Planta (2010) 231:729–740
DOI 10.1007/s00425-009-1083-3

ORIGINAL ARTICLE

Genetic and biochemical analysis of the *TLA1* gene in *Chlamydomonas reinhardtii*

Mautusi Mitra · Anastasios Melis

Received: 4 October 2009 / Accepted: 24 November 2009 / Published online: 15 December 2009

Abstract The *Chlamydomonas reinhardtii* genomic DNA database contains a predicted open reading frame (*ORF-P*) without an apparent stop-codon and unknown coding sequence, located in close proximity and immediately upstream of the *TLA1* gene (GenBank Accession No. AF534570). The latter was implicated in the regulation of the light-harvesting chlorophyll antenna size of photosynthesis (Tetali et al. Planta 225:813–829, 2007). To provide currently lacking information on *ORF-P* and its potential participation in *TLA1* gene expression, thus in the regulation of the chlorophyll antenna size, genetic and biochemical analyses were undertaken. The coding and UTR regions of the *ORF-P* were defined and delineated from those of the adjacent *TLA1* gene. *ORF-P* is shown to encode a protein with a distinct RING-like zinc finger domain that is present in numerous eukaryotic proteins, believed to play a role in cellular ubiquitination, leading to regulation of cellular processes like signaling, growth, transcription, and DNA repair. It is further shown that the two genes share a 74-bp overlap between the 3′ UTR region of *ORF-P* and the 5′ UTR region of *TLA1*. However, they possess distinct start and stop codons and separate coding sequences, and transcribed as separate mRNAs without any trans-splicing between them. Complementation experiments showed that the *TLA1* gene alone is sufficient to rescue the truncated chlorophyll antenna size phenotype of the *tla1* mutant. Protein sequence alignments in *C. reinhardtii* and the colorless microalga *Polytomella parva* suggested that TLA1 defines the relationship between nucleus and organelle in microalgae, indirectly affecting the development of the chlorophyll antenna size.

Keywords *Chlamydomonas* · Chlorophyll antenna size · Chl-deficient mutant · Photosynthesis · *Polytomella parva* · *TLA1* gene

Abbreviations

Chl	Chlorophyll
LHC	Light harvesting complex
MSA	Multiple sequence alignment
PS	Photosystem
RACE	Rapid amplification of cDNA ends
RING	Really interesting new gene
RDP1	RING like domain protein 1
TLA	Truncated Light-harvesting chlorophyll Antenna
UTR	Untranslated region

Introduction

The chlorophyll (Chl) antenna size of the photosystems is defined genetically by unknown genes and regulated molecularly by a mechanism that is not well understood. Up to 300 Chl (*a* and *b*) molecules can be associated with PSII, whereas the Chl antenna of PSI may contain up to 250 Chl molecules (Melis 2002, 2005). The number of Chl molecules associated with each photosystem is not fixed but could vary substantially depending on environmental, developmental, genetic, or physiological conditions (Bjorkman et al. 1972; Anderson 1986; Melis 1991). A long-term acclimation mechanism by plants and algae regulates the

Electronic supplementary material The online version of this article (doi:10.1007/s00425-009-1083-3) contains supplementary material, which is available to authorized users.

M. Mitra · A. Melis (✉)
Department of Plant and Microbial Biology,
University of California, 111 Koshland Hall MC-3102,
Berkeley, CA 94720-3102, USA
e-mail: melis@berkeley.edu; planta@berkeley.edu

expression of nuclear chlorophyll *a–b* light-harvesting complex (*Lhcb*, *Lhca*) and chlorophyllide *a* oxygenase (*CAO*) genes (Jansson et al. 1992; Ohtsuka et al. 1997), thereby attenuating the Chl antenna size. For example, upon growth under light-limiting conditions, up-regulation of the *Lhc* and *CAO* gene expression results in a larger Chl antenna size (Masuda et al. 2003). Conversely, under high irradiance, conferring excitation pressure to the photosynthetic apparatus (Maxwell et al. 1995; Huner et al. 1998; Wilson and Huner 2000), the reverse occurs. Earlier efforts to elucidate the molecular mechanism for the dynamic regulation of the Chl antenna size postulated involvement of the redox state of the plastoquinone pool (Escoubas et al. 1995) and/or the operation of a cytosolic signaling transduction pathway for the rapid (order of minutes) regulation of both *Lhc* and *CAO* gene expression by irradiance (Masuda et al. 2003). The latter implicated activation of a specific Ca^{2+}/CaM-dependent protein kinase in this cytosolic signal transduction pathway. Acclimation of the Chl antenna size enables photosynthetic organisms to better balance rates of the light versus the carbon reactions, so as to optimize the overall efficiency of photosynthesis in diverse irradiance ecotypes (Melis 2009). The above-described Chl antenna size regulatory mechanism is highly conserved and functions in all organisms of oxygenic and anoxygenic photosynthesis (Anderson 1986; Nakada et al. 1995; Escoubas et al. 1995; Huner et al. 1998; Yakovlev et al. 2002; Masuda et al. 2002, 2003). Physiological and biochemical consequences of the function of this molecular mechanism for the regulation of the Chl antenna size are well understood (Anderson 1986; Melis 1991; Melis et al. 1999). However, unknown are still the nuclear genes that dynamically modulate the development and define the size of the Chl antenna in the chloroplast (Escoubas et al. 1995; Melis 1991, 1996, 2002, 2005).

