Summary of the PhD thesis

THE DETECTION, ELIMINATION AND DAMAGING EFFECT OF SINGLET OXYGEN IN THE PHOTOSYNTHETIC APPARATUS OF PLANTS AND MICROALGAE

Presented by

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INTRODUCTION

Photosynthesis is a fundamental biochemical mechanism by which plants, green algae and cyanobacteria produce carbohydrates from carbon dioxide and water using solar energy. The main complexes of the photosynthetic apparatus are Photosystem II, Photosystem I, cytochrome b6/f complex and the ATP synthase, which perform the initial, light-dependent steps of the photosynthetic process. Light is an important factor in photosynthesis and light absorption by chlorophyll (Chl) molecule has various fates; driving the photosynthesis process, dissipated as heat or re-emitted as a fluorescence. The absorbed energy of light by Chls in the light harvesting complex (LHCII) is transferred to the PSII protein complex, which leads to excitation of the P680 reaction center Chl. The charge separation between the excited reaction center Chl assembly (P680*) and the pheophytin (Phe) molecule, i.e. the formation of the primary radical pair/charge separated state ($P680^{+\bullet}Phe^{-\bullet}$), is a primary event during PSII electron transfer, which is further followed by rapid charge stabilization processes.

Light drives photosynthesis, but light can be harmful to any photosynthetic organism if it is in excess. Excess absorbed energy that cannot be used to drive photosynthesis can enhance the production of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$). $^1O_2$ is a highly reactive excited state of molecular oxygen. It is an important ROS in biological systems because it damages proteins, lipids, and nucleic acids. It is less stable than triplet oxygen ($^3O_2$) and can be created in various ways, but the most common way is by transferring energy from a photosensitized pigment or triplet state of dye molecule to triplet oxygen (Hirakawa *et al.*, 2011; Fischer *et al.*, 2013).

PSII is especially vulnerable to light-induced damage, in which electron transport activity is impaired and the D1 (and D2) reaction centre protein is degraded. Plants evolved a protective repair mechanism, by which light induced loss of photosynthetic activity can be restored. This repair mechanism proceeds via *de novo* synthesis of the light-damaged D1 (and D2) subunit(s) of PSII. Although the exact mechanism of photodamage and its repair is not fully clarified yet there is a consensus in the literature that ROS are involved in the overall photoinhibition process.

Several ROS scavenging molecules are found in plant cells, and they are used to eliminate or inactivate harmful reactive oxygen species. Several studies have suggested that proline (Pro) has antioxidant properties, implying that it can scavenge ROS and act as a $^1O_2$ quencher. Pro reduces the levels of various ROS types in intact plant systems and leads to cellular osmotic change as a compatible solute. While there is consensus in the literature that

Pro can directly or indirectly remove hydroxyl radical (˙OH) and hydrogen peroxide (H_2O_2) in vivo, its function in scavenging $^1O_2$ is debated: some studies reported that Pro is an effective $^1O_2$ quencher (Alia *et al.*, 1997; Matysik *et al.*, 2002). In contrary, Signorelli and colleagues found that Pro did not scavenge $^1O_2$ (Signorelli *et al.*, 2013). The debate over Pro's ability to interact with $^1O_2$ most likely stems from a misunderstanding of the physical and chemical mechanisms of $^1O_2$ removal.

Previous studies have shown that increased production of $^1O_2$ seems to occur primarily under high light stress conditions, which will ultimately result in the inactivation of PSII and photoinhibition. Previously the $^1O_2$ detection methods, such as electron paramagnetic resonance (EPR), His-mediated chemical trapping and fluorescent probes were developed to study $^1O_2$ formation in isolated PSII membranes, cyanobacteria and plant leaves, but methods for detection of intracellular $^1O_2$ in *Symbiodinium* cells were largely unknown due to the impermeability of fluorescent probes in *Symbiodinium* cells.

