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Characterization of High Light Inducible (Hli) Proteins of Chlorophyll-protein Complexes of Cyanobacteria

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Abstract. Stress Hli proteins (Hlips) of cyanobacterium *Synechocystis* sp. are necessary for the survival of cells and their adaptation to high light. Nonetheless, the whole picture of Hlips functioning and distribution in these cells is not completely understood and the data for other cyanobacteria such as *Arthrospira platensis* is missing. Studies of *Synechocystis* sp. have shown that HliA protein is associated with the main chlorophyll-protein complexes: with trimers and monomers of photosystem I and photosystem II complexes. According to the NCBI database, there are three *Hli* genes in *A. platensis* genome that encode proteins of 47, 64, and 69 amino acids long. In current study MALDI-TOF mass spectrometry analysis of *A. platensis* revealed presence of Hli 47 amino acids long only. Identified Hli protein is associated with photosystem I and photosystem II and is a homologue of HliC *Synechocystis* sp. Bioinformatics analysis of the amino acid sequence of the identified Hli protein of *A. platensis* revealed a high degree of homology with the amino acid sequences of proteins of a number of other multicellular cyanobacteria and a lesser degree with the Hli amino acid sequence of unicellular cyanobacteria. Results of current study confirms the importance of Hlips for the photoprotection of the photosynthetic apparatus of cyanobacteria.

Key words: high light inducible proteins, stress proteins, abiotic stress.

Introduction

Cyanobacteria are widely used as a model organism either in applied or basic research. Cyanobacterium *Synechocystis* and its transformed strains are used in biotechnology to produce various industrial chemical compounds (Gale et al., 2019; Pope et al., 2020). The photosynthetic apparatus of cyanobacteria and higher plants has a high similarity, which makes cyanobacteria a convenient model for studying plant photosynthesis. Light is essential for photosynthesis, but excess of absorbed light energy can disrupt photosynthetic apparatus and lead to photoinhibition and photodestruction. When absorbed energy cannot be fully used in electron transport reactions the content of singlet oxygen and other reactive oxygen species are increasing. Thus, photosynthesis and photoprotection are two interrelated processes.

Cyanobacteria must have the ability to acclimate to changing of light conditions. Protection mechanisms of photosynthetic apparatus of cyanobacteria under light stress can be divided into short-term and long-term processes. Short-term processes are associated with quenching of excited

chlorophyll molecules and energy migration in photosynthetic apparatus. These responses occur rapidly and are usually completed within several minutes. The long-term processes are associated with changes in gene expression of proteins that play an important role in cell defense responses, in particular against photo-oxidative damage. The long-term reactions are much slower and may take up to several days to complete. These processes involve changes in photosystem I (PSI) / photosystem II (PSII) ratio during adaptation to high light, synthesis of antioxidant enzymes and biosynthesis of light stress proteins: Elip (Early Light Induced Protein) in plants and Hlip (High Light Inducible Protein) in cyanobacteria.

Cyanobacterial Hli proteins are necessary for maintaining normal cell activity. Mutants with inactivated *hli* genes die from high light (Xu et al., 2004; Yurina et al., 2013). Intended functions of Hli proteins are the next: regulation of chlorophyll biosynthesis, transport and binding of free chlorophyll molecules, quenching of singlet oxygen, assembly and repair of PSII, non-photochemical quenching of absorbed light energy (Havaux et al., 2003; Yao et al., 2012). However, the main function of these proteins is still unclear.

Most studies of Hli proteins were performed on cyanobacterium *Synechocystis* sp. PCC 6803. Four *hli* genes were identified in the genome of *Synechocystis* sp. - *hliA, hliB, hliC* and *hliD* (Kilian et al., 2008; Yurina et al., 2013). It is known that: the synthesis of these proteins is increased by 3-4 times under light stress, Hli proteins are localized in the thylakoid membrane, they belong to the family of chlorophyll a/b binding proteins (LHC) and have a molecular weights about 10 kDa (Kilian et al., 2008). It was shown that stress Hli proteins of cyanobacterium *Synechocystis* are necessary for cell survival and adaptation in high light. However a lot of question about their structure, localization, functioning and role in wide range of cyanobacteria are remained unsolved. In current research the characteristics of high light inducible proteins of chlorophyll-protein complexes of cyanobacteria unicellular *Synechocystis* sp. and multicellular *Arthrospira platensis* were performed.

