Thesis examination report on the PhD thesis: *Proteins involved in the tetrapyrole pathway in Synechocystis sp. PCC 6803 and their localization in the proximity of PSII biogenesis* by Mgr. Petra Skotnicová.

General Comments

The thesis reports the characterization of the BtpA, HemJ, Sll1106 and CurT proteins in *Synechocystis* sp. PCC 6803. In each case the data on these proteins indicate a close co-regulation of pigment biosynthesis with regulation of the assembly of Photosystems I and II. As such the thesis represents a coherent investigation of the chosen topic. The thesis has already led to the publication of one paper in the *Journal of Biological Chemistry* describing the novel findings surrounding the role of HemJ. In addition the work described in sections 4.1 (on BtpA), 4.3 (Sll1106) and 4.4 (CurT) would be very likely to make substantial contributions to papers based on the characterization of these proteins. The depth and range of the work presented is sufficient to meet the requirements of the PhD degree. The Introduction and Discussion both display a broad knowledge of the relevant literature and an ability to exercise critical and analytical judgement of the literature. The quality of the reported work also demonstrates a sound mastery of the relevant methodology used in this project. Based on the above I believe the candidate should be congratulated on completing an excellent study.

Specific comments

The "Annotation" at the beginning of the thesis was somewhat brief – is a more complete abstract of the findings warranted?

Similarly the methods are quite succinct. This perhaps undersells the amount of work performed and it also means that future students wishing to follow up must seek out other literature to understand exactly what was done. There is also, at times, a lack of precise detail: for example, we are told *hemG* was cloned but we do not know exactly what was cloned (e.g. how much flanking sequence was incorporated: it would be good to know this, since this might influence regulation?). In fact I don't recall a single primer being described in the thesis. If we consider the construction of the ΔBtpA mutant we do not know if the plasmid supplied by the Pakrasi lab was checked to make sure it was as reported in their paper – did the candidate sequence the plasmid before transforming their *Synechocystis* sp. PCC 6803 strain? This becomes immediately relevant when the phenotype is somewhat different to that reported for the original strain? Is the original strain available – are there plans to sequence the original strain? I note that another scientist has been studying this strain and obtained suppressor mutants and genomic sequencing of these appears to have been done. If someone else's work depends on this thesis it would good to have all details clearly documented and in this case confirmed prior to the start of a subsequent

project. It would be interesting to compare the sequencing of the suppressors, and the ΔBtpA strain of this work, with that of the original Zak and Pakarsi strain. On another note, the inferred interconnection between GluTR and ArJ and BtpA and urease which has been identified by this aspect of the project are particularly interesting: are there plans to follow this up post PhD?

The HemJ work is fully presented in the included paper and section 4.2 nicely presents the candidates contribution to what is a very nice study. Table 4 refers to Sll1106 as a hypothetical protein – I guess it is hypothetical no more.

The choice of inserting protein for expression into the *psbA2* locus followed the use of this approach in an earlier paper by Chidgey et al. (2014) (missing from the reference list!). What controls exist to confirm that this is a "neutral" site for such a modification? We have had suppressor mutants map to *psbA2* that completely blocked photoautotrophic growth: while this might be explained by a misfolded D1 clogging up biogenesis it was still surprising to us that the *psbA3* gene, that did not contain the suppressor mutation, was unable to step up and rescue the phenotype. It does at least suggest the possibility that there is regulation between these two loci and expressing something at the *psbA2* loci using the *psbA2* promoter may in fact be doing more than is immediately apparent. Why did you not use a more commonly used "neural site" such as *slr0168*?

The section on the characterization of Sll1106 is succinct and it is perhaps premature to seemingly suggest on page 76 that the only condition where Δsll1106 exhibited impaired growth was salt stress – particularly when this protein appears to be associated with such an interesting collection of proteins as reported in Tables 5 and 6. What was the rationale to call it a day on this part of the project when there is clearly much to discover here? Likewise the CurT section opens up many potential leads for understanding the role of this protein in biogenesis – was it a time factor that left so many open questions to follow up? Did you actually demonstrate that your R1 suppressor produces a PsbE:PsbL fused peptide as opposed to such a foreign peptide being rapidly turned over? It was suggested that Figure 36B clearly shows the impact of the fused protein but doesn't such a claim require a demonstration of the presence of the fused peptide using, for example, antibodies? Perhaps the evidence is there and I missed it?

Incidentally in the Methods you mention that you considered variants with a 60% or higher frequency – in our experience that is quite relaxed. Did you confirm the suppressor mutations you discus in this thesis by Sanger sequencing?

This thesis is a nice piece of work and certainly opens up many avenues for future research. Do check your references carefully. Besides not finding Chidgey et al. (2014) when I looked up Malavath et al. (2018) I noticed the volume and page numbers were missing. This suggests there may be other omissions since I found two errors in two cross checks (although subsequent spot checks were Ok). Although it is perhaps a matter of personal preference, I think putting your

reference strings in the text in chronological order is better than alphabetical order.

In general the English is well written but there are a few things that could be edited (e.g., page 80 last paragraph line 3 – do you mean "that" rather than "what"? and on page 74 it should be "proof" not "proofs (line74). On page 2 line 8 add the "s" to "chloroplast". All of these examples should be picked by Word spell and grammar checks so I recommend running these over your text if you are able to make corrections to the text at this point.

