

**TRUNCATED CHLOROPHYLL ANTENNA SIZE OF THE PHOTOSYSTEMS –
A PRACTICAL METHOD TO IMPROVE MICROALGAL PRODUCTIVITY AND
HYDROGEN PRODUCTION IN MASS CULTURE**

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ABSTRACT

Unicellular microalgae hold the promise of commercial exploitation in mass culture for hydrogen and biomass production. In any microalgal production system, the achievable photosynthetic productivity and light utilization efficiency of the algae are the single most important factors in the determination of cost. Microalgal mass cultures growing under full sunlight have a low per chlorophyll (Chl) productivity since, at high photon flux densities, the rate of photon absorption by the Chl antenna far exceeds the rate at which photons can be utilized for photosynthesis. Excess photons are dissipated as fluorescence or heat. Up to 80% of absorbed photons could thus be wasted, reducing light conversion efficiencies and cellular productivity to fairly low levels. This shortcoming could possibly be alleviated by the development of microalgal strains with a limited number of Chl molecules in the light-harvesting antenna of their photosystems, i.e.,

strains that have a *truncated Chl antenna size*. It is expected that individually, such microalgae will not be able to saturate rates of photosynthesis and, thus, will not be subject to wasteful dissipation of excitation energy. In turn, the productivity of the mass culture will be improved. The method of choice to reach the objective of a “truncated light-harvesting Chl antenna” size (*tl*a) employed DNA insertional and chemical mutagenesis of the unicellular green algae *Chlamydomonas reinhardtii* and *Dunaliella salina*, followed by a rigorous screening protocol to identify mutants with a smaller light-harvesting Chl antenna size. Molecular and genetic analyses of isolated *tl*a strains were performed. Biochemical and physiological analyses in terms of photosynthetic productivity and light conversion efficiencies are presented. The results show that a truncated Chl antenna size of PSII is more important than that of PSI in terms of the photosynthetic productivity of a mass culture. A list of genes that confer a “truncated light-harvesting Chl antenna” size to green algae is being compiled for future application in algal hydrogen and biomass production.

INTRODUCTION

Over 60-years ago, Gaffron and co-workers [1,2] discovered that unicellular green algae such as *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* could, depending on experimental conditions, either assimilate or photo-produce molecular hydrogen (H₂). Anaerobically incubated *Chlamydomonas reinhardtii* expresses a [Fe]-hydrogenase enzyme and, upon a subsequent illumination, evolves H₂ gas for short periods of time. Under physiological conditions, such H₂ gas production could not be sustained beyond a minute or two because of the photosynthetic evolution of oxygen. The latter is a powerful positive suppressor of the [Fe]-

hydrogenase [2]. However, a newly developed two-stage photosynthesis and H₂-production process [3], which temporally separates O₂ from H₂ production, holds the promise of commercial exploitation of green algae in hydrogen production upon growth in mass culture [4,5,6].

For production of renewable H₂ with microalgae, the costs must be affordable to compete with other sources of renewable energy. In any microalgal bio-hydrogen production system the single most important factor critical for low cost generation of H₂ and biomass is the achievable photosynthetic productivity and light utilization efficiency of the algae. Since in dense microalgal mass cultures light is rapidly attenuated, the cells acclimate to a low light environment. Cells acclimated to low light accumulate a large number of chlorophyll (Chl) molecules in the photochemical apparatus and have a large Chl antenna size in association with their photosystems. This evolutionary trait is driven in the wild by the need of the algae to absorb as many photons as possible. Under bright sunlight (high photon flux densities), this antenna configuration results in a low per Chl productivity [7,8,9], as the rate of photon absorption by the Chl antenna far exceeds the rate at which photosynthesis can utilize them (Figure 1A). This over-excitation causes dissipation of excess photons as fluorescence or heat. Thus, up to 80% of absorbed photons could be lost for photosynthesis, reducing light conversion efficiencies and cellular productivity to unacceptably low levels. Myers [10] and later Radmer and Kok [11] recognized this shortcoming several decades ago. Radmer and Kok [11] suggested development of microalgal strains with a limited number of Chl antenna molecules in the photosystems, i.e., strains that have a truncated light-harvesting Chl antenna size. It was speculated that such microalgae would not be subject to wasteful dissipation of excitation energy and that they would operate with higher light conversion efficiencies (Figure 1B) and productivity in mass culture [12, 13, 14]. In contrast to this prediction, Sukenik et al. [9] proposed that a larger Chl antenna

size of the photosystems would increase light conversion and quantum yield of photosynthesis in algae under mass culture.

