Review of the Ph.D. thesis by Jana Kopečná „Regulation of chlorophyll biosynthesis in the cyanobacterium Synechocystis sp. PCC 6803"

The thesis deals with molecular processes involved in the biosynthesis of chlorophyll molecules and in their incorporation into proteins of photosynthetic complexes. This work is based on four papers published in highly impacted journals, which implies that the scientific topic is up-to-date and the quality of work is of very high level. Jana Kopečná is the first author in two papers. At the beginning of the thesis it is clearly stated what was her contribution in particular papers. I admit that I would have expected more than "a participation in writing" in the papers where Jana Kopečná is the first author. Did you write at least a draft of these manuscripts?

The thesis consists of a review (about 30 pages), focusing on the chlorophyll biosynthesis in cyanobacteria and its regulation, and from the reprints of author's papers. The review, in which the author describes individual processes of chlorophyll biosynthesis, represents a deep and detailed overview of the studied research topic and is supported by many recent citations. The citations of author´s own results/papers is an organic part of the review text. However, I would prefer the author's papers denoted as Paper I – IV in the text. The text includes also author´s data and interpretations that are not presented in the enclosed papers. The review is written in very good English with minimum of mistakes and typing errors. In my opinion, this form of Ph.D. thesis is very suitable and should be generally demanded from PhD students in the faculties of science in the Czech Republic.

The spectrum of experimental methods and techniques that Jana Kopečná had to master during her work was unusually broad. It included physical as well as (bio)chemical methods and covered spectroscopy, microscopy, various purification and electrophoretic techniques, construction of mutants, radioactive labeling, chlorophyll fluorescence technique, thin layer chromatography, etc.

I have several questions which I would like to be addressed during the oral examination:

1) In many parts of the thesis you state that the chlorophyll biosynthesis and the incorporation of chlorophyll into proteins in living organisms must be under perfect control, because the excited free chlorophylls or their precursors can lead to the formation reactive oxygen species (ROS). Can you describe the mechanism of the ROS formation by free tetrapyrrols? Why the incorporation of tetrapyrrols into polypeptides solves of the problem of ROS formation?
2) Conifers, like other evolutionary lower organisms, synthesize chlorophylls in the dark due to the activity of light-independent protochlorophyllide oxidoreductase (DPOR). What is namely interesting for me is the fact that in conifers the chlorophyll synthesis and incorporation into proteins and in particular the complete final assembly of chlorophyll-protein complexes (as deduced from 77 K chlorophyll emission spectra that are similar to that of light-grown conifers) do not require any light regulation. Is this also the case for the Synechocystis cultivated in complete darkness?
3) At the page 22 you state that the L-subunit of DPOR from *Rhodobacter capsulatus* accepts electrons from ferredoxin. Does it mean that the L-subunit can participate in the linear electron flow in thylakoid membranes?
4) In your work you have used native electrophoresis for the separation of PSI supercomplexes (PSI trimers). In such method, a mild detergent with low concentration is usually used in order to preserve the native protein complexes. How stable is the trimeric form of PSI separated from the membranes of Synechocystis? Can you avoid the aggregation of monomeric PSI into an artificial supercomplex?

How would you prove that the separated trimeric PSI represents a native protein structure?

Finally, I congratulate the author on the considerable scientific contributions to the regulatory mechanisms of chlorophyll biosynthesis in cyanobacteria and strongly **recommend, in case of successful oral defense, the award of Ph.D. to Jana Kopečná.**

In Olomouc, 20/11/2012

Prof. RNDr. Petr Ilík, Ph.D.

