Improving the Photosynthetic Productivity and Light Utilization in Algal Biofuel Systems: Metabolic and Physiological Characterization of a Potentially Advantageous Mutant of *Chlamydomonas Reinhardtii*

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Abstract: In this study, we report initial biophysical and biochemical characterization of a spontaneous 'mutant' (referred to as 'IM') of the green alga *Chlamydomonas reinhardtii* with several unique attributes that has potential for improving photosynthetic productivity, light utilization efficiency, and, perhaps, even protection against environmental stress. Growth rate experiments showed that under low light intensity (10 μmol photons m^{-2} s^{-1}), IM showed 36% higher cell density and 25% higher dry cell weight than the wild type cells (WT), while at higher light intensity (640 μmol photons m^{-2} s^{-1}), the IM did not show any advantage. Chlorophyll *a* fluorescence transient measurements and subsequent analysis indicated that IM cells grown at a light intensity of 20 μmol photons m^{-2} s^{-1} had a higher light utilization efficiency in comparison to the WT cells. Interestingly, metabolite profiling analysis showed that during the exponential growth phase with both low and high light intensities (10 and 640 μmol photons m^{-2} s^{-1}), the IM cells had higher concentrations than WT cells for several important metabolites that have been previously shown to help protect against environmental stress.

Keywords: *Chlamydomonas reinhardtii*; Mutant; Growth; Metabolite profiling; Fluorescence transient

Introduction

Fossil fuels have been widely recognized as unsustainable over the long-term, and algae offer some of the best potential for a sustainable supply of renewable biofuels (Chisti, 2007). Algae have several key advantages including: higher growth rates than plants, the ability to grow on marginal areas or in low quality water sources and ability to consume excess nutrients in eutrophic waters; further, high oil content can be achieved with certain species. Despite these significant advantages, the promise of algal biofuels remains largely unfulfilled because of several practical bottlenecks in the process. A spontaneous 'mutant' of the green alga *Chlamydomonas reinhardtii* (referred to as 'IM') showed several unique attributes that have potential for improving algal biofuel production in terms of biomass productivity, light utilization efficiency, and, perhaps, even protection against environmental stress. Thus, elucidating these distinctive characteristics of the mutant could help accelerate development of practical biofuel production processes to meet global fuel demands.

Materials and Methods

Strains and culture conditions

The 'immortal mutant' (IM) algal strain used in this study descended from the previously described ptx2 mutant of *Chlamydomonas reinhardtii* that was

constructed by insertional mutagenesis to be lacking in light-induced flagellar currents, which resulted in defects both phototaxis and photoshock responses (Pazour *et al*., 1995). Wild type (WT) and 'immortal mutant' (IM) cultures of *Chlamydomonas reinhardtii* were maintained under room light and temperature in Tris–acetate–phosphate (TAP) agar culture medium plates (20 mmol Tris; 17.4 mmol acetate; 7 mmol NH_4Cl; 0.4 mmol $MgSO_4$; 0.3 mmol $CaCl_2$; 1 mmol phosphate buffer; 1 ml/L Hutner's trace metal solution; 15 g/L Bacto agar). Prior to each experiment, an inoculum of these cultures was grown in 250 ml Erlenmeyer flasks containing 50 ml liquid TAP medium (without Bacto agar) on an orbital shaker at 24 °C and under 20 μmol photons $m^{-2} s^{-1}$ photosynthetically active radiation (PAR) provided by fluorescent lamps.

Growth rate experiments

WT and IM cells were cultured in TAP medium at two different light intensities. For lower light intensity (10 ± 1 μmol photons m^{-2} s^{-1}) experiments, the light was from two linear fluorescence lamps, placed on top of the shaker; its intensity was measured at 9 evenly distributed points on the surface of the shaker. For higher light intensity experiments (average 640 ± 5 μmol photons $m^{-2} s^{-1}$), 6 compact fluorescence lamps were used to provide light for 6 culture flasks individually, and the light intensity was measured at 10 points on the surface of the flasks. The maximum was 1270 ± 35 μmol photons m^{-2} s^{-1} in the center, and the minimum was 285 ± 20 μmol photons m^{-2} s^{-1} on the edge. The initial cell density was 10,000 cells/ml. Duplicates of each of the algal strains were cultivated and the growth data was averaged to determine the growth curve of each strain.