We generated strains of the unicellular green algae *Chlamydomonas reinhardtii* and *Dunaliella salina* that exhibited a truncated Chl antenna of the photosystems. Biochemical and physiological analyses in terms of photosynthetic productivity and light conversion efficiencies are reported. Our results show that a truncated Chl antenna size of PSII is more important than that of PSI in terms of increasing photosynthetic productivity of a mass culture. A number of genes that confer a “truncated Chl antenna size” to green algae has been compiled and is being considered for future applications in algal bio-hydrogen production.

MATERIALS AND METHODS

Cultivation of Algae

Cells of *Chlamydomonas reinhardtii* strains CC125, *cw15*, *cbs-3*, *npq2* *lor1*, and *tlal* were maintained photoheterotrophically on Tris-Acetate-Phosphate (TAP) medium agar plates [15] under $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ continuous light (cool-white fluorescent). The strain *cw15* is a cell wall-less strain, but has the wild type phenotype with respect to photosynthesis. Because the mutant strains *cbs-3* and *tlal* have a *cw15* strain background and photosynthetic parameters are measured, in this study the strain *cw15* is also referred to as wild type control. For measurements cells were grown in liquid culture at $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 22° Celsius in flat 1 L Roux bottles (3 cm optical path length) in bicarbonate enriched medium [16]. Stirring of the cultures

prevented cell settling and ensured uniform illumination. Cells were harvested at a cell density of about 3.0×10^6 cells mL⁻¹.

Dunaliella salina Teod. (UTEX collection strain LB1644, [17]) was grown photoautotrophically in hypersaline medium [18] in the presence of 25 mM NaHCO₃ as a supplemental inorganic carbon source. Cells were cultivated in flat 1 L Roux bottles at 30°C under continuous illumination of 2,000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (high-light) or 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (low-light). Irradiance was measured with a LI-COR Model LI-185B radiometer. The cultures were shaken to ensure a uniform illumination of the cells. Cells were harvested when they were at a density of 2-2.5x10⁶ cells mL⁻¹.

Mutagenesis and Screening

DNA insertional mutagenesis for the generation of nuclear transformants of *C. reinhardtii* was done according to Polle et al. [19]. Transformation of *D. salina* was described by Jin et al. [20]. Screening of transformants for truncated Chl antenna size of the photosystems was performed as in Polle et al. [19].

Pigment Determination

For pigment determination, cells or thylakoid membranes were extracted in 80% acetone and debris removed by centrifugation at 10,000g for 5 min. The absorbance of the supernatant was measured with a Shimadzu UV-160U spectrophotometer. The Chl (*a* and *b*) concentration of the samples was determined according to Arnon [21], with equations corrected as in Melis et al. [22].

Thylakoid Membrane Protein Analysis

Cells of *C. reinhardtii* and *D. salina* were harvested and thylakoid membranes prepared following the procedures described in Polle et al. [23] and Polle and Melis [24], respectively. SDS-polyacrylamide gel electrophoresis and immunoblot analysis was performed according to Polle et al. [24].

Biophysical Measurements

The initial (F_o), variable (F_v) and maximum (F_{max}) Chl fluorescence yield of intact cells was measured upon excitation of the cultures with green light (CS 4-96 and CS 3-69 Corning Filters, actinic light intensity of $75 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) as described in Polle et al. [23].

For spectrophotometric measurements the thylakoid membrane pellet was resuspended in a buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM MgCl_2 . The concentration of the photosystems in the thylakoid membranes was estimated from the amplitude of the light *minus* dark absorbance difference signal at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII [25]. The functional light-harvesting Chl antenna size of PSI and PSII was measured from the kinetics of P700 photo-oxidation and Q_A photoreduction, respectively [26].

