Scientific report

*regarding the implementation of project "**Functional diversity of D1 proteins in photosystem II in cyanobacteria**" code PN-II-ID-PCE-2011-3-0765 during January-December 2014*

Following the previous implementation stages of the project, the initial phase in 2011, when the project was started, the one in 2012 when the studies on the functional characterization of D1 protein were done and the phase in 2013, when studies regarding the functional characterization of D1 protein in different cyanobacteria communities were started, in 2014 we:

- Continued the studies regarding the characterization of photosystem II functionality in model cyanobacteria cultures, which have a completely sequenced genome; in this respect, we identified a reporter gene specific to *Synechococcus sp*. PCC 7002 cyanobacteria strain, and we also searched for new aspects of the electron transport chain in this specific strain.
- Continued the studies regarding the functional characterization of D1 protein forms from different cyanobacteria communities with the purpose of establishing the optimal environmental conditions for expressing the different isoforms of D1 protein.
- Continued the studies regarding specific modifications in the primary structure of D1 protein, characteristic for different isoforms of the protein.
- As a result of these experiments, a large volume of experimental data was collected, which allowed us to elaborate and publish 3 articles in different scientific journals, ISI (1 article) and BDI (2 articles) rated. Other articles are in the process of writing. A synthesis of these date are presented below.

1. Results of the expression of *psbA* genes which codify different isoforms of D1 protein

Synechococcus sp. PCC 7002 is a unicellular cyanobacteria strain, photoheterotroph, euryhaline, tolerant to a wide range of light intensities, to oxidative stress exposure and temperature changes. In the experiments elaborated with our collaborators from The Institute of Biological Research in Cluj-Napoca, we investigated the selection of some reference genes for this cyanobacteria, using quantitative PCR experiments. For this purpose, 6 reference genes were analyzed. The cyanobacteria cultures were exposed to different forms of stress, then the data was processed using 3 different algorithms, resulting in a list of reference genes which can be used in quantitative PCR experiments. In quantitative PCR reactions, many factors can affect the results, which requires their standardization in order to reveal significant modifications at the mRNA level (Nolan and colab., 2006). In order to facilitate the comparison of transcript concentrations among different samples, a normalization of the data is also required. The most used method is the normalization of an endogenous reference gene, as both the gene of interest and the reference gene are quantified during the same PCR amplification reaction. Thus, through these experiments we evaluated the stability during expression of some reference genes in *Synechococcus sp*. PCC 7002 under the influence of 3 types of stress (microaerobiotic conditions, incubation in dark and UVB). The reference genes were selected based on previous studies which employed this cyanobacteria strain in PCR experiments. PCR reactions were performed, with the results obtained from IQ5 system from Bio-Rad, the values of gene expression were calculated. The data were normalized and we concluded that there are 4 reference genes which can be used for expressing *Synechococcus sp*. PCC 7002 in different environmental conditions. These results were published in the scientific article "**Selection of proper reference genes for the cyanobacterium *Synechococcus* PCC 7002 using real-time quantitative PCR- FEMS Microbiol Lett 359 (2014) 102–109**".

2. Fluorescence and immunodetection measurements of D1 protein in different experimental conditions

In these studies, we used a mutant *Synechococcus* sp. PCC 7002 strain which has an inhibited gene that codifies D1' isoform of D1 protein, by comparison with the normal *Synechococcus* sp. PCC 7002 strain which has 3 genes that codify different isoforms of D1 protein. The mutant strain was a gift from our collaborators at the University of Berlin. The purpose of these experiments was understanding the role of D1 protein isoforms in different environmental conditions. We tried to understand the nature and origin of

the modifications we observed in the fluorescence curves, as well as the linear electron transfer in cyanobacteria. The standard growing conditions were: light intensity 50 µE x m^{-2} x $s^{-1}$and 38°C. The light stress was applied for 120 minutes, followed by a return to the initial light conditions for 60 minutes. For another culture, the initial light conditions were replaced with UVB radiation for 120 minutes, followed by a return to the initial light conditions for 60 minutes. In order to induce microaerobiotic conditions, N_2 was added to the culture for 120 minutes, while during the 60 minutes recovery period, air was added. The chlorophyll fluorescence measurements were performed in both the absence and presence of DCMU ((3-(3,4-dichlorophenyl)-1,1-dimethylurea), a herbicide which blocks the electron transfer between Q_A and Q_B and allows the visualisation of the donor part of the electron transport chain functionality in photosynthesis. The measurements were done using a FL3500 fluorometer. Also, samples for the isolation of nucleic acids and proteins were taken.

