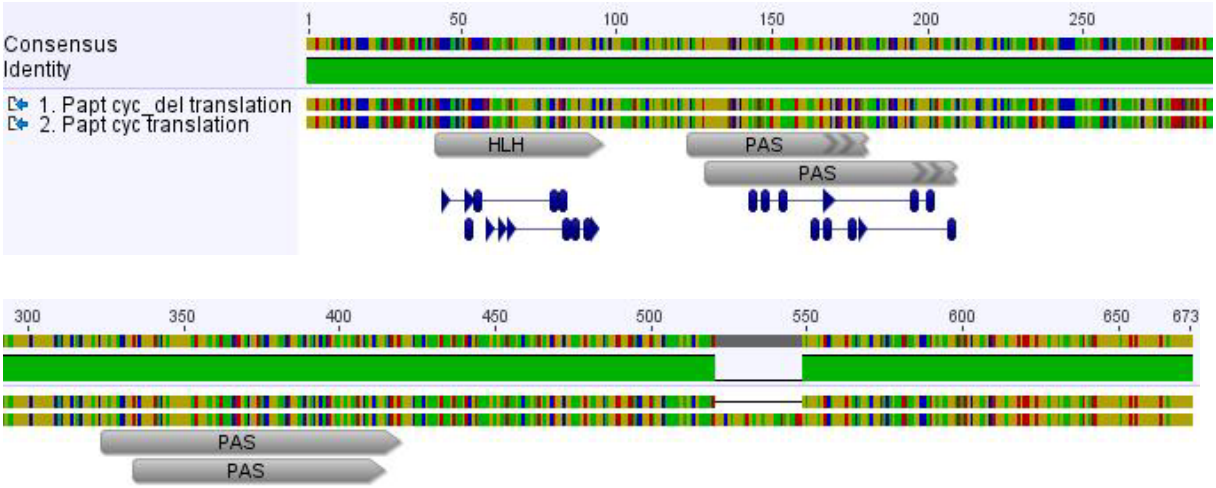

Figure 23: Alignment of the translated sequencing result and the annotated (hypothetical annotation, transferred from *Athalia rosae*) CYC protein from *P. apterus*. This shows that the mutation shutting down the interaction lies outside the annotated domains.

These experiments show, that *tgo* is not a reliable negative control. Nevertheless, the interactions we found are interesting. They might very well be physiologically relevant. Either *tgo* is in fact a circadian factor, but has so far not been identified as such, or the interactions serve a different purpose in the insect body. This would not be surprising, because proteins involved in the circadian system in general also have other functions.

Pdp1_iso1 as bait

A very strange phenomenon which we discovered in this study is that *Pdp1_iso1* used as bait seems to interact strongly with all the other candidate genes. This is not the case if it is used as prey plasmid. Therefore we tried its interaction with two of the control genes provided with the kit: *Krev1* and *RalGDSwt-m2*. Those two genes together are used as negative interaction control by the kit. Since the Y2H essays of *Pdp1_iso1* + *Krev1* and *Pdp1_iso1* + *RalGDSwt-m2* also lead to a strong interaction as result, we assume that the first isoform of the PDP1 protein is very sticky and therefore likely to interact with almost any given partner.

Since this is not the case for the second isoform, the reason for this behavior has to lie in the N-terminal part of the protein which differs among the two isoforms (see figure 24). To elucidate this phenomenon further, we are now planning to produce hybrids of the two isoforms and investigate their behavior in Y2H. This way we hope to find out which part of the protein is responsible for this behavior. Also it would be interesting to try interaction studies in insect cells with this protein to find out if this stickiness occurs only in the presence of the large Y2H constructs or also if the protein is in its native state.

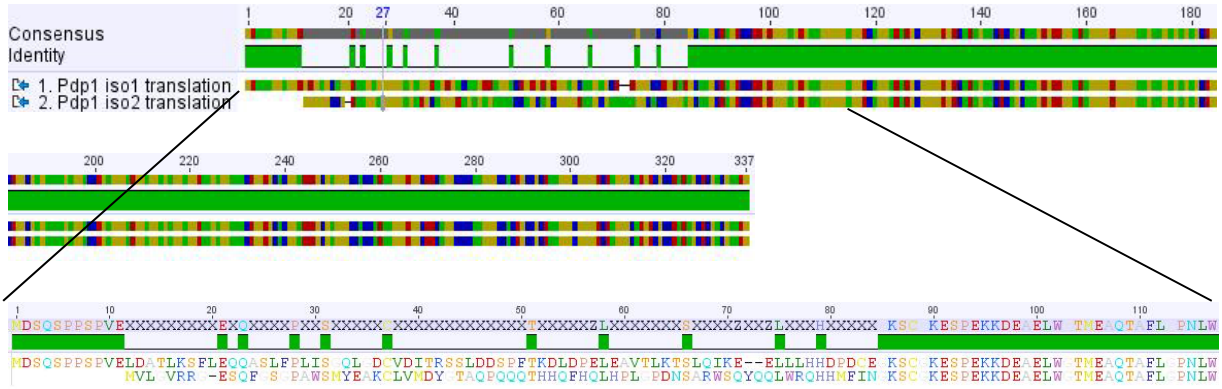

Figure 24: Alignment of the two isoforms of the PDP1 protein, highlighting the differences between them in the N-terminal part.