Oxygen Evolution Measurements

Oxygen exchange activity by the cultures was measured at 22°C with a Clark-type oxygen electrode illuminated with a slide projector lamp as described for *Chlamydomonas* in Polle et al. [16] and for *Dunaliella* in Masuda et al. [27]. The quantum yield of photosynthesis of the samples was calculated from the initial slope of the light-saturated curve of photosynthesis.

RESULTS AND DISCUSSION

Comparison of photosynthetic characteristics of mutant strains

To test the hypothesis that a “truncated Chl antenna size” of the photosystems results in improved light utilization efficiency of the microalgal mass culture, we generated mutants with a truncated Chl antenna size of the photosystems and analyzed their photosynthetic performance. The approach used to achieve the objective of “truncated Chl antenna size” entailed DNA insertional and chemical mutagenesis of the unicellular green algae *Chlamydomonas reinhardtii* and *Dunaliella salina*, followed by a rigorous screening protocol to identify mutants with a small light-harvesting Chl antenna size [19].

Table 1 shows a comparison of the photosynthetic apparatus characteristics of three different mutants of *C. reinhardtii* and one mutant of *D. salina* with their corresponding wild type strains. The three mutants of *C. reinhardtii* are a Chl *b*-less strain (*cbs 3*, [29]), a Chl deficient strain (*tlal*), and a carotenoid deficient strain (*npq2 lor1*, [23]) that lacks neoxanthin, violaxanthin and antheraxanthin. The mutant of *D. salina* (*dcd1*, [20]) is also Chl deficient. As shown in Table 1, all mutants have a significantly higher Chl *a*/Chl *b* ratio than their corresponding wild type. Since Chl *b* is exclusively associated with light harvesting complex proteins, this result indicated lower amounts of light harvesting complex in the mutants as compared to the wild type. Moreover, the amount of Q_A per Chl and P700 per Chl was higher in all mutants when compared to that of the corresponding wild type, suggesting that in the mutants less Chl was associated with the antenna complex of the photosystems.

Analysis of LHC content and determination of Chl antenna sizes of the photosystems

Since the above results indicated that all mutants have a truncated Chl antenna size of the photosystems, we next investigated the amount of light harvesting proteins in the mutants. The level of light harvesting complex proteins in the different wild type and mutant strains was determined by isolation of thylakoid membrane proteins, protein separation by SDS-PAGE, followed by immunoblot analysis and quantification. Table 2 shows the relative amounts of LHCII protein per Chl for the *Chlamydomonas* mutants and the *Dunaliella* mutant in comparison to their corresponding wild type. Not all mutants showed the expected reduction in the amount of LHCII proteins. The Chl *b*-less and *npq2 lor1* mutants maintained an almost unchanged level of LHCII proteins despite the lower amount of Chl *b* and higher per Chl Q_A and P700 contents [16, 23]. These results suggested that some of the LHC may not contain pigment and/or may not be connected to a reaction center in the Chl *b*-less and *npq2 lor1* mutants. Alternatively, it is possible that LHCII in the Chl *b*-less and *npq2 lor1* mutants assemble and function with less than the full complement of Chl. To obtain a better insight into the organization of the photosystems, we measured the functional Chl antenna size of PSI and PSII with the kinetic/spectrophotometric method [25, 26]. In this approach, PSI and PSII are assigned functional Chl molecules in direct proportion to the rate of light absorption/utilization by their respective reaction centers. The rates for PSI and PSII were measured in isolated and DCMU poisoned thylakoids from the kinetics of P_{700} photooxidation and Q_A photoreduction, respectively [26]. The results, summarized in Figure 2, showed that *C. reinhardtii* mutants have a substantially smaller (by about 50%) Chl antenna size in PSII [16, 23]. The PSII Chl antenna size of the *dcd1* mutant of *D. salina* was also smaller but only by about 20% [20]. The Chl antenna size of PSI was reduced (about 20%) only in the Chl deficient mutants *tlal* of *C. reinhardtii* and *dcd1* of *D. salina*. These

results are evidence that it is possible to truncate the Chl antenna size of PSII and PSI through genetic manipulations. However, it is also obvious that the approaches employed resulted in a greater effect on the antenna size of PSII than of PSI.

