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ORIGINAL ARTICLE

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tla1, a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size

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Abstract DNA insertional mutagenesis and screening of the green alga *Chlamydomonas reinhardtii* was employed to isolate *tla1*, a stable transformant having a *t*runcated *l*ight-harvesting chlorophyll *a*ntenna size. Molecular analysis showed a single plasmid insertion into an open reading frame of the nuclear genome corresponding to a novel gene (*Tla1*) that encodes a protein of 213 amino acids. Genetic analysis showed co-segregation of plasmid and *tla1* phenotype. Biochemical analyses showed the *tla1* mutant to be chlorophyll deficient, with a functional chlorophyll antenna size of photosystem I and photosystem II being about 50% and 65% of that of the wild type, respectively. It contained a correspondingly lower amount of light-harvesting proteins than the wild type and had lower steady-state levels of *Lhcb* mRNA. The *tla1* strain required a higher light intensity for the saturation of photosynthesis and showed greater solar conversion efficiencies and a higher photosynthetic productivity than the wild type under mass culture conditions. Results are discussed in terms of the *tla1* mutation, its phenotype, and the role played by the *Tla1* gene in the regulation of the photosynthetic chlorophyll antenna size in *C. reinhardtii*.

Keywords *Chlamydomonas* · DNA insertional mutagenesis · Chlorophyll-deficient mutant · Light-harvesting antenna · Photosynthesis · Solar conversion efficiency

Abbreviations *Chl:* chlorophyll · *CP26 and CP29*: chlorophyll proteins 26 and 29 · *PS I and PS II*: photosystems I and II · *LHC I and LHC II*: light harvesting complex of PS I and PS II, respectively · *PMSF*: phenylmethylsulfonyl fluoride · *tla*: *t*runcated *l*ight-harvesting chlorophyll *a*ntenna

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Introduction

The biotechnology of microalgal mass culture has become a commercially viable industry serving the food, chemical and energy sectors (Zaborsky 1998). In a photobiological production process, important consideration is given to the solar conversion efficiency and photosynthetic productivity of the mass culture. Green algae growing under full sunlight, when photosynthetic productivity ought to be at a maximum, have disappointingly low solar conversion efficiencies (Kok 1953; Myers 1957; Radmer and Kok 1977). The reason for this inefficiency is that green algae have a tendency to assemble large arrays of light-absorbing chlorophyll (Chl) antenna molecules in their photosystems (Melis 1996; Melis et al. 1999). At high solar intensities, the rate of photon absorption by the Chl antennae of the first few layers of cells in the mass culture far exceeds the rate at which photosynthesis can utilize them, resulting in dissipation and loss of the excess photons as fluorescence or heat. Up to 95% of absorbed photons could thus be wasted, reducing solar conversion efficiencies and cellular productivity to unacceptably low levels. In addition to the wasteful dissipation of excitation, and due to the high rate of photon absorption by the photosynthetic apparatus, cells at the surface of the mass culture are subject to photoinhibition of photosynthesis (Powles 1984; Melis 1999), a phenomenon that compounds losses in productivity. Meanwhile, cells deeper in the culture are deprived of much needed sunlight, as this is strongly attenuated due to filtering (Naus and Melis 1991; Neidhardt et al. 1998; Melis et al. 1999). A genetic tendency of the algae to assemble large arrays of light-absorbing Chl antenna molecules in their photosystems is a survival strategy and a competitive advantage in the

wild, where light is often limiting (Kirk 1994). Obviously, this property of the algae is detrimental to the yield and productivity in a mass culture.

A smaller, or truncated, Chl antenna size in microalgae could alleviate the optical shortcomings associated with a fully pigmented Chl antenna, because it will minimize the over-absorption of bright incident sunlight by the photochemical apparatus (Myers 1957; Radmer and Kok 1977). A truncated Chl antenna will diminish the over-absorption and wasteful dissipation of excitation energy by the cells, and it will also diminish photoinhibition of photosynthesis at the surface while permitting for greater transmittance of light deeper into the culture. Such altered optical properties of the cells would result in greater photosynthetic productivity and better solar conversion efficiency in the mass culture. Indeed, actual experiments (Nakajima and Ueda 1997, 1999) and laboratory simulations (Melis et al. 1999) showed that a smaller Chl antenna size results in a relatively higher light intensity for the saturation of photosynthesis in individual cells but, concomitantly, in an overall greater productivity of the mass culture. Thus, genetic approaches by which to truncate the Chl antenna size of photosynthesis in green algae merit serious consideration.

The Chl antenna size of the photosystems is not fixed but could vary substantially depending on developmental, genetic, physiological and even environmental conditions. The acclimation of the photosynthetic Chl antenna size to irradiance has received considerable attention in the literature (Anderson 1986; Melis 1996). In general, low light intensity during growth promotes a large Chl antenna size. Growth under high light intensities elicits the assembly of a smaller Chl antenna size (Melis 1991; Maxwell et al. 1995; Webb and Melis 1995; Tanaka and Melis 1997). Such adjustments of the Chl antenna size in response to irradiance are a compensation reaction of the chloroplast, as they are inversely related to the incident intensity (Melis 1996). This molecular regulatory mechanism is highly conserved and functions in all organisms of oxygenic and anoxygenic photosynthesis (Anderson 1986; Nakada et al. 1995; Yakovlev et al. 2002; Masuda et al. 2003). Under high cell densities in mass culture, which is a condition required for production purposes, and due to the mutual shading of the cells, this regulatory mechanism causes green algae to assemble a large Chl antenna size. As explained above, a large Chl antenna size is detrimental to photosynthetic productivity and solar conversion efficiency. Thus, elucidation of the molecular mechanism for the regulation of the Chl antenna size is of fundamental importance to the field and of practical importance to the algal biotechnology industry. More specifically, in order to alleviate over-absorption and dissipation of energy in green algae, a genetic interference with the regulation of this highly conserved mechanism is required. The present study describes functional properties of *tla1*, a *Chlamydomonas reinhardtii* DNA insertional transformant having a *t*runcated *l*ight-harvesting chlorophyll *a*ntenna size. The plasmid insertion into the nuclear DNA of this transformant interrupted *Tla1*, a newly identified gene, which is apparently responsible for the regulation of the Chl antenna size in green algae. To the best of our knowledge, this is the first cloning and functional characterization of a Chl antenna size regulatory gene in photosynthesis.

Materials and methods

Growth of the algae

Chlamydomonas reinhardtii strain *cw15* (*Chlamydomonas* Genetics Center, Duke University) and the chlorophyll-deficient mutant *tla1* were grown in liquid cultures in flat 1-l Roux bottles at 22 °C under continuous illumination at 200 μmol photons m^{-2} s^{-1} of cool-white fluorescent light. The cultures were stirred to ensure uniform illumination and to prevent cell settling. Cells were grown photoautotrophically to the mid-logarithmic phase in modified 40 mM Tris–HCl–phosphate medium (pH 7.4), supplemented with 25 mM sodium bicarbonate (TBP medium) according to Polle et al. (2000).