Material and Methods

Cells and growth conditions. The object of the study were the cyanobacterium *Synechocystis* sp. PCC 6803: wild-type cells, PSII-less mutant (ΔpsbDI, ΔpsbDII, ΔpsbC) and *Arthrospira platensis* IPPASB-256. Cells were cultivated in BG-11 medium (Akulinkina et al., 2015) at 28°C. The culture was bubbled with air under normal light conditions (40 μmol photons/m2 • s) and under stress conditions (150 μmol photons/m2 • s, 1 h). PSII-less mutant cells were grown at low light intensity (5 μmol photons/m2 • s) and supplemented with 5 mM glucose, and antibiotics (chloramphenicol 20 mg/ml, erythromycin 15 mg/ml, spectinomycin 20 mg/ml).

Isolation of thylakoid membranes. Thylakoid membranes of *Synechocystis* sp. and *A. plathensis* were isolated as described by (Shubin et al. 1993) with modification. Briefly, the cells were disrupted using a French-press (three times for each sample) in a medium A (50 mM MOPS, pH 7.0; 0.4 mM sucrose; 10 mM NaCl; 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged consistently at 5000 g for 10 min and at 50 000g at 4°C for 60 min. The membranes were resuspended in thylakoid buffer A to a chlorophyll a concentration of 1 mg/ml. To fractionate the chlorophyll-protein complexes, 10% dodecyl maltoside was added to the thylakoid membranes to achieve a detergent to chlorophyll ratio of 15:1. The membrane was solubilized at 4°C for 30 min. After incubation the lysate was centrifuged at 18 000 g for 10 min. The supernatant was subjected to Clear Native PAGE (CN PAGE) and SDS-PAGE.

SDS-PAGE. Proteins were fractionated by 12,5% SDS-PAGE. Protein concertation in

the sample was determined by the method of Bradford (1976). Before applying the samples heated at 95^0C for 10 min and then centrifuged at 18 000 g for 10 min. SDS-PAGE was performed as described (Akulinkina et al., 2015).

Two-dimensional electrophoresis and MALDI-TOF protein identification. Proteins were fractionated using two-dimensional electrophoresis: in the first direction CN PAGE was used, in the second direction - 12.5% SDS-PAGE (Wang et al., 2008). Mass spectrometry MALDI-TOF was used for proteins identification. Protein identification was performed using the Mascot program (www.matrixscience.com). The mass spectra were processed using the FlexAnalysis 3.3 software package (Bruker Daltonics, Germany). MALDI-TOF analysis was performed in the center for collective use "Industrial biotechnologies" Research Center of Biotechnology of the Russian Academy of Sciences.

The photochemical activity of PSI was determined as described earlier (Wang et al., 2008). The data presented are mean values from at least three independently grown cultures and experiments. Error bars represent standard errors of mean values. The statistical assessment of the results was performed using the Student's t-test.

Results and Discussion

One of the nowadays-discussed questions of *Synechocystis* biology is which of the chlorophyll-protein complexes in thylakoid membranes is associated with HliA protein? Conflicting data of localization of Hli proteins in the chlorophyll-protein complexes photosystem I and photosystem II were published (Wang et al., 2008; Komenda, Sobotka, 2016). The fractionation of thylakoid membrane of wild-type *Synechocystis* cells using CN PAGE/SDS PAGE was performed. It revealed the next fraction: PSI trimers, and monomers, PSII dimers, and monomers. Western blot analysis revealed the presence of HliA protein in fractions containing PSI trimers, PSI monomers, and PSII complexes that is consistent with previously obtained by us data (Akulinkina et al., 2015). To check the conflicting published data on the association of Hli proteins with PSI, a mutant lacking PSII complex was used. CN PAGE/SDS PAGE with Western blotting of mutant cells reveled that HliA protein was associated with the fraction of PSI complex.

For understanding the role of HliA in functioning of PSI *Synechocystis* the wild-type and ΔPSII mutant cells were used. Grown under low light ΔPSII mutant cells accumulates HliA protein meanwhile in wild-type cells Hli A is absent under these conditions. Both cell types were cultivated in low light and methylviologen-dependent oxygen absorption was measured. It was detected that the electron transport rate through the PSI in ΔPSII mutant cells was significantly higher than in wild-type cells grown under the same conditions (Fig. 1). The photochemical activity of the PSI complex in the membranes of a ΔPSII mutant with HliA protein was three to four times higher than in the membranes of wild-type cells without HliA protein. This data indicates that HliA protein is necessary for optimal PSI activity. The results on the low rate of photochemical absorption of PSI oxygen in the membranes of wild-type cells that contain almost none of them were consistent with previously published (Wang et al., 2008).