Photosynthesis and quantum yield of photosynthesis

The effect of a truncated Chl antenna size on the photosynthetic capacity and quantum yield of photosynthesis was determined in the mutants by measuring their light-saturation curve of photosynthesis. Figure 3A provides an example of the light-saturation curve of photosynthesis for the Chl *b*-less mutant of *C. reinhardtii*. Loss of Chl *b* resulted in a truncated Chl antenna size of PSII and in a two-fold increase in the P_{max} value ([16], Figure 3B). However, at the same time, the quantum yield of photosynthesis of the Chl *b*-less strain was slightly lower than that of the wild type (Figure 3C). In addition, the Chl *b*-less strain displayed a slightly lower efficiency of PSII charge separation (measured from the F_v/F_m ratio, data not shown), suggesting a minor impairment in the process of excitation energy transfer from the light-harvesting Chl antenna to the reaction center of PSII in this strain. The lower quantum yield of photosynthesis and the lower efficiency of PSII charge separation could be the results of pigment vacancy in the PSII Chl antenna of the Chl *b*-less strain. The effect of this impairment on the light conversion efficiency of the mass culture under bright sunlight is discussed below. All other mutant strains exhibited higher P_{max} than their corresponding wild type but, in contrast to the Chl *b*-less strain, they displayed quantum yields of photosynthesis similar to that of the corresponding wild type (Figure 3B,C). The value of P_{max} was not the same in the different pigment mutants but followed the order *cbs3* > *tlal* > *npq2 lor1* > *dcd1* > WT (Figure 3B).

Dependence of P_{\max} on Chl antenna size of the photosystems

The above results showed that all mutants had a smaller Chl antenna size of PSII, but the Chl *b*-less and the *npq2 lor1* mutants did not exhibit a concomitant truncation in the Chl antenna size of PSI. Since all mutants showed an increased P_{\max} , we plotted P_{\max} against the Chl antenna size of PSII (Figure 4A) and PSI (Figure 4B). As shown in Figure 4A, there is a strong linear correlation between P_{\max} and the Chl antenna size of PSII. The smaller the PSII Chl antenna size the larger the increase in P_{\max} . There was no correlation between P_{\max} and the Chl antenna size of PSI. This unexpected result was confirmed independently with cultures of *D. salina* when the inhibitor of Chl biosynthesis gabaculine was used to prevent Chl biosynthesis in HL-grown cultures that were transferred to low light. In the presence of gabaculine cells shifted to low-light developed a full complement of the PSI Chl antenna, but did not recover their PSII Chl antenna size [27]. In contrast, cells shifted to low-light in the absence of gabaculine developed a full complement of the PSI and PSII Chl antenna. Since cells transferred to low-light in the presence of gabaculine maintained a similar P_{\max} as high-light grown cells, it is concluded that reduction of the PSII Chl antenna size only is sufficient to obtain a higher photosynthetic productivity. Taken together, these results are evidence that P_{\max} depends more directly on the Chl antenna size of PSII and less on that of PSI.

Genes for the regulation of the Chl antenna size

As shown above, mutations in genes coding for enzymes of the carotenoid and chlorophyll biosynthetic pathways can lead to a truncated Chl antenna size of the photosystems. It was also demonstrated that the Chl antenna size of PSII is linearly correlated with P_{\max} . However, an increase in P_{\max} is not the only parameter that must to be considered in terms of the

