The role of type II NAD(P)H dehydrogenase in the photosynthetic cyclic electron transport of microalgae

FINAL REPORT

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The overarching aim of the project was to reveal the role of type II NADPH dehydrogenase (NDH-2) in alternative electron processes in several microalgae species, particularly in cyclic electron flow (CEF).

It is known from previous literature that under anaerobic conditions the cyanobacterium *Synechocystis* sp. PCC6803 exhibits a characteristic wave phenomenon in the flash-induced fluorescence relaxation kinetics, which is associated with the transient oxidation then re-reduction of plastoquinone (PQ) pool, mediated by type-I NADPH dehydrogenase, NDH-1. NDH-1 is an essential component of cyclic electron flow (CEF) in cyanobacteria, whereas in microalgae CEF pathways are mediated either by the NDH-1/2 pathway or the PGR5/PGRL1 pathway. However, this wave phenomenon and its association with alternative electron transport pathways was uncharacterized prior the research project. Therefore, the first aim was to establish a physiological screening of microalgae for the potential involvement of NDH-pathways. It was investigated whether a characteristic wave phenomenon appears in microalgae that have high importance in ecosystems (e.g. the species in the family *Symbiodiniaceae*, which live in endosymbiosis with corals), or serve as important source of biofuels or valuable metabolites (*Chlorella sorokiniana*, *Haematococcus pluvialis*, *Nannochloropsis limnetica*, *Dunaliella salina*). Under anaerobic conditions, it was found that although the high reduction level of PQ pool is evident, the wave phenomenon observed previously in *Synechocystis*, related to transient oxidation and re-reduction of PQ pool mediated by CEF, did not appear in any of the studied microalgae species. To reveal the properties of the fluorescence relaxation phenomena in microalgae, we performed first a detailed investigation of the wave phenomenon in the model species *Chlamydomonas reinhardtii*. We showed the appearance of the wave phenomenon only under the condition when the activity of Photosystem II (PSII) relative to the activity of Photosystem I (PSI) decreased (by applying chemical inhibition - hydroxylamine treatment, which inactivates the donor side of PSII - or photoinhibition of PSII) along with microaerobic treatment, which caused a strong reduction in plastoquinone (PQ) pool. This indicated that in *Chlamydomonas*

the significantly imbalanced PSII and PSI activity in favor of PSI is an equally important condition for the appearance of the fluorescence wave as the highly reduced state of the PQ pool. Furthermore, we showed that the wave phenomenon could be inhibited with the NDH-2 inhibitor polymyxin B, but not with antimycin A, indicating the involvement of NDH-2 (rather than PGR5/PGRL1) in the induction of the wave phenomenon. Based on these results we proposed that that refilling of the PQ pool following its transient oxidation by PSI after the flash is due to an electron flow from stromal components to the PQ pool, which is mediated by NDH-1 in cyanobacteria and NDH-2 in *Chlamydomonas*. This work has been published in **Photosynthesis Research (Patil et al. 2022, impact factor: 3.7)**.

Based on the rationale of our previous work in *Chlamydomonas*, we screened the wave phenomenon in different species. We found that the decreased PSII activity relative to PSI activity (by hydroxylamine treatment), was an essential condition to induce the wave phenomenon, however, in addition, different conditions (anaerobiosis or preillumination) was required to induce the wave to its largest extent in the different species. The wave phenomenon appeared in different algae to different extents, it was particularly remarkable in red cells of *Haematococcus pluvialis*, in *Dunaliella salina*, and in *Chlorella sorokiniana*, but it was absent in the green cells of *H. pluvialis* and in *Nannochloropsis limnetica*. Inhibitors of components of cyclic electron flow such as the NDH-2 or the PGR5 pathway blocked the wave, but to different extent in the different species, indicating the involvement of distinctive pathways in microalgae. The wave phenomenon is therefore a species-specific indicator of the regulation of electron transport and alternative electron transport pathways such as cyclic electron flow. This work has been published in **International Journal of Molecular Sciences (Patil et al. 2022, impact factor: 5.6**) and presented at national and international conferences (Patil et al. 2021, Characterization of the wave phenomenon in flash-induced fluorescence decay in microalgae, XIII. Hungarian Congress of Plant Biology, August 24-27, 2021, Szeged) and oral talk (Szabó 2021, XIII. Hungarian Congress of Plant Biology, August 24-27, 2021, Szeged), The 18th International Congress on Photosynthesis Research, July 31 - August 5, 2022, Dunedin, New Zealand (virtual poster presentation), 11th International Conference "Photosynthesis and Hydrogen Energy Research for Sustainability-2023" July 3-9 2023 Istanbul, Turkey (poster presentation).

