MAXIMIZING PHOTOSYNTHETIC EFFICIENCIES AND HYDROGEN PRODUCTION BY MICROALGAL CULTURES

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Abstract

For algal mass cultures and H_2 production, conditions that maximize photosynthetic productivity and solar conversion efficiency are important in determining sustainability and profit. We have shown [Melis et al. (1999) Journal of Applied Phycology 10: 515-525] that photosynthetic efficiencies and hydrogen production by microalgal cultures can be increased upon minimizing the number of the light-harvesting chlorophyll (Chl) antenna pigments of photosynthesis. A highly truncated light-harvesting Chl antenna size in green algae could result in: (a) 6-7 times greater photosynthetic productivity (on a per Chl basis), compared to that of normally pigmented cells, and (b) ~3 times greater yields of photosynthesis and H_2 production under mass culture, compared to that of normally pigmented cells.

We report here the application of molecular genetic approaches for the generation of transformant green algae with a permanently truncated Chl antenna size. Upon generating and screening a library of 6,500 DNA insertional transformants in the green alga *Chlamydomonas reinhardtii,* 155 mutants aberrant in Chl fluorescence, i.e., possibly aberrant in Chl antenna size, have been isolated. Three distinct classes of mutants were identified: mutants aberrant in Chl *b* biosynthesis, and mutants aberrant in the regulation of the Chl antenna size (both down-regulated and up-regulated). Initial biochemical characterization of some of these mutants is presented. The work provides evidence that a smaller and stable Chl antenna size in green algae can be achieved through the application of molecular genetic techniques. Moreover, some unique insights were gained from a detailed examination of the Chl *b*-less mutant. This mutation was partially overcome through a nearly quantitative substitution of Chl *b* with Chl *a* in photosystem-I (PSI), and by a partial substitution by Chl *a* in PSII. These substitutions resulted in a PSI Chl antenna size almost as large in the mutant as in the control, but a PSII antenna size in the mutant that was less than half of that in the control. Genetically engineered algae with a *'truncated Chl antenna* 'can increase the productivity of the culture under moderate to high irradiance. Immediate future plans include the biochemical analysis of additional isolates in search of the smallest possible Chl antenna size for PSII and PSI, and the cloning and sequencing of the genes that regulate the Chl antenna size of photosynthesis.

Introduction

Microalgal mass cultures growing under high irradiance, such as direct sunlight, have significantly lower photon use efficiencies than when grown under low irradiance. The reason for this fundamental inefficiency is that, at moderate to high irradiance, the rate of photon absorption by the antenna chlorophylls far exceeds the maximal rate of photosynthesis. The excess absorbed photons are dissipated as fluorescence or heat. Thus, in algal mass cultures, the first few layers of cells absorb and waste a large proportion of the incident photons, while strongly attenuating the light received by cells deeper in the culture [Naus and Melis 1991, Neidhardt et al. 1998]. More than 90% of absorbed photons can thus be wasted [Melis et al. 1999], reducing photon use efficiencies and photosynthetic productivity.

Theoretically [Kok 1953, Myers 1957], a truncated chlorophyll (Chl) antenna size of the photosystems (PS) is expected to increase the photon use efficiency of microalgae in mass culture as it would minimize the wasteful dissipation of absorbed sunlight, diminish mutual cell shading, permit a greater transmittance of light through the culture and, thus, result in a more uniform illumination of the cells. Overall, this should result in a higher photosynthetic productivity of the microalgal culture [Kok 1960]. These theoretical considerations have been quantitatively tested in the laboratory, supporting the prediction that cells with a highly truncated Chl antenna size will exhibit superior photosynthetic productivity and solar use efficiency compared to that of normally pigmented control cells [Melis et al. 1999].

Thus, for purposes of industrial application, it would be necessary to develop microalgal mutants with a permanently truncated light-harvesting Chl antenna size. To achieve this goal, we took advantage of recent progress in the fields of the Chl antenna organization and regulation of assembly in chloroplasts. The work employed recently developed molecular genetic approaches to generate transformant green algae with a permanently truncated Chl antenna size. Preliminary results show that, indeed as expected, green algae with a permanently truncated Chl antenna size exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented control cells.

Materials and Methods

Cell Cultures and Growth Conditions

Chlamydomonas reinhardtii, strains *cw15* and *CC425* (*arg7.8 cw15 mt*$^{+}$ *sr-u-60*, an arginine auxotroph; Chlamydomonas Genetics Center, Duke University), were cultivated in Tris-Acetate-Phosphate (TAP) [Gorman and Levine 1965] or high salt (HS) [Sueoka 1960] media. Cultures of strain *CC425* were supplemented with 50 μg mL^{-1} arginine. Liquid cultures were grown in flat Roux bottles upon stirring under continuous illumination (200 μmol photons m^{-2} s^{-1}) provided by cool-white fluorescence lamps.

E. coli cells transformed with plasmid pJD67 [Davies et al. 1996] were grown in a 37°C incubator/shaker in LB media supplemented with 100 μg mL^{-1} ampicillin. The plasmid DNA (pJD67), containing the argininosuccinate lyase gene, was isolated from liquid *E. coli* cultures using a Qiagen midiprep kit (Qiagen Inc, CA). Plasmids were linearized upon digestion with HindIII prior to been used for insertional mutagenesis of *C. reinhardtii* strain *CC425*.

Cell Count and Chlorophyll Determination

The cell density in the cultures was measured by counting with a Hemacytometer (improved Neubauer chamber) and an Olympus BH-2 compound microscope. Cells were immobilized and stained by addition of several μL of Lugol solution to a 1 mL aliquot of the culture. Pigments from cells or thylakoid membranes were extracted in 80% acetone and debris was removed by centrifugation at 10,000g for 5 min. The absorbance of the supernatant at 720, 663 and 645 nm was measured by a Shimadzu UV-visible spectrophotometer. The chlorophyll (*a* and *b*) concentration of the samples was determined according to Arnon [1949], with equations corrected as in Melis et al. [1987].

