Summary of the Ph.D. thesis

HEAVY METAL AND SINGLET OXIGEN SENSING IN *SYNECHOCYSTIS* PCC 6803 CYANOBACTERIA

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INTRODUCTION

The experimental model organism of my thesis is the *Synechocystis* sp. PCC 6803 (Synechocystis), which is a blue-green bacterium, also known as a cyanobacterium. *Synechocystis* is a single-celled, freshwater, oxygen-producing photosynthesis, photoautotrophic Gram-negative bacteria, which is the only known prokaryotic strain whose members are able to carry out oxygen-producing photosynthesis. Thanks to its small genome and easy handling experienced during molecular biological and other physiological measurements, it receives a great attention in basic and applied research.

Cyanobacteria are able to respond to many environmental stressors and pollutants with specific gene expression changes, which is the main reason why they use them to create bioreporters. The term "bacterial bioreporter" has gradually become equivalent to genetically modified bacteria in which one or more regulatory networks are created to produce recognizable and quantifiable results.

Various metal-sensing bacterial systems have been used several times to produce whole-cell bioreporters. In the genome of the cyanobacterium *Synechocystis*, there is a group of genes involved in zinc, cobalt and nickel homeostasis. The promoters of these special heavy metal-responsive genes can be used to produce whole-cell heavy metal bioreporters by cloning them in front of reporter genes. However, this promising topic presents many challenges. It is extremely difficult to find genes that can specifically and with high sensitivity detect the given heavy metal pollution, even in a slightly polluted environmental sample. Because of this, it is very important to improve the sensitivity of biosensors, which based on genes that have already been discovered and have a well-known role, in order to detect low, but still harmful to health, concentrations of heavy metals in an environmentally friendly way with the possibly greatest sensitivity and selectivity.

In addition to heavy metals, other substances can also be detected with whole-cell bioreporters. This can be the singlet oxygen, which is a very strong ROS and its signaling pathway in *Synechocystis* is unclear.

Singlet oxygen easily oxidizes different molecules, therefore it is cytotoxic. Its formation is inevitable during photosynthetic processes, which can be formed by the type II light-dependent photodynamic reaction. As part of this process, the absorbed photon can excite

a singlet-state pigment, such as chlorophyll, and convert it into triplet-state chlorophyll after an interconversion.

In the last decade, the recognition that singlet oxygen can participate in signal transmission pathways and can affect gene expression has received more attention.

However, the monitoring of this process is very difficult, since the continuous *in vivo* monitoring of intracellular singlet oxygen within the cells is impossible with current detection techniques. Whole-cell singlet oxygen bioreporters, which require promoters of specific singlet oxygen-sensitive genes, can be a solution to this. Such reporter cells would make it possible to monitor the signaling relationships of singlet oxygen within the cell, which would allow us to learn more about the role of singlet oxygen as a signaling molecule in cyanobacteria.

AIMS

Our work was determined by two main topics, which were connected by one point: the development of whole-cell bioreporters in *Synechocystis* PCC 6803 cyanobacterial cells.

1. One of our main research topic is to increase the sensitivity of heavy metal (HM) biosensor strains. Our working hypothesis was that by inactivating the transporters that remove heavy metals from the cells, the heavy metal concentration inside the cells can be increased, which can amplify the gene induction response by heavy metals.

2. Our other main objective was to study the role of singlet oxygen ($^1O_2$) as a signaling molecule in *Synechocystis*. For this, our goals included the identification of genes specifically induced by $^1O_2$. We plan to phenotyping these genes with deletion mutants, and develop $^1O_2$-specific whole-cell biosensor strains using their promoters, which enables the continuous detection of intracellular $^1O_2$ *in situ* and *in vivo*.