AIMS

The general aim of the PhD work was to gain knowledge on the mechanisms of photodamage and photoprotection of the photosystem II complex in plants and microalgae with special emphasis on the singlet oxygen detection, damage and scavenging processes. The specific aims of this study were:

1. To develop a method for the detection of intracellularly produced singlet oxygen ($^1O_2$) in microalgal cells by preparing physiologically competent protoplasts.

2. To investigate the role of proline as a singlet oxygen scavenger and understand the mechanism of quenching.

3. To characterize the damaging effect of externally produced $^1O_2$ on Photosystem II in isolated thylakoid membranes and intact *Chlorella sorokiniana* cells.

MATERIALS AND METHODS

Symbiodinium sp. CCMP2467 cells were grown in F/2 medium at 25 °C at a light intensity of 50 µmol photons m^{-2} s^{-1}, with a light: dark period of 12 h: 12 h. The cells were collected during their exponential growth phase by centrifugation at 2000 g for 4 min and resuspended in the fresh F/2 medium for the experiments. *Chlorella sorokiniana* cells were propagated in BG-11 growth medium and grown at 24 °C at the irradiance of 60-70 µmol photons m^{-2} s^{-1} white light, in 500 mL flasks containing 200 mL of BG-11 kept on an orbital shaker. The chlorophyll concentration (Chl a+b) was determined by a UV-1601 (SHIMADZU) spectrophotometer after extracting the pigments with acetone: DMSO 1:1. Cells were harvested by centrifugation at 6500 g for 5 min and re-suspended in fresh BG-11 medium at a concentration of 5 µg of Chl mL^{-1}. Thylakoid membranes were isolated from fresh spinach leaves and stored in −80 ˚C. Thylakoid was resuspended in the thylakoid buffer and chlorophyll concentration was set to 5 µg of Chl mL^{-1}.

In order to produce *Symbiodinium* protoplast, cells were incubated in the digestion medium (containing 4 % cellulase Onozuka RS, 1 % macerozyme R-10 and 0.5 M D-sorbitol in pre-cooled sterile F/2 medium) and incubated at 30 °C in the dark for 24 hours on the shaking incubator at 100 rpm. After washing, protoplasts were added in the regeneration medium and finally transferred in the culture medium. Single cell chlorophyll fluorescence of the intact cells and protoplast was determined by pulse-amplitude modulated (PAM) chlorophyll fluorescence imaging microfluorometry (Imaging-PAM M-series Chlorophyll Fluorometer Heinz Walz GmbH, Germany). In order to analyze the morphological changes of protoplasts, an automated image analysis procedure was developed (Matlab version 2018b).

To investigate the role of Pro as a $^1O_2$ quencher, O_2 uptake measurements were performed by a Hansatech DW2 O_2 electrode. The rate of light-induced $^1O_2$ production in solution was detected by measuring the rate of O_2 uptake in the presence of the $^1O_2$ sensitizer methylene blue (MB) and chemical scavengers histidine (His) and physical quenchers Sodium azide (NaN_3) or Pro. EPR spin trapping was used for $^1O_2$ detection using TEMPD as a spin trap. TEMPD was mixed at a concentration of 100 mM with 10 µM MB and with different concentrations of Pro, and the production of $^1O_2$ was induced by illumination of samples for 3 min with 2300 µmol photons m^{-2} s^{-1} intensity of visible light.

A multiwell plate-based screening method combined with chlorophyll fluorescence imaging was used to characterize the effect of externally produced $^1O_2$ on the photosynthetic

activity of isolated thylakoid membranes and intact *Chlorella sorokiniana* cells. For photoinhibitory treatment, the cell suspensions were incubated with different concentrations of Rose Bengal (RB) ($^1O_2$ photosensitizer) with and without lincomycin (300 µg mL^{-1}), which block the protein synthesis dependent repair of PSII and allows determination of the rate of photodamage. The illumination was provided from the top by a LED array using green+ white light (total intensity 240 µmol photons m^{-2} s^{-1}) in order to excite the RB dye without providing excess excitation to the photosynthetic processes. Chlorophyll fluorescence of the samples was assessed by pulse-amplitude modulated (PAM) chlorophyll fluorescence imaging (Imaging-PAM MAXI Chlorophyll Fluorometer Heinz Walz GmbH, Germany). Oxygen evolution/uptake measurement were carried out by using a 4-channel FireStingO2 (FSO2-4) fiber optical oxygen meter with optode sensors (Robust oxygen probe, OXROB10).