A genetic approach toward identification of the genes and elucidation of the mechanism for the regulation of the Chl antenna size employed DNA-insertional mutagenesis and screening for the isolation of mutants with a "truncated light-harvesting Chl antenna size" (*tla* mutants) in the model organism *Chlamydomonas reinhardtii.* The *tla1* mutant strain was isolated from such a DNA-insertional mutagenesis library. It possessed a smaller than wild-type Chl antenna size for both photosystems, with the PSII and PSI Chl antenna size of the mutant being 50 and 65% of those found in the wild type, respectively. It also showed lower levels of light-harvesting proteins and of Chl *b* relative to the wild type (Polle et al. 2003). The *tla1* strain required a higher light intensity for the saturation of photosynthesis and showed greater solar conversion efficiency and a higher photosynthetic productivity than the wild type under mass culture conditions. Molecular and genetic analyses revealed that, in the *tla1* mutant, the exogenous plasmid DNA was inserted at the end of the 5′ UTR and just prior to the ATG start codon of a hitherto unknown gene (termed *TLA1)*, which encodes a protein of 213 amino acids. Expression of the *TLA1* gene was substantially down-regulated as a consequence of this plasmid insertion (Tetali et al. 2007).

Work in this manuscript was necessitated by the presence of a *C. reinhardtii* genomic DNA "predicted open reading frame" (*ORF-P*), which lacked an apparent stop-codon, and was located in close proximity and immediately upstream of the *TLA1* gene. Lack of ESTs for *ORF-P,* and lack of a stop codon, coupled with the close proximity of *ORF-P* to *TLA1* raised questions as to whether *ORF-P* is expressed, whether a portion or all of the *ORF-P* extends into and might be part of the *TLA1* gene, or whether trans-splicing between coding regions of *ORF-P* and *TLA1* mRNA plays a role in the expression of the *TLA1*. These possibilities were not previously investigated (Tetali et al. 2007). The work provides new information on the genetic, molecular and functional characteristics of the *TLA1* gene. It addressed the relationship of *TLA1* with the upstream proximal *ORF-P* gene, reports on *tla1* mutant rescue experiments performed separately with the *ORF-P* and *TLA1* genes, and probes TLA1 protein similarities between two divergent microalgae, the green *C. reinhardtii* and the colorless *Polytomella parva*. Insights gained from this work advance our knowledge of *C. reinhardtii* genomic DNA organization in the vicinity of the *TLA1* gene and provide evidence on the function of the TLA1 protein.

Materials and methods

Growth of the algae

Chlamydomonas reinhardtii CC425, the arginine-requiring strain (http://www.chlamy.org); *tla1,* the chlorophyll-deficient *tla1* mutant (Polle et al. 2003); and *tla1*-complemented strains of the *tla1* mutant (Tetali et al. 2007) were employed in this work. Strains were grown to the mid-exponential growth phase either in TAP [Tris acetate phosphate, pH 7.4], TAP + Arg (Sueoka 1960; Harris 1989), or in modified minimal media containing 40 mM Tris–HCl, pH 7.4, supplemented with 25 mM sodium bicarbonate with or without Arg (TBP medium, Polle et al. 2000) in flat 1-L Roux bottles at 25°C under continuous illumination at 200 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps. The cultures were stirred continuously to ensure mixing, a uniform illumination of the cells, and to prevent settling.

Cell density was estimated upon counting the number of cells per mL culture using a Neubauer ultraplane hemacytometer. Pigments from intact cells 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 (Chl *a* and Chl *b*) concentration of the samples was determined according to Arnon (1949), with equations corrected as in Melis et al. (1987).

Nucleic acid extractions

Chlamydomonas genomic DNA was isolated using a combination of Qiagen's (Valencia, CA, USA) DNeasy plant mini kit and phenol chloroform extraction. *Chlamydomonas* total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Small- and large-scale isolation of plasmid preps were done using the QIAprep spin miniprep and Hispeed plasmid Midi kit (Qiagen), respectively. Gel purifications of amplified PCR and RT-PCR products were performed using QIAEX II kit (Qiagen).

RACE analysis of *ORF-P* and *TLA1* cDNAs

3′ and 5′ RACE analyses were performed on isolated RNA samples from the wild-type strain using the Gene Racer kit primers (Invitrogen) and respective *TLA1*/*ORF-P* gene-specific primers (Table 1 and 1S). The Hot Star Taq Plus PCR kit (Qiagen) was used for this PCR work. The amplified RACE PCR products were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen). The cloned constructs were sequenced at the UC Berkeley DNA Sequencing Facility.

Cloning of full-length *TLA1* gene for complementation of the *tla1* mutant

The full-length *TLA1* gene (genomic DNA sequence including two exons and a single intron) was amplified using "primer 2" and "primer 3R" (Exon-1 and Exon-2; Table 1) on extracted wild-type genomic DNA. The "primer 2" and "primer 3R" had *Nde*I and *Xba*I restriction sites added at the 5′ ends, respectively. The 768 bp genomic-DNA PCR product (*TLA1* gene with the added base pair from the restriction sites) was digested with *Nde*I and *Xba*I and cloned into vector pSL18 (Fischer and Rochaix 2001; Pollock et al. 2003). Platinum Taq high fidelity DNA polymerase (Invitrogen) was used for the PCR amplification. A 1-kb plus DNA ladder was used as DNA size markers (Invitrogen). The pSL18 plasmid incorporates 5′ and 3′ UTR regions from the *PsaD* gene of *C. reinhardtii*, and also contains the ampicillin and paromomycin (Sizova et al. 2001) resistance genes as selectable markers (Fig. 1S). The latter are useful for screening transformants of *Escherichia coli* and *C. reinhardtii* on LB + ampicillin and TAP + paromomycin agar plates, respectively. The recombinant pSL18-*TLA1* construct from one of the positive *E. coli* clones was sequenced to confirm the *TLA1* gene sequence present. The sequenced pSL18-TLA1 construct was used to transform the *tla1* mutant strain by the glass-bead method (Debuchy et al. 1989; Kindle 1990).