Review of the PhD thesis entitled
"Regulation of the chlorophyll biosynthesis in the cyanobacterium *Synechocystis* sp. PCC 6803" by Jana Kopečná

The aim of this thesis has been to unravel how the regulation of the chlorophyll biosynthetic pathway is coordinated with biogenesis of chlorophyll-binding proteins in the cyanobacterium *Synechocystis* sp. PCC 6803. One specific aim was to analyze the correlation between biogenesis of the photosystems and synthesis of chlorophyll in Synechocystis cells. A second aim was to elucidate the role of two different enzyme systems involved in catalyzing protochlorophyllide reduction. In addition, the role of two thylakoid proteins, Ycf54 and Psb27, in chlorophyll biosynthesis and photosystem II assembly was investigated.

The thesis comprises a well written literature review (Introduction, ~32 pages) which describes and discusses the current existing knowledge on chlorophyll biosynthesis, its regulation and current status of the coordinated synthesis of the photosynthetic pigments with the assembly and accumulation of photosynthetic protein complexes. The chapters on biosynthesis of chlorophyll, regulation and coordination represent an exhaustive account on the information available from the literature. This demonstrates an excellent overview of the existing scientific literature. The Introduction nicely set the stage for the topic of this thesis: the coordination between chlorophyll biosynthesis and biogenesis of the pigment-binding photosynthetic proteins.

Formally a few spelling mistakes are found and in some cases the English is a bit unclear but none of them disturbs the overall impression of the Introduction.
Some example off the unclear English:

Page 1: *It is important to mention that although the last step of Chl biosynthesis is the moment when the Chl molecule is made, from a regulatory point of view Chl itself is just a toxic intermediate until it is not attached to a protein.* – this is rather unclear what is meant.

Page 17: ***Regulation signals originating from the magnesium branch*** - "Regulatory" would have been the correct word.

Page 19: *However, Gun4 probably uses a mechanism that depends on binding both porphyrins and activated ChlH to associate with chloroplast membranes; the ChlH association with chloroplast membranes seems to be influenced rather by MgCH activity than Gun4 activity (Adhikari et al. 2011). Moreover, accumulation and membrane binding of ChlH and Gun4 is differently regulated than that of ChlI and, even more strikingly, ChlD, thus they both can play more roles than just Mg2+ chelation (Kopečná et al. 2012b).* Again it gets a bit unclear what is actually meant.

Page 20: *Light 'sensitivity' thus makes LPOR an ideal sensor of an actual light intensity, which the cell exposed and this enzyme has also a potential to modulate other parts of the tetrapyrrole biosynthesis.* – a bit unclear English.

Page 23: *This however does not say nothing why an atypical, light-powered mechanisms, was chosen just for this step of Chl biosynthesis.* – unclear English.

Page 31: *Cells thus handle with tetrapyrroles very carefully, which is especially true for photosynthetic organisms accumulating high concentration of chlorophyll (Chl) to utilize light energy.* – what is actually meant?

Specific questions to the thesis:

1. On page 1. Please explain the 'perfect control' – I am just wondering whether there is such a thing as perfect control in biological systems and I would very much like to hear your opinion on this.
2. On the same topic – on page 2 you write that it is rational to expect the same regulatory aspects operate in similar ways in both cyanos and chloroplasts – is that always true? Please explain.
3. On page 9 you write that 'By binding to a variety of different proteins, the level of free tetrapyrroles within a cell is kept to a minimum.' Please explain where in the pathway there can be these binding or storage capacities. What is known and what is not?
4. On page 11 you discuss the role of FLU and you end the section with the sentence: 'However, the exact role of PChlide in modulation of FLU activity needs to be elucidated' – what is known about FLU, i.e. what has been tested and how do you envisage this problem could be addressed experimentally?
5. On page 11 where you discuss 'free' heme I lack a more quantitative approach. What concentration ranges are we talking about in vivo? What concentrations ranges are being tested in vitro? What are the requirements with respect to equipment and methods of choice?
6. On page 23 you write: 'The benefit from ability to sense light directly with no need for a signaling cascade from photoreceptors might be the reason.' Could you please elaborate a bit on this?
7. On page 24 you start the chapter nicely with the observed fact that supply and demand should be balanced. How is this achieved at the different levels?
8. Further down on page 24 you state something about PSII core complexes (RCa and RC*). Could you please outline the composition of the PSII core complexes (protein subunits and Chl pigments).
9. On page 25 you arrive to some very interesting conclusions based on your own results which apparently are in contrast to previous results. Please elaborate and explain the contrasting results as it is quite complex and deserves some discussion. Is the same or comparable methods used?
10. On page 25 it is stated that the lifetime of Chl is much longer than that of proteins… How stable is PSI in cyanos? Is it turned over at all?
11. On page 27 you write: 'Given the critical need for Chl during Chl-protein synthesis it sounds sensible that the terminal steps of Chl pathway are placed in the cellular or chloroplast compartment where Chl-proteins are translated and then assembled into photosystems.' – How do you envisage this? Feel free to speculate.
12. How can a potential "assembly line" be shared between PSI and PSII biogenesis? Or should there be different "lines"?
13. The ycf54 work is highly interesting and you suggest that the Ycf54 is an auxiliary factor essential for the assembly of MgPMC complex or facilitates formation of a catalytic complex between cyclase and preceding and/or following enzymes that would be required for MgPMC activity. This sounds very plausible but how can it be proven? Please feel free to speculate.