Detailed investigations of the electron transfer pathways were carried out under the condition when the Calvin-Benson cycle was inhibited. CO_2 limitation is known to induce CEF, therefore CO_2 limitation was induced with natural depletion of inorganic carbon (Ci) of the

medium due to photosynthetic uptake or by applying the Calvin-Benson cycle inhibitor glycolaldehyde. For the purpose of inducing CO_2 limitation in the medium by photosynthetic Ci uptake without disturbing the Ci content of the samples, a cuvette-based system was utilized, in which parallel measurements of photosynthetic electron transfer activity, oxygen evolving capacity and NADPH production and consumption (by means of NADPH fluorescence) could be applied along with flash-induced Chl fluorescence relaxation and fast-fluorescence induction (OJIP) transients. We found that under Ci depletion PSII quantum yield (Y(II)) along with O_2 evolution capacity decreased, and PQ pool became slightly reduced (indicated by the increased J:P ratio from OJIP curves and the elevated middle phase of the fluorescence relaxation), and the NADPH uptake capacity by Calvin-Benson cycle was diminished, indicating the presence of Ci limited state. These changes were largely reversible by supplementing the medium with sodium bicarbonate. The analysis of the impact of CO_2 limitation on electron transport pathways has been completed in several species; in the model cyanobacterium *Synechocystis* PCC6803 and several microalgae species (*Chlorella sorokiniana, Nannochloropsis limnetica, Dunaliella salina*). These integrated assays would potentially facilitate the screening for stress- or condition-specific traits in microalgae and cyanobacteria. The work using the multi-parametric cuvette system for studying the effect of CO_2 limitation has been published in **(Patil et al. 2020, PLoS ONE 15(7): e0236188, impact factor: 3.24)**. The article was highlighted by the Editors as a featured article on the main homepage of the journal. These results were also presented at the conference 9th Symposium on Microalgae and Seaweed Products in Plant/Soil-Systems, Mosonmagyaróvár, Hungary, 25-26 June 2019 (oral presentation). Related to this work, another publication was completed about the optimization of growth and photosynthesis by CO_2 dosage and enhancing light availability in the diatom *Chaetoceros muelleri*. By applying various combination of CO_2 and light levels, we showed that the biomass production and photosynthesis of *Chaetoceros* can be significantly improved, which is important to consider for applied research when adjusting the environmental conditions for enhancing algal biomass is a priority (published in: **Iwasaki et al. 2021, Improving light and CO2 availability to enhance the growth rate of the diatom, Chaetoceros muelleri, Algal Research 55 p. 102234, impact factor: 5.1**).

The results of the aims addressed in the project have important connections to other works which analyzed the alternative electron transfer pathways. The investigation of cyclic electron flow (CEF) was performed using a specific thermoluminescence signal, the so-called afterglow (AG) band. In our study, the AG band has been identified in *Synechocystis* with a peak

temperature of +40 °C, emerging after far-red preillumination at -10 °C. This band was observed when the samples were grown at ambient CO_2 levels, but it was absent in the M55 line, devoid of the NDH-1 complex, an essential component of CEF in *Synechocystis*. Therefore, our study indicated for the first time that the AG band detected at +40 °C could be assigned to the NDH-1 complex mediated CEF in *Synechocystis* (published in: **Kodru et al. 2021, Physiologia Plantarum, 171: pp. 291-300, impact factor: 5.1)**.