Isolation of *tla1*-complements

The *tla1* mutant was transformed with the above-mentioned pSL18-*TLA1* construct (Fig. 1S) and transformant lines were screened on TAP + 7.5 μM paromomycin-containing agar plates (Sizova et al. 2001). About 100 colonies were thus isolated and screened for strains showing high Chl/cell and low Chl *a*/Chl *b* ratios compared with that of the *tla1* mutant. These paromomycin-resistant colonies, having a dark green coloration, were tested for the presence of the transforming *TLA1* gene by PCR/RT-PCR and TLA1 protein levels by western blot analysis. Five different *tla1*-complemented lines were isolated on the basis of visual/

Table 1 RT-PCR and 5′ and 3′ RACE primers used for RT-PCR and RACE analysis of *TLA1* and *ORF-P* cDNA in the wild-type strain

Primers	Expected product
"Primer 6" (5′ GTACGACAAGGGCAATGATAATGG 3′) "Primer 7" (5′ CGTAGCAGAATGACGCGC 3′) Probing for the ORF-P gene and its cDNA	772 bp genomic DNA product; 296 bp cDNA product
"Primer 2" (5′ ATGACTTTCAGCTGCTCCGCTGACCAAA 3′) "Primer 3R" (5′ TTGTTGTCCAGCACCAGCAC 3′) Probing for TLA1 cDNA	359 bp cDNA product; 768 bp genomic DNA product
"Primer X" (5′ CGACTGGAGCACGAGGACACTGA 3′) and "Primer Y" (5′ TTAAGAGCGCGGTTTGGTCAGCGGAGCAGC 3′) Used for 5′ RACE of TLA1 cDNA	207 bp
"Primer A" (5′ CAACACCAGGGGCAACACCAGGGGCA 3′) and "Primer B" (5′ CGCTACGTAACGGCATGACAGTG 3′) Used for 3′ RACE of ORF-P cDNA	847 bp
"Primer X" (5′ CGACTGGAGCACGAGGACACTGA 3′) and "Primer C" (5′ CCATTATCATTGCCCTTGTCGTAC 3′) Used for 5′ RACE of ORF-P cDNA	473 bp

microscopic inspection and Chl analysis of the cultures. From these, two lines with similar to the wild-type Chl content and light-harvesting Chl antenna size and were selected for further analysis.

PCR and RT-PCR analysis of *ORF-P*

Primers (Table 1) were designed on the basis of the *ORF-P* genomic DNA sequence in the database and used for PCR and RT-PCR reactions with the wild-type DNA and RNA, respectively. The amplified PCR and RT-PCR products were cloned in pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen). The cloned constructs were sequenced at the UC Berkeley DNA sequencing facility.

Analysis of the *TLA1* gene

Primers for PCR and RT-PCR reactions were designed on the basis of wild type, transforming, and mutant *tla1* gene structure. These features are summarized below:

- Wild-type *TLA1* gene possess the following distinct DNA regions: 5′ UTR, Exon 1, Intron, Exon 2, 3′ UTR.
- Mutant *tla1* gene shows: 3′ end of pJD67 plasmid, Exon 1, Intron, Exon 2, 3′ UTR. (Note that the *TLA1* mutant lacks the wild-type 5′ UTR region.)
- Transforming *TLA1* gene for mutant complementation contains: *PsaD* 5′ UTR, Exon 1, Intron, Exon 2, *PsaD* 3′ UTR.

tla1 mutant strains complemented with the wild-type *TLA1* gene (*tla1*-complements) were first tested by PCR and RT-PCR to check for the presence and expression of two distinct *TLA1* genes (transforming and endogenous). To test for the presence of the transforming *TLA1* gene and transcripts, a *PsaD* 5′ UTR-specific forward primer ("primer 5"; Fig. 1S, Table 1S; supplemental material) and a reverse primer specific to the second exon of the *TLA1* gene ("primer 4"; Fig. 1S, Table 1S; supplemental material) were used. To test for the presence of the wild-type *TLA1* gene, PCR and RT-PCR were also performed on the genomic DNA and cDNA by using a forward primer specific to the 5′ UTR of the *TLA1* gene ("primer 1"; Table 1S; supplemental material) and a reverse primer specific to the second exon of the *TLA1* gene ("primer 3"; Fig. 1S, Table 1S; supplemental material). Exon-1-specific forward primer beginning with the start codon of the *TLA1* gene ("primer 2"; Table 1S; supplemental material) and the Exon-2-specific reverse primer ending with the stop codon of the *TLA1* gene ("primer 4"; Fig. 1S, Table 1S; supplemental material) were used to probe for the full-length *TLA1* transcript.

The one-step RT-PCR kit (Qiagen) was used for RT-PCR experiments. A 1 kb plus DNA ladder was used as DNA size markers for all DNA gel electrophoresis (Invitrogen).

Cell protein analysis

Chlamydomonas reinhardtii cells were harvested, washed twice with fresh growth medium and resuspended in TEN buffer, containing 10 mM Tris–HCl, 10 mM EDTA and 150 mM NaCl, pH 8. Following sonication, the crude cell extract was incubated in the presence of solubilization buffer (Smith et al. 1990). Gel lanes were loaded with an equal amount of Chl, in the range 1–2 nmol Chl, as indicated. SDS-PAGE analysis was performed according to Laemmli (1970) on a 12.5% gel, using either the Fermentas prestained (Glen Burnie, MD, USA) or Benchmark unstained protein ladder (Invitrogen). A constant current of 15 mA was applied to the electrophoresis for a 3-h period. Gels were stained with 1% Coomassie brilliant Blue R for protein visualization.

