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Referee comments on Ph.D. thesis

Title: The regulatory role of cyanobacterial High light inducible proteins

Ph.D. candidate: Mahendra Kumar Shukla

Ph.D. thesis aims to clarify the protective role of high light inducible proteins (Hlips) in cyanobacterium *Synechocystis sp.* PCC 6803. The high light inducible proteins were previously studied by the group and the current Ph.D. thesis provides deep insight into the understanding of their role. The focus is given predominantly on HliC (exists as pure oligomer) and HliD (forms complex with a putative alcohol dehydrogenase Ycf39). Ph.D. thesis comprises of well-organized introduction which summarizes the current knowledges on PSII biogenesis with the focus on de novo PSII assembly, PSII repair cycle, associated chlorophyll biosynthesis and synthesis of high light inducible proteins. Result section is based on five articles (three published in peer-reviewed journals and two manuscripts). Major contribution to understanding of the protective role of high light inducible proteins is finding that these proteins bind pigments (chlorophylls and β-carotene). Even if this fact was previously postulated in literature, sophisticated experimental evidence has not been provided. More detail specification shows that HliD binds two β-carotenes, one with highly twisted β-ring allowing more effective conversion of excess light to heat. In general, it is concluded that the topic of thesis is up-to-date and questions answered in the thesis are very important for the understanding of PSII protection against excess light.

Papers presented in Ph.D. thesis were published in peer-reviewed journals highly ranked in the field of plant biochemistry. One paper was published with the first authorship and other two papers as co-author publications. The Ph.D. student´s contribution to the published and submitted papers clearly stated by the corresponding author shows that Ph.D. student contributed significantly to the obtained results. His impressive contribution to our current ideas of how high light

inducible proteins protect PSII against high light reveals high scientific quality of Ph.D. candidate. As the most conclusions made in Ph.D. thesis were already published, I have no serious objections to the content of Ph.D. thesis. I have just few questions and comments for discussion related to submitted manuscripts:

1) Ph.D. thesis shows that singlet excited chlorophyll is effectively quenched in high light inducible proteins by β-carotene which converts excitation energy to heat. However, when singlet excited chlorophyll is not properly quenched, triplet excited chlorophyll is formed by intersystem crossing. It is well established that quenching of triplet excited chlorophylls by carotenoids occurs in green bacteria, algae and higher plants. Does triplet-triplet energy transfer from triplet chlorophyll to carotenoid and subsequent energy dissipation occur in high light inducible proteins?
2) It was proposed that effective quenching of singlet excited chlorophyll by β-carotene occurs due to decrease in S1 energy level caused by specific pigment-protein or pigment-pigment interaction. I might predict that more detail evidence on this interaction has not been provided yet. Can Ph.D. candidate briefly design experiment which might help to more specify these interactions?
3) Apart to single-helix high light inducible proteins, multiple-helix LHC-like protein were shown to play protective role of PSII against excess light. It is well accepted that four-helix PsbS protein has no capability to bind chlorophylls or carotenoids. Can authors explain why single-helix high light inducible protein binds pigments, whereas four-helix PsbS protein has no such capability?

Ph.D. candidate succeeded to learn a number of experimental methods, obtain scientific results of deep significance, interpret experimental data, discuss properly observed results and make conclusions. Thus, I recommend Mahendra Kumar Shukla for receiving Ph.D. degree.

Pospíšil

In Olomouc, 27th April 2018

Doc. RNDr. Pavel Pospíšil, Ph.D.

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Review on the doctoral thesis of Mahendra Kumar Shukla

Mr. Mahendra Kumar Shukla submitted a doctoral thesis entitled "The regulatory role of cyanobacterial High-light inducible proteins". The thesis is a cumulative dissertation, also defined as a thesis by publication or article thesis. It includes a self-written introductory part and contains five attached scientific manuscripts. Among these manuscripts, one is published with first authorship, two are published with co-authorship; a fourth manuscript with co-authorship is submitted and a fifth first author manuscript is apparently in a preliminary state and at present not submitted.

A cumulative thesis entails the difficulty to specify the own contribution of each author, here the doctoral candidate, to the work as a whole, the experimental part of the publications and the preparation of the manuscripts. In particular, the contribution of the doctoral candidate to the preparation of the manuscripts is difficult to judge. As confirmed by the supervisor, the survey about the doctoral candidate's contributions to five papers notifies only for one manuscript the involvement in the preparation of the manuscript. Therefore, an entirely autographic doctoral thesis would be more appreciated.