Cell count and Chl determination

Cell density was estimated upon counting the number of cells per milliliter of culture using a Neubauer ultraplane hemacytometer. Pigments from intact cells or thylakoid membranes were extracted in 80% acetone and cell debris removed by centrifugation at 10,000 *g* for 5 min. The absorbance of the supernatant was measured with a Shimadzu UV-160U spectrophotometer, and the chlorophyll (*a* and *b*) concentration of the samples was determined according to Arnon (1949), with equations corrected as in Melis et al. (1987).

Mutagenesis and screening protocols

Mutants of *Chlamydomonas reinhardtii* were obtained upon DNA insertional transformation by the glass-bead method, as described in Debuchy et al. (1989). Parental strain CC-425 was transformed with linearized plasmid pJD67, containing the structural gene (*ARG7*) of the argininosuccinate lyase to complement the *arg2* locus (Davies et al. 1994, 1996). Since a truncated Chl antenna size would result in lower yields of chlorophyll fluorescence in vivo, *C. reinhardtii* transformants were first screened for aberrant Chl fluorescence yield properties via a fluorescence video imaging apparatus (Niyogi et al. 1997) as described in Polle et al. (2001a). Mutants showing lower Chl fluorescence were isolated and analyzed for their Chl *a*/Chl *b* ratio.

Nucleic acid extractions and analyses

Genomic DNA was isolated using Stratagene's DNA purification kit. Total RNA was isolated using Qiagen's Plant RNeasy Kit and mRNA was isolated from the total RNA using Qiagen's Oligotex mRNA midi kit. 10 μg of genomic DNA was used for restriction digestion, separated on 0.8% agarose gels for Southern blot analyses. 1 μg of mRNA was separated in 7.4% formaldehyde and 1.0% agarose gels for Northern blot analysis. After separation, nucleic acids were blotted onto positively charged nylon membranes (NEN Life Science Products). The blotted membranes were hybridized with appropriate ^{32}P-labeled probes (Random oligonucleotides DNA Labeling System; Roche).

For Southern blot analysis, probes were obtained upon digesting linearized pJD67 with different restriction enzymes and excising the proper DNA fragments. For Northern blot analysis, a CAB-2 cDNA probe was obtained upon digesting the plasmid p453

with *Hind*III and *Eco* RI. An actin cDNA probe was obtained upon digesting plasmid p728 with *Eco* RI and *Xho*I. Plasmids p453 and p728 were kindly provided by Dr. Elizabeth Harris, *Chlamydomonas* Genetics Center, Duke University.

Genetic analyses

Progeny of *tla1* (*ARG7 cw15 sr-u-2-60 mt*$^+$) were obtained by crossing with a cell walled mt$^-$ strain CC1068 (*arg2 CW*$^+$ *nr-u-2-1 mt*$^-$). The genetic crosses were performed according to the protocol of Harris (1989). Gametes of *tla1* and CC1068 were induced under nitrogen stress, mated and the mating mixture was spread onto zygospore maturation plates [4% agar TAP (Tris–Acetate–Phosphate; Harris 1989) plates supplemented with arginine]. After maturation, the zygospores were isolated and transferred onto germination plates (1.5% TAP agar + arginine). Colonies that appeared on these agar TAP plates with arginine were spread again onto new TAP agar plates with arginine to obtain single colonies. The phenotypes of single-progeny colonies were tested by doing spot colonies on agar TAP plates with and without arginine. Progeny colonies were also tested for the presence of cell wall by treating cells with 1% NP40 followed by observation under a compound microscope.

Isolation of thylakoid membranes

Cells were harvested by centrifugation at 1,000 *g* for 3 min at 4 °C, the pellet was 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 ice-cold sonication buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone 40, 0.2% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 μM phenylmethylsulfonyl fluoride (PMSF). Cells were broken by sonication in a Branson 250 Cell Disrupter operated at 4 °C, three times for 30 s each time (pulse mode, 50% duty cycle, output power 5) with 30-s cooling intervals. Unbroken cells and starch grains were removed by centrifugation at 3,000 *g* for 4 min at 4 °C. The thylakoid membranes were collected by centrifugation of the supernatant at 75,000 *g* for 30 min at 4 °C. The thylakoid membrane pellet was resuspended in a buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ for spectrophotometric measurements, or 250 mM Tris–HCl (pH 6.8), 20% glycerol, 7% SDS and 2 M urea for protein analysis. Thylakoid membrane proteins were solubilized for 30 min in the dark at room temperature, a procedure designed to prevent the formation of protein aggregates during denaturation. Samples were then centrifuged in a microcentrifuge for 4 min to remove unsolubilized material and then stored at −80 °C.

Chlorophyll fluorescence measurements

The initial (F_O), variable (F_V) and maximum (F_M) chlorophyll fluorescence yields of intact cells were measured upon excitation of the cultures with green actinic light (CS 4-96 and CS 3-69 Corning Filters, light intensity of 75 μmol photons m^{-2} s^{-1}). Green actinic excitation was employed in order to equally sensitize Chl *a* and Chl *b* molecules (Ghirardi and Melis 1984). For these measurements, an aliquot from the culture was incubated in the dark for 5 min prior to illumination and the chlorophyll fluorescence was recorded in the absence or presence of 3-(3′-4′-dichlorophenyl)-1-1-dimethylurea (DCMU; 2.5 μM final concentration).

Spectrophotometric analyses

The concentration of the photosystems in thylakoid membranes was estimated spectrophotometrically from the amplitude of the light *minus* dark absorbance difference signal at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII (Melis and Brown 1980; Smith et al. 1990). 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 (Melis 1989).

Analysis of thylakoid membrane proteins

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 (4 nmol Chl per lane). SDS–PAGE analysis was carried out according to Laemmli (1970), with resolving gels containing 12.5% acrylamide, at a constant current of 9 mA for 16 h. Gels were stained with 1% Coomassie brilliant blue R for protein visualization.

