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Algal glycerol accumulation and release as a sink for photosynthetic electron transport

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ABSTRACT

Responses of freshwater and marine species of *Chlamydomonas* to salinity and light intensity were compared in the context of potential biomaterial and bioenergy production. While the freshwater species *C. reinhardtii* arrested growth at a salinity of 0.2 M NaCl, the marine species *C. euryale* showed vigorous growth and high levels of internal glycerol accumulation between 0.2 and 2 M NaCl. The freshwater species exhibited no downregulation of photosynthesis between 0 and 0.2 M NaCl; the marine species exhibited significant upregulation of photosynthesis between 0.2 and 2.0 M NaCl, indicating that internal glycerol, unlike sugars, does not induce photosynthetic downregulation. The freshwater species released considerably more glycerol than the marine species at the common salinity of 0.2 M NaCl, suggesting either possible limitations in the freshwater species to internal retention of glycerol as an osmoregulant, or formation and active release of high glycerol levels as a sink for photosynthetic electron transport when growth is not available as a sink. Continuous release of glycerol into the medium, or internal glycerol accumulation followed by periodic glycerol release via hypo-osmotic shock, are attractive potential options for continuous production of biomaterials and bioenergy from renewable sources in algae with high rates of solar energy conversion to photosynthetically produced energy carriers.

1. Introduction

The overall efficiency of solar-energy conversion into energy-rich biomass by living organisms is relatively low [1,2], meaning that plant or algal biomass contains only a small fraction of the solar energy collected by the photosynthetic organism over its lifetime, because the vast majority of harvested energy is expended to fuel the myriad metabolic reactions the organism carries out during growth, cell division, and reproduction. In contrast, a photosynthesizing organism's efficiency of solar-energy conversion into the initial energy-rich chemical products of photosynthesis can be quite high [3–5]. Consequently, withdrawing energy carriers before the energy is utilized for the organism's own growth and development is a promising approach for increasing overall efficiency and productivity of energy-carrier production by photosynthetic organisms.

Examples of such an approach can be found in nature in the symbioses between photosynthetic microbes and their non-photosynthetic host organisms. Such photosynthetic symbionts expend little energy for cell division and growth, instead releasing a large fraction of the energy-rich products of photosynthesis to the host in exchange for CO_2 and nutrients [6–11]. A major energy carrier released by photosynthetic symbionts for utilization by their host is glycerol produced directly from photosynthesis; glycerol is, in turn, readily used as the main energy source by a multitude of non-photosynthetic organisms [12–14]. Attractive aspects of copying this process in a nature-inspired approach for producing biomaterials and bioenergy include (i) elimination of the need to harvest, extract, and regrow algae by virtue of simply harvesting energy carriers released by the algae to the surrounding medium and (ii) the prospect of driving up photosynthetic rates by circumventing feedback inhibition acting on photosynthesis.

A photosynthetic organism's maximal capacity for photosynthesis is controlled in a feedback loop by the organism's rate of utilization of energy carriers produced by photosynthesis [15,16]. When production exceeds utilization, resulting sugar accumulation in leaves or algae induces repression of multiple photosynthetic genes, which lowers (downregulates) photosynthetic capacity [17]. Conversely, efficient sugar removal from the site of photosynthesis leads to upregulation of photosynthetic genes and an increased maximal capacity for photosynthesis (for plants, see [15,16]; for algae, see [18]). This phenomenon is known as source-sink regulation, with photosynthesis representing the energy source and all processes that withdraw, store, and/or consume sugars produced in photosynthesis acting as sinks for photosynthate [19].

The present study focuses on green algae of the genus *Chlamydomonas* and their production of glycerol, which is strongly stimulated by salinity in the external medium [20–23]. Internal glycerol accumulation within the alga functions in osmotic adjustment, which

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lowers internal osmotic potential and thereby prevents excessive water loss from the alga to the saline external medium [24]. We test the hypothesis that formation and/or accumulation of glycerol, unlike that of sugars, is not associated with photosynthetic repression and may instead act as a major sink for electrons from photosynthetic electron transport. This postulate is based on the notion that conversion of photosynthetically produced triose dihydroxyacetone phosphate to glycerol (via glycerol-phosphate dehydrogenase) produces an osmoregulant and energy carrier (glycerol) that, unlike glucose, is presumably not perceived as part of the pool of photosynthetic products (sugars) by the signaling network that exerts feedback on photosynthesis [25]. We predict that maximal capacity of photosynthetic electron transport can thus be positively correlated with internal glycerol accumulation and/or release from the algae into the external medium.

In addition, we compare and contrast the responses of a freshwater versus a marine species and show that the proportion of internally accumulated glycerol to released glycerol is higher in the marine compared to the freshwater species, while the freshwater species loses more glycerol to the external medium than the marine species at a common salinity. We place these results into the context of approaches to continuously siphon off released glycerol and of non-destructive removal of internally accumulated glycerol.

2. Materials and methods

2.1. Species

The freshwater species *Chlamydomonas reinhardtii* (UTEX #2244) and the marine species *Chlamydomonas euryale* (UTEX #2274) were obtained from the Culture Collection of Algae at the University of Texas, Austin (https://utex.org). Culture maintenance and pre-culturing in Roux bottles was performed as described in [23], except that the marine species was maintained in flasks and pre-cultured in Roux bottles at 0.5 M NaCl.

