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Higher packing of thylakoid complexes ensures a preserved Photosystem II activity in mixotrophic *Neochloris oleoabundans*

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ABSTRACT

A better understanding of the microalgal basic biology is still required to improve the feasibility of algal bio-products. The photosynthetic capability is one of the parameters that need further progress in research. A superior PSII activity was previously described in the green alga *Neochloris oleoabundans*. In this study, *N. oleoabundans* was grown in a glucose-supplied culture medium, in order to provide new information on the organisation and interaction of thylakoid protein complexes under mixotrophy. Fluorescence measurements suggested a strong association of light harvesting complex II (LHCII) to PSII in mixotrophic samples, confirmed by the lack of LHCII phosphorylation under growth light and the presence of PSI-PSII-LHCII megacomplexes in Blue-Native gel profile. The chloroplast ultrastructure was accordingly characterised by a higher degree of thylakoid appression compared to autotrophic microalgae. This also affected the capability of mixotrophic microalgae to avoid photodamage when exposed to high-light conditions. On the whole, it emerged that the presence of glucose affected the photosynthetic performance of mixotrophic samples, apparently limiting the dynamicity of thylakoid protein complexes. As a consequence, PSII is preserved against degradation and the PSI:PSII is lowered upon mixotrophic growth. Apparent increase in PSII photochemical activity was attributed to a down-regulated chlororespiratory electron recycling.

1. Introduction

Photosynthesis supports almost all life on Earth and involves several light-dependent reactions, which start with the absorption of light energy for the synthesis of NADPH and ATP [28], used during the Calvin-Benson cycle for CO_2 fixation [22]. Important features of the light reactions of photosynthesis are: collection of photons by light-harvesting antennae, migration of excitation energy to the reaction centers, electron transfer from H_2O to $NADP^+$, and ATP generation [28]. Light-harvesting pigment-protein complexes (LHC) deliver the absorbed light energy to the reaction centers of Photosystem II (PSII) and Photosystem I (PSI) [53]. The major LHC of PSII, LHCII, is also essential for maintaining thylakoid membranes stacked and promoting distribution of absorbed light energy between photosystems [56,70]. PSII transfers electrons from water to plastoquinone (PQ) using light energy as a driving force [16,19,53]. The electrons from plastohydroquinone reach PSI *via* cytochrome (Cyt) b_6f complex and plastocyanin. PSI is involved in a light-dependent electron transport to ferredoxin and to $NADP^+$ [16]. ATP synthase (ATPase) is the highly-conserved complex that catalyses ATP synthesis using the trans-membrane proton gradient created during the electron flow [54].

Important for understanding the molecular basis of the photosynthetic process is a detailed knowledge of the structure of its components [11,21,55]. All protein complexes are composed of several protein subunits coordinating a large number of cofactors, which show a tendency to form higher-order associations, the so-called supercomplexes [14,17,21,52,67]. The dynamic organisation of the pigment-protein complexes in the thylakoid membrane plays important roles in

Abbreviations: 2D, second dimension; ATPase, ATP synthase; BN-PAGE, Blue-Native polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Chl, chlorophyll; Cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_0, basal fluorescence level excited by a very low measuring light after dark incubation; F_M, maximum fluorescence level obtained by a saturating light pulse after dark incubation; F_M', maximum fluorescence level during a light-adapted state; F_t, basal fluorescence level during a light-adapted state; F_V, variable fluorescence level obtained by the difference of F_M and F_0; F_V/F_M, maximum photochemical quantum yield of Photosystem II; LHC, light-harvesting pigment-protein complexes; PAM, pulse amplitude modulation; PQ, plastoquinone; PQH_2, plastohydroquinone; PSI, Photosystem I; PSII, Photosystem II; Q_A, quinone A; Q_B, quinone B; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy

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maintaining an optimal photosynthetic efficiency under several conditions, including different light regimes, temperature and nutrient supply [4,16]. In green microalgae, whose cell volume is mainly occupied by the chloroplast, the photosynthetic efficiency is an indicator of their wellness conditions [80]. This is an important factor to be taken into account, considering the importance of green microalgae for biotechnological purposes [13,15]. In this scenario, mixotrophic microalgae have been largely investigated for their capability to highly increase their biomass content, benefitting from the exogenous organic carbon source assimilation together with light harvesting and CO_2 fixation for growth [46,63,66,81]. However, there are few works concerning the interaction between photosynthetic complexes in thylakoid membranes during the assimilation of organic carbon by microalgae; in general, a specific reduction in PSII photochemistry was observed [48,57,73]. Very differently, mixotrophy promoted a very high PSII maximum quantum efficiency in the Chlorophyta *Neochloris oleoabundans* [9,30]. In this work, the effects of glucose supplied in the culture media of *N. oleoabundans* were assessed in order to provide new information on the photosynthetic metabolism and to understand the interaction of the different pigment-protein complexes during the organic carbon source assimilation. Immunodetection of different subunits of thylakoid multi-protein complexes was employed to identify differences in their relative abundance between autotrophic and mixotrophic samples, whereas Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) was employed to obtain information on native interactions of photosynthetic protein complexes in thylakoids [33,61]. In parallel, chlorophyll (Chl) fluorescence measurements were performed *in vivo* on freshly-collected samples to identify differences in photosynthetic electron transport in autotrophic and mixotrophic cells.