Due to the fact that overall 56 different interactions were tested in this study, not all of them are described here in detail. A comprehensive overview of all results is shown in table 8 and 9 on the following 2 pages. *cwo_iso2* and *cwo_iso4*, for which we also obtained the prey and bait plasmids, have not been used for Y2H so far, therefore they are not included in the tables. We are planning to use them for further experiments though.

Summary of methoprene independent interactions

Table 8: Summary of all interactions in the absence of methoprene. (- No interaction, + Weak interaction, ++ Strong interaction, * self-activation, ? no convincing result, / not tested)

Prey \ Bait	*Clk*	*cyc*	*Met*	*Pdp1_iso1*	*Pdp1_iso2*	*vri*	*tgo*	*tai_short*	*kay*
Clk	-	+	-	++	-	-	*	-	-
cyc	+	-	+	++	-	-	*	/	/
Met	-	-	-	++	/	-	*	?	-
Pdp1_iso1	-	-	-	++	-	-	*	-	-
Pdp1_iso2	-	-	/	++	-	-	*	/	/
vri	-	-	-	++	-	+	*	/	/
tgo	+	+	-	++	/	/	*	-	-
tai_short	-	/	?	++	/	/	*	-	-
kay	-	/	-	++	/	/	*	-	-

Summary of methoprene dependent interactions

Table 9: Summary of all interactions in the presence of methoprene (1 µM). (- No interaction, + Weak interaction, ++ Strong interaction, * self-activation, / not tested)

Prey \ Bait	*Clk*	*cyc*	*Met*	*Pdp1_iso1*	*Pdp1_iso2*	*vri*	*tgo*	*tai_short*	*kay*
Clk	/	+	-	/	/	/	*	/	/
cyc	+	-	+	/	/	/	*	/	/
Met	-	-	-	++	/	-	*	+	-
Pdp1_iso1	/	/	-	/	/	/	*	-	-
Pdp1_iso2	/	/	/	/	/	/	*	/	/
vri	/	/	-	/	/	+	*	/	/
tgo	/	/	-	++	/	/	*	-	-
tai_short	/	/	+	++	/	/	*	/	-
kay	/	/	-	++	/	/	*	-	-

CONCLUSION

This study initiated our effort to elucidate protein-protein interactions of *P. apterus* clock factors. As a pilot study, we intentionally employed an approach which allows for fast and cost effective screening for interactions between and among many factors. In total, coding regions of 11 isoforms corresponding to 9 genes were cloned into pENTR plasmids, from which they were transferred into bait and pray constructs. Yeast two hybrid experiments then revealed protein-protein interactions (CLK-CYC, MET-CYC, MET-TAI) that have been demonstrated in different model organisms before. We also identified that methoprene influences the interactions between MET and TAI as well as between MET and CYC in the same way as previously demonstrated.

Surprisingly we found that VRI forms homodimers in Y2H and TGO can interact with both, CKL and CYC. Particularly the interaction of TGO with two classical circadian transcription factors suggest to explore this phenomenon further, including *in vivo* behavioral experiments and alternative biochemical approaches. We also discovered that the different N-terminal part of the two isoforms of PDP1 leads to a very different behavior in Y2H. While PDP1_ISO1 seems to bind almost any given partner, PDP1_ISO2 does not. Since the N-terminal region specific for isoform 1 is small (83 AA), an approach aiming at identification of the specific region responsible for these “promiscuous” interactions is feasible.

In addition to the protein-protein interactions we were looking for, we found that mutations can occur also within the yeast cells, having an impact on the interaction results. This means that even sequencing the constructs twice does not prevent mutations from influencing the results. On the other hand, it allowed for identification of several mutations, which disturbed certain interactions. This way we can now tell which protein regions are necessary for dimer formation.

In the end I have to say that this study was a successful start for the overall goal to propose a model for JH reception in *P. apterus*, but there is still a lot of work to be done. We have many more interactions to test among the proteins we have tried so far to completely fill the tables (8 and 9) shown above. Moreover there are the two isoforms of *cwo*, which we already have prey and bait plasmids for, but have not used in Y2H yet. Also, there are many genes, for which we have not obtained proper pENTR clones so far. The clones generated in course of this project are promising research material for alternative approaches to further elucidate protein-protein interactions and also investigate their interaction with promoter sequences. Since all constructs are cloned in Gateway vectors, fast and reliable subcloning into various new vectors is easy.

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