The role and significance of alternative electron transport in microalgae

Ph.D. Dissertation

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Szeged, 2025

Background

The family Symbiodiniaceae comprises unicellular microalgae that form crucial mutualistic relationships with cnidarian hosts, including corals, sea anemones, and jellyfish. These algae provide energy to their hosts through photosynthesis, receiving inorganic nutrients such as nitrogen and phosphorus in return. This symbiotic partnership constitutes the foundation of reef ecosystems, which thrive even in nutrient-poor marine environments. These algae are equipped with light-harvesting complexes composed of chlorophyll *a*, chlorophyll c_2, and carotenoids such as peridinin, which enable them to capture solar energy and drive photosynthetic electron transport. In this process, electrons are transferred within the thylakoid membrane from Photosystem II (PSII) to Photosystem I (PSI) and protons transfer across the thylakoid membrane respectively, leading to the generation of NADPH and ATP which are crucial for the process of carbon fixation in the Calvin-Benson cycle. However, this delicate balance can be disrupted under conditions of elevated sea temperatures and excessive light exposure which leads to expulsion of these algal symbionts from the host, a phenomenon known as coral bleaching. This expulsion deprives the host of its primary energy source, threatening its survival and ultimately destabilizing the entire reef ecosystem.

In order to survive under diverse environmental and metabolic conditions, photosynthetic organisms such as Symbiodiniaceae employ alternative electron transport pathways, including Cyclic Electron Flow (CEF), the Alternative Oxidase (AOX) pathway, the Mehler reaction, Chlororespiration, and Extracellular Electron Transport (EET). These pathways serve critical functions in maintaining the ATP/NADPH balance, mitigating oxidative stress, and ensuring the flexibility and resilience of the photosynthetic machinery. The CEF pathway, for example, facilitates the recycling of electrons around PSI, thereby generating additional ATP without the production of NADPH, thus contributing to the maintenance of energy balance during periods of stress. Depending on the route of the electrons, CEF in microalgae usually occurs in two different pathways, which are mediated by either Protein Gradient Regulator-Like 1 (PGRL1)/ Protein Gradient Regulator 5 (PGR5) or type II NADPH dehydrogenase (NDH-2).

The EET pathway, although less extensively studied, has been hypothesized to play a role in the dissipation of excess reducing power and the stabilization of the redox state within the system.

However, the stability of this mutualism is increasingly threatened by coral bleaching, a phenomenon driven by rising sea temperatures. Heat stress disrupts photosynthesis, impairing PSII and Calvin-Benson cycle enzymes like type II RuBisCO, resulting in diminished carbon fixation and increased production of reactive oxygen species (ROS), which leads to oxidative stress. Prolonged exposure to such stress can result in the expulsion of the algal symbionts by the cnidarian host, leaving the coral vulnerable and causing widespread destruction of reef ecosystems. Interestingly, species within the Symbiodiniaceae family exhibit significant variation in their tolerance to thermal and oxidative stress, as well as in the operation of photoprotective and alternative electron transport mechanisms. Some species are better equipped to manage the damage caused by the heat through various alternative electron transport pathways, which could mitigate the impacts of bleaching. A comprehensive investigation of the processes that occur under stress is therefore essential to understanding species-specific adaptations of the symbiont and their potential contribution to stress adaptations and resilience of the coral in a changing environment.

Aims

The aims of the present study are:

i) Understand the impacts of acute heat stress on the photosynthetic efficiency of coral endosymbiont algae Symbiodiniaceae, using chlorophyll fluorescence techniques like OJIP curves and flash-induced fluorescence relaxation.

ii) Determine how electron transport is affected under heat stress, focusing on fluorescence changes and their reversibility when normal conditions are restored. This will help uncover how the photosynthetic machinery adapts to heat stress.

iii) Explore alternative electron transport pathways by utilizing the fluorescence "wave phenomenon", and $P700^+$ reduction kinetics measurements to probe the roles of specific pathways like PGR5/PGRL1 and NDH-2 in heat tolerance.

iv) Study the fluorescence relaxation wave in intact corals, specifically the slow 1-400 second fluorescence wave, to see how it links to electron transport and the overall stress response in the coral host and the algae.

v) Assess the role of extracellular electron transport (EET) in the coral-Symbiodiniaceae system, investigating its potential contribution to stress adaptation and coral resilience in a changing environment.