2.2. Experimental conditions and sampling protocol

After pre-culturing in Roux bottles, 30-mL culture volumes were standardized to an Optical Density at 750 nm (OD_{750}) of 2.25 and transferred to 50-mL test tubes. Test tubes and growing conditions were as described in [23], with a continuous stream of 5% CO_2 (in ambient air) delivered to cultures and an incident light intensity at the surface of the test tubes of either 200 or 500 μmol photons m^{-2} s^{-1}. Experimental media for *C. reinhardtii* contained 0.0, 0.1, or 0.2 M NaCl, while media for *C. euryale* contained 0.2, 0.5, or 2.0 M NaCl. Cultures were grown in test tubes for approximately 1.5 days for acclimation to the experimental conditions, and subsequently diluted (by visual assessment) to a similar greenness as initial cultures prior to the 1.5 days of growth. Thirty mL of these diluted cultures were transferred to sterile 50-mL centrifuge bottles, gently centrifuged (2 mins, 706*g*), and pellets resuspended in 30 mL of fresh medium and returned to test tubes and experimental conditions for the duration of the subsequent 24-h experiment. Samples for OD_{750}, cell counts, chlorophyll, and intracellular and released glycerol were collected directly before and 24 h after experimental exposure of diluted cultures in test tubes. Samples for determination of rates of CO_2– and light-saturated photosynthetic oxygen evolution and respiration were collected after 24 h. Samples for the quantification of intracellular glycerol levels were prepared from algal pellets using centrifugation, rinsing, and boiling protocols described by [18], but with a lower centrifugation g-force (10 mins, 1476*g*) to avoid rupturing the cells. Supernatants were frozen for subsequent analysis of glycerol released into the medium. Samples were processed and analyzed as described in [23] for OD_{750}, cell count, chlorophyll, and rates of photosynthetic oxygen evolution and respiratory oxygen uptake.

2.3. In-situ chlorophyll fluorescence

In-situ chlorophyll fluorescence measurements were obtained with a portable PAM-2000 fluorometer (Walz, Effeltrich, Germany) by appressing the end of the fiber optic cable against the test tubes perpendicular to the incident growth light, and fluorescence yields were recorded on a chart recorder (Kipp & Zonen B.V., Delft, The Netherlands). Effective energy conversion efficiency of photosystem II under the incident light intensity ($[F_m' - F]/F_m'$, where F_m' is the maximal fluorescence yield during illumination and F is the steady-state yield of fluorescence under the incident light) was calculated as described in [3]. Measurements were taken 2.5 h (200 μmol photons m^{-2} s^{-1} only) and 24 h (for both 200 and 500 μmol photons m^{-2} s^{-1}) after the start of the 24-h experimental period with diluted cultures in fresh medium.

2.4. Glycerol analysis

Glycerol concentration was determined colorimetrically using an EnzyChrom Glycerol Assay Kit (BioAssay Systems, Hayward, CA, USA). For assessment of glycerol in the external medium, 100 μL of supernatant was diluted 1:10 with 900 μL of deionized water in 2 mL-microcentrifuge tubes, thoroughly mixed, and 4 μL of each sample were placed in wells on a flat-bottom 96-well plate, with colorimetric readings obtained using a Tecan Safire^2 plate reader (Männedorf, Switzerland). For the determination of intracellular glycerol levels, samples were diluted 1:20 to account for the higher glycerol concentrations.

2.5. Glycerol addition to the culture medium

Cultures with glycerol added to the experimental medium were prepared as above, but after the cultures were diluted and gently centrifuged, they were resuspended in fresh sterile medium containing 25 mM glycerol. Twenty-five mM glycerol was chosen to provide slightly more glycerol in the surrounding medium than would be accumulated after several days of glycerol release by a dense culture (data not shown), but was also a low enough concentration as to not significantly change the osmotic potential of the culture medium compared to cultures without glycerol added.

2.6. Statistical analyses

Data were subjected to ANOVAs coupled with Tukey-Kramer tests or non-paired, two-tailed *t*-tests in order to determine differences among mean values using JMP software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Response of the freshwater species Chlamydomonas reinhardtii to external salinity

The freshwater species *C. reinhardtii* was grown over a range of external salinities, from optimal growth conditions in the absence of NaCl to near-complete inhibition of algal growth. Algal growth rate (rate of cell divisions per day) was strongly inhibited by 0.2 M NaCl, which corresponds to approximately 40% of seawater salinity (Fig. 1A). Chlorophyll content per cell increased strongly with increasing salinity (Fig. 1B), and the efficiency of the conversion of light absorbed in photosystem II into photochemistry (measured via chlorophyll fluorescence as $[F_m' - F]/F_m'$) decreased with increasing salinity (Fig. 1C).

Both the maximal capacity of photosynthesis (light- and CO_2-saturated rates of linear electron transport as net oxygen evolution) and cellular respiration rate decreased with increasing salinity when expressed relative to the (increasing) algal chlorophyll content (Fig. 2A, C), but