Western blot analysis

Electrophoretic transfer of the SDS–PAGE-resolved proteins onto nitrocellulose was carried out for 3–5 h at a constant current of 800 mA, in transfer buffer containing 50 mM Tris, 380 mM glycine (pH 8.5), 20% methanol and 1% SDS. Identification of thylakoid membrane proteins was accomplished with polyclonal antibodies raised against the light-harvesting complex (LHC) of PSI of *Zea mays* (Di Paolo et al. 1990; Bassi et al. 1992), and against the chlorophyll protein CP26 (*Lhcb5*). These antibodies were kindly provided by Dr. R. Bassi (LHC) and Dr. K.K. Niyogi (CP26). Cross-reaction with the antibodies was visualized by a chromogenic reaction with anti-IgG secondary antibodies conjugated with alkaline phosphatase (BioRad) and it was quantified by densitometry using an HP-ScanJet 6100C optical scanner on-line with a PowerMacintosh/G3 computer. The software programs DeskscanII in combination with Adobe Photoshop were utilized for scanning of the western blots and the programs NIH Image version 1.6 in combination with Microsoft Excel 98 were employed for deconvolution and quantification of the bands.

Measurement of photosynthetic activity

The oxygen evolution activity of the cultures was measured at 22 °C with a Clark-type oxygen electrode illuminated with a slide projector lamp. Yellow actinic excitation was provided by a CS 3-69 Corning cut-off filter in combination with an Ealing 35-5453 VIQ5-8 filter. An aliquot of 5 ml cell suspension (2 μM Chl) was transferred to the oxygen-electrode chamber. Sodium bicarbonate (100 μl of 0.5 M solution, pH 7.4) was added to the suspension prior to the oxygen evolution measurements to ensure that oxygen evolution was not limited by the carbon source available to the cells. After registration of dark respiration by the cells, samples were illuminated with gradually increasing light intensities. The rate of oxygen exchange (uptake or evolution) under each of these irradiance conditions was recorded continuously for a period of 2.5 min. Rates of oxygen exchange were plotted as a function of incident irradiance to generate the light-saturation curve of photosynthesis.

Mass-culture growth conditions of the algae were simulated in the greenhouse in glass bottles (2.5 l capacity, 15 cm internal diameter). Cells were grown under ambient conditions in TAP medium until they reached a variety of different densities. When the incident solar irradiance was about 1,500 μmol photons m^{-2} s^{-1}, or higher, 50 ml of 0.5 M $NaHCO_3$ solution (pH 7.4) was added to the culture. Gaseous O_2 evolved under these conditions was collected for volume measurement in upside-down graduated cylinders by the method of water displacement.

Results

Isolation of *C. reinhardtii* strains with a truncated Chl antenna size

Strains with a truncated Chl antenna size of the photosystems are expected to show a phenotype with a low yield of Chl fluorescence. Chl fluorescence of *ARG7* transformant colonies was used to screen a library of 6,500 strains. In this case, strain CC425 (*cw15 arg7-8 mt*$^{+}$ *sr-u-2-60*) was used as the host strain for the insertional mutagenesis. *Chlamydomonas reinhardtii* Arg^{+} transformants on TAP-agar index plates were screened for aberrant Chl fluorescence yield properties via a fluorescence video imaging apparatus (Niyogi et al. 1997). Index plates were kept under a light intensity of about 10 μmol photons m^{-2} s^{-1} for at least 18 h, followed by a 30-min dark incubation immediately prior to the measurement. Actinic illumination of approx. 2,000 μmol photons m^{-2} s^{-1}, sufficient to induce the F_M 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, mutants with a yield of Chl fluorescence lower than that of the controls were identified. Color images of chlorophyll fluorescence were calibrated with the CC425 host strain prior to screening the mutant index plates.

Figure 1 shows a fluorescence video image of an index plate containing 24 *C. reinhardtii* DNA insertional transformants. All colonies, except one, displayed Chl fluorescence yields similar to that of the control (greenish color). The exception was one 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 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 mutants. Therefore, these colonies were selected for further screening analysis.

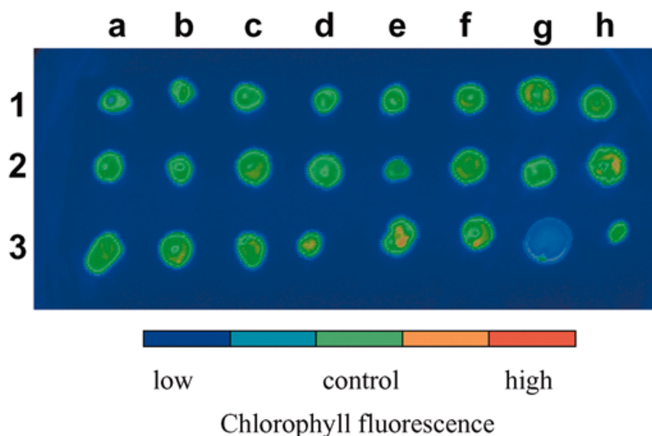

Fig. 1 False-color fluorescence video imaging analysis of an index plate containing 24 *Chlamydomonas reinhardtii* DNA insertional transformants. All colonies, except one, displayed Chl fluorescence yields equivalent to that of the control (*greenish color*). The exception was a colony in position "g-3", which showed a substantially lower yield of Chl fluorescence (*dark-blue color*), possibly indicating a smaller than normal Chl antenna size

All 129 mutant colonies, showing fluorescence yield lower than the control, tested positive for photoautotrophic growth in HS media (agar plates as well as liquid media; Harris 1989) under low-illumination conditions. Of those, four mutants showed higher than the control Chl *a*/Chl *b* ratio (Polle et al. 2001a). Among those four, one mutant (*tla1*) had the highest Chl *a*/Chl *b* ratio, a pale-green color and a single plasmid insert in its nuclear genome (see below). The other three mutants turned out to have multiple plasmid inserts and were not further analyzed in this work.

Southern blot analysis to determine copy number of *ARG7* inserts in *tla1*

Southern blot analysis of *tla1* in comparison to its host strain CC425, using various probes derived from pJD67 (the *ARG7*-containing transforming plasmid), confirmed the presence of a single copy of *ARG7* insert in *tla1*. The probe *Sal* I-*Sal* I (1.3 kb representing the 5′-end of the *ARG7* structural gene) was used in Southern blots against genomic *tla1* DNA digested with the *Kpn* I, *Apa* I or *Hind* III restriction enzymes (Fig. 2). Such hybridizations of the *tla1* genomic DNA upon digestion with different restriction enzymes indicated, in addition to the endogenous copy of *ARG7*, the presence of a single copy of insert DNA (Fig. 2, left panel). When re-probed with *Pvu* I-*Pvu* I (approx. 1.0 kb obtained from the 5′-end of the vector sequence) the same *tla1* genomic DNA fragment was recognized (data not shown).

The probe *Nde* I-*Nde* I (0.75 kb) derived from the 3′-end of the *ARG7* plasmid also indicated, in addition to the endogenous *ARG7* copy, a single plasmid insert (Fig. 2, right panel). This section of the Southern blot, when re-probed with *BamH* I-*Hind* III (0.35 kb) obtained from the 3′-end of the vector sequence hybridized with the same *tla1* genomic DNA fragment as did the *Nde* I-*Nde* I probe (results not shown).