Cell growth was determined by cell density and dry cell weight. Cell counting was conducted using Neubauer hemacytometer and repeated twice for each replicate to determine an average value. Dry cell weight was determined as total suspended solids according to standard methods (Clesceri *et al*., 1999).

Chlorophyll a fluorescence transient analysis

Chlorophyll *a* fluorescence transients were measured at ambient temperature (20−22 ºC) for 3 s with a portable fluorimeter (PEA, Plant Efficiency Analyzer, Hansatech Inst., UK) with excitation light (620 nm) intensity of 3,000 μmol photons $m^{-2} s^{-1}$ after cells were dark adapted for 6 min. The algal cultures used in these tests were taken from the exponential phase of growth under a light intensity of 20 μmol photons $m^{-2} s^{-1}$ (48 h after inoculation), and the cells were diluted (or concentrated) to a chlorophyll concentration of 15 μg/ml. Before the measurement began, the cell suspension was placed in flasks with stirring under room illumination. Three separate cultures of each of the strains were used for measurement, and triplicate measurements were conducted for each culture replicate ($n = 9$). Analysis of these data was carried out according to the JIP test procedure as previously described by Strasser *et al*. (2004) and reviewed by Stirbet and Govindjee (2011).

Metabolite profiling

Metabolite profiling of the IM and WT algal cells, both in the exponential growth phase and stationary growth phase, was conducted using GC-MS (Gas Chromatography-Mass Spectroscopy) as described previously by Rupassara (2008) with the following modifications. In the methanol extraction step, the samples were vortex-mixed and incubated at 65 °C instead of 70 °C. After the initial methanol extraction, pellets were further extracted using methanol: chloroform: 0.1mol HCl in water (5:2:3) instead of water: methanol (1:3). The GC-MS interface temperature was 310 °C, and the ion source was kept at 220 °C.

Results and Discussion

Growth rate experiments

After 337 hours of cultivation at light intensity of 10 μmol photons m^{-2} s^{-1}, the dry weight of IM cells (370 mg/L) was 25% higher than the WT cells grown under the same conditions (See Fig. 1, panel A), and the density of the IM cells ($1.41 \pm 0.46 * 10^7$ cells/ml) was 36% higher than WT cells ($1.03 \pm 0.40 * 10^7$ cells/ml). At a higher light intensity of 640 μmol photons $m^{-2} s^{-1}$, IM and WT cells showed very similar biomass production as shown in panel B of Fig. 1, but IM cells did have a noticeably lower cell density ($0.93 \pm 0.39 * 10^7$ cells/ml) than the WT cells ($1.47 \pm 0.39 * 10^7$ cells/ml).

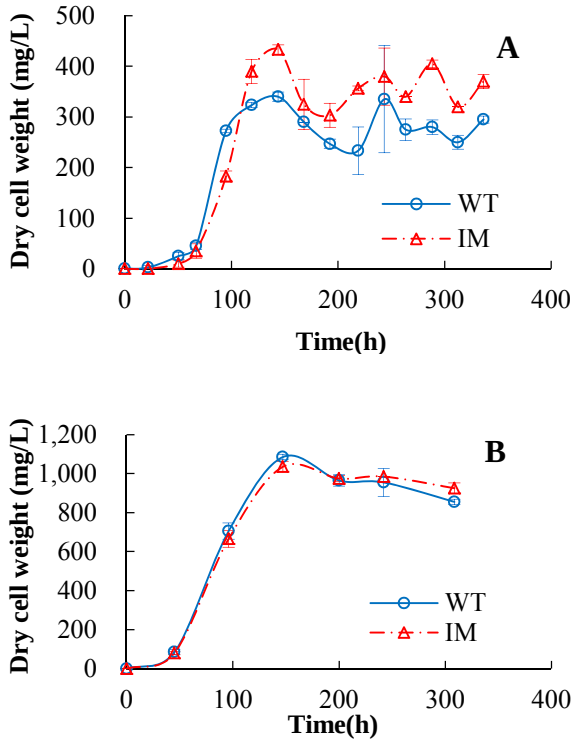

Fig. 1 Dry cell weight of wild type (WT) and 'immortal mutant' (IM) cells at two different light intensities.(**A**) At light intensity of 10 μmol photons m^{-2} s^{-1}; (**B**) At light intensity of 640 μmol photons m^{-2} s^{-1} .