The aim of Mahendra Kumar Shukla's thesis was the analyses of functions of the High-light inducible protein (HLIP) family of cyanobacteria for the assembly of the photosynthetic complexes. To gain further information about the protein, the doctoral candidate's approaches were to unravel the specific functions of two of the four variants of cyanobacterial HLIPs by exploring the binding capacities of HLIPs with photosynthetic pigments, such as chlorophyll and carotenoids, and its interaction with other proteins, which are required for the assembly process of photosynthetic complexes or the transfer and association of pigments into the photosynthetic pigment-binding proteins. Therefore, the analysis of pigmented complexes consisting of HliD and the auxiliary factor Ycf39 and chlorophyll synthase, the last enzyme of chlorophyll synthesis are very substantial.

Datum:
23.04.2018

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In detail, the aims include the stepwise biogenesis of photosystem assembly, mainly of photosystem II, in which several factors are involved. The specific contribution of cyanobacterial Hlips were examined for this photosystem assembly. Thereby, intermediate complexes were identified in the formation of the photosynthetic complexes. Additionally, a series of interactions of Hlips with assistant proteins during this assembly process were described, which are required for the supply and transfer of chlorophyll to the photosynthetic complexes.

According to my estimates, it belonged to the main research contributions of the doctoral student to explore the diverse capacities of HliD and HliC for pigment binding. Moreover, it should be acknowledged that new insights were given to the HliC role upon stress conditions to support the organisation of the complex consisting of chlorophyll synthase and Ycf39, as a number of variants in protein complex formation was found. Moreover, as previous studies mainly focussed on the role of auxiliary factors to the assembly and association of different subunits of photosystem II, it is worth to mention that initial findings were introduced about the supportive involvement for and the protein interaction of HliC during the photosystem I assembly. All in all, these are substantial contributions to the function of cyanobacterial Hlips, however, it seems that these accomplishments are rather a sign of productivity of the entire research team.

The candidate performed experiments with recombinant Hlips and with cyanobacterial mutant strains. He applied biochemical methods (i.a. clear-native 2D gels, affinity chromatography, spectroscopic methods). He contributed material for more advanced spectroscopic analyses of the pigment-Hlip interactions. Apart from these experiments he masters the entire spectrum of experimental methods to cultivate and analyse cyanobacterial strains by molecular biology and genetics approaches.

Thus, the doctoral studies comprise a small, but fairly substantial role of two variants of the Hlip family in cyanobacteria. Considering the time frame between 2013 and 2018, it would be eligible to be more responsible in depth and completely for the scientific questions addressed, here the characterisation of one or both member(s) of the Hlip family. It would have been preferable to obtain an entire overview about a self-prepared complete discussion on the potential contribution of Hlips to the formation of photosynthetic complexes and chlorophyll metabolism. This discussion could include own or new figures and models and a more extensive reference to publications of other contributors. Moreover, to demonstrate the own overview about the entire subject area of the biogenesis of photosynthetic complexes, even a comparative discussion would be appreciated about the contributions and functions of Hlips and other auxiliary factors of the huge light-harvesting-like family as well as other chaperones, assistant/assembly or auxiliary factors for the co- and posttranslational assembly of photosynthetic proteins at the thylakoid membranes. In fact, at the end of the doctoral studies, the candidate could acknowledge the contributions of Hlips in context to these other assembly factors. This own debate could be embedded and completed in an own model for the entire processes of thylakoid biogenesis.

Thus, a broader discussion would be desirable and could be substantiated in the following doctoral defence and disputation. A few points can immediately be mentioned:

- What are the technical, the biological, the structural prerequisites to study photosystem assembly?
- What are potential experimental evidences for the chlorophyll recycling process? What is known about chlorophyll re-utilization?
- Hlip function as a monomer or homo/heterodimer? Molecular processes of the interaction and functional roles as a monomer with other proteins, such as the chlorophyll synthase of YCF39? Can the model be improved when only Hlip dimer formation has to be considered?

I declare without reservation, that the doctoral candidate fulfilled the requirements for successful doctoral studies and his doctoral thesis. I recommend the doctoral admissions committee to approve the submitted doctoral thesis.

Bernhard Grimm