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Review of the Diploma thesis of Marion Sieber 'Protein-protein interaction of photoperiodic clock factors in *Pyrrhocoris apterus*'

The proposed goal of this thesis was to test direct interactions between several circadian clock proteins and juvenile hormone (JH) receptors of the Linden bug, *Pyrrhocoris apterus*. The yeast two-hybrid assay (Y2H) was employed as the method of choice and nine proteins were screened in total in presence and absence of the JH analogue methoprene.

The thesis is written in the classical format separated to Introduction, M&M, Result and Discussion and Conclusion parts. In the introduction section the author described the current knowledge about protein factors involved in circadian rhytmicity, the role of JH in photoperiodism and the principle of Y2H screening used for the study. I would just pointed out that the introduction a bit 'animal-centric', examples of model organisms where the circadian clock are best studied should include also the cyanobacterium *Synechococcus* and the plant *Arabidopsis thaliana*. Perhaps too brief is the part about photoperiodic clock in insects, as this is the main topic of the thesis. I would also welcome two-three paragraphs about recently published results (or potentially unpublished) from author's home laboratory. The whole introduction section is however well written and is sufficient, even for readers unfamiliar with the topic, to understand the sense of experiments.

What I found missing is a kind of hypothesis or a clearly stated question(s) the proposed experiments should help to resolve. It is not clear what it would be the dream result. It is just my guess that the main idea behind this work is that the tested protein interactions in Linden bug are more sensitive to JH than in Drosophila. Is it this the case?

Experiments are described intelligibly and it is easy to follow the work. I have no doubt Marion mastered preparation of RNA from Linden bug, reverse transcription, cloning techniques, RT-qPCR, Y2H and using of basic bioinformatic tools. Particularly, the Y2H is considered to be challenging and requiring robust controls and replications to exclude false-positive hits. In this thesis the Y2H experiments are very well done. Although no new JH-dependent interactions were revealed yet, the identification of several mutations, which affected protein-protein interactions, are valuable and if confirmed, these data can be useful for further studies. It is apparent that the Marion's thesis just initiated more extensive study and a number of combinations of selected proteins have not been assessed yet.

I can conclude that that the thesis contains solid scientific data, is written in good English with a minimum of formal errors. The thesis fully complies with all general demands for the diploma thesis and I would evaluate it by degree between *outstanding* and *very good*.

Questions for the student (as an input for discussion):

Instead of using a chemical analogue, is it possible to purify, or at least partly, the JH from the Linden bug for protein-interaction studies? Is it there any standardised protocol for JH purification?

1μM methoprene was used for Y2H. Is it known in what physiological concentrations JH oscillates in insect cells?

Does methoprene absorb in visible light or does exhibit any fluorescence that could be used to detect a binding of this compound to a recombinant protein by e.g. tryptophan quenching or by chromatographic approaches?

(not the serious one). One of proteins involved in the study is called Clockwork Orange. Is it known the name was intentionally chosen to be the same like the famous Stanley Kubric's movie? If so, why?

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Review of the Master Thesis "Protein-protein interaction of photoperiodic clock factors in *Pyrrhocoris apterus* " submitted by Ms. Marion SIEBER

Ms. Sieber's thesis deals with the molecular basis of the photoperiodic clock. As a model organism, the linden bug, *Pyrrhocoris apterus,* that features a (robust) day length induced diapause, was used in her work. In *P. apterus* diapause includes reproductive arrest that is accompanied by changes in the physiology and gene expression in the digestive section. The laboratory in which she performed her studies could recently show that juvenile hormone (JH) together with circadian clock genes (or gene products) regulates the switch between reproductive (non-diapause) and reproductive arrest (diapause) phases (Bajgar *et al.,* 2013; *Proc. Natl. Acad. Sci. USA*, 110, 4416). However, the downstream signaling is not yet fully elucidated. In order to shed some more light on the pathway, and the proteins involved Ms. Sieber tried to identify (using the yeast two hybrid technique, Y2H) physical interactions between several circadian clock proteins and whether they were dependent on JH (or the JH analogue methoprene).