MATERIAL AND METHODS

Investigation of the growth conditions of the applied cyanobacterial strains and the effects of heavy metal salt treatments

During our experiments, we worked with the wild-type PCC 6803 *Synechocystis* cyanobacterial strain (WT), as well as the HM cluster-deficient Δ*nrsSRBACD*: Δ*coaRT*: Δ*ziaRA* (NiCoZia for short) strains created by us. In addition, we created the Δ*hliB* strain, in which the *hliB* (*ssr2595, SGL_RS16470*) gene was deleted, as well as several luminescent biosensor strains creted by us, which are based on the use of the pILA promoter probe vector (Kunert et al., 2000). *Synechocystis* cells were grown in photoautotrophic 3% CO_2-containing atmospheric conditions, 30-40 μmol photon m^{-2} s^{-1} white light and 30 °C temperature, in BG-11 (Rippka et al., 1979) liquid media.

Escherichia coli DH5α strain was used for routine DNA manipulations and creation of plasmid constructs. *E. coli* cells were grown in Luria broth (LB) media at 37 °C (J. Sambrook, D.W. Russell, 2001).

The heavy metal salt treatments were performed in a 96-well black, low autofluorescence (Perkin-Elmer Opti-Plate) cell culture plate at 25°C; in an atmosphere with normal CO_2 content, under illumination of 40 μmol photon m^{-2} s^{-1}. Each well contained 200 μL of log-phase cyanobacterial cell culture (OD_{720} 0.8-1) and 100 μL of heavy metal salt solutions with different concentrations. After the 3-hour treatment, luminescence was measured.

The singlet oxygen treatments were carried out in a 10 mL volume in a water bath system equipped with an LED light source, at 30°C, in an atmosphere with a normal CO_2 content. During the low educational light treatments (LL) we used illumination with an intensity of 40 μmol photons m^{-2} s^{-1}, while for the induction of light stress we used illumination with an intensity of 200 μmol photons m^{-2} s^{-1} (HL). During our experiments, we used Rosebangal or Metileneblue as a photosensitizer dye at a final concentration of 0.5 μM, and His as a $^1O_2$ inhibitor at a concentration of 5 mM.

Gene expression studies

To examine the transcripts, total RNA was purified from the cell cultures under treatment, and then using this as a sample, complementary DNA (cDNA) was synthesized in a reverse transcription reaction. The resulting cDNA was used as a template in a qPCR reaction

and the C_T value of the amplified amplicons was compared to the C_T values of the *rrn16S* gene encoding 16S ribosomal RNA. The latter gene, due to its constitutive expression, was used as a control gene. In addition, the values of the transcript levels shown in the figures are normalized to the C_T values of the untreated samples. For qPCR reactions, the reaction volume was 5 µL, which was 1 µL 5 X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne), 0.1 µL cDNA sample, 3.8 µL MilliQ water, 0.1 µL gene-specific F+R primer mix (5 pmol /µL) formed. The obtained C_T values were extracted and evaluated using the Bio-Rad CFX Maestro 1.1 sequence detection software.

cDNA libraries were prepared from 0.5 µg of total RNA using the NEBNext rRNA depletion Kit for Bacteria #E7850, #E7860 (Biolabs). Sequencing was performed using the Illumina platform.

Bioluminescence measurement

Treatments were performed in a 96-well black (Perkin-Elmer Opti-Plate) cell culture plate with low autofluorescence at 25°C. Each well contained 300 µL of log-phase treated cyanobacterial cell culture (OD_{720} 0.8-1). The plates were covered with perforated transparent film (Peca et al. 2008). After the treatment, 6 µL of 50 mM decanal dissolved in 50% methanol was added to the samples placed in the wells. Decanal was present in the 300 µL cyanobacterial and heavy metal salt solution in a final concentration of 1 mM (Kunert et al., 2000). After adding the substrate, the samples were incubated in the dark for 2 min before the luminescence measurement. The tests were performed with four parallel samples. Luminescence was determined using a Top Count NXT luminometer (Packard Instruments) in counts per second (CPS).