Linkage of the Arg^{+} phenotype with the *tla1* phenotype

Selected random progeny from the cross of the *tla1* strain with CC1068, a normally-pigmented arginine auxotroph and cell wall-containing strain, showed co-segregation of the Arg^{+} phenotype with the Tla

phenotype. An example of such genetic analyses is shown in Fig. 3. When grown on TAP supplemented with arginine (Fig. 3, TAP + Arg), both Arg^{+}/Arg^{-} and normal green/pale-green phenotypes were grown. In replica plates with TAP only (Fig. 3, TAP only), the pale-green phenotypes grew but the dark-green phenotypes did not. The pale-green phenotypes showed a lower Chl fluorescence and a higher Chl *a*/Chl *b* ratio than the normally pigmented cells (data not shown), suggesting co-segregation of the Arg^{+} and *tla1* phenotypes and indicating that the *ARG7* insertion is responsible for the Tla phenotype. Cell wall phenotypic analysis of the progeny colonies showed independent segregation with their Arg^{+} phenotype, confirming that the progeny colonies obtained in such crosses were different and distinct from their parental strains.

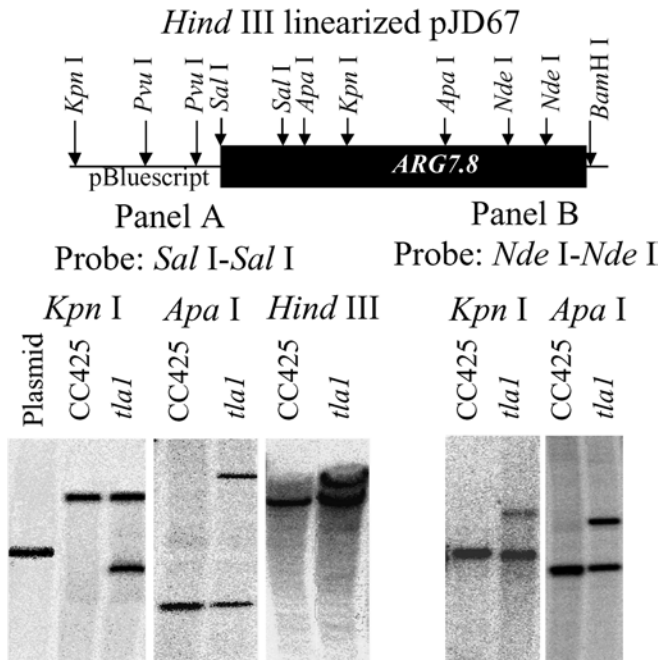

Fig. 2 Southern blot analysis of *tla1* and its host strain, CC425, probing for the number of *ARG7* genes. *Thin lines* of the pJD67 DNA map represent vector sequences while the *solid black box* represents the *ARG7* gene sequence. Isolated genomic DNA was digested with restriction enzymes *Kpn* I, *Apa* I or *Hind* III and hybridized with either the 5′ (*Sal* I-*Sal* I) fragment, or digested with *Kpn* I or *Apa* I and hybridized with the 3′ (*Nde* I-*Nde* I) fragment of the *ARG7* gene. Hybridizations showed the presence of the inactivated endogenous *ARG7* gene in both CC425 and *tla1*, and the presence of a single *ARG7* insert in the *tla1*. The band from *tla1* aligning with the band of CC425 corresponds to the endogenous copy of *ARG7*. Upon re-probing *Panel A* with the 5′-end of vector sequence (*Pvu* I-*Pvu* I), the same size of insert fragments to that of *Sal* I-*Sal* I probe were obtained (data not shown). Upon re-probing *Panel B* with the 3′-end of the vector sequence (*Bam*H I-*Hind* III), the same size of insert fragments to that of the *Nde* I-*Nde* I probe were obtained (data not shown). Full plasmid pJD67 size: 11.28 kb; plasmid restriction size when probed with *Sal* I-*Sal* I fragment: 5.8 kb (*Kpn* I digestion). Endogenous *ARG7* gene restriction size when probed with *Sal* I-*Sal* I fragment: approx. 20 kb (*Kpn* I digestion); 2.1 kb (*Apa* I digestion); approx. 20 kb (*Hind* III digestion). Endogenous *ARG7* gene restriction size when probed with *Nde* I-*Nde* I fragment: approx. 13 kb (*Kpn* I digestion); 2.5 kb (*Apa* I digestion)

Photosynthetic apparatus organization in the wild type and *tla1* mutant

The low Chl fluorescence phenotype and pale-green coloration of the *tla1* strain indicated alterations in the organization of the photosynthetic apparatus in this mutant. Therefore, we studied the photosynthetic apparatus organization of *tla1* in greater detail. Table 1 shows that, when grown in liquid cultures at 200 μmol photons m^{-2} s^{-1}, the Chl *a*/Chl *b* ratio of the *tla1* mutant (8.1:1) was higher than that of the wild type (3:1). At the same time, the amount of Chl per cell in *tla1* was only 35–40% of that in the wild type (Table 1).

Table 1 also presents the thylakoid membrane content in Q_A (PSII) and P700 (PSI). Quantification of photochemically active PSII and PSI reaction centers was obtained from the light-induced absorbance change at 320 nm (Q_A) and 700 nm (P700). The wild type contained 2.9 mmol Q_A per mol Chl, whereas the amount of PSII was nearly 2-fold higher in *tla1*

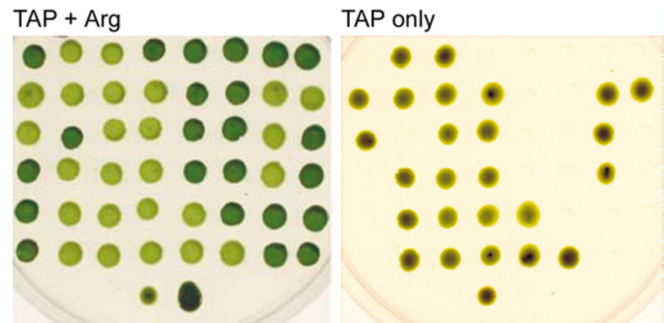

Fig. 3 Random progeny analysis of the cross between *tla1* and CC1068 [(*ARG7 cw15 sr-u-2-60 mt*$^{+}$) × CC1068 (*arg2 CW*$^{+}$ *nr-u-2-1 mt*$^{-}$)]. Random progeny analysis was performed to assess whether the pale-green, or low Chl fluorescence mutant-phenotype, co-segregates with the insert phenotype, i.e. arginine prototrophy. Progeny colonies from the cross of *tla1* × CC-1068 were tested for their phenotypic segregation. Of 48 progeny colonies grown in TAP supplemented with arginine (TAP + Arg, *left panel*), 23 colonies were dark green with wild-type yield of Chl fluorescence while 25 were pale green with *tla1* levels of Chl and yields of Chl fluorescence. In replica plates with TAP in the absence of arginine, only the latter germinated into viable colonies (TAP only, *right panel*). These results indicate that insert DNA co-segregates with the 'Tla' phenotype