Thylakoid Membrane Isolation

Cells were harvested by centrifugation at 1,000g for 3 min at 4°C. Pellets were resuspended in 1-2 mL of growth medium and stored frozen at -80°C until all samples were ready for processing. Samples were thawed on ice and diluted with sonication buffer containing 100 mM Tris-HCl (pH 6.8), 100 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone-40, 0.2% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 μM phenyl-methylsulfonylfluoride (PMSF). Cells were broken by sonication in a Branson 200 Cell Disruptor operated at 4°C. The samples were sonicated three times for 30 s (pulse mode, 50% duty cycle, output power 5). Unbroken cells and starch grains were removed by centrifugation at 3,000g for 4 min at 4°C. Thylakoid membranes were collected by centrifugation of the supernatant at 75,000g for 30 min at 4°C. The thylakoid membrane pellet was resuspended in a buffer containing 250 mM Tris-HCl (pH 6.8), 20% glycerol, 7% SDS and 2 M urea. Solubilization of thylakoid proteins was carried out for 30 min at room temperature, a procedure designed to prevent the formation of protein aggregates during denaturation. Samples were centrifuged in a microfuge for 4 min to remove unsolubilized material, β-mercaptoethanol was added to yield a final concentration of 10% and the samples were stored at -80°C.

SDS-PAGE and Western Blot Analysis

Samples were brought to room temperature prior to loading for electrophoresis and diluted accordingly to yield equal Chl concentrations. Gel lanes were loaded with an equal amount of Chl (1 nmol Chl per lane). SDS-PAGE was carried out according to Laemmli [1970], with the resolving gel containing 12.5% acrylamide, at a constant current of 9 mA for 16 h. Electrophoretic transfer of the SDS-PAGE resolved proteins onto nitrocellulose was carried out for 4 h at a constant current of 800 mA. The transfer buffer contained 50 mM Tris, 380 mM glycine (pH 8.5), 20% methanol and 1% SDS. Identification of thylakoid membrane light-harvesting proteins was accomplished with specific polyclonal antibodies kindly provided by Dr. R. Bassi [Di Paolo et al. 1990]. Cross-reaction with the antibodies was detected by a chromogenic reaction with anti Ig-G secondary antibodies conjugated with alkaline phosphatase (BioRad, Hercules, CA). The blots were scanned with an HP-scanner and quantified with an NIH Imaging program.

Photosynthetic Apparatus Activity Measurements

The concentration of functional PSI and PSII reaction centers was estimated from the amounts of P700 and Q_A, respectively, present in the various samples. The amounts of P700 and Q_A were determined from the amplitude of the light-*minus*-dark absorbance change at λ=700 nm (ΔA_{700}) and at λ =320 nm (ΔA_{320}), respectively [Melis 1989, Smith et al. 1990]. The functional Chl antenna size of PSI and PSII was measured from the kinetics of P700 photooxidation and Q_A photoreduction, respectively [Melis and Anderson 1983, Melis 1989].

The initial non-variable (F_o), variable (F_v) and maximum (F_{max}) yield of chlorophyll fluorescence was measured with intact cells suspended in their growth medium. Actinic excitation was provided in the green region of the spectrum by CS 4-96 and CS 3-69 Corning filters at an intensity of 35 μmol photons m^{-2} s^{-1}.

Photosynthetic activity of the cells was assessed from measurements of the light saturation curve of photosynthesis, obtained with a Clark-type oxygen electrode as described earlier [Melis et al. 1997]. Actinic excitation was provided in the yellow region of the spectrum by CS 3-69 Corning filter in combination with a 35-5453 VIQ 5-8 Ealing filter.

DNA Insertional Mutagenesis of *C. reinhardtii*

Strain *CC425* was used as the host strain to generate nuclear transformants of *Chlamydomonas reinhardtii*. *CC425* was grown in TAP medium supplemented with 50 μg mL^{-1} of arginine at ~50 μmol photons m^{-2} s^{-1}. Plasmid pJD67, containing the argininosuccinate lyase (*ARG7*) gene [Debuchy et al. 1989], was linearized with HindIII and subsequently used to transform strain *CC425* by a procedure similar to that described by Davies et al. [1994, 1996]. Arg^+ transformants were selected on TAP agar media lacking arginine. A library of 6,500 independent nuclear transformants were generated with the Arg^+ phenotype in *C. reinhardtii* and maintained on grid in 175 TAP agar index plates. Independent transformant colonies were streaked onto TAP agar index plates and grown to a size of ~10 mm^2 under cool-white fluorescent illumination of ~50 μmol photons m^{-2} s^{-1} intensity. Subsequently, plates were transferred to weak light (5 μmol photons m^{-2} s^{-1}) until further processing.

Screening of Transformants by Fluorescence Video Imaging Analysis

C. reinhardtii transformants on TAP agar index plates were screened for aberrant chlorophyll fluorescence yield properties *via* a fluorescence video imaging apparatus [Niyogi et al. 1997]. Prior to screening, the index plates were kept under a light intensity of about 10 μmol photons m^{-2} s^{-1} for at least 18 h. Actinic illumination of ~ 2,000 μmol photons m^{-2} s^{-1}, sufficient to induce the F_{max} emission from the algal colonies, was employed in this fluorescence video imaging analysis. The actinic illumination was administered for a period of 1 s and the resulting fluorescence images were captured by the digital video camera of the apparatus. From the displayed color image, transformants with a yield of Chl fluorescence either lower or greater than the control were identified. Color images of chlorophyll fluorescence were calibrated with the *CC425* host strain prior to screening the mutant index plates.

Transformants showing fluorescence yields either lower or greater than the control were identified, isolated from the index plates and tested for photoautotrophic growth in HS media (agar plates as well as liquid media) under low illumination conditions.