Comparison of growth curves

Growth differences caused by intracellular accumulation of metal ions in WT and NiCoZia cyanobacterial cultures were quantified by measuring the optical density at 680 nm and 720 nm for 2–4 days at a light intensity of 30–40 µmol photon m^{-2} s^{-1} and 30°C. The optical density of the starter *Synechocystis* culture was set to the same initial value for each measurement (OD_{720} 0.15), and heavy metal salt was added in 8 different concentrations in a final volume of 50 mL.

Differences in growth rate caused by excess $^1O_2$ in WT and Δ*hliB* cyanobacterial cultures were determined by measuring the optical density at 680 nm with a white light intensity

(LL) of 40 μmol photon m^{-2} s^{-1}, or 200 μmol photon m^{-2} s^{-1} in white light (HL) at 30 °C for 4 days. The optical density of the starter *Synechocystis* culture was set to OD_{720} 0.15 in a final volume of 50 mL, then grown under LL and HL conditions, with or without the addition of a suitable photosensitizer dye (0.5 μM Mb or Rb).

For the measurement, we used a photo-multicultivator MC-1000 (Photon Systems Instrument) with automatic OD protocol.

Determination of the intracellular heavy metal concentration

The intracellular cobalt, zinc and nickel contents were determined in the WT and NiCoZia S*ynechocystis* strains, which served as the basis of the bioreporter treatments. Cell cultures were treated with 5 μM heavy metal salt in addition to the control (0 μM added heavy metal salt); after 3 hours of incubation with $ZnSO_4$, $CoCl_2$ or $NiCl_2$, than collected by centrifugation (6000 rpm for 10 minutes), washed with BG-11 solution and freeze-dried. From 50 mL cultures, we were able to extract 40 ± 5 mg wet weight cell pellets, from which 5 ± 1 mg dry material could be obtained, and then we determined their heavy metal content by ICP-MS.

Data analysis and representation

The results of our experiments were evaluated using the Microsoft Office Excel 2016 program and the OriginPro 2021b data analysis program. The *in silico* design of the molecular biology work and the analysis of our whole transcriptome data were performed using the CLC Genomics Workbench 20.0.2 program.

Student's t-test was used for statistical analysis, where the obtained results are significantly different from each other at $P \leq 0.05$ (*), 0.001 (**), or 0.001 (***) significancy levels.

RESULTS

Increasing the sensitivity of heavy metal bioreporters

1) Cell responses to individual metal ions depend on their intracellular concentration, which is determined by the combined operation of the exporter and importer systems and ion channels. By eliminating the gene cluster responsible for the export of the three previously mentioned heavy metals, we created a mutant that showed inhibited growth compared to the wild-type strain, even below the previously described IC_{min} concentrations. This growth inhibitory effect can be up to 20 times higher for some heavy metals.

2) The inhibited growth of the NiCoZia strain was the effect of the increased intracellular heavy metal concentration, which background was the lack of the appropriate transporters, this is also confirmed by the ICP-MS determination of the intracellular HM content

3) The heavy metal sensitivity of the NiCoZia strain could be used to improve the sensitivity of specific bioluminescent reporter constructs. Based on the luminescence measurements, the cell response to all three examined heavy metals increased. In the case of zinc, a tenfold increase in sensitivity was observed in the NiCoZia bioreporters compared to the reporters with WT background.

4) The *nrsRS*-1 orientation, where the original gene and promoter direction in *Synechocystis* was preserved, only the original transporter genes were replaced by the genes of the luciferase reporter protein, results in a higher level of induction and seems to be more efficient than the opposite, *nrsRS*- 2 orientations.

5) The internal Zn^{2+} concentration is approximately 20% increase resulted in a tenfold increase in the detection limit of the corresponding bioreporter strain. This result shows that a significant improvement can be achieved by limited manipulation of the genome, which may make the application of biosensors even more competitive and valuable in environmental monitoring.

6) Among the currently described whole-cell Ni^{2+}, Co^{2+}, Zn^{2+} bioreporters using reporter protein systems, the construct we created stands out in terms of sensitivity.