2. Materials and methods

2.1. Algal strain and culture condition

The Chlorophyta *Neochloris oleoabundans* UTEX 1185 (syn. *Ettlia oleoabundans*, *Sphaeropleales*, *Neochloridaceae*) was obtained from the Culture collection of the University of Texas (UTEX, USA; www.utex.org). Cells were grown and maintained in axenic liquid BM medium [8] in a growth chamber (24 ± 1 °C temperature, 80 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ PAR and 16:8 h of light-darkness photoperiod), without shaking and external CO_2 supply. For experiments, cells were inoculated at least in triplicate at a density of 0.6 ± 0.1 × 10^6 cells mL^{-1} in BM medium containing 0 (autotrophic cells) or 2.5 g L^{-1} of glucose and grown in 500 mL Erlenmeyer flasks (300 mL of total volume) in the growth chamber described above, with continuous shaking at 80 rpm. The glucose concentration of 2.5 g L^{-1} was selected in previous experiments in which the microalga was grown in the presence of increasing concentrations of glucose from 0 to 30 g L^{-1}, comparing among them growth rates, cell morphology, glucose consumption and lipid accumulation inside cells, as reported in Giovanardi et al. [30]. Growth was estimated measuring the optical density at 750 nm with a Pharmacia Biotech Ultrospec®2000 UV–vis spectrophotometer (1 nm bandwidth; Amersham Biosciences, Piscataway, NJ, USA) and counting cells with a Thoma's haemocytometer under the light microscope (Zeiss, Axiophot, Jena, DE), on 1 mL of culture samples at days 0, 2, 3, 4, 7, 9, 11.

2.2. Fluorescence measurements

2.2.1. Modulated chlorophyll fluorescence: slow kinetics

In vivo Chl*a* fluorescence was determined from liquid cultures at the late exponential phase of growth, *i.e.* at the 6th day from the inoculum, harvested by centrifugation to contain 15 μg mL^{-1} Chl. Chlorophyll quantification was performed according to Wellburn [79]. Cell suspensions were pre-incubated in darkness for 10 min and samples were subsequently exposed to actinic blue light. The following program was triggered: 90 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, 11 min; dark, 11 min; 1000 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, 15 min; dark, 5 min. Light saturating pulses (0.6 s) were given every 40 s. Initial fluorescence F_0 and maximum fluorescence F_M after dark incubation were used to calculate the maximum quantum yield of PSII (F_V/F_M ratio), according to Lichtenthaler et al. [47]. Time course of Chl fluorescence parameters F_M', *i.e.* the maximum fluorescence in the light-adapted state measured applying the pulse, and F_t, *i.e.* the steady-state fluorescence yield, were determined with a DUAL-PAM-100 (Walz, Germany).

The effects of far red light on PSII fluorescence were determined using an ODC OS1-FL portable fluorimeter (ADC Bioscientific Ltd., Hoddesdon, Hertfordshire, UK) on cell pellets prepared as described in Ferroni et al. [24]. Measurements were performed on 10 min dark-adapted samples. Cells were excited with far red light (740 nm) for 10 min. After that, recovery was followed for 10 min in darkness. During the experiment, light saturating pulses were given every minute during the far red light exposure and at times 1, 2, 5 and 10 min during dark relaxation. The F_M'/F_M ratio was calculated and used to determine variations of PSII fluorescence.

2.2.2. Fast chlorophyll fluorescence

Q_A^- reoxidation kinetics was determined by flash-induced Chl fluorescence relaxation kinetics. The single turnover flash-induced increase in Chl*a* fluorescence yield and its subsequent relaxation in darkness (FF-relaxation) were measured with a double-modulation fluorimeter (Photon System Instruments, Brno, Czech Republic). For analyses, 1 mL of samples containing 8 μg mL^{-1} Chl was incubated in darkness for 10 min and then Q_A^- reoxidation kinetics was recorded, after a single-saturating flash (10 μs) provided by red LED, in the 150 μs–100 s time range. Analyses were carried out either in the presence or absence of 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [2]. For easier comparison, the fluorescence relaxation curves were averaged and normalised to the same amplitude. The relative Q_A^- concentration was estimated according to the model of Joliot [40]. Multicomponent deconvolution of the relaxation curves was performed according to Vass et al. [74].

2.2.3. 77 K fluorescence emission spectra

Fluorescence emission spectra measured *in vivo* from samples containing 8 μg mL^{-1} Chl were recorded at 77 K using a diode array spectrophotometer (S2000; Ocean Optics, Dunedin, FL, USA) equipped with a reflectance probe as described in Keränen et al. [83]. The spectra were obtained by excitation with light at 440 nm, defined using LS500S and LS700S filters (Corion, Holliston, MA, USA) placed in front of a slide projector, whereas the emission between 600 and 800 nm was recorded. For each biological replicate, at least 3 measurements were recorded.

2.3. Thylakoid isolation

Thylakoid membranes were isolated according to Järvi et al. [39], with modifications. For extraction, 300 mL of cultures in late-exponential phase of growth were harvested by centrifugation at 600*g* for 10 min. Pellets were transferred to an ice-cold mortar containing sand quartz. The extraction was performed grinding cells with liquid N_2, then the lysate was resuspended in a grinding buffer (330 mM sorbitol, 50 mM Tricine-NaOH pH 7.5, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 5 mM ascorbate, 0.05% bovine serum albumin, 10 mM NaF) and transferred to 15 mL tubes. Samples were centrifuged at 300*g* for 5 min at 4 °C and then at 700*g* for 5 min at 4 °C, to remove sand quartz and cell debris. Pellets were discarded and the thylakoids present in the supernatant were collected by centrifugation at 7000*g* for 10 min at 4 °C. The supernatant was discarded and thylakoids were resuspended in 1 mL of shock buffer (5 mM sorbitol, 50 mM Tricine-NaOH pH 7.5, 2 mM Na_2EDTA, 5 mM $MgCl_2$, 10 mM NaF) and centrifuged at 7000*g* for 10 min at 4 °C. After that, the supernatant was removed and around 100 μL of storage buffer (100 mM sorbitol, 50 mM Tricine1-NaOH

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