productivity of algal cells in mass culture. Ideally, useful mutations affecting the Chl antenna size of PSII should not in any way compromise the quantum yield of photosynthesis of the green algae. In the Chl *b*-less mutant, absence of Chl *b* and a probable rearrangement of Chl *a* in the Chl-proteins resulted in a slightly lower quantum yield of photosynthesis, probably originating from a lower efficiency of PSII charge separation. In mass culture under conditions of bright sunlight, such shortcoming will inevitably subtract from, and thus lower the solar conversion efficiency and productivity of the Chl *b*-less strain. Nevertheless, even with a lower quantum yield of photosynthesis, we estimated that a mass culture of the Chl *b*-less strain would still outperform the productivity of the wild type under direct sunlight conditions. This is because, under light-saturating conditions, a two-fold greater P_{\max} of the Chl *b*-less mutant as compared to the wild type, would more than compensate for the lower by about 25% quantum yield of photosynthesis.

Wasteful dissipation of absorbed energy is a major problem that lowers productivity in microalgal mass cultures. Mutants with a small Chl antenna size of the photosystems mitigate this problem. We thus compiled a list of genes that should be considered for future application in algal bio-hydrogen production. Table 3 summarizes the effect various mutations have on the photosynthetic productivity and the quantum yield of photosynthesis. Table 3 also shows whether mutant strains would be beneficial for improving the light utilization efficiency of microalgae. Such a comparative study of various mutants revealed that strains carrying the *tlal* mutation would be most promising for use in microalgal mass cultures.

CONCLUSIONS

In summary, our comparative study of Chl antenna size and light conversion efficiency in *C. reinhardtii* and *D. salina* showed that a truncated Chl antenna size of the photosystems may lead to improved light utilization efficiency of the microalgae under mass culture conditions. Recently, Nakajima and co-workers [13, 14] validated the notion that microalgal pigment mutants had somewhat increased productivity as compared to wild type when cultivated under continuous high light intensities. Also, as a conclusion of our mutant study, we were able to compile a list of genes that may find application in algal biotechnology industries and for hydrogen photoproduction.

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TABLES

Table 1: Photochemical apparatus characteristics of different strains of the green algae *Chlamydomonas reinhardtii* and *Dunaliella salina*. The standard deviation for the Q_A /total Chl and P700/total Chl values is less than 10%.

Parameter	WT (<i>C. r.</i>)		Chl b-less	Chl def.	Car def.	WT	Chl def.
	<i>(cw15)</i> (CC125)		<i>(cbs 3)</i>	<i>(tla1)</i>	<i>(npq2 lor1)</i>	<i>(D. s.)</i>	<i>(dcd1)</i>
Chl <i>a</i> / Chl <i>b</i>	3.0 ±0.12	2.9 ±0.2	-	8.1 ±0.33	4.1 ±0.4	4.5 ±0.1	9.9 ±1.1
mol x10 ⁻¹⁵	2.4 ±0.5	4.4 ±0.7	3.8 ±1.1	0.89 ±0.06	3.1 ±0.33	1.48 ±0.2	0.85 ±0.1
Chl/cell							
Q_A / total Chl	1.0	1.0	1.41	1.86	1.55	1.0	1.15
(relative units)							
P700/ total Chl	1.0	1.0	1.35	1.51	1.24	1.0	1.23
(relative units)							

Table 2: Relative amounts of light harvesting complex proteins (LHCII), measured on a per Chl basis, in wild type and various mutant strains of *C. reinhardtii* and *D. salina*.

Parameter	WT (<i>C. r.</i>)		Chl b-less	Chl def.	Car def.	WT	Chl def.
	<i>(cw15)</i> (CC125)		<i>(cbs 3)</i>	<i>(tla1)</i>	<i>(npq2 lor1)</i>	<i>(D. s.)</i>	<i>(dcd1)</i>
LHCII	1.0	1.0	0.9	0.6	1.0	1.0	0.7
(relative units)							

Table 3: Genes conferring a “truncated Chl antenna size” to green algae and potential for improved productivity in algal mass cultures.