Materials and methods

Symbiodiniaceae strains used

- Three different species of *Symbiodiniaceae* were used: *Symbiodinium tridacnidorum* (CCMP2465), *Symbiodinium microadriaticum* (CCMP2467), and *Fugacium kawagutii* (CS156). Cultures were grown at 24°C under 50 µmol photons m^{-2} s^{-1} white light in F/2 media for a week, reaching the mid-log growth phase.

Coral species used

- Coral species *Pocillopora damicornis, Acropora sp.* and *Seriatopora* sp. were used. Corals fragments were obtained from a controlled tank with water parameters maintained and monitored.

Chlorophyll fluorescence methods

- Flash-induced chlorophyll fluorescence yield (FF)

Measured using a double modulation fluorimeter (PSI, FL3000). Four 8 µs flashes (620 nm) at 200 µs intervals were used to determine the minimum fluorescence (F_0) in the dark, then a 30 µs saturating actinic flash (639 nm) was applied to induce the formation of Q_A^-. The fluorescence decay was then observed, by using non-actinic measuring flashes, in three distinct phases with a fast phase (few hundreds of µs, indicating Q_A^- to Q_B electron transfer), a middle phase (5-10 ms, indicating reoxidation of Q_A^- by PQ), and a slow phase (1-10 s, arising from the $S_2Q_AQ_B^-$ charge recombination).

- Kautsky induction curve or OJIP

This method uses red actinic light (650 nm, 22 nm half-width) at an intensity of 3500 µmol m^{-2} s^{-1} to induce fluorescence. It records the OJIP transient, measuring the shift from the dark adapted state of the PSII reaction centres (O step/F_0 at 20 µs) to the light saturated state of the centres (P step/F_m ~300 ms). The OJ phase reflects the redox state of PQ pool under different stress conditions.

- Post-illumination chlorophyll fluorescence transient (PIFT)

The measurement utilizes weak measuring light for 30 seconds, followed by actinic light (50 µmol photons m^{-2} s^{-1}) for 3 minutes. After actinic light exposure, the chlorophyll fluorescence rise in darkness was recorded, attributed to DHAP accumulation during the light phase, which is re-metabolized via the Calvin cycle, releasing NADPH that donates electrons to plastoquinone (PQ).

- Activities of photosystem I and II (Y(I) and Y(II))

A saturation pulse method was used to determine Y(I) and Y(II). In the dark-adapted state, PSII activity is calculated as $(F_m - F_0) / F_m$ and Y(I) as $(P_m' - P) / P_m$ where P_m is the maximal P700 signal obtained under far-red illumination when P700 is fully oxidized.

- $P700^+$ reduction kinetics

Measurement was done by applying actinic illumination to induce the formation of photo-oxidized $P700^+$. This was followed by a dark phase during which the kinetics of $P700^+$ re-reduction was measured. In the presence of DCMU, which blocks electron transport from PSII, it serves as an indicator of cyclic or alternate electron transport around PSI.

Spectrophotometric analysis of FeCN reduction

Reduction of 1 mM FeCN was monitored by observing absorbance change at 420 nm after 2 hours. The rate of FeCN reduction was used to determine the rate of extracellular electron transport (EET).

Results

Flash-induced chlorophyll fluorescence relaxation kinetics provide critical insights into photosynthetic electron transport, including PSII-PSI dynamics, cyclic electron flow (CEF), and plastoquinone (PQ) pool redox changes. This technique revealed species-specific responses to acute heat stress in Symbiodiniaceae, with strain 2465 showing most pronounced effects. Acute heat stress caused an elevated reduction of the PQ pool and a decrease in PSII activity, and in some species it slightly accelerated the activity of PSI. The effect of the acute heat stress was found to be largely reversible, highlighting its potential for stress tolerance. Temperature dependency studies further demonstrated that heat alone, up to 40°C, was insufficient to induce the fluorescence wave phenomenon, but microaerobic conditions and a partial loss of PSII activity relative to PSI activity facilitated the wave formation, which is related to the transient oxidation and re-reduction of the PQ pool and was more pronounced in the 2465 strain. The wave phenomenon, linked to PSII:PSI activity ratio, reflecting a complex interplay of different electron flow processes and has been previously observed in cyanobacteria and in microalgae like *Chlamydomonas reinhardtii*. However, its presence and characteristics in Symbiodiniaceae is were not explored previously. These findings underscore the importance of fluorescence relaxation studies in understanding heat stress responses and the resilience of photosynthetic organisms in dynamic environments.