Detection of singlet oxygen sensitive genes in *Synechocystis*

7) We managed to perform a complete transcriptome analysis, where the endogenous $^1O_2$ formed during HL illumination, and where we also treated the cyanobacterial cells with exogenous $^1O_2$. For this, we used Mb and Rb photosensitizer dyes in low concentration (0.5 μM), which are not toxic to the cell, but generated enough $^1O_2$ to induce stress. The specificity of the responses to $^1O_2$ was verified by using His, which inhibits the formation of $^1O_2$.

8) With this complete transcriptome analysis, it was possible to find gene candidates that showed induction in the presence of endogenously and exogenously generated $^1O_2$, while they repressed in the presence of the $^1O_2$ inhibitor His, thus confirming/supporting their $^1O_2$ dependence. Among the genes, the high-light-induced gene *hliB* proved to be an outstanding candidate, and its endogenous and exogenous $^1O_2$-induced gene expression in addition to HL induction was repeatedly proven.

9) In the absence of the *hliB* gene, Δ*hliB Synechocystis* cells show strong growth inhibition during constant exogenous $^1O_2$ treatment compared to the growth of WT cells.

10) Except for the HL induction of the other members of the *hli* gene family (*hliA*; *hliC* and *hliD*), we found no significant indication that the other members of the gene family, expect the *hliB* gene, are related to $^1O_2$ and its signal transduction regulation. These genes partially bind to $^1O_2$ signaling, may do a very small extent.

11) The HL induction of the *hliB* gene was clearly visible during the luminescence measurements with the pILA*hliB* reporter, similar to the gene expression experiments. The luminescence experiments confirmed the results of previous gene expression experiments, according to which, in addition to the induction of *hliB* HL, endogenous and externally added exogenous $^1O_2$ is also able to induce the gene. The luminescence in the Mb and Rb samples was much higher than in the samples treated only with HL. In parallel, the presence of His resulted in a decrease in luminescence in these samples, which confirms the $^1O_2$ specificity of the phenomenon.

12) Whit these results, it was possible to verify the $^1O_2$ dependence of the *hliB* gene by gene expression and bioluminescence measurements, and by monitoring the decrese in growth of the Δ*hliB* strain in the presence of $^1O_2$. From the growth observation, we also obtained evidence that the HliB protein participates in reducing the cell-damaging effects of $^1O_2$, since in its absence, cells exhibit strong growth inhibition. These results enabled us to achieve our goal of developing a specific, selective and sensitive $^1O_2$ measurement method in cyanobacteria, precisely in *Synechocystis* PCC 6803.

13) Beside of *hliB*, among the genes induced and inhibited by $^1O_2$, several interesting genes already associated with oxidative stress. For example *idiA* and *idiB*, or *isiA*, which next to their iron sensitivity are also sensitive to $^1O_2$, and in our work these genes were also induced in some cases (Rb treatment), and even *isiA* was inhibited by the presence of His.

14) Among the genes induced by $^1O_2$ there was an Scp-like, high-light-dependent and Chl-binding protein encoding gene; a gene encoding a protein containing a fasciclin domain, a gene encoding the sigma factor of RNA polymerase, and a gene encoding a threonine synthase, which genes have already been linked (together or separately) several times in different *Synechocystis* stress processes, but at the same time their overall function and their relationship to each other still unclear.

15) We have successfully demonstrated that there are genes inhibited by $^1O_2$, which show a specific decrease in gene expression in the presence of $^1O_2$, although in much smaller numbers than the $^1O_2$-induced genes. This side of the gene expression of $^1O_2$ has not been dealt significantly in the literature so far, but we have shown that the repression effect of $^1_{O2}$ is worth the attention. Among the genes inhibitied by $^1O_2$, there were some, which were previously associated with oxidative and other stress effects in *Synechocystis*. Among these genes, except for *hspA* and *coaT*, all are hypothetical protein-coding genes, like the *sll0846* and the *slr7008* gene.