Genes	Truncation of Chl antenna size		Mutants promising for mass culture
	PSII	PSI	
Carotenoid Biosynthesis:			
ϵ -cyclase (<i>lor1</i>)	yes	no	yes
zeaxanthin epoxidase (<i>npq2</i>)	minimal	no	?
Chl Biosynthesis:			
Chl a oxygenase (<i>cao</i>)	yes	no	yes
<i>TLA1</i>	yes	yes	yes

FIGURES

Fig. 1: A) The light-saturation curve of photosynthesis in wild type *Chlamydomonas reinhardtii* is compared to the curve corresponding to the rate of light absorption as a function of incident intensity. B) A theoretical light-saturation curve of photosynthesis expected from a mutant with a truncated Chl antenna size of the photosystems is compared to that of the wild type.

Fig. 2: The relative Chl antenna size of photosystem II and I in wild type and various mutant strains of *C. reinhardtii* (1 – 4) and *D. salina* (5+6). *C. reinhardtii* wild type (1), Chl *b*-less (2), Carotenoid deficient (3), Chl deficient (4). *D. salina* wild type (5), Chl deficient (6). The standard deviation is about 10%.

Fig. 3: A) Light saturation curves of photosynthesis for wild type and a Chl *b*-less mutant of *Chlamydomonas reinhardtii*. Summary of oxygen evolution parameters for *Chlamydomonas* and *Dunaliella* strains: B) photosynthetic productivity (P_{\max}) and C) quantum yield of photosynthesis. The numbers in Figure B and C refer to *Chlamydomonas* wild type (1), Chl *b*-less (2), Car deficient (3), Chl deficient (4), and *Dunaliella* wild type (5), Chl deficient (6).

Fig. 4: Dependence of P_{\max} on the Chl antenna size of PSII (A) and PSI (B). The smallest possible Chl antenna size of PSII (about 19% of the control) is indicated by the vertical dotted line in panel (A). The intercept between the P_{\max} function and the dotted line (open circle) indicates the maximal increase in P_{\max} that could be achieved on the sole basis of a PSII Chl antenna size truncation. MT1, Chl *b*-less mutant; MT2, *Chlamydomonas* Chl def. mutant; MT3,

Car def. Mutant; MT4, *Dunaliella* Chl def. Mutant; G, *Dunaliella* wild type cells after HL to LL shift in the presence of Gabaculine.

Figure 1: Polle et al.

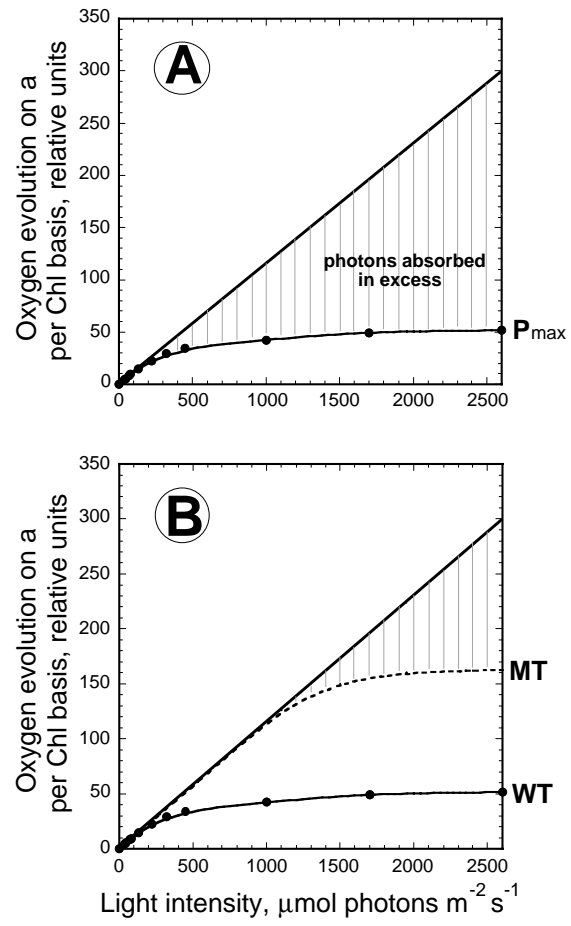


Figure 2: Polle et al.