Our data show that the wave phenomenon in flash-induced chlorophyll fluorescence relaxation in Symbiodiniaceae is linked to CEF, and induced heat stress, especially when heat is accompanied with microaerobic conditions, indicating reduced PSII activity and a strongly reduced PQ pool. Although genes for CEF-related pathways are present, evidence linking specific components to wave induction was lacking in Symbiodiniaceae. Therefore, inhibitors like DCMU, DMBQ, and MV were used to probe the underlying mechanisms. DCMU, blocking electron transfer from Q_A^- to Q_B, abolished fast and middle phases, emphasizing the importance of linear electron flow. DMBQ partially reduced the wave, and MV, inhibiting CEF at PSI, abolished the wave, highlighting the need for an intact electron transport chain. Inhibition of the PSII donor side with hydroxylamine under microaerobic conditions did not induce the wave. Glycolaldehyde, a CBB cycle inhibitor, increased the $P700^+$ reduction rates similarly to heat and also induced a slower wave-like phenomenon, suggesting a role of the CBB cycle in the wave. Further investigation into the roles of NDH-2 and PGR5/PGRL1 pathways revealed that in cells with partially digested cell wall, antimycin A had no effect, while polymyxin B inhibited the wave and slowed $P700^+$ re-reduction, indicating that in Symbiodiniaceae NDH-2 mediates in channeling the electron from stromal components back to the PQ pool under stress conditions.

Chlorophyll fluorescence patterns in corals were influenced by oxygen levels and displayed a wave-like phenomenon under low oxygen conditions (below 200 μmol/L), lasting up to 400 seconds. This pattern occurs faster in intact corals than in isolated *Symbiodinium* and depends on a reduced PQ pool. As oxygen levels drop (below 100 μmol/L) due to host respiration, the fluorescence dip and rise intensify reflecting an increased reduction level of the PQ pool. A slow rise of the F_0 fluorescence in corals can be observed in the 1-400 seconds time range even in the dark. This phenomenon indicates that there is an inflow of electrons from the coral host to the symbiont. FeCN, an artificial electron acceptor which does not penetrate inside the cells, eliminated the rise of F_0 in the 1-400 seconds time range and was reduced gradually in the presence of cultured *Symbiodinium* cells showing that *Symbiodinium* can transfer electrons to external acceptors, i.e. capable of extracellular electron transport (EET). EET was observed to be more pronounced in intact corals than in isolated *Symbiodinium*, suggesting that the coral host enhances EET, possibly through redox-active compounds. This mutualistic relationship plays a key role in coral health, potentially by contributing to stress adaptation and coral resilience in a changing environment.

Thesis Points

- Heat susceptibility varies from species to species in Symbiodiniaceae, hence the intact corals that host the specific species, may also respond differently.

- Acute heat stress makes the PQ pool more reduced, decreases Photosystem II efficiency, and in some species accelerates Photosystem I activity slightly.

- Flash-induced Chl fluorescence decay exhibited a wave phenomenon under heat treatment and microaerobic conditions, which is related to the transient oxidation and re-reduction of the PQ pool.

- These fluorescence phenomena are not only potentially associated with decreased relative activity of PSII:PSI, but also with a decreased Calvin-Benson-Bassham cycle activity and increased alternative electron flow, which can be correlated well with $P700^+$ reduction kinetics.

- The NDH-2 complex was observed as a potential mediator in channeling the electrons from stromal components back to the PQ pool under stress conditions in Symbiodiniaceae.

- Stress conditions usually intensify from intact coral to the symbiosomes, as high respiration of the host might lead to hypoxia in the symbiosomes much before the ambient oxygen reaches zero.

- FeCN reduction shows extracellular electron transport from the symbiont and the host to its environment, while the rise of the F_0 fluorescence in the dark indicates electron inflow from the coral host to the symbiont. These phenomena indicate a novel, two-way electron transport between the symbiont and the host.

List of publications

List of publications relevant to thesis (MTMT: 10075181):

- Mohammad Aslam, S., Vass, I., & Szabó, M. (2023). Characterization of the Flash-Induced Fluorescence Wave Phenomenon in the Coral Endosymbiont Algae, Symbiodiniaceae. International Journal of Molecular Sciences, 24(10), 8712. (Impact Factor: 4.9).

- Mohammad Aslam, S., Patil, P. P., Vass, I., & Szabó, M. (2022). Heat-induced photosynthetic responses of Symbiodiniaceae revealed by flash-induced fluorescence relaxation kinetics. Frontiers in Marine Science, 9, 932355. (Impact Factor 2.8).