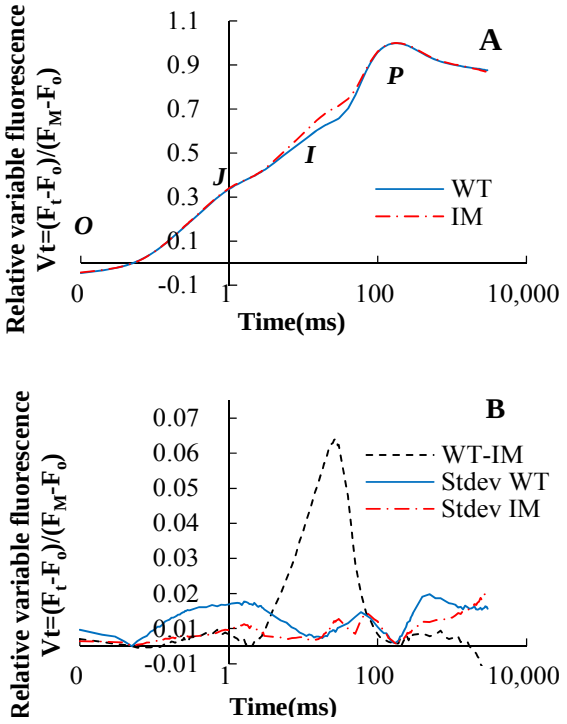

Fig. 2 (**A**) Chlorophyll *a* fluorescence transients for wild type (WT) and 'immortal mutant' (IM) cells after double normalization. O stands for origin, J and I for inflection points, and P for peak. Each curve was obtained from the average of nine independent experiments. (**B**) The difference between the relative variable fluorescence of 'immortal mutant' cells and wild type cells (IM-WT) and the standard deviation for the relative variable fluorescence of wild type cells (Stdev WT) and 'immortal mutant' cells (Stdev IM) over nine experimental replicates.

Chlorophyll a fluorescence transient analysis

Chlorophyll *a* fluorescence transients were measured and analyzed to characterize Photosystem II activity. The fluorescence transient curves were double normalized to get relative variable fluorescence, which allows for a comparison of transients measured on different samples (Strasser *et al*., 2004). As shown in panel A of Fig. 2, a small difference in the relative variable fluorescence intensity (~5%−10%) at the 'I' level was observed between IM and WT cells during the exponential growth phase at light intensity of 20 μmol photons m^{-2} s^{-1}. Similar differences were also observed during the stationary phase. In order to better highlight the difference at the 'I' level, we subtracted the relative variable fluorescence intensity of WT cells from that of IM cells as shown in panel B of Fig. 2. This difference was consistent in all of the nine replicate samples, and it was much larger than the standard deviations of the nine replicates for both the WT and IM cells, which are also shown in panel B of Fig. 2. Thus, this relatively small difference at the 'I' level indicates that there are some real differences in the Photosystem II activity of the IM cells.

The fluorescence transient data was analyzed using the JIP test method as previously reviewed (Strasser *et al*., 2004; Stirbet and Govindjee, 2011), and selected parameters are shown in Table 1. This analysis revealed that the IM cells had ~9% higher variable fluorescence ratio F_v/F_o, which is an indicator of higher capacity for photosynthetic quantum conversion and CO_2 fixation (Lichtenthaler and Babani, 2004). The IM cells also showed ~9% higher efficiency in primary photochemistry, $\varphi_{Po}/(1-\varphi_{Po})$, and a ~7% lower dissipation of energy per reaction center, DI_o/RC. These two values reflect that after the photons are absorbed by the antenna pigments, less excitation energy is lost to heat dissipation or fluorescence in the IM cells as compared to the WT cells. Thus, more excitation energy is channeled to the reaction centers and further converted to redox energy, which ultimately leads to CO_2 fixation. The performance index on an absorption basis PI(ABS), which reflects the energy conservation from photons

absorbed by the antenna to the reduction of Q_B, is also higher (~10%) in the IM cells than in WT cells. This indicates a stronger overall photosynthetic driving force. These differences could help explain the higher IM biomass yields under low light shown previously in Fig. 1.

Table 1 Selected fluorescence parameters.