For in vitro detection of $^1O_2$ in aqueous solutions and thylakoid membranes, a fluorescence stereo microscope (Olympus, SZX12) was used for $^1O_2$ imaging. Singlet Oxygen Sensor Green (SOSG) reagent was used for $^1O_2$ imaging in cultured *Symbiodinium* cells. SOSG was added to *Symbiodinium* intact cells and protoplast followed by light illumination for 5 min under 2300 µmol photons m^{-2} s^{-1} visible light and imaging was performed by a Leica SP5 AOBS (Leica, Heidelberg, Germany) confocal laser scanning microscope using excitation at 488 nm and emission detection at 510-590 nm. The distribution pattern of $^1O_2$ in the intact cells and protoplast was quantified by using Leica Microsystems LAS-X software.

RESULTS

Development of method for the detection of intracellularly produced $^1O_2$ in *Symbiodinium* cells

1) Previous studies demonstrated that production of $^1O_2$ could be measured with the His uptake method and SOSG fluorescent probe (Rehman *et al.*, 2013, 2016). However, they were unable to detect intracellular $^1O_2$ in *Symbiodinium* and found that SOSG dye did not penetrate the cell wall, therefore intracellularly produced $^1O_2$ could not be detected in *Symbiodinium* cells. We established a method for the investigation of intracellularly produced $^1O_2$ by using the fluorescent probe (SOSG) via protoplast technology. Protoplasts were successfully isolated from *Symbiodinium* by using a combination of enzymes. For the real time monitroing of the process of protoplast

formation and regeneration, we have employed microfluidic system, with trapping and morphometric analysis of single cells throughout the whole protoplast formation and regeneration time.

2) With the presented method, we showed that the protoplast formation process and its associated morphological parameters could be monitored on single cell level, with high time resolution, which is also essential for further biotechnological and environmental applications. We investigated and quantified the morphological changes over time during the protoplast formation and observed that after addition of digestion solution, the area, major and minor axis of the cells increased, until an equilibrium state in these parameters were attained. The eccentricity of the cells exhibited a decrease, indicating that the cells became more rounded over time.

3) We also investigated the physiological or photosynthetic competency of the cells across the applied range of the flow rate to test whether the applied flow rate influenced the Photosystem II activity (F_v/F_m). Our results revealed that F_v/F_m of the cells without enzyme treatment was not impacted by the applied flow rate; however, during enzyme treatment, higher flow rate resulted in reduced F_v/F_m particularly in the 80-100 µL h^{-1} flow range. F_v/F_m largely (but not fully) recovered in the regeneration phase, when the digestion solution was replaced with regeneration medium, indicating that the enzyme digestion procedure did not impair PSII quantum yield.

4) Our results regarding the SOSG labeling of the *Symbiodinium* cells confirmed the previous findings of (Rehman *et al.*, 2016) that intact cells does not show any SOSG fluorescence intracellularly, fluorescence could only be detected externally. The protoplast were labelled intracellularly and the green fluorescence of SOSG was observed to be co-localized with the red fluorescence of chlorophyll indicating the localization of the SOSG dye in the chloroplast. However, in some cases the SOSG dye was evenly distributed in the cytoplasm and it did not show considerable co-localization with chlorophyll autofluorescence. Nevertheless, our results demonstrated for the first time that SOSG is penetrated to the protoplasts of *Symbiodinium* and therefore protoplasts are amenable to investigate singlet oxygen signaling in *Symbiodinium*.