The Introduction is followed by four papers published in international peer reviewed journals. Jana Kopečná is first author on two, second and third author on two, respectively.

***I. Kopečná J**, Komenda J, Bučinská L, Sobotka R (2012). Long-term acclimation of the cyanobacterium Synechocystis PCC 6803 to high light is accompanied by an enhanced production of chlorophyll that is preferentially channeled to trimeric PSI.*
Plant Physiology (DOI:10.1104/pp.112.207274) (IF = 6.535).

In this paper, the correlation between synthesis of PSI and PSII and Chl biosynthesis in Synechocystis cells acclimated to different light intensities was investigated. As method radioactive labeling of proteins and Chl by 35S and 14C, respectively, was used and in combination with physiological, biochemical and spectroscopic methods it was found that the rate of de novo Chl formation, as well as synthesis of both photosystems is significantly enhanced in high light, despite the markedly reduced cellular level of PSI. Interestingly, the data suggests that there is no simple correlation between the actual synthesis of PSI and PSII core subunits and the synthesis and distribution of de novo Chl; de novo Chl was found to be predominantly directed to the PSI trimer, whereas the Chl binding PSII subunits seem to be mostly synthesized using the recycled Chl molecules previously released during Chl-protein degradation. From an organismal perspective it appears that the level of PSI needed for optimal photosynthetic performance at a given light intensity is reached by a precise equilibrium between the rate of cell proliferation, the rate of Chl biosynthesis and the distribution of Chl into individual Chl-proteins. Overall the data presented in this paper are of very high technical quality and clearly demonstrates highly developed experimental skills. The biological questions asked and answered in this work are rather complex as it entails molecular details in a dynamic physiological context and therefore also demonstrate excellent analytical skills.

***II. Kopečná J**, Sobotka R, Komenda J (2012). Inhibition of chlorophyll biosynthesis at the protochlorophyllide reduction step results in the parallel depletion of Photosystem I and Photosystem II in the cyanobacterium Synechocystis PCC 6803.*
Planta (DOI: 10.1007/s00425-012-1761-4) (IF = 3.000).