Results

Isolation of DNA Insertional Transformants with Aberrant Chlorophyll Fluorescence

Figure 1 shows a fluorescence video image of an index plate containing 24 *Chlamydomonas reinhardtii* DNA insertional transformants. All colonies, except one, displayed Chl fluorescence yields similar to that of the control (greenish color). The exception was a colony in position "**g-3**", which showed a substantially lower yield of Chl fluorescence (blue-violet color). From 6,500 transformants that were screened with this fluorescence video imaging technology, 129 transformant colonies displayed Chl fluorescence yield properties similar to that of the colony in position "**g-3**". The lower yield of Chl fluorescence from such colonies may be a consequence of a truncated Chl antenna size for the photosystems in these transformants. These colonies were selected for further study.

Figure 2 shows a fluorescence video image of an index plate containing 33 *Chlamydomonas reinhardtii* DNA insertional transformants. All colonies, except one, displayed chlorophyll fluorescence yields similar to that of the control. The exception in this case was a colony in position "**c-4**", which showed a substantially greater yield of Chl fluorescence (red color). From 6,500 transformants that were screened with this fluorescence video imaging technology, 26 transformant colonies displayed a high chlorophyll fluorescence yield, similar to that of the colony in position "**c-4**". The significantly greater yield of Chl fluorescence in these colonies may signify an unusually large Chl antenna size for the photosystems in these transformants. These transformants may be impaired in the regulation of the Chl antenna size in a way that causes the unregulated formation of large Chl antenna sizes in the cells. Such impairment is useful because it may lead to the genes that regulate the Chl antenna size of photosynthesis. Colonies with a high yield of Chl fluorescence were also selected for further study.

Table 1 shows initial characterization of a small fraction of the isolated transformants. Strain # 1 was the "control", strains # 2-6 were DNA insertional transformants that displayed relatively low Chl fluorescence, and strain # 7 was a transformant with a relatively high Chl fluorescence. The Chl content of the cells and the Chl *a*/Chl *b* ratio were measured following strain cultivation in a small volume of liquid culture. A common feature of these transformants was the significantly lower than the control Chl content of the cells. Interestingly, this was the case for the five low-Chl fluorescence (strains # 2-6) as well as the sole high Chl fluorescence strain (# 7).

Table 1. *Chlamydomonas reinhardtii* DNA insertional transformants with aberrant chlorophyll fluorescence properties.

Strain Number	Cell type/ Mutant No.	Fluorescence intensity	Chl/cell, x10^{-15} mol/cell	Chl *a*/Chl *b* ratio
1	control	control	6.7	2.7/1
2	Chl *b*-less	very low	3.4	infinity
3	KS-061-16	very low	0.9	5.8/1
4	KS-032-18	low	2.3	2.8/1
5	KS-017-04	low	2.2	2.5/1
6	KS-009-23	low	3.3	2.4/1
7	KS-032-27	high	2.2	2.2/1