Numerous protective mechanisms have been developed in photosynthetic organisms in evolution for adaptation under light stress. Transition from single to multicellular organization cells could develop additional approaches of adaptation to light conditions. As compared to unicellular *Synechosystis* the multicellular *A. platensis* has another mechanism of photoprotection due to presence of long-wave chlorophyll (Karapetyan et al., 2014). It is also interesting that the spectral characteristics of chlorophyll-protein complexes of trimers and monomers of PS I *A. platensis* differ from

Synechocystis. Long-wave forms of chlorophyll were found in the thylakoid membranes of multicellular cyanobacterium *A. platensis* (Karapetyan et al., 2014). Probably, this may indicate the presence of specific features in the mechanism of protection of *A. platensis* from light stress. Long-wave chlorophyll with a fluorescence band at 760 nm was also found in other multicellular cyanobacteria - *Pseudoanabaena* sp., *Phormidium uncinatum* and *Nostoc muscorum*, but not in unicellular cyanobacteria *Synechocystis* sp. and *Synechococcus elongatus*, often used for research in the physiology and biochemistry of photosynthesis (Gobets et al., 2001). Apparently, this may indicate the presence of features in the mechanism of protection *of A. platensis* and other filamentous cyanobacteria from light stress.

In current study association of chlorophyll-protein complexes of thylakoid membranes with Hli proteins *Arthrospira platensis* were investigated. According to the NCBI database, there are three Hli genes in *A. platensis* genome that encode proteins of 47, 64, and 69 aa (amino acids) long. Clear Native PAGE and two-dimensional electrophoresis followed by mass spectrometry MALDI-TOF were used to determine the association of Hli proteins with chlorophyll protein complexes of thylakoid membranes. The identification of all colored protein spots on 2D-electrophoregram was performed. On Fig. 2 identified low-molecular-weight protein are shown. In our study MALDI-TOF mass spectrometry analysis of *A. platensis* proteins fractionated by two-dimensional PAGE revealed presence of Hli 47 aa long only (Fig. 2). It was observed that the identified Hli protein is associated with PSI and PSII complexes.

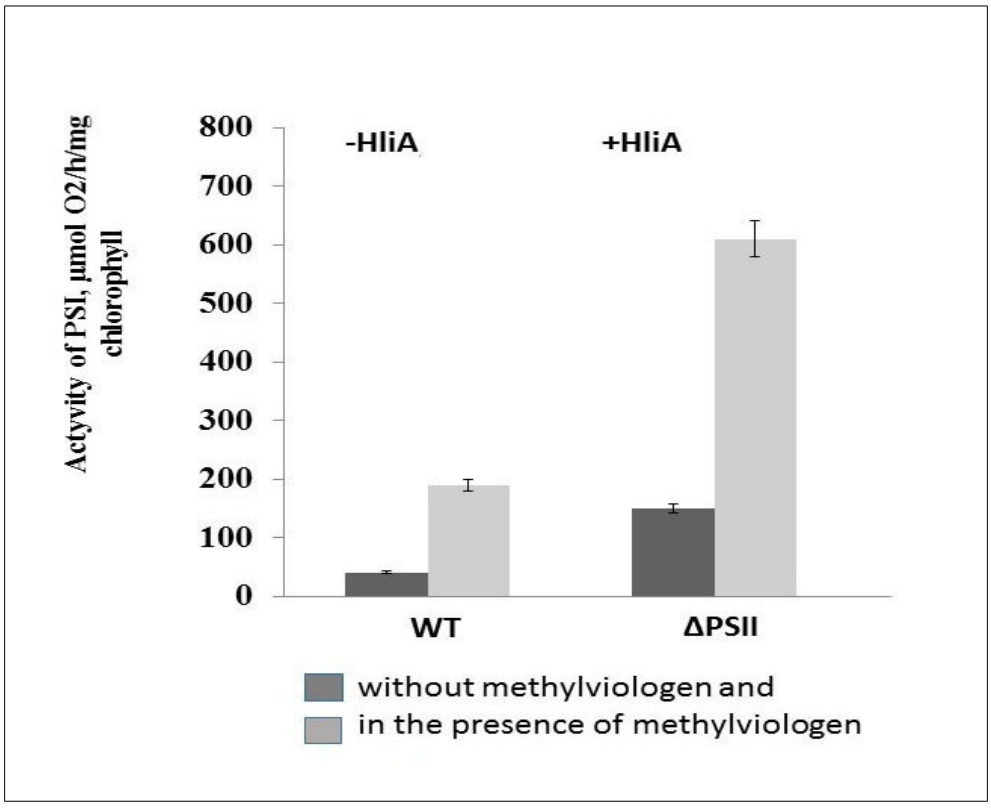

Fig. 1. Photochemical activity of PSI complex of thylakoid membranes of *Synechocystis* sp. wild-type (WT) cells and mutant without PSII in the absence and in presence of methylviologen.