Table 1 Chlorophyll parameters and photosynthetic apparatus organization of the wild type and the *tla1* mutant of *Chlamydomonas reinhardtii* (*n* = 3-5). *cw15*, a cell wall-less and arginine prototroph strain was employed as the wild type. Means ± SD

Parameter	Wild type	*tla1*
mol×10^{-15} Chl/cell	2.4 ± 0.5	0.9 ± 0.06
Chl *a*/Chl *b*	3.0 ± 0.12	8.1 ± 0.33
Q_A/total Chl (mmol:mol)	2.86 ± 0.09	5.38 ± 0.65
P700/total Chl (mmol:mol)	1.63 ± 0.17	2.46 ± 0.52
×10^{-18} mol Q_A/cell	6.9	4.8
×10^{-18}mol P700/cell	3.9	2.2
PS II/PS I	1.8	2.2

(5.4 mmol Q_A per mol Chl). Similarly, the wild type contained 1.6 mmol P700 per mol Chl, whereas the amount of PSI was about 50% higher in *tla1* (2.5 mmol P700 per mol Chl). These results suggested that the Chl antenna size of the photosystems was smaller in the *tla1* mutant relative to that in the wild type.

On a per-cell basis, the amounts of Q_A and P700 in *tla1* were lowered to about 70% and 56% of that in the wild type, respectively. These results show that, in addition to the substantially lower level of Chl per cell in the mutant (down to about 30–35% of that in the wild type), *tla1* has a smaller number of electron-transport chains in its thylakoid membranes (down to about 65–70% of that in the wild type). Moreover, the concentration of the two photosystems was not equally affected by the mutation, with PSI dropping quantitatively more than PSII. In consequence, the PSII/PSI ratio was slightly elevated in the *tla1* mutant relative to that in the wild type (Table 1). Altogether, these results showed that the phenotype of *tla1* is similar to that of the so-called Chl-deficient regulatory mutants in several higher-plant species, which have been described in the literature from this and other laboratories (Melis 1996; Falbel et al. 1996; and references therein). These Chl-deficient mutants are known to have a truncated Chl antenna size for PSII and PSI.

Photosystem Chl antenna size, LHC composition and Lhcb gene expression

The absolute size of the light-harvesting Chl antenna of PSI and PSII in the wild type and *tla1* mutant was determined by the so-called kinetic and spectrophotometric method (Melis and Anderson 1983). The approach was to assign Chl to each reaction center in direct proportion to the rate of light utilization by the respective photosystem. Rates of light utilization by the photosystems were measured from the kinetics of Q_A photoreduction and P700 photo-oxidation in isolated thylakoid membranes suspended in the presence of DCMU (Melis 1989).

The photoreduction of Q_A in wild-type and *tla1* thylakoids occurred with biphasic kinetics and revealed the existence of two populations of PSII reaction centers, PSIIα and PSIIβ, respectively (Melis and Duysens 1979). Photo-oxidation of P700 occurred with monophasic exponential kinetics, showing the presence of a uniform population of PSI units. Table 2 shows the weighed average of the Chl antenna size of PSIIα and PSIIβ relative to that in PSI in the wild type and *tla1*. On the average, PSII in the wild type had about 220 Chl (*a* and *b*) molecules functioning in its light-harvesting antenna. This was lowered to about 110 Chl molecules in the light-harvesting antenna of *tla1*, a reduction of about 50%. Similarly, PSI in the wild type had about 240 Chl (*a* and *b*) molecules functioning in its light-harvesting antenna. This was lowered to about 160 Chl molecules in the light-harvesting antenna of *tla1*, a reduction of about 35%.

It is commonly believed that changes in the functional Chl antenna size of photosynthesis occur via the regulated association of variable amounts of LHC with the periphery of the antenna in each of the photosystems, whereas the composition of the photosystem core remains constant. This basic premise was tested by SDS–PAGE and Western blot analysis of the LHC apoprotein composition in the thylakoid membrane of the *tla1* mutant relative to that in the wild type. For Western blot analysis of the protein composition, different specific polyclonal antibodies were used, which recognized different LHC apoproteins in *C. reinhardtii*. As shown in the SDS–PAGE analysis of Fig. 4, the amount of CP26 is drastically reduced in *tla1* as compared to the wild type. Figure 5 shows a Western blot analysis with specific polyclonal antibodies that cross-react with different LHC apoproteins in *C. reinhardtii* (Bassi et al. 1992; Polle et al. 2000). It is evident that the level of all LHC

Table 2 Photosystem Chl antenna size andrelative amounts of various LHC proteins in wild-type and *tla1* mutant of *C. reinhardtii* as determined by densitometric scans of results similar to those in Fig. 5 ($n=5$). Means ± SD

Protein	Wild type	*tla1*
Chl-PS II	222 ± 26	114 ± 36
Chl-PS I	240 ± 4	159 ± 12
CP26 (%)	100	18 ± 6
CP29 (%)	100	64 ± 2
LHCII (%)	100	62 ± 6

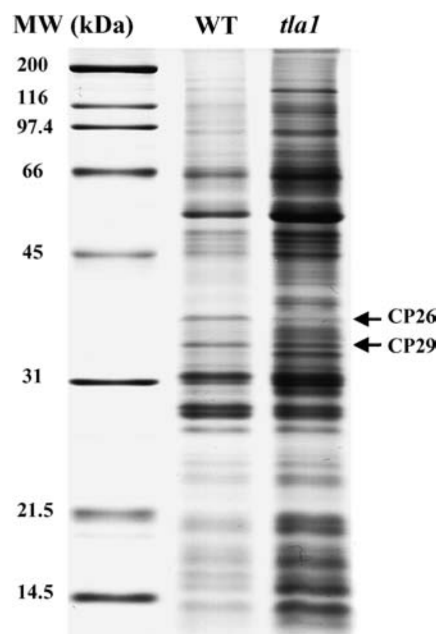

Fig. 4 SDS–PAGE analysis of wild type (*WT*) and *tla1* thylakoid membrane proteins from *C. reinhardtii*. Lanes were loaded on an equal-Chl basis (4 nmol Chl per lane). The various LHC proteins were labeled on the basis of their electrophoretic mobility/apparent molecular weight