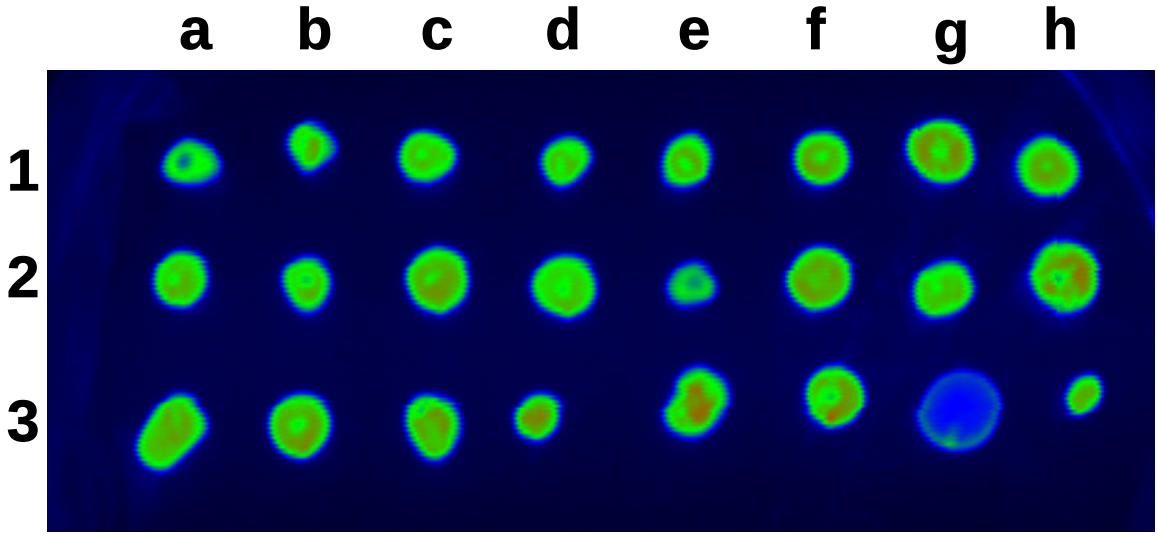

Figure 1 – Fluorescence Video Imaging Analysis of *C. reinhardtii*

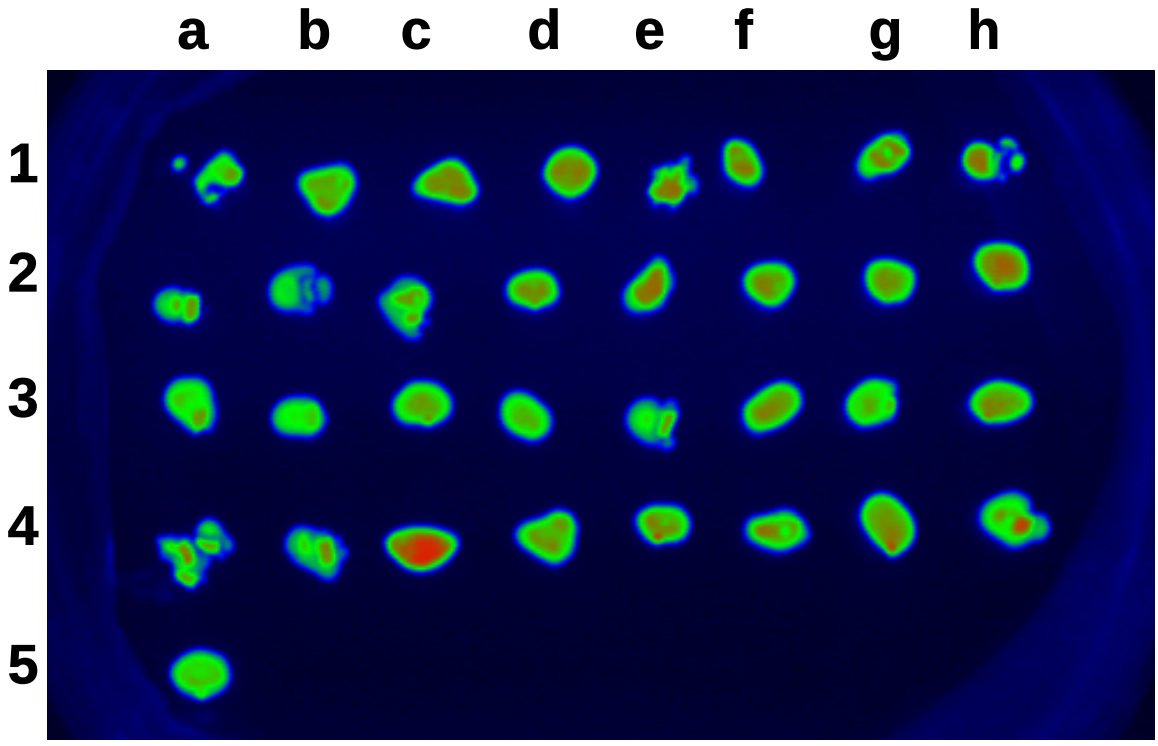

Figure 2 – Fluorescence Video Imaging Analysis of *C. reinhardtii*

The intensity of Chl fluorescence by the transformant strains was calibrated against that of the control. Green color signifies Chl fluorescence yield similar to that of the control, blue-violet is lower and red is higher yield of Chl fluorescence.

Measurements of the Chl *a*/Chl *b* ratio (Table 1) showed that strain # 2 [Tanaka et al. 1998] was aberrant in Chl *b* biosynthesis (Chl *b*-less mutant). Strain # 3 had a Chl *a*/Chl *b* ratio of 5.8/1 (Chl-deficient mutant), whereas strains # 4-7 had either similar or lower than the control Chl *a*/Chl *b* ratios. It is obvious that strains # 2 and # 3 are good candidates of a truncated Chl antenna size and, therefore, suitable for the objectives of the DOE H_2 program. The work below provides a more detailed characterization of the Chl *b*-less transformant (strain # 2, Table 1). Work currently in progress seeks to also characterize the remainder of the isolated transformants.

Photochemical Apparatus Organization in Control and Chl *b*-less Mutant

Table 2 shows the result of quantitative measurements of P700 and Q_A in isolated thylakoid membranes. Control *C. reinhardtii* exhibited a P700/Chl ratio of 2.14/1 (mmol/mol) and a Q_A/Chl ratio of 1.96/1. Relative to total Chl, the Chl *b*-less mutant had a greater content in P700 and Q_A (P700/Chl=3.1/1 and Q_A/Chl=2.95/1). This is consistent with a depletion of Chl from the Chl antenna of this mutant. The ratio of Q_A/P700 provided an estimate of PSII/PSI ratio in the thylakoid membrane of the two strains. This ratio was 0.92:1 for the control and 0.95:1 for Chl *b*-less (Table 2). The efficiency of PSII primary photochemistry was also estimated from the *in vivo* variable to maximal Chl fluorescence (F_v/F_{max}) yield ratio [Kitajima and Butler 1975]. This ratio was 0.65 for the control and 0.52 for the Chl *b*-less mutant.

Table 2: Photochemical apparatus organization in control and Chl *b*-less mutant of *C. reinhardtii.*

The standard deviation of the mean is given for n=3-5.

Parameter measured	control	Chl *b*-less
P700/Chl (mmol/mol)	2.14±0.13	3.1±0.28
Q_A/Chl (mmol/mol)	1.96±0.03	2.95±0.22
PSII/PSI (mol/mol)	0.92	0.95
F_v/F_{max}	0.65±0.06	0.52±0.02

Determination of the Chl Antenna Size of PSII and PSI

Chlorophyll antenna sizes were estimated from the kinetics of the primary photochemical activity of PSII (chlorophyll fluorescence induction) and PSI (P700 photooxidation) upon illumination of the samples by continuous green actinic light of limiting intensity [Melis and Anderson 1983]. In this approach, functional Chl antenna sizes are assigned to each photosystem in direct proportion to the rate of the respective photochemical activity [Melis 1989]. **Figure 3** (left panels) shows light-induced changes in the absorbance of the reaction center P700 at λ=700 nm, occurring as a result of P700 photooxidation in thylakoid membranes of control and the Chl *b*-less mutant. **Figure 3** (right panels) shows the respective semilogarithmic plots of the ΔA_{700} kinetics, revealing single exponential functions of time with rate constants K_{PSI} of 9.0 s^{-1} for control and 7.0 s^{-1} for the Chl *b*-less mutant. The slower P700 photooxidation kinetics for the Chl *b*-less mutant suggest a slightly smaller PSI Chl antenna size than in the control.