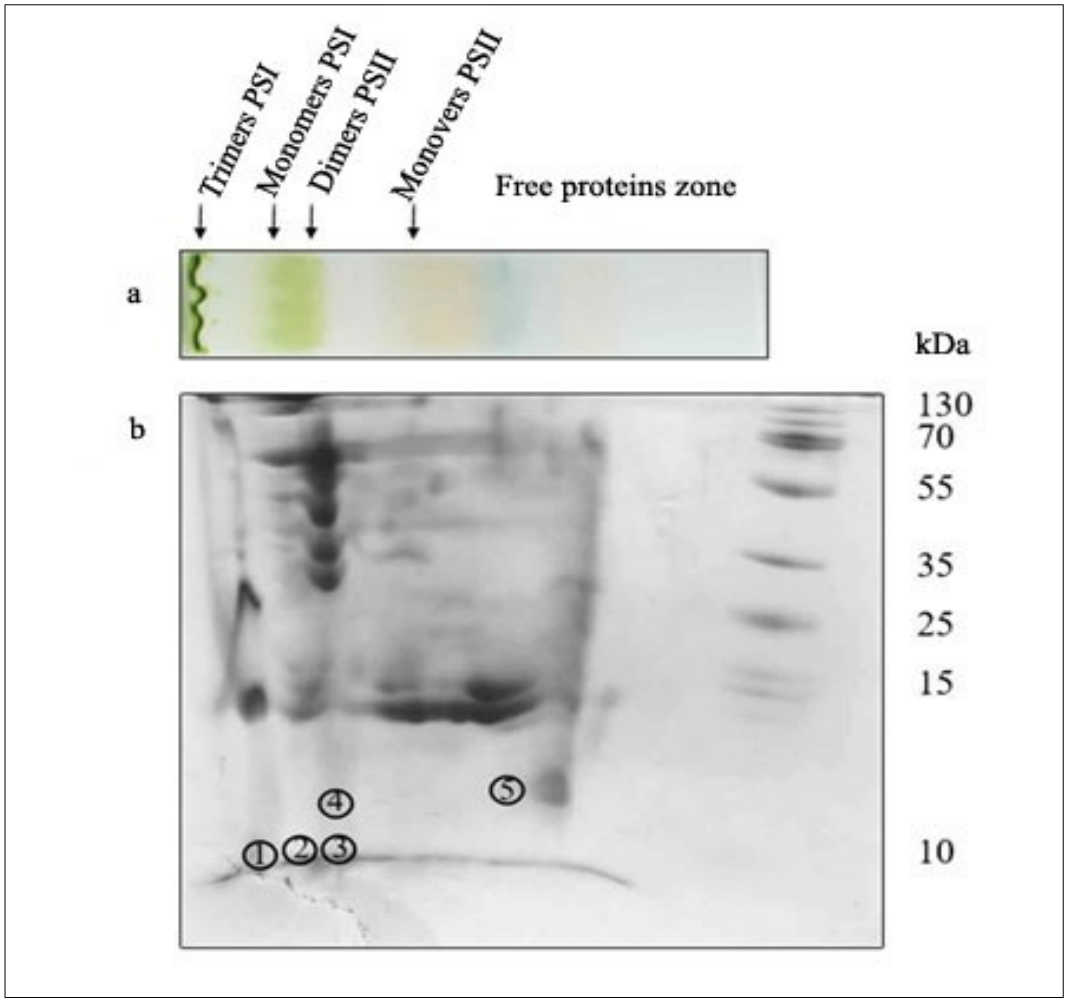

Fig. 2. 2D-electrophoresis of thylakoid membrane proteins of *A. platensis* after light stress: (a) PAAG under native conditions of chlorophyll-protein complexes; (b) SDS-PAGE of thylakoid membrane proteins (1-5 – protein spots identified by MALDI-TOF mass spectrometry): 1-3- Hli 47 aa proteins; 4 – Psb27 protein of PSII; 5 – alpha subunit of phycocyanin.