	WT	IM	Difference (IM-WT)/WT
F_v/F_o	3.04	3.31	8.7%
$\varphi_{Po}/(1-\varphi_{Po})$	3.04	3.31	8.7%
DI_o/RC	0.64	0.59	7.0%
PI(ABS)	18.31	20.10	9.8%

F_v/F_o: variable fluorescence ratio
$\varphi_{Po}/(1-\varphi_{Po})$: efficiency in primary photochemistry
DI_o/RC: dissipation of energy per reaction center
PI(ABS): performance index on absorption basis

Metabolite profiling

Metabolite profiling also revealed some potentially advantageous differences of IM cells over WT cells under certain conditions. During the exponential growth phase (Fig. 3, panel A), the IM cells had higher concentrations than WT cells of several potentially important metabolites, such as total tocopherols, which has been previously shown to help protect against various environmental stress conditions (Paul, 2007; Maeda Della, 2007). During stationary growth (Fig. 3, panel B), IM cells had higher levels of tocopherols and α-tocopherolhydroquinone than WT cells, but only under low light conditions (10 µmol photons m^{-2} s^{-1}). Trehalose and inositol were lower in the IM cells during the stationary phase. Overall, the IM cells had better production of protectant compounds during exponential growth but showed poorer production during the stationary phase.

Conclusion

In this study, a spontaneous mutant of the green alga *Chlamydomonas reinhardtii* exhibited certain unique biophysical and biochemical characteristics including higher biomass production, light utilization efficiency, and production of protective compounds under certain conditions. These unique attributes are potentially advantageous for enhancing algal biofuel production capabilities. Future research should be directed at a more detailed characterization of the IM cells including direct measurement of photosynthesis rate, non-photochemical quenching measurements and its resistance to other environmental stress conditions. Ultimately, gene sequencing could be used to identify the specific genes associated with desirable photosynthetic differences and then transfer of those genes to other algae species (or other strains of Chlamydomonas) could be pursued.

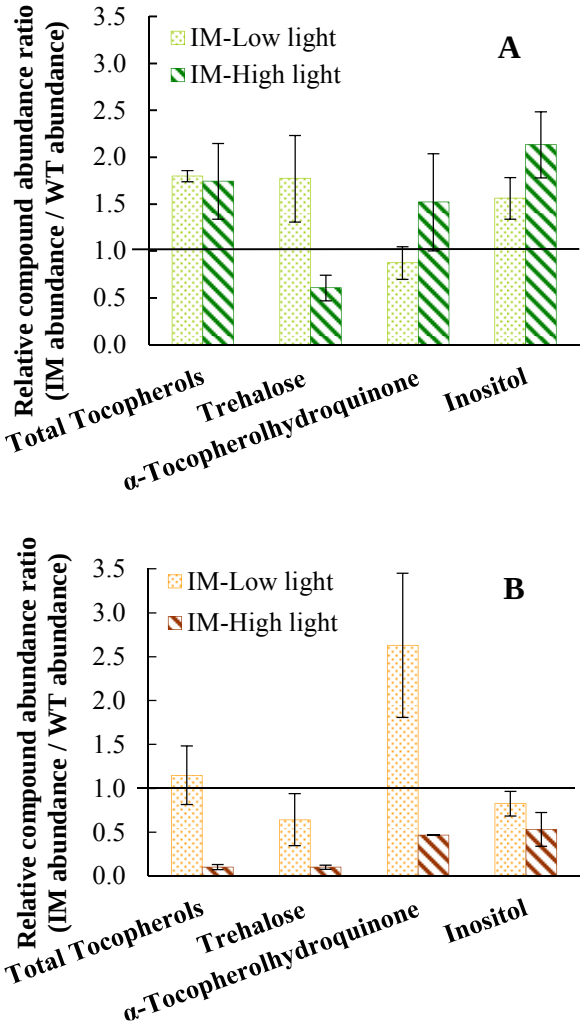

Fig. 3 Metabolite profiling data for 'immortal mutant' (IM) cells grown at low and high light intensity (20 and 640 µmol photons m^{-2} s^{-1}, respectively). All IM values have been normalized by dividing by the respective values for wild type cells indicated by the lines at value '1'. (**A**) Results for exponential growth phase cells. (**B**) Results with stationary growth phase cells.

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