5) In the microfluidcs system, the main advantage of the preparation of protoplasts from trapped *Symbiodinium* cells is that single protoplasts can be retained in a fixed position, therefore the SOSG fluorescence level can be precisely measured before and after illumination with strong light (i.e. signal changes before and after $^1O_2$ production). This is particularly important to consider when semi-quantitative assays of fluorescence changes are required, which is the case for the SOSG dye assay. Furthermore, parallel assays of Chl autofluorescence and SOSG fluorescence was performed on the same trapped protoplast, which enabled the co-localization of the two fluorescence signals and therefore the site of $^1O_2$ production in relation of chloroplast position can be revealed. The microfluidic method allowed the localization of $^1O_2$ inside *Symbiodinium* protoplasts using the $^1O_2$ sensitive dye SOSG, creating therefore several possibilities for the investigation of $^1O_2$-mediated signaling pathways and oxidative stress impacts.

Proline is a $^1O_2$ quencher both in vitro and in isolated thylakoids

6) By using three different approaches: EPR spin trapping by TEMPD, the fluorescent reporter dye SOSG and O_2 uptake measurements, we could clearly show that Pro does quench $^1O_2$ both in vitro and in photosynthetically active plant thylakoid membranes.

7) In our experiments, we used a slightly modified version of the spin trap, TEMPD, instead of the TEMP used by Mohanty and coworkers and added an aqueous solution, as used by (Signorelli *et al.*, 2013). The EPR line shape did not change under our conditions, indicating no significant interaction between TEMPO-D and Pro. Our findings support the original interpretation by Alia et al. (1997) that the explanation for the decreased signal of the TEMPO-D product is the removal of $^1O_2$ by Pro, which reduces the likelihood of $^1O_2$ being trapped by TEMPD.

8) We could also demonstrate that $^1O_2$ quenching by Pro occurs via a physical mechanism, i.e., it does not include a chemical (oxidation) reaction. Physical quenching of $^1O_2$ by Pro most likely proceed via forming a charge transfer complex, due to the low ionization potential of Pro, which relaxes O_2 back to its triplet ground state via inter system crossing.

9) Our results confirm the original findings of Mohanty and coworkers (Alia *et al.*, 1997, 2001) regarding the $^1O_2$ quenching ability of Pro, which was questioned on the basis of a result, that excluded only the chemical scavenging mechanism (Signorelli *et al.*, 2016), but not the physical quenching of $^1O_2$ by Pro.

10) We compared the rate constants of $^1O_2$ removal and total ROS-detoxifying capacities of proline and other antioxidants from previous literature of osmotic or salt-stressed wild-type *Arabidopsis*. From these data, it is revealed that $^1O_2$-scavenging capacity of Pro can reach up to two third of that of α-tocopherol and one fourth or more of that of Ascorbate. Therefore, the $^1O_2$-quenching potential of Pro can provide physiologically relevant contribution to $^1O_2$ detoxification in stressed plants, in cell compartments having low levels of more efficient antioxidants. The scavenging efficiency of Pro is relatively small on a molar basis, but considering its presence in high amounts in plant cells under stress conditions it may provide a physiologically relevant contribution to ROS scavenging, supplementing other nonenzymatic ROS scavengers of plant cells.

Singlet oxygen mediated photodamage to PSII in isolated thylakoid membranes and *Chlorella sorokiniana*

11) Our data show that illumination of thylakoids and intact *Chlorella* cells in the presence of RB can damage PSII activity even when relatively low concentrations of RB (1–10 μM) are used. The loss of PSII activity by externally generated $^1O_2$ is in full agreement with previous studies, which showed the impact of $^1O_2$ in vitro can be related to the fragmentation of D1 protein, i.e. the scission of peptide bonds in the D1 protein (Miyao, 1994; Lupínková & Komenda, 2004), and consequently inactivation of electron transport (Mishra *et al.*, 1994), and also with the degradation of the D1 protein during illumination of RB infiltrated leaves (Hideg *et al.*, 2007; Kovács *et al.*, 2014).