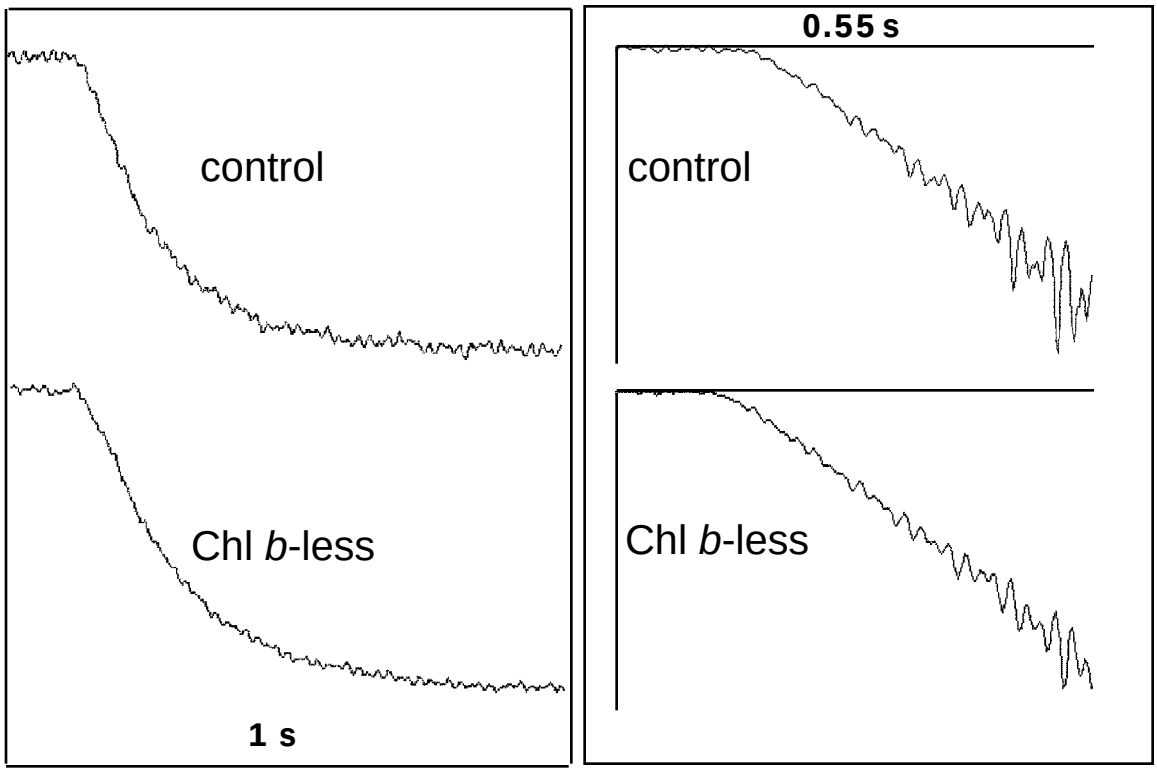

Figure 3 – Light-induced absorbance change measurements

(Left panels) Kinetics of P700 photooxidation(ΔA_{700}) with thylakoid membranes of control and a Chl *b*-less mutant of *C. reinhardtii*. Upper trace, control; lower trace, Chl *b*-less mutant.

(Right panels) Corresponding semilogarithmic plot of theΔA_{700} kinetics.

Figure 4 (left panels) shows light-induced fluorescence induction kinetics, the variable part of which reflects the photoreduction of Q_A in the thylakoid membranes of control and the Chl *b*-less mutant [Melis and Duysens 1979]. The fluorescence induction kinetics of the control strain were faster than that of the Chl *b*-less mutant, suggesting a larger PSII Chl antenna size for the former. **Figure 4** (right panels) shows the respective semilogarithmic plots of the area over fluorescence induction kinetics. This parameter (area over fluorescence) is directly proportional to the amount of Q_A that becomes photoreduced [Melis and Duysens 1979, Melis 1989]. The analysis (Fig. 4, right panels) revealed biphasic Q_A reduction kinetics for the control, occurring with rate constants $K\alpha=9.7\ s^{-1}$ and $K\beta=4.0\ s^{-1}$. These biphasic kinetics reflect a PSII heterogeneity and the existence of two populations of PSII (PSIIα and PSIIβ) with significantly different Chl antenna sizes. In the Chl *b*-less mutant, Q_A photoreduction occurred as a single exponential function of time with rate constant $k_{PSII}=2.9\ s^{-1}$, suggesting lack of PSII heterogeneity and the occurrence of a uniform and small Chl antenna size for the mutant. Heterogeneity in the PSII Chl antenna size is well known in the literature [Melis 1991, Lavergne and Briantais 1996]. The relative amounts of PSIIα and PSIIβ centers in the control strain were 41% and 59%, respectively. In contrast to the control, the kinetic analysis of the fluorescence induction revealed only a slow, monophasic first order function of time for the Chl *b*-less mutant (Fig. 4, right panel). This is evidence for only one population of photosystem II with a uniform Chl antenna size in this mutant.