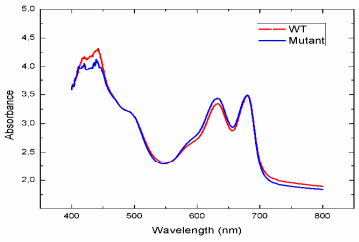

Figure 1: The total absorption spectrum of the cell in normaland mutant *Synechococcus sp*. PCC 7002 strains.

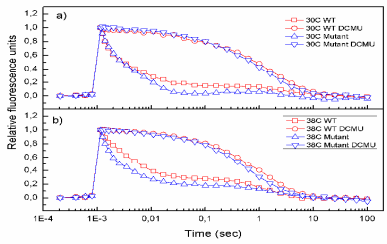

Figure 2: Q_A reoxidation measured through chlorophyll fluorescence in presence and absence of DCMU in normal and mutant *Synechococcus sp*. PCC 7002 strains.

It can be observed that during normal growth, the mutant strain shows an accelerated transfer between Q_A and Q_B, modification emphasized especially at 38ºC (Figure 2) and also that the mutant strain presents a high ratio between chlorophyll and phycobilisomes (Figure 1), which suggest a higher light energy harvesting capacity. From Figure 3 and 4, it can be observed that there are no special differences between normal and mutant cells durin treatments with low oxygen concentrations and UVB stress.

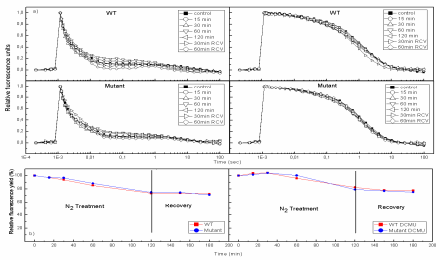

Figure 3: The effect of microaerobiotic conditions on the donor and acceptor parts of PSII in normal and mutant *Synechococcus sp*. PCC 7002 strains.

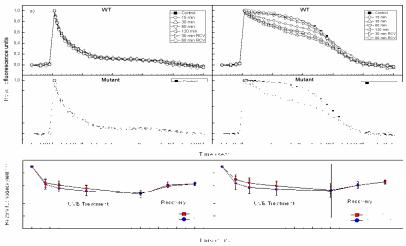

Figure 4: The effect of UVB stress on the donor and acceptor parts of PSII in normal and mutant *Synechococcus sp*. PCC 7002 strains.

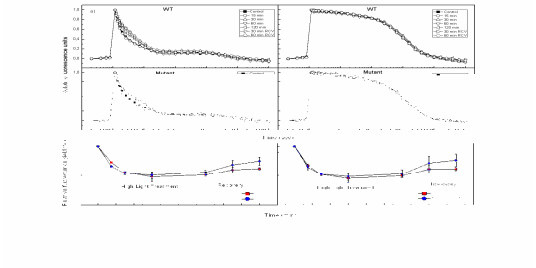

Figure 5: The effect of high light intensity stress on the donor and acceptor parts of PSII in normal and mutant *Synechococcus sp*. PCC 7002 strains.

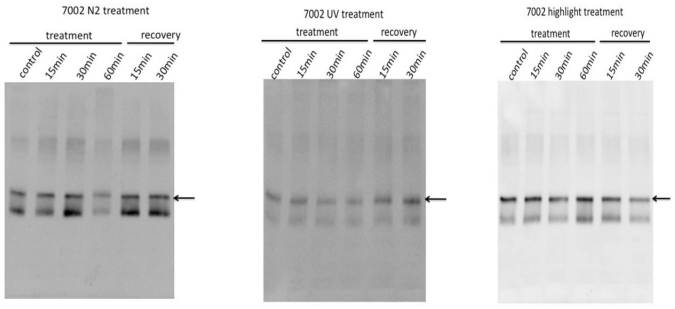

Figure 6: D1 protein detection using a global anti D1 antibody in *Synechococcus sp*. PCC 7002 during microaerobiotic, UVB and light treatments.