During her work Ms. Sieber first cloned several circadian clock genes from *P. apterus* via RT-PCR and the Gateway system into suitable Y2H activator domain (AD, "prey") and DNA binding domain (BD, "bait") plasmids. After sequence verification, yeast (MaV203) cells were co-transformed with pairs of AD and BD plasmids, each containing one of the proteins which possibly interacted. Using these strains, Ms. Sieber finally performed (more than 50!) Y2H assays to check for interaction.

For a master thesis this is an enormous and unusual amount of laboratory work what is highly appreciated. Furthermore the thesis points to interesting and novel results that certainly could be published (in a high quality journal) after some elaboration / verification.

In her written thesis, Ms. Sieber gives a well written introduction that makes the reader familiar with circadian and photoperiodic clocks and describes the involved proteins as well as the important messenger, the juvenile hormone. She then justifies the usage of Y2H for testing the interactions between the "players" and explains the principle of the assay.

The "Methods" section is also in general written in a clear and concise way. However (probably to keep this part short), Ms. Sieber rather often refers to "the xxx manual, page yy". This is in principle fine, but makes it difficult for the reader to understand where and why technical problems might have had occurred.

Ms. Siebers then continues with a combined "Results and Discussion" part that starts with a result from a side project, the attempt to check by qPCR, whether a predicted second CLOCK (CLK) isoform Clk_iso2) is expressed in *P. apterus* gut tissue. The results presented here seem for me contradictory: On one hand it is stated that "The experiment showed, that both predicted isoforms are expressed -...". On the other hand "we were not able to amplify the Clk_iso2 isofom by PCR".

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This part is followed by a concise description of the results from clonings, the verification of the generated plasmids, the generation of yeast strains and their testing.

The main results are presented in the chapter "Yeast Two-Hybrid assays". This chapter is unfortunately not very easy to read. For example the figure legends hardly allow understanding what is shown without having carefully read the main text. Additionally, it seems that many of the yeast plates shown in the figures are contaminated, possibly compromising the validity of the interpretation of the results. Both points can be illustrated already with the first subchapter "Clock and cycle" (Fig. 16). The figure legend does not tell which interaction was tested. An indication of the streaks that indicate CLK/CYC interaction is missing. Furthermore, all plates (except maybe the X-Gal assay plate, where it is not clear from the picture shown) are contaminated. From the figure, I also cannot depict that there are "slightly different responses" in different streaks of cells in which interaction is claimed to be observed. Finally, the interpretation that there is interaction is further compromised that Ms. Sieber was not able to recover CYC expressing plasmids from the yeast strains she analyzed.

Another example of the technical difficulties that occurred is shown in the next subchapter "Methoprene tolerant and cycle". Here the growth of some non identifiable microorganisms is interpreted as strong growth of "*Met + cyc* interaction yeast cells" and furthermore as "weak interaction" without discussing the fact that there was not -as in that case expected- at least a weak induction of galactosidase was observed,

There are various other details where (it seems to me) that the conclusions are not fully supported by the results shown.

In general the results and discussion section also suffers a somewhat from the lack of a "real" discussion that tries interpret the results in the context of the published literature in the field.

Finally, the conclusion nicely summarizes the whole thesis.

Due to the huge amount of work done and the tremendous number of experiments performed and despite the criticism above, I clearly suggest that Ms. Siebers' Master thesis is accepted by the Faculty of Science of the University of South Bohemia.

Some questions that I'd 'like to discuss during the defense are:

(i) (p24) How can it be that qPCR seemed to indicate the expression of Clk_iso2 whereas "standard PCR" did not yield a product?

(ii) (p29) What could be the explanation for the failure to recover plasmids expressing CYC?

(iii) (p36 - p38) What could explain the fact that Table 8 (and Table 9) are not symmetrical, i.e., that the observed interactions are in many cases dependent on which of the analyzed genes (proteins) was fused to AD or to BD?

(iv) (p33, also assumed on p29) It seems that during the work a rather high (unusual) number of spontaneous mutations of plasmids in yeast occurred. Is the strain used known to be genetically unstable? How could one determine the frequency of spontaneous mutations in general?

Yours sincerely

(Jost Ludwig)