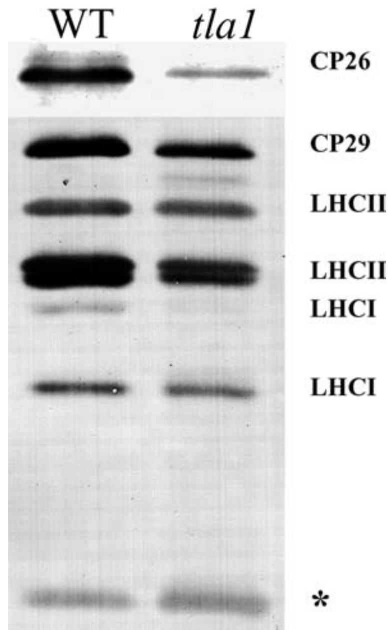

Fig. 5 Western blot analysis of wild type (*WT*) and *tla1* thylakoid membrane proteins from *C. reinhardtii* with CP26-specific polyclonal antibodies (CP26) and with polyclonal LHC antibodies. Lanes were loaded on an equal-Chl basis (4 nmol Chl per lane). The monomeric forms of the various LHC apoproteins were labeled on the basis of their electrophoretic mobility/apparent molecular weight. *Denotes a possible degradation product of the LHC

apoproteins is lowered in the *tla1* mutant as compared to the wild type, consistent with the notion of variable amounts of LHC associated with the periphery of each of the photosystems. Table 2 summarizes quantitative results on the level of different LHC apoproteins that are associated with PSII in the wild type and *tla1*. It is observed that the amount of CP26 in *tla1* was only about 18% of that in the wild type. CP29 and the rest of LHCII were lowered to the 60–65% level as compared to the wild type. Thus, the *tla1* mutation does not affect all LHC proteins to the same extent but appears to have a disproportionately greater effect on CP26.

The association of variable amounts of LHC with the periphery of each of the photosystems implies regulation of the biosynthesis/assembly of the LHC by physiological, environmental and genetic conditions. It is possible that the *ARG7* plasmid insertion in the nuclear genome of the *tla1* strain caused interference with the expression of the *Tla1* gene, thereby resulting in the truncated Chl antenna size of the *tla1* strain (Table 2). In this case, expression of the *Lhcb* genes should be affected in the *tla1* strain, as the signal transduction pathway for the regulation of the Chl antenna size is thought to involve, among other things, regulation of the *Lhcb* gene expression (Masuda et al. 2003). Therefore, analysis of the steady-state transcript levels of the Lhcb was undertaken. Figure 6 compares the *Lhcb* mRNA abundance in wild-type and *tla1* strains. The steady-state *Lhcb* mRNA level in the mutant was only 30–50% of that in the wild type. Levels of actin mRNA, the gene of which is constitutively expressed, were not affected by the insertion of the *ARG7* plasmid in the *Tla1* locus (Fig. 6). Thus, it is likely that the *Tla1* gene plays a role in the regulation of expression of other genes that directly code for components of the light-harvesting complex of photosynthesis, thereby influencing the size of the functional chlorophyll antenna size (please see Discussion).

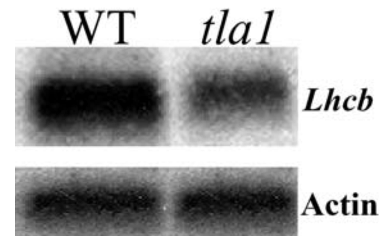

Fig. 6 Northern blot analysis of steady-state *Lhcb* mRNA levels in wild-type (*WT*) and *tla1 C. reinhardtii*. mRNA was isolated from WT and *tla1* grown in 1-l Roux bottles upon continuous stirring in TBP (Tris–Bicarbonate–Phosphate) medium, at a light intensity of 200 μmol photons m^{-2} s^{-1}. *Lhcb* mRNA levels were determined by using an *Lhcb* cDNA probe and normalized using an actin cDNA probe (for probe details, see Materials and methods). The steady-state *Lhcb* mRNA level in *tla1* was about 30–50% of that in the wild type

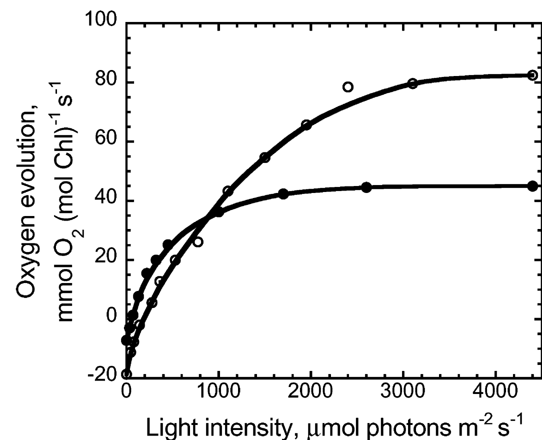

Fig. 7 Light-saturation curves of photosynthesis obtained with the wild type (*solid circles*) and the *tla1* mutant (*open circles*) of *C. reinhardtii*. Note the similar initial slopes and the substantially greater P_{max} value in *tla1* as compared to the wild type

Chlorophyll antenna size and the photosynthesis–irradiance relationship

The Chl antenna size of the photosystems influences the light-saturation curve of photosynthesis and defines the solar conversion efficiency and productivity of green algae under mass-culture conditions (Melis et al. 1999; Polle et al. 2000). To compare these parameters in the wild type and *tla1*, the light-saturation curve of photosynthesis was measured from the rate of oxygen evolution in the two strains and plotted as a function of the incident light intensity in the two samples (Fig. 7). In

such presentation, the slope of the initial linear increase of the light-saturation curve is a measure for the photon-use efficiency (Φ) of photosynthesis (Bjorkman and Demmig 1987; Neale et al. 1993). Figure 7 shows the light-saturation curves of photosynthesis for wild-type and *tla1* strains. An approximately 2-fold higher P_{max} for the mutant than for the wild type follows similar initial slopes in the light-saturation curves. Table 3 presents in greater detail the analysis from such measurements. On a per-Chl basis, *tla1* (Φ = 0.129) had a similar slope in the initial linear rise of the light-saturation curve when compared to the wild type (Φ = 0.125). This is direct evidence that, under light-limiting conditions, the *tla1* mutant has the same quantum yield of photosynthesis as the wild type. A similar conclusion was drawn from the PSII photochemical charge-separation efficiency, obtained from measurements of the fluorescence yield ratio (F_V/F_M) in the two samples (Butler and Kitajima 1975). Thus, the high efficiency of charge separation and electron transport per se were not affected by the *tla1* mutation.

The rate of cellular respiration was about 3-fold higher in the *tla1* mutant than in the wild type (Table 3, Chl basis). On a per-cell basis, cellular respiration was about the same in the two strains. The light-saturated rate of oxygen evolution (P_{max}) was about 2-fold higher in *tla1* than in the wild type (Table 3, Chl basis). On a per-cell basis, the light-saturated rate of photosynthesis in the *tla1* mutant was only about 70% of that in the wild type (Table 3). This is consistent with the lower PSII (Q_A) content of *tla1* relative to the wild type (Table 1) and underlines an additional effect of this mutation, namely a reduction in the number of electron-transport chains in *tla1* (by about 30%) relative to the wild type.