12) The damaging effect is obviously more substantial in the isolated thylakoids than in the intact *Chlorella* cells, but even in the latter case the enhancement of PSII activity loss is statistically significant and increases with increasing RB concentrations, especially in the presence of lincomycin, which blocks protein synthesis dependent PSII repair. The reason for the weaker inhibition of PSII in the intact *Chlorella* cells in comparison to the isolated thylakoids should be related to the lower local RB concentration inside the

chloroplasts in the algal cells as compared to the bulk medium, and/or the presence of efficient $^1O_2$ scavenging processes in the intact system. This idea is supported by the observation that even though a large amount of $^1O_2$ was produced at 1 μM RB concentrations in the bulk medium PSII activity loss was significant only in the higher concentration range of 5–10 μM.

13) The addition of His together with RB in thylakoids provided a significant protection against the loss of PSII activity when compared to the effect of RB addition alone, confirming the involvement of $^1O_2$ in PSII damage. In the case of *Chlorella* cells, the protective effect of His was not conclusive most likely due to the relatively small extent of PSII activity loss, which was observed as a result of illuminating *Chlorella* cells in the presence of RB. It is also possible that the penetration of histidine to the vicinity of PSII complexes inside the *Chlorella* cells, where it could exert its protective action, was limited. However, based on the strong protective effect of histidine in thylakoids, we can assume that histidine would ameliorate PSII activity loss in *Chlorella* as well, provided that it can reach the thylakoids inside the cells.

14) Considering the contradiction of our results concerning the damaging effect of externally produced $^1O_2$ with those of earlier studies by Nishiyama and coworkers in which no effect of RB induced $^1O_2$ on PSII damage rate was observed, it is important to clarify the possible causes of this disagreement. There are four main differences of the experimental conditions in our study and in the previous investigation (Nishiyama *et al.*, 2004): (i) Isolated spinach thylakoids and *Chlorella sorokiniana* cells in our study vs. the cyanobacterium *Synechocystis* PCC 6803 in the previous study. (ii) Relatively weak (240 mol photons m^{-2} s^{-1}) green dominated light to specifically excite RB without significant excitation of Chl in our case vs. strong illumination by white light (1500 mol photons m^{-2} s^{-1}) by Nishiyama et al. (iii) Lincomycin vs. chloramphenicol as protein synthesis inhibitor in the present study. (iv) PSII activity was assessed here by Chl fluorescence imaging to determine F_v/F_m vs. O_2 evolution measurements by a Clark type oxygen electrode by Nishiyama et al. (Nishiyama *et al.*, 2004).

15) We conclude that the failure of previous study to identify the RB-induced increase in PSII photodamage rate (Nishiyama *et al.*, 2004) was most likely due to numerous effects: (i) Since the $^1O_2$ produced by the RB could not reach the thylakoid-embedded

functional components of PSII, it lowered the possibility of detecting the PSII activity loss. (ii) Using strong visible light instead of selective excitation of RB for photodamage studies, together with a high chloramphenicol concentration enhanced the photodamage rate, which could easily mask the relatively small enhancement of $^1O_2$-induced PSII damage. These technical issues were solved in our study by selective excitation of RB and the use of lincomycin as a protein synthesis inhibitor that does not interfere with PSII electron transport, allowing to detect $^1O_2$-induced PSII activity loss not only in isolated thylakoids but also in intact *Chlorella* cells.