Electrophoretic transfer of the SDS-PAGE resolved proteins onto nitrocellulose was carried out for 2 h at a constant current of 400 mA in the transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). The TLA1 immune serum was used to detect the TLA1 protein in the total cell extracts of wild type (CC425), *tla1* mutant, and *tla1*-complements. The TLA1 immune serum was diluted with the antibody buffer [Tris-buffered saline, 0.005% Tween 20 and 1% bovine serum albumin (pH 7.4)] to a ratio of 1:3,000 before used as the primary probe. The secondary antibody employed for this western blot analysis was conjugated to horseradish peroxidase (BioRad, Hercules, CA, USA) and diluted to a ratio of 1:30,000 with the antibody buffer. Western blots were developed for visualization by using the Supersignal West chemiluminescent substrate kit (Pierce, Rockford, IL, USA). Image processing software (ImageJ, http://rsb.info.nih.gov/ij/) in conjunction with Microsoft Excel 2007, were used for optical density quantification of Lhcb bands from the western blot analysis experiments.

Bioinformatic analysis

Webserver BLAST-P (http://www.ncbi.nlm.nih.gov/) was used for identifying TLA1 homologs using the non-redundant protein sequence database. ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used for alignment of TLA1 homologous sequence from *C. reinhardtii* and *P. parva*.

Accession numbers

The GenBank Accession numbers for the *TLA1* gene are AF534570 and AF534571. The GenBank Accession numbers for the *RDP1* (*ORF-P*) gene are EU717142 and EU717143.

Results

Identification of an open reading frame (*ORF-P*)

Examination of the *C. reinhardtii* genomic DNA sequence in the JGI Chlre3, Scaffold 36 database (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), which contains the *TLA1* gene (Polle et al. 2003; Tetali et al. 2007) revealed the occurrence of a putative gene (*ORF-P*) of about 792 bp (chlre3/scaffold 36: 146,926–147,461 bp). The predicted start codon of the *ORF-P* was about 1,882 bp upstream of the ATG start codon of the *TLA1* gene (Fig. 1a). Three putative exons were identified to be 129, 158 and 29 bp long, respectively, with two putative introns of 249 and 227 bp long (Fig. 1a). The 316 bp of the putative *ORF-P* cDNA would normally encode a polypeptide of 105 amino acids, but *ORF-P* revealed no apparent stop codon in the JGI *C. reinhardtii* database, suggesting a longer product. Further, the 5′ and the 3′ un-translated regions of the *ORF-P* were not specified in the database. Due to lack of detailed information in the database, and lack of a corresponding EST, a question was raised as to whether the *ORF-P*-encoding sequence might be longer than the three putative exons described above, and whether it could be part of the *TLA1* gene, which is immediately downstream of the *ORF-P* sequence (Fig. 1a). These questions pertain to the genetics and regulation of the *TLA1* gene, and bear upon the mode of action of the TLA1 protein in the regulation of the Chl antenna size (Polle et al. 2003; Tetali et al. 2007). Accordingly, a detailed investigation was undertaken to better define *ORF-P* gene and product, and to clarify its relationship with the *TLA1* gene.

Effort was directed toward testing for the expression of the *ORF-P*. An initial set of primers was designed based on the putative *ORF-P* genomic DNA sequence and used for PCR and RT-PCR analyses. When *ORF-P* Exon-1-specific "primer 6", and *ORF-P* Exon-3-specific "primer 7" (Fig. 1a; Table 1), were used for PCR and RT-PCR, products of 772 and 296 bp, respectively, were obtained (Fig. 1b). These PCR products are consistent with those