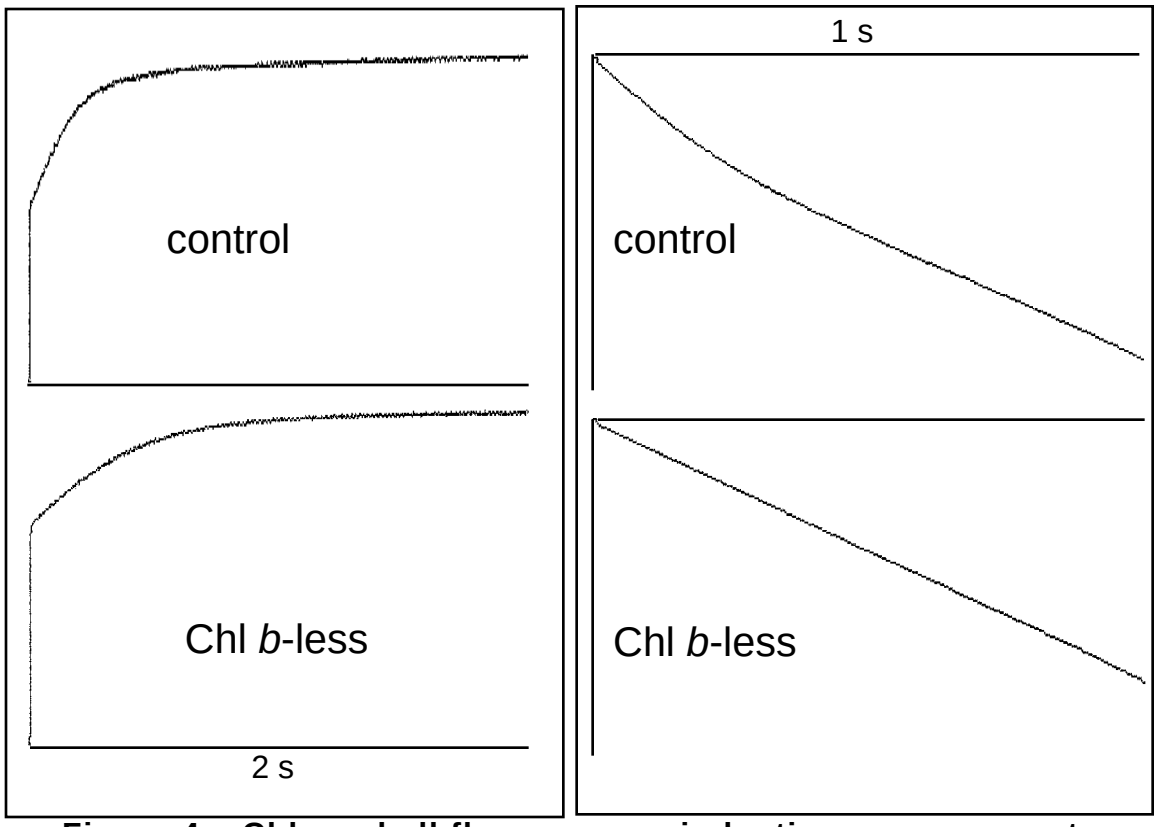

Figure 4 – Chlorophyll fluorescence induction measurements
Left panels: Chl fluorescence induction kinetics of control (upper) and a Chl *b*-less mutant (lower). Right panels: Corresponding semilogarithmic plots of the area over the fluorescence induction curve.

From the measured kinetics of P700 photooxidation and "area over fluorescence induction" we determined the functional chlorophyll antenna size of PSI and PSII, respectively [Melis 1989], for the control and Chl *b*-less mutant (**Table 3**).

Table 3: Chl Antenna Size of PSII and PSI in control and a Chl *b*-less mutant.
Number of chlorophyll molecules per reaction center.

Photosystem	control	Chl *b*-less
PSIIα	322	-
PSIIβ	127	-
PSII	-	93
PSI	290	246

The functional Chl antenna size of PSIIα and PSIIβ in the control was determined to be 322 and 127 Chl molecules, respectively, with 93 Chl molecules in PSII of the Chl *b*-less mutant. The drastic reduction in the PSII Chl antenna size of the mutant was evidently caused by the lack of Chl *b*. In contrast, the PSI Chl antenna size of 246 Chl molecules in the Chl *b*-less mutant was only slightly smaller than the 290 Chl measured in the control. It is concluded that the major portion of LHC-I complexes can assemble and functionally associate with PSI in the absence of Chl *b*.

Characterization of the Light-Harvesting Complex Proteins of the Photosystems in Control and Chl *b*-less Mutant

The results described above show a truncated Chl antenna size for PSI and PSII in the Chl *b*-less mutant. Consequently, the amount of light harvesting complex proteins should be accordingly reduced in the mutant relative to the control. The amount and composition of the LHC proteins was determined in western blots by using polyclonal antibodies that cross-react with the LHC proteins of both PSII and PSI [Bassi and Wollman 1991, Bassi et al. 1992]. **Figure 5** shows the cross-reaction of at least 8 protein bands with these polyclonal antibodies. These bands originated either from LHC-II or LHC-I polypeptides.

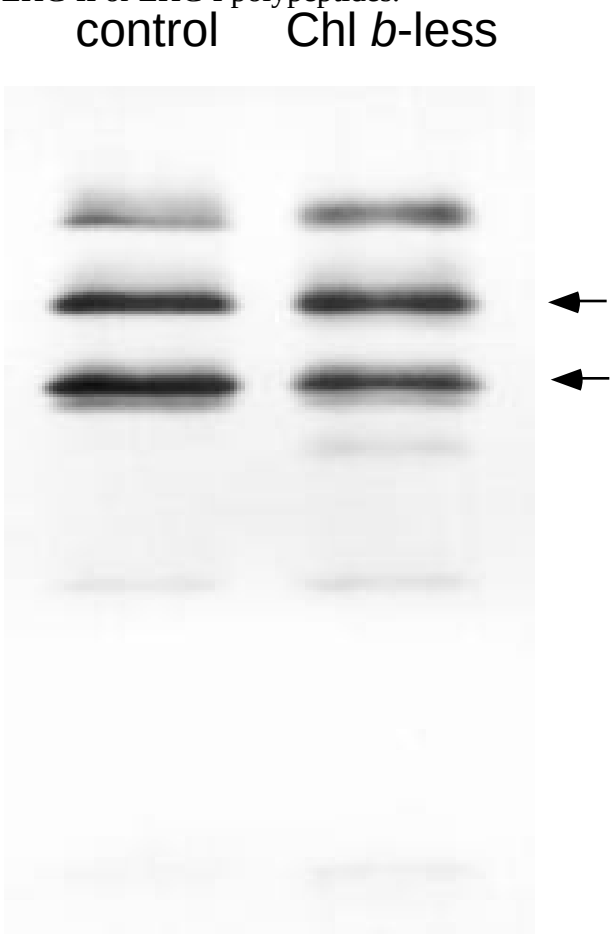

Figure 5 – Western Blot Analysis of Chl Antenna Proteins
Thylakoid membrane proteins were isolated from control and Chl *b*-less *C. reinhardtii.* Arrows mark the position of the major constituents of the LHC-II.

A comparison between control and Chl *b*-less in Fig. 5 revealed that the thylakoid membranes of the Chl *b*-less mutant contained all LHC proteins. However, loss of Chl *b* is

correlated with a reduction in the amount of the major LHC-II proteins (shown by arrow in Fig. 5) [Webb and Melis 1995, Tanaka and Melis 1997]. Since the major LHC-II proteins form the peripheral antenna of PSII, the reduction in their amount is consistent with the spectrophotometric and kinetic results (Table 3) showing a truncated Chl antenna size for PSII in the Chl *b*-less strain.