Bioinformatics analysis of amino acid sequence of identified Hli 47 aa protein of *A. platensis* revealed a high degree of homology with amino acid sequences of proteins of a number of other multicellular cyanobacteria and a lesser degree with the Hli amino acid sequence of unicellular cyanobacteria. Alignment of amino acid sequences of Hli proteins of different species revealed a conservative region corresponding to transmembrane helix (Fig. 3). The aa (Glu, Asn, and Arg) presumed to be involved in chlorophyll binding (Funk et al., 2011) were located. This allows as to conclude that identified Hli 47 aa protein *A. platensis* is homologue of HliC protein *Synechocystis* sp. (Fig. 3).

Structural model of low-molecular light-induced protein Hli 47 aa *A. platensis* was calculated basing on the crystal structure of photoprotective protein PsbS of spinach (Fig. 4). The structural data pointing out that at least four chlorophyll molecules and two β-carotene molecules can bind to the HliC protein and to its homologue Hli 47 aa. Hydrophobicity profile shows that identified Hli 47 aa protein is a membrane protein.

Fig. 3. Alignment of amino acid sequences of Hli proteins of *Synechocystis* sp. and *A. platensis* using the UniProt program.

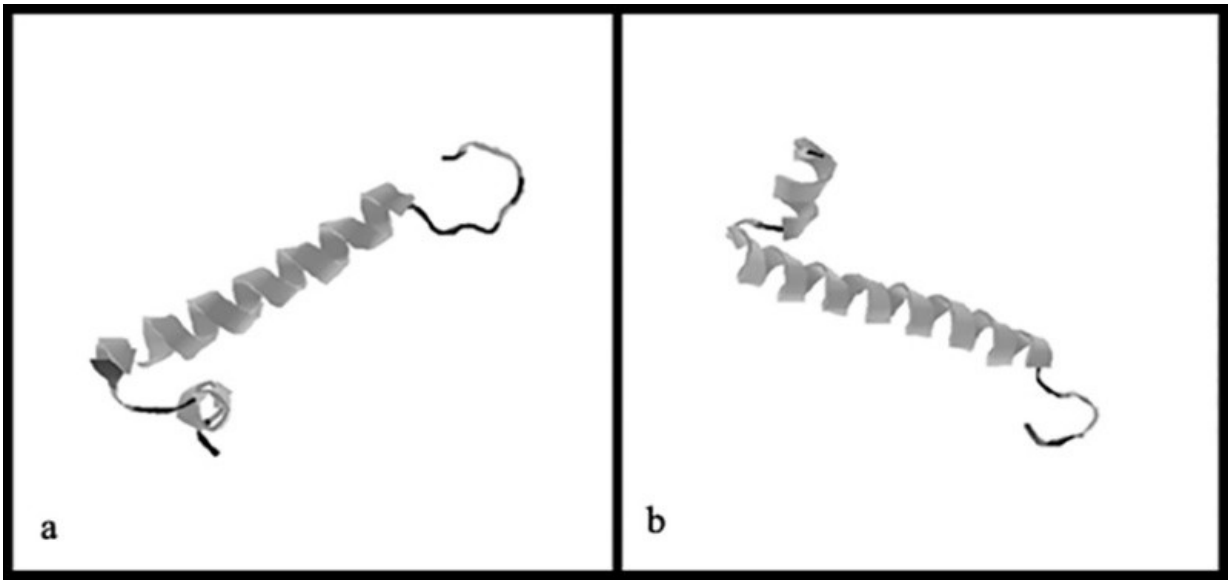

Fig. 4. Structural model of low-molecular light-induced protein Hli 47 a.a. of *A. platensis* in different projections. The model is calculated basing on the crystal structure of the photoprotective protein PsbS of spinach (*Spinacia oleracea*). (a) side view along the plane of the thylakoid membrane, (b) view from the side of the stroma. RCSB PDB database website.

Conclusions

It is shown that light-induced protein HliA *Synechocystis* sp. PCC 6803 is associated with photosystem I complex, as well as with photosystem II complex and is necessary for optimal photochemical activity of PSI cells of cyanobacterium.

High light-induced protein Hli 47 a.a. *Arthrospira platensis* was first identified. This protein was found to be associated with photosystem I and II under light stress.

Comparative analysis of Hli proteins of cyanobacteria *A. platensis* and *Synechocystis* sp. revealed the structural and functional

homology of Hli 47 a.a. *A. platensis* and HliC *Synechocystis* sp.

Results of current study involving data of association of Hli proteins with complexes of photosystems I and II, their necessity for optimal photochemical activity, as well as the presence of chlorophyll-binding domains, indicate the importance of these proteins for the photoprotection of the photosynthetic apparatus.

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