As a final comment I thought the quality of the figures in this thesis are about the best I have ever seen in a PhD thesis. Well done!

Julian Eaton-Rye
29 November 2019

Ph.D. thesis review

Title: **Proteins involved in the tetrapyrrole pathway in *Synechocystis* sp. PCC 6803 and their localization in the proximity of PSII biogenesis**

By Mgr. Petra Skotnicová

The dissertation thesis of Mgr. Petra Skotnicová presents results of the complex study focused on the tetrapyrrole biosynthetic pathway in Synechocystis. This pathway produces essential pigments hemes and chlorophylls (and also bilins). Tetrapyrrole pigments and many of their biosynthetic precursors (and degradation products) are photoactive and can generate free radicals and ROS when excited under oxic conditions. So, the synthesis of these pigments has to be precisely coordinated with protein synthesis and assembly of protein-pigment complexes harbouring these pigments. Tetrapyrrole pigments in these complexes are essential for the capture of photons and conversion of their energy to fuel carbon assimilation. On the other hand, synthesis of tetrapyrroles can utilize significant portion of cellular nitrogen (some tetrapyrolles can also serve as a nitrogen store). This connection between nitrogen consumption and carbon assimilation mediated by terapyrrole pigments suggests that the C/N ratio can be an important factor in their biosynthetic and degradation pathways as was also indicated by Petra´s results.

Though several mechanisms of the feed-back control of the tetrapyrrole synthesis are known from plants, the knowledge of this regulation in cyanobacteria is incomplete. From these reasons, the Ph.D. study of Petra was focused mainly on the regulatory mechanisms of the tetrapyrrole synthesis. The research was smartly designed and fully utilized the advantages of the model cyanobacteria Synechocystis, which has high multiplication rates and allows simple production and complementation of mutant strains. Synechocystis is also prone to create suppressor mutations that can seriously complicate characterization of mutants and interpretation of the observed phenotypes, but as demonstrated in Petra´s thesis the suppressor mutants can be utilized as a unique tool providing direct valuable insights into the regulatory networks (when the suppressor mutations are identified by WGS). The thesis was focused mainly on protein regulation by protein-protein interaction, so the primary method that Petra used was affinity purification and characterization of multiprotein complexes.

Based on these methodologies, Petra succeeded to identify new metabolic-regulatory modules present within specific region of the thylakoid membranes. She demonstrated that non-enzymatic components (BtpA, Sll1106 and CurT) are essential for formation of these complexes since they mediate specific association of various proteins/processes (tetrapyrrole synthesis pathway, photosystem assembly and repair, nitrogen metabolism, ...). Physical interactions mediated by analysed proteins are required for proper regulation and tight coordination of the above mentioned processes to avoid phototoxicity and likely also to adopt to nutrient availability.

The thesis is nicely written, like a detective story. By reading the abstract it seems that there are three more or less separate stories, then all protagonists are introduced in detail, but their connection are unclear. They start to appear when going through the results and discussion to finally provide complex data showing that the story is beautifully complete.

The thesis contains huge amount of novel data: characterization of HemJ, role of BtpA in regulation of GluTR, role of CurT and Sll1106 in organizing specific thylakoid membrane domains involved in coordination and regulation of tetrapyrrole synthesis and incorporation and the potential role of the convergence zone for the transformation competence. Only small part of these results has been already published in highly impacted journal (JBC) with Petra being the first author. Additional papers will surely follow in the near future.

I can state that Petra has clearly demonstrated the ability to design and conduct experiments, process data, perform correct interpretations and summarize them in a readable scientific text. Petra has all the skills needed to be awarded with the Ph.D. title.

Comments and questions:

There are only very few points that can be criticized (and none of them is really significant):

the list of abbreviations should be presented in the alphabetical order, archebacteria is an old name for Archea and should not be used, Archea is a sister group to bacteria, so the statement "from archea to eukaryotes" which was used in the thesis is misleading (Archea cannot be considered to be more primitive compared to bacteria), in the title I would use "tetrapyrrole biosynthetic pathway" instead of "tetrapyrrole pathway", pigment molecules are incorporated not only to photosystems, but also to cytochrome b6f complexes.

1) It was noted in the Methods section that hemJ in the plasmid in E. coli frequently contained frame-shift mutations. How do you interpret this observation?
2) What can be the reason of high rate of mutations in the btpA mutant?
3) In the btpA mutant suppressor lines the GluTR levels were recovered by various ways. Do you plan to use these lines for deeper understanding the role of BtpA in C/N regulatory processes?
4) Do I understand well that based on your data, Hik33/Rre26 signalling likely serves to provide a positive feed-back regulation from PSII assembly to induce tetrapyrrole synthesis?
5) The performance of hemJ mutant complemented by HemG strongly differed depending on the presence of glucose in the medium. Can be expected that mixotrofic cultivation with active water splitting leads to deep decrease in oxygen level? Did you try to demonstrate dependence of the growth inhibition on glucose concentration gradient and on light?
6) The level of FeCH in HemJ.f eluate was very low compared to the crude WT thylakoid membranes – can be this detection of FeCH considered as a positive result? Is possible that the increased signal in PSI mutant eluate results from overall increased level of FeCH in this strain (control sample of TM from PSI mutant is not shown)?

In Krušovice, 9.12. 2019

Lukáš Fischer

Fischer