In the context of this work, it is worth noting that photosynthesis in the wild type saturated at about 1,000 μmol photons m^{-2} s^{-1}, whereas in the *tla1* mutant it saturated at about 2,500 μmol photons m^{-2} s^{-1} (Fig. 7). This difference is a direct consequence of the truncated Chl antenna size in the latter versus that of the former (Table 2).

Photosynthetic productivity measurements under mass culture conditions

To compare the photosynthetic productivity performance of wild-type and *tla1* strains under mass culture conditions, it was first necessary to cross the "truncated light-harvesting Chl antenna size" property from the CC425 cw(−) background into a CW^+, cell wall-containing background. This was necessary because cell wall-less strains (CC425, *cw15*, etc.) tend to break upon mechanical stirring of the culture. Crosses between CC1068, a wild type (CW^+) and *tla1* were performed for the isolation of a *tla1* CW^+ offspring. One of the progeny strains showing a *tla1* phenotype and containing a wild-type cell wall (*tla1* CW^+) was isolated. This strain was used for comparative photosynthetic productivity measurements of the wild type and *tla1* under mass-culture mini scale-up conditions.

Figure 8 shows the greenhouse experimental setup used in these measurements. Wild-type and *tla1* CW^+ strains were grown under ambient conditions in 2.5-l bottles having an internal diameter of about 15 cm (approx. 6 inches). It has been determined (Melis et al. 1999) that tubular bioreactors of this internal diameter would be optimal for mass culture conditions. Hence, the simple experimental setup, shown in Fig. 8, is a model for such scale-up measurements as it provides the basic geometry of full-scale photobioreactors. This is useful as it affords measurements of solar conversion efficiency and photosynthetic productivity in the laboratory. At variable cell densities, bottles were sealed with a silicone stopper that was perforated by a syringe and, via Teflon tubing, were connected to upside-down graduated cylinders for the collection and measurement of oxygen. During late morning or early afternoon, when the incident solar intensity reached about 1,500 μmol photons m^{-2} s^{-1}, oxygen production measurements were performed over a fixed period of 2 h.

Figure 9 shows results from a rigorous productivity analysis with the above setup. Shown are the cell density (Fig. 9a), Chl *a*/Chl *b* ratio (Fig. 9b) and rate of photosynthetic O_2 accumulation (Fig. 9c) as a function of the Chl concentration in the respective culture at the time of measurement. Results from this detailed analysis showed that the *tla1* CW^+ culture had consistently lower Chl per cell and a higher Chl *a*/Chl *b* ratio (Fig. 9a, b). The results in Fig. 9c show that, under ambient conditions, productivity of green algae in a mass culture increases linearly as a function of cell density and Chl concentration. The linear increase is observed under conditions when the amount of the biomass, but not irradiance, is the yield-limiting factor. This initially linear increase in the yield of the culture levels-off, as the green algal biomass reaches a certain density. The 'saturation' occurs because, at a threshold Chl concentration, cells in the culture would absorb all incoming irradiance. From that point on, photon

Table 3 Photosynthetic oxygen exchange rates and quantum yield efficiency in wild type and *tla1* strains of *C. reinhardtii* on a per-Chl basis (*n* = 3–5). Means ± SD

Parameter	Wild type	*tla1*
Quantumyield (relative units)	0.125	0.129
F_V/F_M	0.73 ± 0.02	0.63 ± 0.04
Respiration (per Chl), mmol O_2 (mol Chl)$^{-1}$ s^{-1}	7 ± 2.8	19 ± 5.8
Photosynthesis (per Chl), mmol O_2 (mol Chl)$^{-1}$ s^{-1}	45 ± 5.8	82 ± 5.7
Respiration (per cell), ×10^{-15} mmol O_2 cell^{-1} s^{-1}	16.8 ± 3.3	17.1 ± 5.9
Photosynthesis (per cell), ×10^{-15} mmol O_2 cell^{-1} s^{-1}	108 ± 6.3	74 ± 5.8

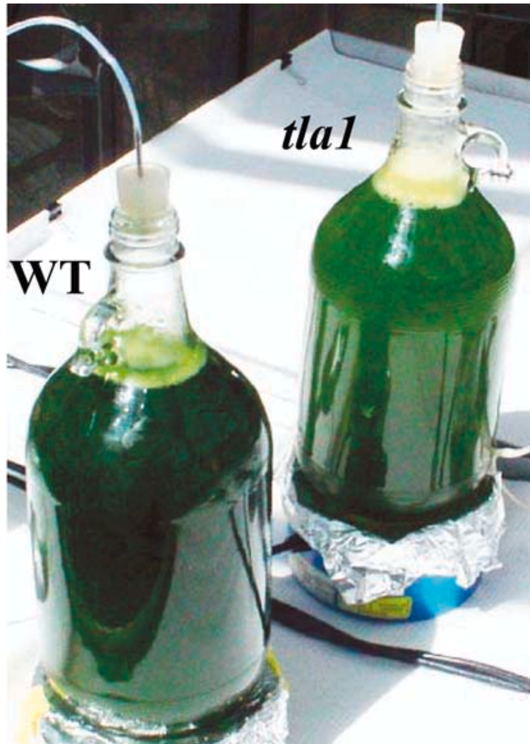

Fig. 8 A mass-culture setup of *C. reinhardtii* for measurements of photosynthetic productivity under ambient conditions. The wild type (WT; CC125, a CW^+ and Arg^+ strain) and the *tla1* CW^+ mutant were grown in 2.5-l bottles having an internal diameter of about 15 cm. Photosynthetic oxygen was drained through a syringe (inserted in the middle of the silicone stopper) and, through Teflon tubing, collected in upside-down graduated cylinders, where the volume of oxygen gas was measured by the method of water displacement. Culture characteristics: WT: 6.4×10^6 cells/ml, 25.6 μM Chl; *tla1*: 10×10^6 cells/ml, 15.4 μM Chl