In the case of light stress experiments, a higher recovery capacity for the mutant strain cells can be observed after 120 minutes of treatment, in both presence and absence of DCMU (Figure 5). Regarding the proteomic analyses, an increase of the total D1 protein quantity during the recovery period can be observed, an increase which is specific to the post stress cell reaction. These results were presented at the IXth European Workshop in Cyanobacteria Molecular Biology, a prestigious international symposium which was held in Holand in September, we presented our poster intitled „**New Insights in PSII Electron Transport Chain in Cyanobacterium *Synechococcus* sp. PCC 7002**". We mention that, due to budget restrictions in 2014, the payment for our participation was acquired from other sources than the present project.

This year, we also continued the studies regarding the influence of the circadian rhytm on PSII in some model cyanobacteria species. In this respect, we continued the chlorophyll fluorescence measurements for *Cyanothece sp.* ATCC 51142 cyanobacteria strain at every 60 minutes for 26 hours, both in presence and absence of DCMU. Also, some fluorescence measurements in the presence of lincomycin (300µg/ml), an inhibitor which blocks the synthesis of new proteins, were performed, using a FL3500 fluorometer from Photon Systems Instruments, with a Q_A^- reoxidation protocol, then the data were processed in Origin.8, but only after applying a Joliot correction on each curve. Samples for the isolation of nucleic acids and proteins were also taken.

Thus, it can be observed that during dark conditions, the acceptor part of PSII is modified due to a slower transfer between Q_A and Q_B, (Figure 7), modification more obvious in the centres which have the Q_B locus free during light impulse. The appearance of a rapid phase on the fluorescence curve in the presence of DCMU (Figure 8) during dark phase suggests an inhibition of the water oxidation complex, modification which is reversed at approximately one hour from the start of the light phase. The lincomycin treatment, applied 80 minutes before the dark phase began, reduced the observed modifications both in the donor part as in the acceptor part of PSII, but didn't change significantly the evolution of the active centres of PSII. This suggests that, at least partly, the modifications observed during dark period are owed to the *de novo* protein synthesis.

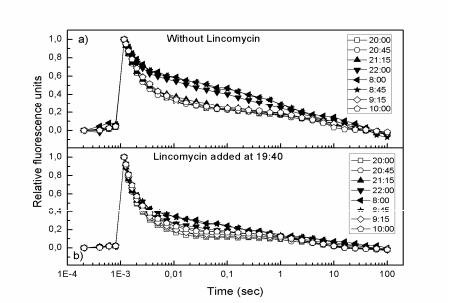

Figure 7: Modifications in the acceptor part of PSII during 12h light/12h dark cycle in *Cyanothece* sp. ATCC 51142 cyanobacteria, in absence (a) and presence (b) of lincomycin.

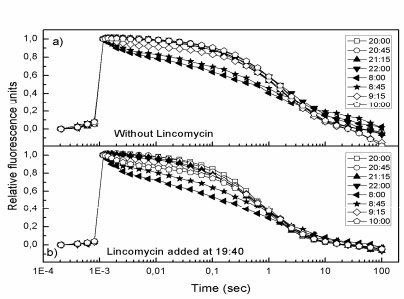

Figure 8: Modifications in the donor part of PSII during 12h light/12h dark cycle in *Cyanothece* sp. ATCC 51142 cyanobacteria, in absence (a) and presence (b) of lincomycin.

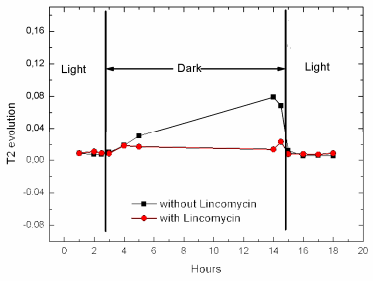

Figure 9: The evolution of T2 parameter in the acceptor part of PSII during 12h light/12h dark in *Cyanothece* sp. ATCC 51142 cyanobacteria, in presence and absence of lincomycin.

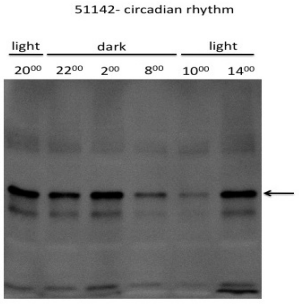

Figura 10: Modifications in the total D1 protein quantity during a 12h light/12h dark cycle in *Cyanothece* sp. ATCC 51142 cyanobacteria.