LIST OF PUBLICATIONS

Patyi, G., Hódi, B., Solymosi, D., Vass, I., & Kós, P. B. (2021). Increased sensitivity of heavy metal bioreporters in transporter deficient Synechocystis PCC6803 mutants. Plos One, 16(12), e0261135. https://doi.org/10.1371/journal.pone.0261135 **IF:3.58**

Poór, Péter, **Gábor Patyi**, Zoltán Takács, András Szekeres, Nikolett Bódi, Mária Bagyánszki, and Irma Tari. 2019. "Salicylic Acid-Induced ROS Production by Mitochondrial Electron Transport Chain Depends on the Activity of Mitochondrial Hexokinases in Tomato (Solanum Lycopersicum L.)." Journal of Plant Research 132 (2): 273–83. https://doi.org/10.1007/s10265-019-01085-y. **IF:2.19**

Poór, P., Z. Takács, **G. Patyi**, P. Borbély, O. Bencsik, A. Szekeres, and I. Tari. 2018. "Dark-Induced Changes in the Activity and the Expression of Tomato Hexokinase Genes Depend on the Leaf Age." South African Journal of Botany 118: 98–104. https://doi.org/10.1016/j.sajb.2018.06.006. **IF:1.63**

TOTAL. IF.:7.4

Other publications

1. Péter Borbély, Péter Poór, Judit Kovács, Zoltán Takács, **Gábor Patyi**, Ágnes Szepesi, Irma Tari (2014) Exogenous sodium nitroprusside alleviates salt-induced changes in photosynthesis of tomato leaves. 11th Congress of the Hungarian Society of Plant Biology, Szeged, Hungary

2. Judit Kovács, Péter Poór, **Gábor Patyi**, Péter Borbély, Ágnes Szepesi, Zoltán Takács, Irma Tari (2014) Investigation of salt stress induced changes in cysteine protease activity in abscisic aciddeficient sitiens tomato (*Solanum lycopersicum*) mutant. 11th Congress of the Hungarian Society of Plant Biology, Szeged, Hungary

3. Péter Poór, Ágnes Gallé, Judit Kovács, Zoltán Takács, Péter Borbély, **Gábor Patyi**, Ágnes Szepesi, Irma Tari (2014) Analysis of light dependent cis-regulatory elements of hexokinase genes in tomato (*Solanum lycopersicum*). 11th Congress of the Hungarian Society of Plant Biology, Szeged, Hungary

4. Péter Poór, **Gábor Patyi**, Irma Tari (2015) In Silico Analysis of cis-Regulatory Elements of Hexokinase Genes in Tomato (Solanum lycopersicum) JOURNAL OF CURRENT PLANT SCIENCE RESEARCH : 1 1 pp 1-10

5. Poór Péter, Németh Edit, **Patyi Gábor**, Czékus Zalán, Takács Zoltán, Szepesi Ágnes, Tari Irma (2015) Fény és sötét által szabályozott oxidatív robbanás és antioxidáns rendszer szalicilsav kezelt paradicsom levelekben. A Magyar Szabadgyök-Kutató Társaság VIII. Kongresszusa : Program és előadáskivonatok Budapest, Magyarország

6. Péter Borbély, Péter Poór, **Gábor Patyi**, Irma Tari (2015) Effect of Ethylene Precursor ACC Pre-Treatment on Photosynthesis Under Salt Stress. Plant Abiotic Stress Tolerance III : Programme and Abstracts Wien, Austria

7. Péter Poór, Zoltán Takács, Péter Borbély, Zalán Czékus, **Gábor Patyi**, Irma Tari (2016) Involvement of ethylene in hydrogen-peroxide metabolism in the leaves of salicylic-acid treated tomato. Plant Biology Europe EPSO/FESPB 2016 Congress : Abstracts