LIST OF PUBLICATIONS (MTMT: 10074886)

Peer-reviewed publications, for PhD thesis:

Bashir, F., Rehman, AU., Szabó, M., Vass, I. (2021) Singlet oxygen damages the function of Photosystem II in isolated thylakoids and in the green alga *Chlorella sorokiniana. Photosynthesis Research* 149, 93-105. **IF: 3.63**

Rehman, A. U., **Bashir, F.**, Ayaydin, F., Kóta, Z., Páli, T., Vass, I. (2020) Proline is a quencher of singlet oxygen and superoxide both in in vitro systems and isolated thylakoids. *Physiologia Plantarum*, *172*(1), 7-18. **IF: 4.14**

Faiza Bashir, Milán Szabó, Sándor Kovács, Ágnes Ábrahám, Ferhan Ayaydin, Péter Galajda, László Sass, Imre Vass (2021) Microfluidic analysis of viable protoplast formation in the coral endosymbiont alga Symbiodinium sp. (submitted to Lab on a Chip)

Other publications:

Mallick, I., Kirtania, P., Szabó, M., Bashir, F., Domonkos, I., Kós, P. B., & Vass, I. (2020). A simple method to produce Synechocystis PCC6803 biofilm under laboratory conditions for electron microscopic and functional studies. *Plos one*, *15*(7), e0236842. **IF: 2.74**

International conference abstracts and poster presentations

1. **Faiza Bashir**, Ateeq Ur Rehmam, Ildiko Domonkos, Ferhan Ayaydin and Imre Vass (2017) $^1O_2$ induced impairment of EPSs in Synechocystis and Symbiodinium at a Straub conference at Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.
2. **Faiza Bashir**, Ateeq Ur Rehmam, Ildiko Domonkos, Ferhan Ayaydin and Imre Vass (2017) Singlet oxygen induced impairment of extracellular polymeric substances in *Synechocystis* and *Symbiodinium* at 19th IUPAB and 11th EBSA Congress, Edinburgh, Scotland
3. Ateeq Ur Rehmam, **Faiza Bashir**, Ferhan Ayaydin, Zoltán Kóta, Tibor Páli and Imre Vass (2018) Proline is an excellent quencher of superoxide, and singlet oxygen in vitro at a Straub conference at Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.
4. Ateeq Ur Rehman, Huiru Li, **Faiza Bashir**, László Kovács, Imre Vass, Christian Wild, Kai Bischof (2018) Effect of high intensity light on Photosystem II function and singlet oxygen production in the brown seaweed *Saccharina latissima* at a Straub conference at Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.
5. **Faiza Bashir**, Ferhan Ayaydin, Ildikó Kelemen-Valkony, Györgyi Ferenc, Péter Kós, Milán Szabó, Imre Vass (2019) Application of protoplast technology for Symbiodinium sp. and other microalgae at a Straub conference at Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.
6. **Faiza Bashir**, Ferhan Ayaydin, Ildikó Kelemen-Valkony, Györgyi Ferenc, Péter Kós, Milán Szabó, Imre Vass (2019) Protoplast technology as an experimental platform for characterizing oxidative stress in Symbiodinium sp. and other microalgae at 9th Symposium on Microalgae and Seaweed products in Plant/Soil-systems at Mosonmagyaróvár, Hungary.
7. **Faiza Bashir**, Milán Szabó, Imre Vass (2021) Singlet oxygen damages the function of Photosystem II in isolated thylakoids and in the green alga Chlorella sorokiniana at 8th Congress of Hungarian Society of Plant Biology, Szeged, Hungary.
8. **Faiza Bashir**, Milán Szabó, Imre Vass (2021). Characterization of singlet oxygen in the photosynthetic apparatus of plants and microalgae at 11th Congress of the Hungarian Free Radical Society, Budapest, 27th August, 2021, Hungary.

Declaration

As the main author of the scientific publications, I certify that the results reported in the Ph.D. dissertation and the following publications were not used to acquire any Ph.D. degree previously and will not be used in future either.

Bashir, F., Rehman, AU., Szabó, M., Vass, I. (2021) Singlet oxygen damages the function of Photosystem II in isolated thylakoids and in the green alga *Chlorella sorokiniana. Photosynthesis Research* 149, 93-105.

Rehman, A. U., Bashir, F., Ayaydin, F., Kóta, Z., Páli, T., Vass, I. (2020) Proline is a quencher of singlet oxygen and superoxide both in in vitro systems and isolated thylakoids. *Physiologia Plantarum*, 172 (1), 7-18.

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