Measurements of Photosynthetic Capacity

A measure of photosynthetic efficiency and productivity can be obtained from the light-saturation curve of photosynthesis. This type of analysis is necessary and sufficient for the measurement of the vital signs of photosynthesis [Melis et al. 1999]. In such measurements, the rate of O_2 evolution, when plotted as a function of irradiance, first increases linearly and then levels off as the saturating irradiance (I_s) is approached [Neale et al. 1993]. The slope of the initial linear increase provides information about the photon use efficiency of photosynthesis (estimated from the number of O_2 evolved per photon absorbed [Björkman and Demmig 1987, Neale et al. 1993]. The rate of photosynthesis is saturated at irradiances greater than I_s. This light-saturated rate (P_{max}) provides a measure of the capacity of photosynthesis for the particular sample [Powles and Critchley 1980]. **Figure 6** shows the light saturation curve of photosynthesis for control and the Chl *b*-less mutant. Control cells showed a light-saturated rate of photosynthesis (P_{max}) of ~30 mmol O_2 (mol Chl)$^{-1}$ s^{-1} with I_s = ~ 400 μmol photons m^{-2} s^{-1}. The Chl *b*-less mutant reached a P_{max} of ~90 mmol O_2 (mol Chl)$^{-1}$ s^{-1}, i.e., ~3 times greater than that of the control. This difference is attributed to the smaller Chl antenna size for the HL-grown cells, translating into higher per Chl productivity of the culture. Consistent with this interpretation is also the difference in the I_s values which was ~3 times greater for the Chl *b*-less mutant than for the control.

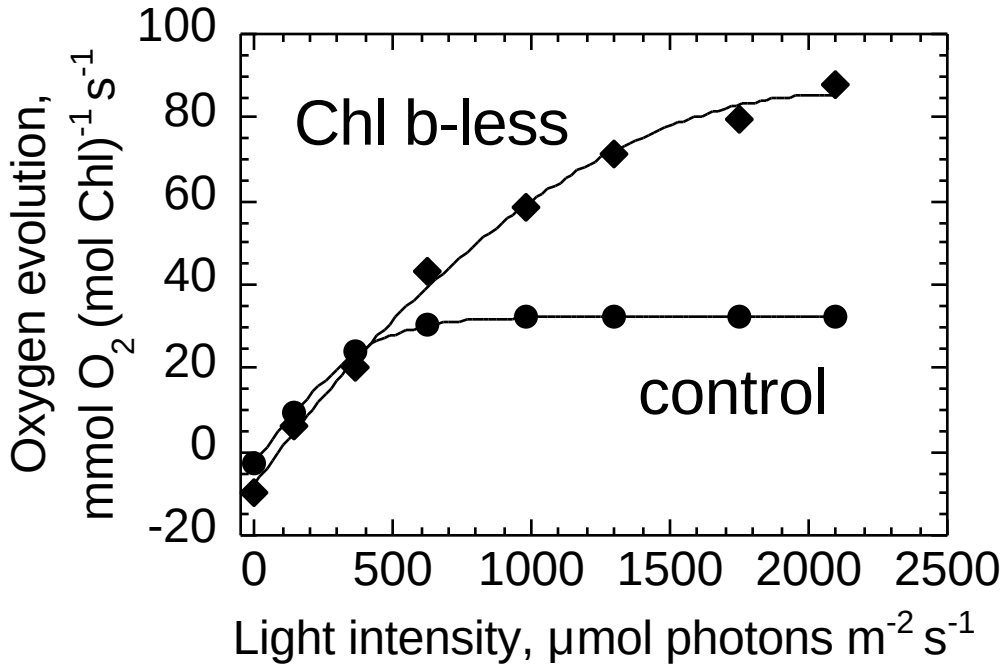

Figure 6 – The light-saturation curve of photosynthesis
Rates of oxygen evolution on a *per Chl* basis. Note the similar initial slopes and the different light-saturated rates between control and Chl *b*-less mutant.

Fig. 6 also compares the initial linear portion of the light-saturation curves for the two strains. It is obvious that the initial slopes, which provide a measure of the photon use efficiency of photosynthesis, are similar in the two samples, suggesting that both samples exhibit a similar 'photon use efficiency' of photosynthesis. It is concluded that the smaller Chl antenna size in the Chl *b*-less mutant does not introduce an adverse effect on the efficiency of photosynthesis under low light-intensity conditions.

Discussion

Work in several laboratories established that the size and composition of the light-harvesting Chl antenna of the photosystems is adjusted and optimized depending on the prevailing growth and irradiance conditions (reviewed in [Anderson 1986, Melis 1991, Melis 1996]). In general, growth under low light promotes larger Chl antenna size for both PSI and PSII (larger photosynthetic unit size). High-light growth conditions elicit a smaller Chl antenna size. This adjustment in the Chl antenna size of the photosystems comes about because of regulated changes in the size and composition of the auxiliary Chl *a*-*b* light-harvesting complex (LHC-II and LHC-I) [Leong and Anderson 1984, Larsson et al. 1987, Sukenik et al. 1988, Morrissey et al. 1989, Smith et al. 1990, Mawson et al. 1994]. The response appears to be highly conserved in all photosynthetic organisms examined, **suggesting the existence of a highly conserved regulatory mechanism that controls the development of the Chl antenna size in the photosystems.**

Mechanistic details of this regulatory mechanism are not known. The regulation could occur at several different steps in the pathway of chlorophyll biosynthesis [Matters and Beale 1995, Reinbothe et al. 1996, Fujita 1996, Falbel et al. 1996, Ohtsuka et al. 1997] resulting in less tetrapyrrole biosynthesis under high irradiance than under low irradiance. In turn, Chl availability may determine the priority of Chl-protein assembly in the chloroplast. According to Greene et al. [1988], Chl-protein assembly occurs with the following distinct hierarchy: PSII-core>PSI-core>LHC-monomers>LHC-inner trimers>LHC-peripheral trimers. A limited availability of Chl under moderate or high irradiance may permit the assembly of the PSII- and PSI-core complexes. However, lack of sufficient Chl will not be conducive to the assembly of LHC-peripheral trimers, resulting in a smaller Chl antenna size.