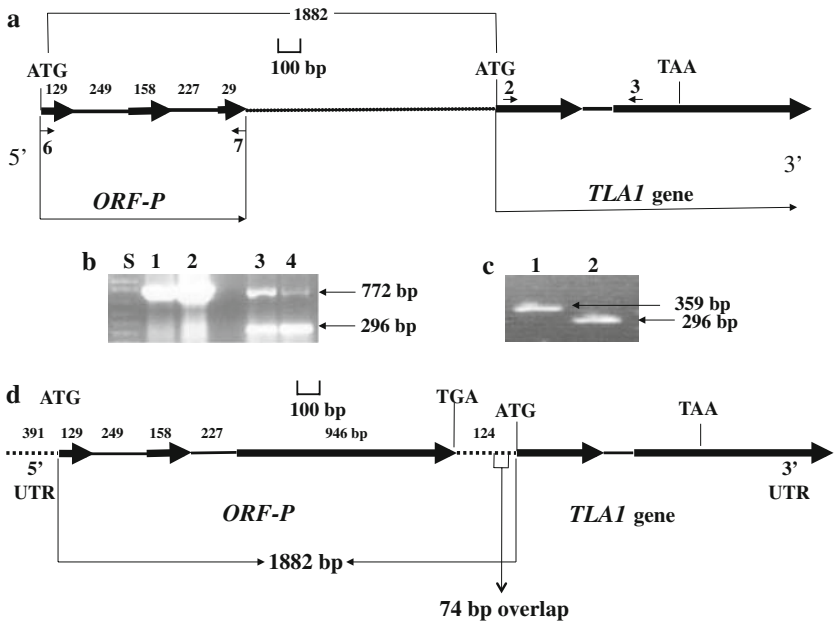

Fig. 1 Identification of an incomplete predicted open reading frame (*ORF-P*) in the *Chlamydomonas reinhardtii* database. **a** Genomic map of the *ORF-P* showing predicted exons (*thick black arrow*) and introns (*thin black lines* in-between exons). The uncharacterized genomic DNA region between *ORF-P* and *TLA1* is depicted by a *dotted line*. The *small black arrows* (6, 7; 2, 3) denote primers used for genomic DNA PCR and RT-PCR. The known start codon (**ATG**) and the stop codon (**TAA**) of the *TLA1* gene are denoted. *Numbers* refer to the base pairs of the putative exons, introns and distance between the start codon of *ORF-P* and start codon of *TLA1*. **b** *Lanes 1* and *2* show the genomic DNA PCR product (772 bp) obtained with *ORF-P*-specific primer 6 and primer 7 (Exon-1/Exon-3 of *ORF-P*; Table 1); *lanes 3* and *4* show the RT-PCR product (296 bp) obtained with the same primer set. (Genomic DNA contamination in the RNA preparation gave rise to the 772 bp product in the RT-PCR reaction in *lanes 3* and *4*.). *Lane S* refers to 1 kb plus DNA ladder. **c** RT-PCR using *TLA1* gene-specific Exon-1 "primer 2" and Exon-2 "reverse primer 3R" (359 bp product; *lane 1*, Table 1) and *ORF-P* gene-specific Exon-1 "primer 6" and Exon-3 "primer 7" (296 bp product; *lane 2*, Table 1). **d** Complete genomic DNA map of the *C. reinhardtii ORF-P* and *TLA1* genes. The positional alignment of the *TLA1* gene with respect to the *ORF-P* is shown. *Thick black arrows* denote exons, while *thin black lines* denote introns. Untranslated regions of the two genes are denoted by *dotted lines*. Note that the 3′ UTR of *ORF-P* overlaps the 5′ UTR of *TLA1* by 74 bp. The start and stop codons of the *ORF-P* and *TLA1* genes are labeled. *Numbers* denote the base pairs of the respective DNA regions, i.e., 5′ UTR, exons, introns and 3′ UTR of the *ORF-P*

expected from the structure of the *ORF-P* putative gene. Moreover, nucleotide-sequencing analysis of the PCR and RT-PCR amplified products (Fig. 1b) confirmed the identity of these products and, therefore, the existence of three exons and two introns as components of the *ORF-P* gene. Figure 1c shows results of RT-PCR analysis performed with RNA from the same sample, but with *TLA1* gene-specific primers, "primer 2" and "primer 3" (Exon-1/Exon-2, Fig. 1c, lane 1; Table 1) and *ORF-P* gene-specific primers, "primer 6" and "primer 7" (Exon-1/Exon-3, Fig. 1c, lane 2; Table 1). This side-by-side comparison showed RT-PCR products of 359 and 296 bp, respectively, providing evidence that both *TLA1* and *ORF-P* are expressed under the customary and physiological *C. reinhardtii* growth conditions employed in this work. To facilitate comparison, Fig. 1d shows the updated complete map of the *ORF-P: TLA1 C. reinhardtii* genomic DNA region. Evidence for some of the features presented in this map is provided by detailed analysis, below.

RACE analyses of *ORF-P* and *TLA1* cDNAs

Detailed RACE analyses for the *ORF-P* and *TLA1* genes were undertaken to define coding and untranslated regions. RACE primers used in this work are listed in Table 1. It was found that the 5′ UTR of *ORF-P* is 391 bp long (Fig. 1d). These 5′ RACE results did not change the translational reading frame that was earlier deduced for *ORF-P*. In order to precisely define the 3′ UTR end of the *ORF-P* and the beginning of the 5′ UTR of the *TLA1* gene, 3′ RACE and 5′ RACE analyses of the *ORF-P* and *TLA1* cDNAs were performed, respectively. Results from these analyses showed that the putative **TGA** stop codon of *ORF-P* is 1,706 bp away from the start codon in the genomic sequence, and 1,230 bp away from the start codon in the cDNA sequence (Fig. 1d). These results suggested a putative *ORF-P*-encoded protein of 410 amino acids. The putative **TGA** stop codon of *ORF-P* is shown in Figs. 1d and 2a along with the 124 bp that comprise the 3′ UTR of this open reading frame. 5′ RACE analysis for the *TLA1* gene revealed a 5′ UTR comprising 123 bp. The **ATG** start codon and upstream 5′ UTR of the *TLA1* gene is also shown in Figs. 1d and 2a. There is overlap of 74 bp between the 3′ UTR of *ORF-P* and 5′ UTR of the *TLA1* gene (Fig. 2a, gray highlighted nucleotides). It was also observed that the **TGA** stop codon of the *ORF-P* and the **ATG** start codon of the *TLA1* gene are outside this overlap region.

Transcriptional analysis of *ORF-P* and *TLA1* genes

To investigate the possibility of trans-splicing between the *ORF-P* and *TLA1* mRNAs, RT-PCR analyses were performed with a combination of *ORF-P*- and *TLA1*-specific forward (F) and reverse (R) primers, with the following outcomes:

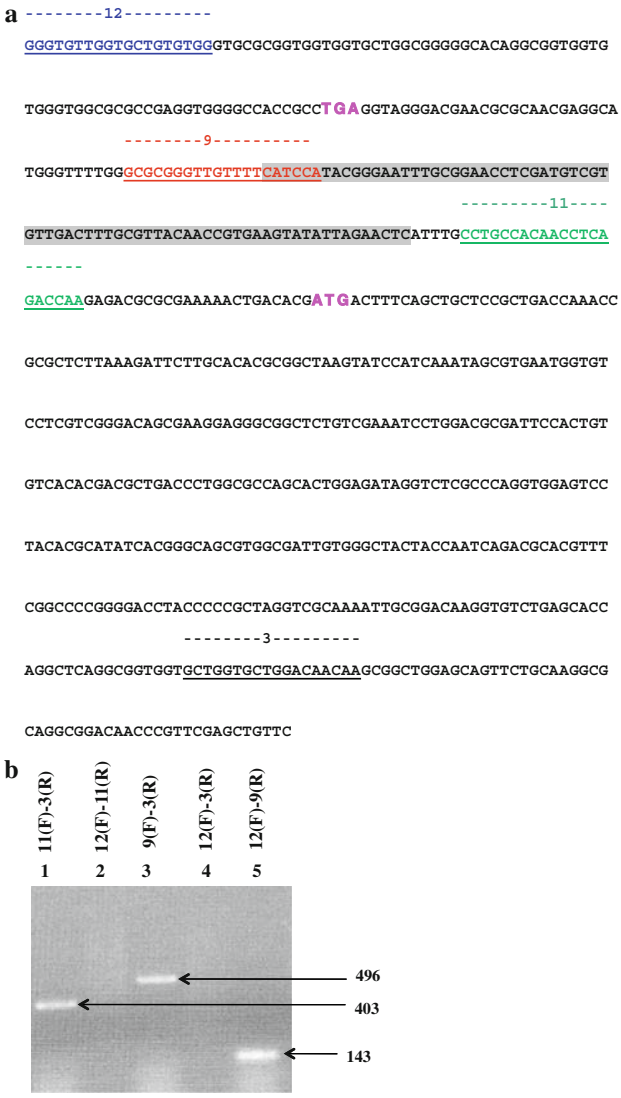

Fig. 2 cDNA sequence of the *ORF-P* 3′ and *TLA1* 5′ ends. **a** Overlap between the cDNA sequence of *ORF-P* and *TLA1* is highlighted in *gray*. Gene-specific primers used for RT-PCR are *color coded* and *underlined*. Shown in *uppercase bold* and underlined fonts are "primer 12F" (*blue*), "primer 9F" (*red*), "primer 11F" (*green*), and "primer 3F" (*black*). The stop codon **TGA** of *ORF-P* and start codon **ATG** of *TLA1* are shown in *uppercase bold magenta font*. **b** RT-PCR results using combinations of *ORF-P*- and *TLA1*-specific primers. *Lane 1* shows 403-bp RT-PCR product obtained with *TLA1* forward "primer 11F" and second exon-specific reverse "primer 3R". *Lane 2* shows no RT-PCR result with *ORF-P* 3′ UTR-specific forward "primer 12F" and *TLA1* 5′ UTR-specific reverse "primer 11R" (reverse sequence of "primer 11F"). *Lane 3* shows 496-bp RT-PCR product obtained with *TLA1* 5′ UTR-specific forward "primer 9F" and second exon-specific reverse "primer 3R". *Lane 4* shows no RT-PCR result with *ORF-P* 3′ UTR-specific forward "primer 12F" and *TLA1* second exon-specific reverse "primer 3R". *Lane 5* shows 143 bp RT-PCR product obtained with *ORF-P* 3′ UTR-specific forward "primer 12F" and *TLA1* 5′ UTR-specific reverse "primer 9R" (reverse sequence of "primer 9F"). *F* and *R* stand for forward primer and reverse primer, respectively

- *TLA1* 5′ UTR-specific forward "primer 11F" and *TLA1* second exon-specific reverse "primer 3R" (Fig. 2a) yielded a 403-bp product (Fig. 2b, lane 1).
- *ORF-P* 3′ UTR-specific forward "primer 12F" (Fig. 2a) and *TLA1* 5′ UTR-specific reverse "primer 11R" (reverse sequence of "primer 11") generated no product (Fig. 2b, lane 2), suggesting that the two genes generate distinct and separate mRNAs.
- *TLA1* 5′ UTR-specific forward "primer 9F" and *TLA1* second exon-specific reverse "primer 3R" (Fig. 2a) resulted in a 496 bp RT-PCR product (Fig. 2b, lane 3).
- *ORF-P* 3′ UTR-specific forward "primer 12F" and *TLA1* second exon-specific reverse "primer 3R" (Fig. 2a) generated no product (Fig. 2b, lane 4), consistent with the notion that *ORF-P* and *TLA1* generate two distinct and separate mRNAs.
- *ORF-P* 3′ UTR-specific forward "primer 12F" and *TLA1* 5′ UTR-specific reverse "primer 9R" (reverse sequence of "primer 9F", Fig. 2a) produced a PCR product of 143 bp (Fig. 2b, lane 5), consistent with the notion of an overlap in the 3′ UTR of *ORF-P* with the 5′ UTR of *TLA1*.

The resulting detailed genomic DNA map and gene structure of *ORF-P* and *TLA1* is shown in Fig. 1d. Thick black arrows represent exons and thin black lines show introns. It is seen that exon 3 of the *ORF-P* is 1,070 bp long including 124 bp of the 3′ UTR of the *ORF-P*. The positional alignment of the *TLA1* gene with respect to the *ORF-P* and the 74-bp overlap between the two genes is also indicated (Fig. 1d).

Characterization of the full-length *ORF-P*

The above analysis showed that the full-length *ORF-P* genomic DNA and cDNA are 2,224 and 1,748 kb long, respectively (Fig. 1d). A *C. reinhardtii* EST database search with the *ORF-P* cDNA did not show significant homology to any existing sequences. This indicates that *ORF-P* is not highly expressed, or it is not expressed under the conditions that were used to grow the cells from which mRNA was extracted to make the cDNAs for the generation of the EST database.

The predicted 1,748 kb *ORF-P* cDNA, when translated in the +2 reading frame, yields a putative protein of 410 amino acids (Fig. 3a). Protein BLAST analysis using the Swiss Prot protein sequences database revealed that this putative protein contains a domain highly similar to eukaryotic really interesting new gene (RING) zinc finger proteins. In general, zinc finger proteins mediate protein–protein interactions with proteins involved in ubiquitination and 26S proteasome degradation pathways or DNA transcription and repair (Capili et al. 2001; Dominguez et al. 2004). Figure 3b shows the canonical RING motif also called the C3HC4 motif. In this motif, one zinc ion is bound to three cysteines and a histidine, while the other zinc ion is bound to four cysteines. The tetrahedral coordination is atypical and referred to as a "cross-brace" motif. Figure 3b compares the consensus sequence of a zinc finger domain (Fig. 3b upper; Mladek et al. 2003 http://www.ebi.ac.uk/interpro/IEntry?ac=IPR001841) with the corresponding sequence of the putative *ORF-P* gene product (Fig. 3b lower), showing that the latter deviates from the canonical zinc finger motif at only one location with regard to the spacing between two cysteine residues (highlighted in red). Given the zinc finger domain and canonical C3HC4 motif in the protein product of the *ORF-P* gene, we named this protein RDP1 (RING-like domain protein 1) and renamed *ORF-P* as the Cr*RDP1* gene. Henceforth, we shall be referring to *RDP1* instead of *ORF-P* as the name of this novel gene.