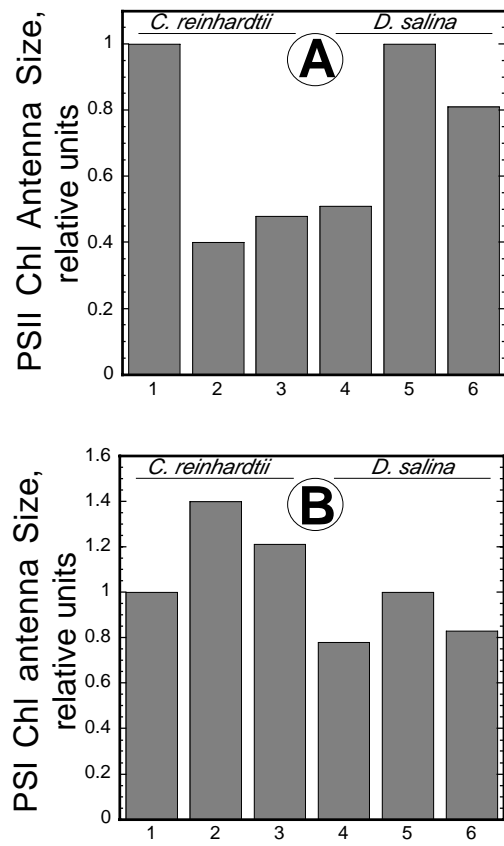


Figure 3: Polle et al.

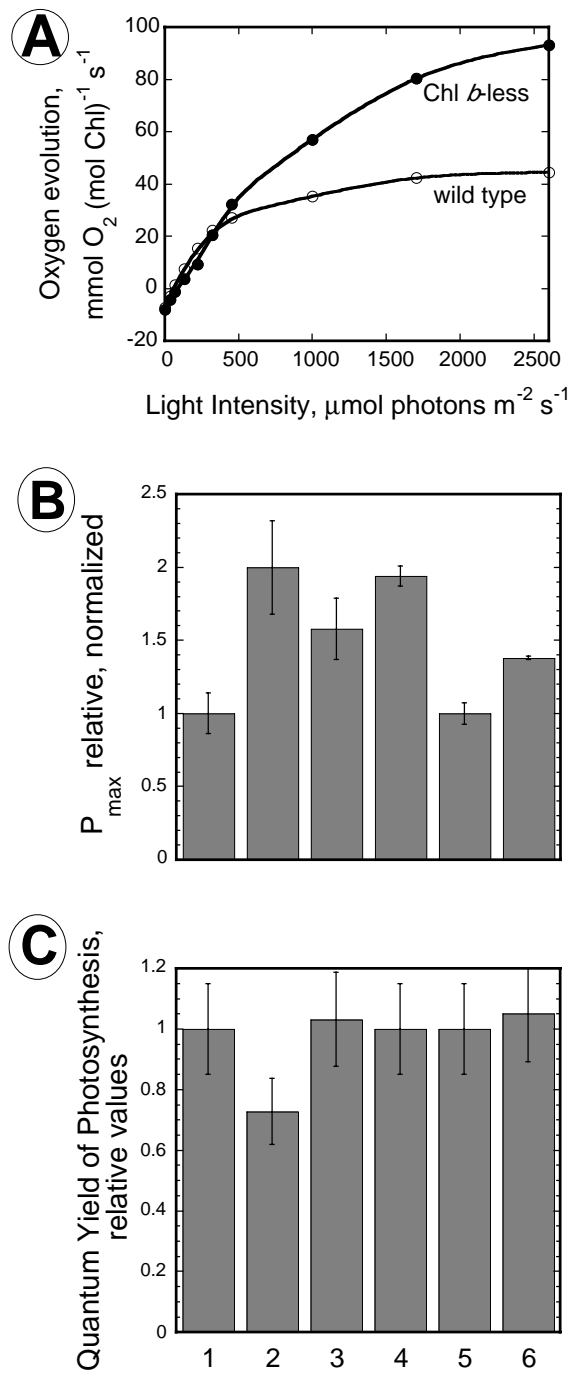


Figure 4: Polle et al.