Using a general antibody for the D1 protein, significant changes in the total D1 protein quantity can be observed during light/dark cycle (Figure 10), modifications which correlate with the changes observed

on the fluorescence measurements. These results were presented at the IXth European Workshop on Cyanobacteria Molecular Biology, a prestigious international symposium which was held in Holand in September, where we participated with the poster „**Changes In Photosystem II (PSII) Function During Dark and Light Cycles In Cyanobacterium *Cyanothece* sp. ATCC 51142"**. We mention that, due to budget restrictions in 2014, the payment for our participation was acquired from other sources than the present project.

3. Agarose gel pictures representing the isolation of DNA from medium samples

For this purpose, the DNA extraction protocol was optimized for the cyanobacteria communities at the Ciocaia hot spring in Bihor district.

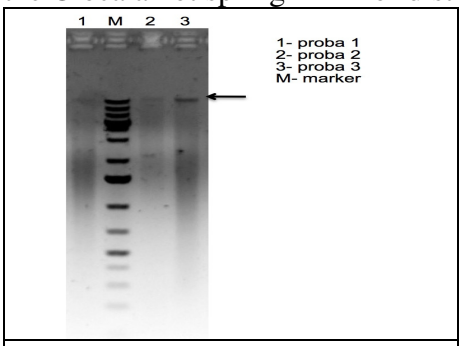

Figure 11: Agarose gel with the samples from Ciocaia, for optimizing the DNA extraction protocol.

Different cyanobacteria culture quantities were employed for DNA extraction (50 and 100mg). DNA extraction was performed using Isolate II Plant DNA kit (Bioline). The three DNA samples obtained were quantified using the Nanodrop (Thermoscientific) spectrophotometer and they were subjected to electrophoresis in agarose gel (Figure 11), in view of checking the obtained DNA. The electrophoresis bands corresponding to the three DNA samples confirm the different concentrations obtained according to the spectrophotometric quantification, which are as follow: sample 1- 18.3ug/ul (50mg starting material, 3 sec. sonication), sample 2- 21.4ug/ul (50mg starting material, 8 sec sonication), sample 3- 40ug/ul (100mg starting material, 8 sec sonication). It can be concluded that the DNA extraction efficiency increases with the quantity of vegetal material and the sonication time. The quantity of vegetal material and the sonication time used for sample 3 in our experiment represent the optimal conditions for isolation of cyanobacteria DNA without substantial decomposing of the isolated DNA. Next step will target the optimization of the conditions for PCR with corresponding primers for the amplification of important gene fragments.

4. Chlorophyll fluorescence measurements which emphasize the presence of different D1 protein forms in samples from the environment.

This year, several trips to the hot spring of Ciocaia were made, in view of monitoring the cyanobacteria communities found there. We mention that one of these trips was funded using money from this project.

Also, preliminary monitoring studies of the hypersaline lakes from Transylvania in view of highlighting the bacteria communities established in this type of ecosystem, were started. The hypersaline lakes are a niche ecosystem which presents a peculiar diversity of cyanobacteria strains that can present interesting conformations of the D1 protein forms in PSII. Below, we shortly present a description of the experiments performed at Ciocaia.

The hot spring at Ciocaia, Bihor district, presents a variety of temperatures, from 65.5°C at the spring, 60.5°C 2 meter from the spring and between 32-38°C in the area where cyanobacteria communities form. It can be observed that very close to the spring, at the highest temperature, a white bacteria community developed, followed by a red and a green one, at lower temperatures. Thus, we made a comparative study of the modifications of PSII at ,the communities which grow around the hot spring and the communities grown in the lab. The lab culture was maintained at a light intensity of 50 $\mu E \times m^{-2} \times s^{-1}$ and 38°C, in BG11 medium, while air was added continuously. The microscope analysis of the culture showed that in the microbial communities at Ciocaia there is a *Phormidium sp*. Strain, which is used as control in the fluorescence measurements.

After optimizing the protocol for measuring the chlorophyll fluorescence outdoor, it can be observed that in the case of green cyanobacteria communities (Figure 12 panel a) there is fluorescence curve simmilar to the control curve, showing a typical Q_A^- reoxidation curve. In this case, a slowdown of the transfer between Q_A and Q_B can be noticed, and the F_M value is close to 1.2.