a

```
MENHAYDKGNDNGEGGGPHDGPATCYACWRVFCPSCLSPGWHH
GFSCAAYAALPAALRSLEDAALLRLGAARGWRRCPACRQMVER
AGGCNHMRCRCGASFCYACGEGCDMEEPAVAAAAAAAAALQ
PQRPAAPWRPPQLQPPPTAAAATRAAGAVVGVAGGGRASASAS
TPAQGQGQQQQGQHQGQHQGQHQGQQQQQQQQQQRQQQQQLGIA
TALPVADQQQQQQQQQQPALAMVAVLRCACPLFDVPQEERGD
GGGRGVAGGGGGRGGGFGFGLRLFGDDANRRGWAVLRGLLGEA
AEAAAHASEAGLERGLVVAGLLLTVMVTGLRVAEAAGAAGRKP
RRGVGEAEVAVCARWAAAAVLRASAVVVVAGSGLALAGVLVLC
GCAVVVLAGAQAVVWVARRGGATA*
```

b

```
Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys-X2-Cys-X4-48-Cys-X2-Cys
Cys-X2-Cys-X9-  Cys-X-  His-X2-  Cys-X- Cys-X4-   Cys-X2-Cys
```

Fig. 3 Deduced amino acid sequence of the *ORF-P*-encoded, RDP1 protein. **a** The *RDP1* gene encodes a protein of 410 amino acids. The C3HC4 RING zinc finger domain is shown in *bold*. **b** Comparison of the conventional C3HC4 RING zinc finger motif (*upper*) with that present in RDP1 (*lower*). *C* and *H* denote conserved cysteine and histidine residues, respectively, and *X* represents generic amino acids. The non-canonical residues are highlighted in *red*. Note that the spacing between the fourth and fifth cysteine is one amino acid in RDP1 in contrast to two amino acids in the conventional C3HC4 RING zinc finger

An interesting feature of the putative RDP1 amino acid sequence (Fig. 3a) is the occurrence of numerous stretches of homopolypeptide repeats of poly-alanine, poly-glutamine and poly-glycine in the RDP1 protein sequence. Similar homopolypeptide repeats also occur in other *C. reinhardtii* proteins, including the *psaA* trans-splicing factors Raa1 and Raa3, Nac2, Mbb1 and Mca1, which are involved in RNA processing and stability, or Tbc2 which seems to be involved in translation (Boudreau et al. 2000; Lown et al. 2001; Rivier et al. 2001; Auchincloss et al. 2002; Merendino et al. 2006). However, in RDP1, the repetitive sequences are longer, more frequent, and involve diverse residues (Ala, Gln and Gly). A polyglycine tract in plant protein Toc-75 (a component of the protein import machinery in the outer chloroplast envelope) has been

shown to be important for targeting this protein to the outer envelope (Inoue and Keegstra 2003; Faux et al. 2007). The functional significance of these repetitive stretches is largely unknown (Merendino et al. 2006). They may play a structural role and may occur in parts of proteins where the precise sequence is not important so that it has drifted during evolution (Katti et al. 2000).

Delineation of the function of *TLA1* and *RDP1* genes in *C. reinhardtii*

To test whether *TLA1* alone is responsible for the regulation of the Chl antenna size in chloroplasts, without involvement by the *RDP1* gene, we constructed a plasmid upon cloning the full-length *TLA1* genomic DNA (758 bp), coupled with the constitutive *PsaD* promoter, in vector pSL18 (Fig. 1S, Table 1S, supplementary material). This construct was used to complement the *tla1* mutant in order to clarify whether the *TLA1* gene by itself is necessary and sufficient to rescue the *tla1* mutation. Transformant colonies were selected on TAP agar plates in the presence of 7.5 μM paromomycin and screened for dark green coloration.

Two independent lines of putative complemented *tla1* mutant strains were randomly selected from a batch of five isolated transformants and further analyzed upon growth autotrophically in minimal TBP liquid media. Figure 4 shows that wild type (Fig. 4a) and complemented strains (Fig. 4c, d) had a dark green coloration when compared with the lighter green phenotype of the *tla1* mutant (Fig. 4b). The *tla1*-comp1 and *tla1*-comp2 strains were tested for their Chl/cell and Chl *a*/Chl *b* ratios, and compared with those of the wild type and the original *tla1* strain. Table 2 shows the Chl antenna characteristics of the strains examined. Chl/cell in the wild type (3.2×10^{-15} mol/cell) was substantially greater than that in the *tla1* mutant (1.1×10^{-15} mol/cell). The complemented strains had Chl/cell values ($2.9–3.0 \times 10^{-15}$ mol/cell) i.e., comparable to that of the wild type. Table 2 also shows that wild-type *C. reinhardtii* had a Chl *a*/Chl *b* ratio of 2.6, whereas the *tla1* mutant had a Chl *a*/Chl *b* ratio of 6. The putative *tla1*-complemeted strains had much lower Chl *a*/Chl *b* ratios, ranging between 2.7 and 2.8. A lower Chl *a*/Chl *b* ratio suggests assembly of peripheral subunits of the Chl *a*–*b* light-harvesting complex, underlying an enlarged photosystem Chl antenna size (Polle et al. 2001, 2003). These results provide evidence for the return of the wild-type levels of Chl in the *tla1*-complemented strains.