The present work employed a mutagenesis approach, based on the random insertion of tagged DNA into *C. reinhardtii* cells, by which to impair the Chl antenna size regulation mechanism**.** This procedure, along with the stringent screening employed (Fig.1 and Fig. 2), will help to unlock the "**black box**" of the developmental regulation of the Chl antenna size in microalgae. Thus, it is expected that mutants with a permanently truncated Chl antenna size, as well as mutants with a permanently large Chl antenna, will be isolated (Table 1). **The advantage of this molecular genetic approach is that it will lead to the identification of genes responsible for the operation of this highly conserved regulatory mechanism. Identification of these genes in *C. reinhardtii* will permit the direct manipulation of the Chl antenna size in other microalgae that may be of equal interest to the DOE Hydrogen Program.**

In green algae, the largest Chl antenna configurations reported contain about 500 Chl (*a* and *b*) for PSII and 350 Chl (*a* and *b*) for PSI [Melis 1996]. The smallest stable Chl antenna configurations for the photosystems are the so-called PSII-core complex (containing 37 Chl *a* molecules) and PSI-core complex (containing 95 Chl *a* molecules). These core-complexes with a minimal Chl antenna size are necessary and sufficient for the stable assembly of functional PSII and PSI in thylakoids [Glick and Melis 1988**]. The goal of this project is to generate, through the application of molecular genetic approaches, green algae with Chl antenna configurations that are as close to the "core" antennae as possible.**

Earlier work showed that *Dunaliella salina* (green algae), grown under continuous illumination of high intensity, had a highly truncated Chl antenna size where PSII contained ~ 60 Chl and PSI contained 105 Chl molecules [Smith et al. 1990, Neidhardt et al. 1998]. Functional analysis of these algae provided a **"proof of concept"**, i.e., the ability of the internal chloroplast regulatory mechanism to generate highly truncated Chl antenna sizes [Neidhardt et al. 1998], and the optimization of photosynthetic productivity and solar conversion efficiency in microalgae by minimizing the light-harvesting chlorophyll antenna size of the photosystems [Melis at al. 1999].

The present work illustrates in some detail the result of a unique mutation, one that impaired the biosynthesis of Chl *b* and resulted in a truncated Chl antenna size for the photosystems [Tanaka et al. 1998]. In the present Chl *b*-less mutant, PSII contained 93 Chl *a* molecules and PSI contained 246 Chl *a* molecules (Table 3). These antenna sizes are significantly larger than the PSII-core and PSI-core antennae, suggesting that Chl *b* may not be absolutely essential for the assembly of all Chl *a*-*b* light-harvesting complexes (see also [Ghirardi et al. 1986]). This was especially true for PSI which, in the Chl *b*-less mutant, had a Chl antenna size almost as large as that of the control (Table 3). It may be concluded that the Chl *b*–less mutation can be overcome by a nearly quantitative substitution of Chl *b* with Chl *a* in the Chl antenna of PSI, and by a partial substitution by Chl *a* in the antenna of PSII.

An explanation of the peculiar features of the PSII and PSI antenna configuration in the Chl *b*-less mutant may be provided upon consideration of the role of Chl *b* in these complexes. This pigment is associated exclusively with the LHC proteins of the two photosystems. Since the core complex of PSII contains only about 37 Chl *a* molecules [Glick and Melis 1988], it follows that the remaining ~56 Chl *a* molecules in PSII of the Chl *b*-less mutant must be associated with LHC-II proteins. Based on the assumption of ~12 Chl molecules per Lhcb protein in *C. reinhardtii* [Thornber et al. 1988, Morrissey et al. 1989, Bassi and Wollman 1991, Harrison and Melis 1992], we estimated that 4-5 LHC-II proteins are assembled and functionally associated with PSII.

Conversely, the core complex of PSI contains about 95 Chl *a* molecules [Glick and Melis 1988]. Since PSI in the Chl *b*-less mutant contains 246 Chl *a* molecules, it follows that about 150 Chl *a* molecules must be associated with LHC-I proteins. Based on the assumption of ~10 Chl molecules per Lhca protein in PSI [Thornber et al. 1988], we estimated that about 15 LHC-I proteins must be assembled and functionally associated with PSI in the absence of Chl *b*. Consistent with these conclusions are the western blot results with polyclonal LHC antibodies (Fig. 5) which showed the presence of significant amounts of LHC proteins in thylakoid membranes isolated from Chl *b*-less cells.

In summary, the work clearly shows that a **permanently truncated Chl antenna size in green algae** can be achieved through the application of DNA insertional mutagenesis and related molecular genetic techniques. It is shown that cells with a permanently truncated Chl antenna size of the photosystems are capable of higher rates of light-saturated oxygen evolution than the wild type. From the preliminary results presented in this report, it is also concluded that a Chl *b*–less mutation does not lead to the maximum truncation of the PSII and PSI Chl antenna size in the green alga *C. reinhardtii*. Rather, given the stable assembly of the LHC without Chl *b*, it appears that the absence of Chl *b* can be overcome, presumably through a nearly quantitative substitution of Chl *b* by Chl *a* in PSI, and through a limited substitution by Chl *a* in PSII [Sukenik *et al.* 1987, Tanaka and Melis 1997].

Since the absence of Chl *b* does not lead to the minimum possible Chl antenna size of the photosystems, it is important to continue to test and analyze transformants in which impairment in the regulation of the Chl antenna size has brought about a highly truncated Chl antenna size for the two photosystems. Accordingly, plans for future work include the analysis of additional transformants in search of the smallest possible Chl antenna size for PSII and PSI, and the cloning and sequencing of the genes that regulate the Chl antenna size of photosynthesis.

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