In this paper the light-dependent (LPOR) and light-independent (DPOR) ways to catalyze the reduction of protochlorophyllide to chlorophyllide was investiaged. The synthesis and accumulation of Chl-binding proteins in mutants of cyanobacterium Synechocystis PCC 6803 that either completely lack LPOR or possess low levels of the active enzyme due to its ectopic regulatable expression was investigated. Biochemical and spectroscopic methods were used to assess the effect on the cells. The LPOR-less mutant grew photoautotrophically in moderate light and contained a maximum of 20 % of the wild-type (WT) Chl level. Both Photosystem II (PSII) and Photosystem I (PSI) were reduced to the same degree. Accumulation of PSII was mostly limited by the synthesis of antennae CP43 and especially CP47 as indicated by the accumulation of reaction center assembly complexes as shown by BN gel electrophoresis in combination with immuno-blotting. The phenotype of the LPOR-less mutant was comparable to the strain lacking DPOR that also contained <25 % of the wild-type level of PSII and PSI when cultivated under light-activated heterotrophic growth conditions. However, in the latter case, no reaction center assembly complexes were detected, indicating that synthesis was almost completely inhibited for all Chl-proteins, including the D1 and D2 proteins. Based on finding in this paper it is excluded that the PSI/PSII ratio in Synechocystis in controlled simply by upregulation/downregulation of the Chl pathway, a model proposed in a recent review (Muramatsu and Hihara 2012). Instead it seems that the regulatory mechanism involve a selective delivery of de novo synthesized and reused chlorophyll according to actual demand. As above the data presented in this paper are of very high technical quality and underlines highly developed experimental and analytical skills.

III. *Hollingshead S,* ***Kopečná J****, Jackson PJ, Canniffe DP, Davison PA, Dickman MJ, Sobotka R, Hunter CN (2012) Conserved chloroplast open-reading frame ycf54 is required for activity of the magnesium-protoporphyrin monomethylester oxidative cyclase in Synechocystis PCC 6803. Journal of Biological Chemistry 287: 27823-27833 (IF = 4.773).*

In this paper the role of the Ycf54 protein in Chl biosynthesis is elucidated. The Ycf54 protein was identified in a tight complex with Mg protoporphyrin IX monomethylester oxidative cyclase - the enzyme catalyzing oxidative cyclization of the 'fifth' ring in the Chl molecule. Ycf54 is apparently required for the enzyme activity since its inactivation dramatically decrease Chl level as well as the step of the cyclase enzyme. Other enzymes of Chl biosynthesis both up- and downstream of the cyclase step are impaired suggesting a role in super-complex formation (a Chl metabolome). Jana Kopečná participated in FLAG-Sll1214 purification, immunodetection analyses and detection of Sll1214 mRNA in the Synechocystis strains by Northern blot analysis and thereby contributed to essential findings in this paper.

IV. *Komenda J, Knoppová J,* ***Kopečná J****, Sobotka R, Halada P, Yu J, Nickelsen J, Boehm M, Nixon PJ (2012). The Psb27 assembly factor binds to the CP43 complex of photosystem II in the cyanobacterium Synechocystis sp. PCC 6803.*
Plant Physiology 158, 476-486 (IF = 6.535).

In this paper the possible roles of the Psb27 protein, an auxiliary protein found in PSII complexes that lacks a functional Mn cluster. The Psb27 forms a complex with the PSII subunit CP43 and probably is also involved in the biogenesis of this subunit. However, deletion of the psb27 gene does not impair the PSII assembly or repair, but affects acclimation of Synechocystis cells to high light. Interestingly, it is also demonstrated Psb27 interact with PSI in a large supercomplex containing also PSII. For this paper Jana Kopečná participated in CP47-His and CP43-His purifications and analyses of the eluates by a 2D - clear native/denaturing electrophoresis.

Jana Kopečná's contribution to the last two papers is clearly stated by the supervisors on page V and there no doubt that her contribution is of high quality and the results obtained has helped to specifically advance our understanding the role of YCF54 and PSB27 and more generally to our understanding of organization of enzymes in the Chl biosynthtiec pathway and the assembly of pigment-protein complexes.

In conclusion, this thesis reports a substantial scientific contribution to our understanding Chl biosynthesis and its interaction with assembly/biogenesis of pigement-protein complexes. From the PhD thesis and published papers it is clear that Jana Kopečná has made significant and independent contributions to the field. In addition Jana Kopečná has organized her experimental work and collaborations in an efficient manner that has enabled her to generate good results that are published in excellent international peer reviewed journals.

Poul Erik Jensen,
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