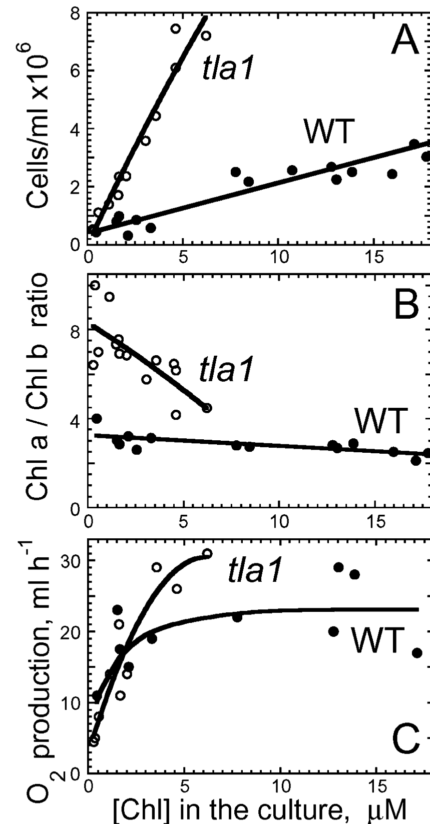

Fig. 9 Cell density, Chl *a*/Chl *b* ratio and O_2 production measurements with the *C. reinhardtii* wild type (*WT*) and the *tla1* CW^+ strain as a function of Chl concentration in the culture. Note the higher cell density, Chl *a*/Chl *b* ratio and greater rates of O_2 production in the *tla1* CW^+ strain than in the CC125 wild type. Productivity measurements were conducted at a solar incident intensity (PAR) of about 1,500 μmol photons m^{-2} s^{-1}

conversion efficiency would define yield. In both the wild type and *tla1* mutant, productivity appeared to become saturated at a Chl concentration of about 5 μM in the culture, with a rate of about 22 ml O_2 per h (WT) and 32 ml O_2 per h (*tla1*). The lower yield of the wild type is attributed directly to the greater fraction of photons that are absorbed but cannot be utilized (Fig. 7), resulting in dissipation and loss of the excess photons as fluorescence or heat (Melis et al. 1999). This is apparently alleviated to some extent by the *tla1* mutation.

The comparative measurements shown in Fig. 9c were obtained at a summertime solar intensity of 1,500 μmol photons m^{-2} s^{-1} in the San Francisco Bay Area, which is less than the maximal solar intensity of 2,500 μmol photons m^{-2} s^{-1}. Obviously, the difference in the productivity of *tla1* and the wild type would be accentuated at light intensities that are greater than 1,500 μmol photons m^{-2} s^{-1}.

Discussion

The Chl antenna size of the photosystems is not fixed. Rather, through a molecular mechanism (Masuda et al. 2003), it responds to imbalance between the supply and consumption of light energy in the photosynthetic apparatus (Anderson 1986; Melis 1991; Escoubas et al. 1995; Huner et al. 1998). In general, low light intensity or shading during growth promotes a large Chl antenna size for both PSII and PSI. Growth under high light intensities elicits the assembly of a smaller Chl antenna size. This regulatory mechanism is known to function in all organisms of oxygenic and anoxygenic photosynthesis. Nuclear mutants, which amplify the function of this regulatory mechanism and which are known as 'Chl-deficient', have been described in earlier studies from several higher-plant species (reviewed in Melis 1991, 1996; Falbel et al. 1996) but not in green algae. Most important, genes that play a role in this regulation of Chl antenna size have not yet been identified. The *tla1* nuclear transformant belongs to this group of 'Chl-deficient' regulatory mutants and the *Tla1* gene (GenBank Accession # AF534570, AF534571) is the first such gene that is apparently involved in the signal transduction pathway leading to regulation of the Chl antenna size. The mode of action of the Tla1 protein in the regulation of the Chl antenna size is now the subject of investigation in this laboratory. It could be functioning as a

component of a novel signal transduction pathway for the regulation of the Chl antenna size, recently described by Masuda et al. (2003). Alternatively, it may act in an as yet unknown manner for the regulation of expression of nuclear genes that are directly responsible for the biosynthesis and assembly of the LHC.

Recent work from this laboratory reported on the Chl antenna size of pigment mutants, specifically on a Chl *b*-less (*cbs3*) and a xanthophyll-deficient (*npq2 lor1*) mutant of the green alga *C. reinhardtii* (Polle et al. 2000, 2001b). Characteristic in those pigment biosynthesis mutants was the unilateral effect of the lesion on the Chl antenna size of PSII and the absence of an effect on the Chl antenna size of PSI. The uniqueness and advantage of the *tla1* mutation in this respect is that it caused a smaller Chl antenna size in both photosystems. However, the truncated Chl antenna size of PSII and PSI in the *tla1* mutant was still larger than that of the minimum Chl antenna size possible. Earlier work from this laboratory (Glick and Melis 1988) demonstrated that the PSII-core and PSI-core complexes are the minimum respective photosystem configurations that can functionally assemble. They contained 37 (PSII-core) and 95 (PSI-core) Chl *a* molecules. In the *tla1* mutant, with a PSII = 114 Chl (*a* and *b*) molecules and a PSI = 159 Chl (*a* and *b*) molecules, the Chl antenna size of the photosystems remains substantially larger than that of the minimum possible. This suggests room for further reduction in the number of the light-harvesting antenna pigments, in order to further maximize solar conversion efficiencies and photosynthetic productivity of green algae in mass culture.

The results in Fig. 9c showed that, under ambient conditions, productivity of green algae in a mass culture depends on cell density, incident light intensity and solar conversion efficiency. A linear increase in productivity was observed under conditions when the amount of the biomass, but not irradiance, was the yield-limiting factor. At greater biomass densities, when quantitative absorption of all incoming irradiance occurs, the productivity of the culture becomes biomass-independent and depends only on the optical properties of the cells. The latter are mainly determined by the size of the light-harvesting Chl antenna of the photosystems and they affect solar conversion efficiency. The smaller chlorophyll antenna size of the photosystems in the *tla1* strain alleviates in part the optical shortcomings associated with a fully pigmented Chl antenna. A truncated Chl antenna minimizes the over-absorption of bright incident sunlight by the photochemical apparatus of individual cells and diminishes the subsequent wasteful dissipation of excitation energy. In addition, a truncated Chl antenna partially alleviates steep light intensity gradients (Naus and Melis 1991; Melis et al. 1999), permitting greater light penetration deeper into the culture, and thus contributing to productivity. This contention was predicted from the light-saturation curves of photosynthesis in the wild type and *tla1* mutant, which showed that a smaller Chl antenna size results in a relatively higher light intensity for the saturation of photosynthesis in individual cells (Fig. 7). It was experimentally demonstrated by the photosynthetic productivity measurements under mass culture conditions shown in Fig. 9c.

It is concluded that the partially truncated chlorophyll antenna size of the *tla1* mutant prevents the over-absorption of incident sunlight by individual cells and alleviates the wasteful dissipation of absorbed irradiance. It diminishes the rather severe gradient of light and mutual cell shading that occurs in the wild type and permits a more uniform illumination of the cells in the mass culture. It thus leads to better solar conversion efficiencies and greater photosynthetic productivity under bright sunlight conditions.

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