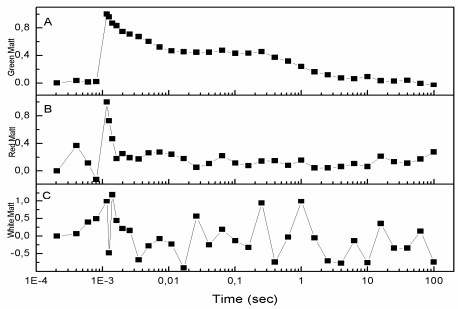

Figure 12: Modifications in the acceptor part of the electron transport chain in PSII at the green (a), red (b) and white (c) cyanobacteria community from the Ciocaia hot spring.

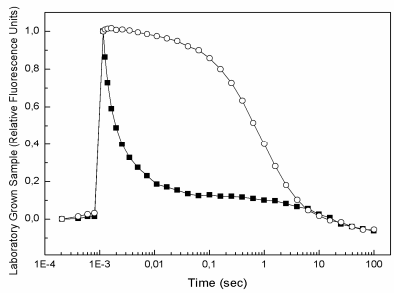

Figura 13: The donor and acceptor parts functional characteristics measured in the absence (black square) and the presence of DCMU (circle) for the culture taken from Ciocaia and grown in the lab.

In the case of the red community (Figure 12 panel b), a clear separation of the charges can be observed at the saturation impulse moment, showing the formation of Q_A^-. Nevertheless, the Q_A^- reoxidation is strongly disturbed, without the typical characteristics of a fluorescence curve. This activity is correlated with the organisms which do not realise a typical photosynthesis process, but rather a bacteriochlorophyll-dependent photosynthesis. In the case of the white community (Figure 12 panel c), no answer to the light impulses is observed.

The *Phormidium sp*. culture from the green cyanobacteria community, grown in the lab, presents a quicker electron transfer between Q_A și Q_B, according to a normal functioning of the acceptor part of PSII. Also, regarding the DCMU curves, they don't change, suggesting a standard donor part of PSII in this cyanobacteria. The fact that, at the lab sample, the fluorescence curve is faster than the outdoor measurements, indicates a deterioration of the electron transfer which normally appears when cells are exposed to light. These are the first measurements of the electron transport chain at a hot spring, a very important observation, considering that most experiments are performed in lab conditions which pretend to mimic environmental conditions. More information regarding these dat were published in an article called „**Forward Electron Transport Measured In Situ In Microbial Mats From A Hot Spring in N-W Romania**" in "Studia Universitatis Babes-Bolyai" journal.

Also, this year we continued our bioinformatics studies in order to synthesize the existing data in the databases regarding the key modifications in the primary structure of different D1 protein isoforms. Furthermore, we presented a possible functional distribution of D1 protein isoforms, based on the accumulated knowledge, and we made an improved and complete Venn diagram, which clearly shows the distribution of D1 protein isoforms in the cyanobacteria strains.

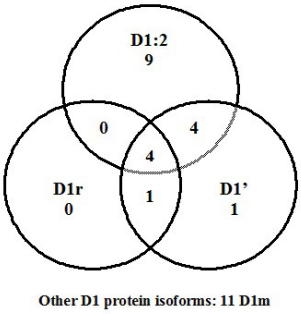

Figure 14: Venn diagram presenting the D1 protein isoforms distribution in the studied cyanobacteria.

We can therefore notice that, from a total of 30 studied cyanobacteria genomes, 4 strains have all 3 isoforms (D1:2, D1' and D1r) of D1, 17 genomes have isoform D1:2, from which 9 genomes possess only this isoform, 4 genomes possess isoforms D1:2 and D1', while 3 genomes contain all the isoforms. The data presented here bring an original interpretation of the existent information regarding the *psbA* gene family and the D1 proteins codified by them, available from the cyanobacteria genomes sequenced so far. The results we obtained were published in an article called **"Comparative Analysis of D1 Protein Sequences in Cyanobacteria"** in "Studia Universitatis Babes-Bolyai" journal.

Project director,
C.S.I Dr.Cosmin Ionel Sicora

Date: 24th November 2014

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