It was also the aim of the project to establish a protoplast preparation and characterization procedure in microalgae. Obtaining physiologically competent protoplasts was crucial for the project in order to assess the intracellular singlet oxygen production using singlet oxygen specific, but cell wall impermeable fluorescent dyes (Singlet Oxygen Sensor Green, SOSG). Furthermore, the cell wall permeability of several photosynthetic inhibitors are ambiguous, which make the studies on alternative electron transport pathways difficult. Protoplast technology therefore have a high potential to assess various fluorophores and inhibitors that could not be successfully applied in intact cells of algae. Successful isolation of physiologically competent protoplasts from coral endosymbiont algae *Symbiodinium* sp. has been achieved. Protoplast formation was confirmed by Calcofluor White staining as it gives blue fluorescence when it reacts with cellulose. Blue fluorescence was observed in the rim of the intact control cells, but in the protoplast this characteristic blue fluorescence was not present, indicating the removal of cell wall as a result of enzymatic digestion. Regeneration of cell wall was also confirmed with the Calcofluor White staining, showing the re-formation of the cell wall in protoplasted cells after 3-4 days. Single cell photosynthetic performance assessment revealed that protoplasted cells were physiologically active throughout the process, and the appearance of cell division during regeneration also indicated that the physiological activity of cells has recovered after enzyme treatment (cell division was largely arrested during protoplast formation stage). Intracellular singlet oxygen production under high light stress could be visualized by the singlet oxygen sensitive fluorescent dye, SOSG in *Symbiodinium* protoplasts, but not in intact cells, because the presence of cell wall prevented the penetration of SOSG into intact cells. The protoplast preparation procedure of *Symbiodinium* cells has been further optimized using microfluidic chambers and a morphological analysis has also been developed to follow the timescale of the morphological changes. By selectively adjusting the flow rate of the enzyme solution, the time for protoplast isolation could be optimized, without compromising photosynthetic activity (assayed using single cell chlorophyll fluorescence kinetics). Intracellular singlet oxygen labeling using SOSG was also established in trapped

protoplasts, potentially allowing the analysis of intracellular singlet oxygen production, as well as revealing intracellular distribution of the SOSG dye. The SOSG showed a certain co-localization with Chl autofluorescence, indicating the localization of the SOSG dye in the chloroplast. To confirm that the SOSG signal is indeed specific to singlet oxygen in the protoplast, SOSG staining was done in combination with a histidine assay. *Symbiodinium* protoplast showed strong SOSG fluorescence when illuminated with light due to the production of singlet oxygen; however, this fluorescence disappeared when SOSG was used together with histidine, indicating singlet oxygen uptake by histidine. The established method and procedure of protoplast preparation allowed the characterization of the morphology and life cycle assessment of protoplast formation and regeneration in *Symbiodinium* sp., therefore a fast and efficient procedure is established for this species. These results were presented at the conferences of the Hungarian Free Radical Society (Bashir et al. online talk, August 27, 2021, Szeged and Szabó 2023 oral presentation, August 24-25, Martonvásár, Hungary). The investigation of protoplast viability and the applicability of protoplasts for studying singlet oxygen and uptake of fluorescent labeled synthetic oligonucleotides in Symbiodiniaceae has been published in **Lab on a Chip (Bashir et al. 2022, impact factor: 6.1)**. The role of singlet oxygen and other reactive oxygen species in coral symbiosis and coral bleaching has also been reviewed during the project (**Szabó M., Larkum A. W. D., Vass I.: A Review: The Role of Reactive Oxygen Species in Mass Coral Bleaching, In: Raven, J.A.; Grossman, A.R.; Larkum, A.W.D. (eds.) Photosynthesis in Algae: Biochemical and Physiological Mechanisms, Springer International Publishing (2020) pp. 459-488**). It was an important finding of this study that *Symbiodinium* protoplasts are able to take up fluorescein-labeled short (53 nucleotides long) DNA oligonucleotides, whereas intact cells did not show uptake of oligonucleotides. This finding opens important new perspectives for antisense silencing of *Symbiodinium* genes; investigations are underway to assess the applicability of the protoplast technology for silencing the psbO gene encoding the PsbO, the Mn-stabilizing subunit of the water-oxidizing complex of PSII, using antisense oligonucleotides. The microfluidic technology was applied for trapping other highly important algae, such as the astaxanthin producing *Haematococcus*. Using a combined light microscopy and single cell Chl fluorescence analysis the physiological and photosynthetic changes during the carotenogenesis process could be followed (manuscript in preparation).

Related to the role of singlet oxygen on PSII activity, the impact of singlet oxygen production on the photosynthetic activity of cyanobacteria and microalgae has been studied

using 24 well plates and chlorophyll fluorescence imaging. The photosensitizer dye Rose bengal was applied in various concentrations to generate singlet oxygen, and the impact of singlet oxygen production on the Photosystem II activity was monitored as a function of time. The results of this study showed that the external singlet oxygen produced by the reactions of the photosensitizer Rose bengal damaged Photosystem II in both isolated thylakoids and in intact *Chlorella sorokiniana* cells in a concentration-dependent manner (published in: **Bashir et al. 2021, Photosynthesis Research, 149: pp. 93-105, impact factor: 3.4)** and presented at the national conference of the Hungarian Free Radical Society, August 27, 2021, Szeged). Relevant to the above point, another publication has been completed, which investigated the physiological and photosynthetic activity of the model cyanobacterium *Synechocystis* PCC6803 grown on biofilms **(Mallick et al. 2020 PLoS ONE 15(7): e0236842, impact factor: 3.24)**. This method opens the potential to investigate the singlet oxygen production in immobilized cells, because the applied photosensitizer dye (methylene blue or Rose bengal) can be easily washed out after the treatment and before the measurement of the quantum yield of Photosystem II.