- Patil, P. P., Mohammad Aslam, S., Vass, I., & Szabó, M. (2022). Characterization of the wave phenomenon of flash-induced chlorophyll fluorescence in Chlamydomonas reinhardtii. Photosynthesis Research, 152(2), 235-244. (Impact Factor 2.9).

Other publication:

- Yadav, R. M., Mohammad Aslam, S., Madireddi, S. K., Chouhan, N., & Subramanyam, R. (2020). Role of cyclic electron transport mutations pgrl1 and pgr5 in acclimation process to high light in Chlamydomonas reinhardtii. Photosynthesis Research, 146, 247-258. (Impact Factor 2.9).

Összefoglalás

A Symbiodiniaceae alga család, mint a korallok szimbiotikus partnere, fontos szerepet játszik az ökoszisztéma egyensúlyának fenntartásában. Azonban a globális felmelegedés a szimbiózisra jelentős károsító hatást gyakorol, amely a korallok kifehéredéséhez és pusztulásához vezethet. Flash-indukálta klorofill fluoreszcencia lecsengés mérésekkel kimutattuk, hogy a Symbiodiniaceae különböző fajainak hőstressz toleranciája eltérő mértékű. Akut hőstressz a plasztokinon (PQ) pool redukálódását, a PSII aktivitás csökkenését és bizonyos fajokban a PSI aktivitás enyhe növekedését okozta. Továbbá, hőkezelés és mikroaerob körülmények során a flash-indukálta klorofill fluoreszcencia lecsengés kinetikájában bizonyos hullámjelenséget figyeltünk meg, amely fajspecifikus sajátságokat mutatott és a PQ pool tranziens oxidációjához és re-redukciójához volt köthető. Ez a jelenség az alternatív elektrontranszport folyamatok hőstressz toleranciában betöltött szerepére utal.

További vizsgálataink kimutatták, hogy a hullámjelenség a kettes típusú NAD(P)H dehidrogenáz aktivitásához köthető, valamint kialakulásában a lineáris elektrontranszport folyamatok működése is kulcsfontosságú. A hullámjelenség kialakulását a PSII:PSI aktivitás arányának relatív csökkenése, a csökkent Calvin-Benson ciklus aktivitás, valamint a felgyorsult alternatív elektrontraszport folyamatok is befolyásolják. Ezért a hullámjelenség az alternatív elektrontraszport folyamatok fontos markere stresszkörülmények során.

Korallokban a flash-indukálta klorofill fluoreszcencia lecsengés egy lassabb (1-400 s) hullámjelenségben nyilvánult meg anaerob körülmények között. Ez a jelenség a PQ pool lassabb redukciójával hozható összefüggésbe az extracelluláris elektronátviteli folyamatok miatt. *Symbiodinium* sejtek és egész korallok 1-400 s-os időtartományban megfigyelhető fluoreszcencia emelkedése, valamint a fluoreszcencia emelkedés aktinikus flash jelenlétében vagy hiányában, különböző oldott oxigéntartalom mellett a stresszviszonyok felerősödését jelzi a korallszövettől a szimbioszómáig. Ferricianid redukció mérések segítségével *Symbiodinium* sejtekben és egész korallokban is megfigyeltük az extracelluláris elektrontranszport (EET) jelenlétét, azonban az EET korallokban nagyobb mértékű volt, mint az izolált sejtekben. Eredményeink alapján először mutattuk ki a korallok és a szimbiotikus algák között megvalósuló kétirányú elektrontranszport folyamatok jelenlétét, amelyek kulcsfontosságúak lehetnek a metabolikus egyensúly és az energiamérleg fenntartásában stresszkörülmények során.

Co-author declaration

Dr. Milán Szabó and Dr. Imre Vass, as the supervisors and co-authors of the publications listed below, hereby declare the following:

We are well acquainted with the dissertation work of the Ph.D. candidate Mohammad Aslam Sabit. The candidate has made a substantial contribution to achieving the results presented in the dissertation and the associated publications. These results have not been utilized previously to obtain any other Ph.D. degree and will not be used for such purposes in the future. The publications are as follows:

1. Mohammad Aslam, S., Vass, I., & Szabó, M. (2023). Characterization of the Flash-Induced Fluorescence Wave Phenomenon in the Coral Endosymbiont Algae, Symbiodiniaceae. International Journal of Molecular Sciences, 24(10), 8712. (Impact Factor: 4.9).

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