8. Péter Poór, Judit Kovács, Ágnes Szepesi, Péter Borbély, **Gábor Patyi**, Zoltán Takács, Irma Tari (2016) Salt stress-induced oxidative stress in ethylene signaling mutant, Never ripe tomato. Joint development of higher education and training programmes in plant biology in support of knowledge-based society, CLOSING CONFERENCE : Book of abstracts Novi Sad, Serbia

9. Péter Poór, Zalán Czékus, **Gábor Patyi**, Péter Borbély, Judit Kovács, Zoltán Takács, Irma Tari (2016) Investigation of Salt Stress-Induced Changes in Water Status, Photosynthetic Parameters and Cysteine Protease Activity in Wild Type and Abscisic Acid-Deficient Sitiens Mutant of Tomato (*Solanum Lycopersicum cv. Rheinland Ruhm*) Plant Model Species: Fundamentals and Applications : Programme and abstracts Wien, Austria

10. **Gábor Patyi**, Ivy Mallick, Imre Vass and Péter Kós (2017) Singlet oxygen sensing in cyanobacteria. Straub Conference at BRC-HAS, Szeged Hungary

11. **Gábor Patyi**, Barbara Hódi, István Zoltán Vass, Imre Vass, Péter Kós (2019) Assessment of intracellular singlet oxygen by GFP fluorescence in Synechocystis PCC6803. Straub Conference at BRC-HAS, Szeged Hungary

12. **Gábor Patyi**, Barbara Hódi, István Zoltán Vass, Imre Vass, Péter Kós (2019) Assessment of intracellular singlet oxygen by GFP fluorescence in *Synechocystis* PCC6803. 9th Symposium on Microalgae and Seaweed Products in Plant/Soil-Systems Mosonmagyaróvár, Hungary.

13. Barbara Hódi, István Zoltán Vass, Ivy Mallick, **Gábor Patyi**, Péter B. Kós, Imre Vass (2019) Effect of singlet oxygen on gene expression profile in *Synechocystis* PCC6803. 9th Symposium on Microalgae and Seaweed Products in Plant/Soil-Systems Mosonmagyaróvár, Hungary.

14. Boglárka Bereczky, **Gábor Patyi**, Lilla Futó-Dékány, Krisztián Laczi, Péter B. Kós (2022) Straub Conference at BRC-HAS, Szeged Hungary

15. **Patyi Gábor**, Hódi Barbara, Solymosi Dániel, Vass Imre, Kós Péter (2022) NEHÉZFÉM BIORIPORTEREK ÉRZÉKENYSÉGÉNEK FOKOZÁSA TRANSZPORTER HIÁNYOS *SYNECHOCYSTIS* PCC6803 CIANOBAKTÉRIUM MUTÁNSOKBAN. Straub napok ELKH-SZBK, Szeged, Magyarország

DECLARATION

As the responsible first author of the following scientific papers, I declare that I did not use the scientific results contained in the following scientific papers to obtain a PhD degree, and I will not use them for same purpose in the future. I acknowledge that Gábor Patyi had a decisive role in achieving the results of the following publications, so he can use the publications as the equirement of the doctoral process.

Poór, Péter, **Gábor Patyi**, Zoltán Takács, András Szekeres, Nikolett Bódi, Mária Bagyánszki, and Irma Tari. 2019. "Salicylic Acid-Induced ROS Production by Mitochondrial Electron Transport Chain Depends on the Activity of Mitochondrial Hexokinases in Tomato (Solanum Lycopersicum L.)." Journal of Plant Research 132 (2): 273–83. https://doi.org/10.1007/s10265-019-01085-y.

Poór, P., Z. Takács, **G. Patyi**, P. Borbély, O. Bencsik, A. Szekeres, and I. Tari. 2018. "Dark-Induced Changes in the Activity and the Expression of Tomato Hexokinase Genes Depend on the Leaf Age." South African Journal of Botany 118: 98–104. https://doi.org/10.1016/j.sajb.2018.06.006.

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