Global temperature increase has detrimental effects on coral ecosystems. The heat tolerance of the corals is strongly determined by the algae species (formerly genetic clades) belonging to the family Symbiodiniaceae, which lives in endosymbiosis with the coral host. We therefore investigated three different strains of Symbiodiniaceae family, i.e., *Fugacium kawagutii* (CS156), *Symbiodinium tridacnidorum* (2465), and *Symbiodinium microadriaticum* (2467), which display different heat tolerance under heat stress conditions. We found that flash-induced Chl fluorescence relaxation exhibited a wave phenomenon under heat treatment and anaerobic conditions, which is related to the transient oxidation and re-reduction of PQ pool. This response was accompanied by a marked post-illumination Chl fluorescence rise, and a slightly elevated quantum yield of PSI relative to the quantum yield of PSII, and the response was strain specific. These fluorescence phenomena are potentially associated with the operation of cyclic electron flow, which was the most expressed in *Symbiodinium tridacnidorum* (2465). Therefore, the flash-induced chlorophyll fluorescence relaxation and its wave phenomenon is a sensitive indicator of heat stress in Symbiodiniaceae, displaying species specific features, and by this means it could be considered as a non-intrusive marker of stress-induced changes e.g. under coral bleaching conditions. This work has been published in **Frontiers in Marine Science (Mohammad Aslam et al. 2022, impact factor 3.7)**. Our next aim was to investigate the flash-induced Chl fluorescence relaxation phenomenon in Symbiodiniaceae by using

inhibitors that are specific to certain electron transport processes, such as NDH-2. By using various inhibitors, we showed that the linear electron transport has a crucial role in the formation of the wave. The inhibition of the donor side of Photosystem II did not induce the wave, whereas inhibition of the Calvin–Benson cycle (using glycolaldehyde) accelerated it. Applying inhibitors of the cyclic electron flow components, antimycin A and polymyxin B did not have effect on the fluorescence wave phenomenon in Symbiodiniaceae, however this may be due to the impermeability of cell wall to these inhibitors. We therefore applied the protoplast technology developed earlier during the project to assess the impact of these inhibitors. We successfully optimized the protoplast preparation procedure to ensure that the protoplasts remain photosynthetically competent and retain the flash fluorescence wave phenomenon. Under these conditions we found that polymyxin B significantly suppressed the formation of the wave and also slowed down the re-reduction of $P700^+$, but antimycin A did not cause inhibitory effect. These results indicated that NDH-2 (and not the PGR5 pathway) contributed to the formation of the wave phenomenon by mediating the electron transfer between NADPH and the PQ pool. These results were published in **International Journal of Molecular Sciences (Mohammad Aslam et al. 2023, impact factor: 5.6)** and were also presented in posters at national and international conferences (Mohammad et al. 2021, XIII. Hungarian Congress of Plant Biology, August 24-27, 2021, Szeged; The 18th International Congress on Photosynthesis Research, July 31 - August 5, 2022, Dunedin, New Zealand (virtual poster presentation); 11th International Conference "Photosynthesis and Hydrogen Energy Research for Sustainability-2023" July 3-9 2023 Istanbul, Turkey – poster award, selected oral presentation).

Publications related to the project

Bashir F, Kovács S, Ábrahám Á, Nagy K, Ayaydin F, Valkony-Kelemen I, Ferenc G, Galajda P, Tóth SZ, Sass L, Kós PB, Vass I, Szabó M (2022) Viable protoplast formation of the coral endosymbiont alga Symbiodinium spp. in a microfluidics platform. Lab on a Chip 22 (16):2986-2999.

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Iwasaki K, Evenhuis C, Tamburic B, Kuzhiumparambil U, O'Connor W, Ralph P, Szabó M (2021) Improving light and CO2 availability to enhance the growth rate of the diatom, Chaetoceros muelleri. Algal Research 55:102234.

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