In contrast, complementation of the *tla1* mutant strain with a pSL18 construct containing the full-length 2.2 kb *RDP1* genomic DNA, also cloned under the control of the *PsaD* promoter, failed to rescue the mutation, as evidenced by inability of such transformants to restore wild-type levels of Chl/cell and Chl *a*/Chl *b* ratio in the mutant strain (not shown). These results suggest that the *TLA1* gene alone is responsible for the regulation of the Chl antenna size of photosynthesis in *C. reinhardtii*. The functional role of *RDP1* was not further investigated in the context of this work.

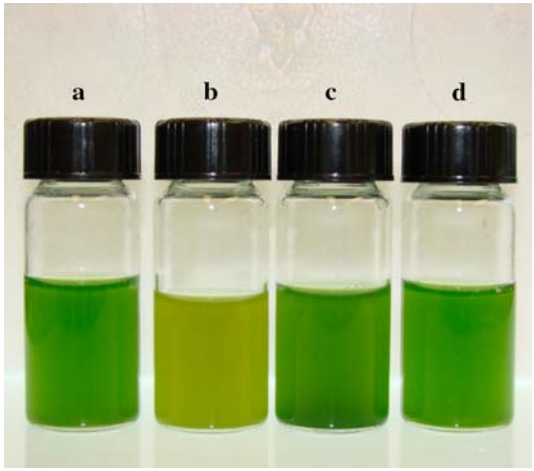

Fig. 4 Phenotype of wild type, *tla1* mutant and *tla1*-complemented strains of *C. reinhardtii*. Minimal media (TBP) liquid cultures of wild type (**a**), *tla1* mutant (**b**) and two complemented strains, *tla1*-comp1 (**c**) and *tla1*-comp2 (**d**) are shown. Cell densities of the four cultures were approximately, 6×10^6 cells/mL. The *tla1* mutant strain was complemented with a single copy of the wild-type *TLA1* gene. The *tla1* mutant showed a *pale green coloration* phenotype (**b**), indicative of the low-level chlorophyll concentration in these cells, whereas the wild type (**a**) and putative complemented strains *tla1*-comp1 (**c**) and *tla1*-comp2 (**d**) had a dark *green coloration* similar to that of the wild type

Table 2 Chl content per cell, Chl *a*/Chl *b* ratios, and LHC protein amount in *C. reinhardtii* wild type (WT), *tla1* mutant and *tla1* strains complemented with the *TLA1* gene (*tla1*-comp1 and *tla1*-comp2)

Strain	Chl ($\times 10^{-15}$ mol/cell)	Chl *a*/Chl *b* ratio	Lhcb amount (%)
WT (*cw15*)	3.2	2.6	100
tla1	1.1	6.0	22
tla1-comp1	2.9	2.7	65
tla1-comp2	3	2.8	80

Quantification of the LHC band from the scanned western blots was achieved with image processing software (ImageJ). Statistical error (±SD) was <10% of the values shown

PCR and RT-PCR analysis of *tla1*-complemented strains

A more detailed molecular and biochemical analysis was undertaken, entailing a comparative and quantitative evaluation of *tla1*-comp1, *tla1*-comp2, *tla1* mutant and wild type. When PCR and RT-PCR were performed on the genomic DNA using forward *PsaD* 5′ UTR "primer 5" and reverse *TLA1* Exon-2 "primer 4" (Fig. 1S and Table 1S supplementary material), strains *tla1*-comp1 and *tla1*-comp2 yielded a 781-bp PCR product (Fig. 5a) and a 665-bp

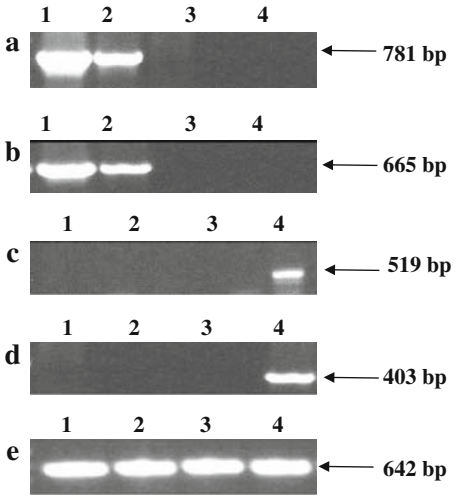

Fig. 5 PCR and RT-PCR analysis of wild type, *tla1* and two *tla1*-complemented strains. *Lane 1 tla1*-comp1, *lane 2 tla1*-comp2, *lane 3 tla1* mutant, *lane 4* wild type. **a** Genomic DNA PCR product of 781 bp was obtained with forward *PsaD* 5′ UTR "primer 5" and reverse *TLA1* Exon-2 "primer 4" (Fig. 1S and Table 1S; supplementary material). No products were obtained with the *tla1* mutant (*lane 3*) or wild type samples (*lane 4*). **b** RT-PCR products of 665 bp were obtained with forward *PsaD* 5′ UTR "primer 5" and reverse (*TLA1* Exon-2 "primer 4" (Fig. 1S and Table 1S; supplementary material) from the cDNA of the *tla1*-complements (*lanes 1* and *2*). No products were obtained from the cDNA of the *tla1* mutant (*lane 3*) or wild type samples (*lane 4*). **c** Genomic DNA PCR product of 519 bp was obtained with forward *TLA1 5′ UTR* "primer 1" and reverse Exon-2 "primer 3" from the wild type sample only (*lane 4*). **d** RT-PCR product of 403 bp was obtained with forward *TLA1 5′ UTR* "primer 1" and reverse Exon-2 "primer 3" with the wild type cDNA only lane 4). **e** RT-PCR products of 642 bp were obtained with all samples when using forward Exon-1 "primer 2" and reverse Exon-2 "primer 4". Genomic DNA and RNA for PCR and RT-PCR were extracted from cells grown photosynthetically in minimal TBP liquid media

RT-PCR product (Fig. 5b), whereas the *tla1* mutant and the wild type failed to yield a product, consistent with the absence of the transforming *PsaD-TLA1* gene. When forward *TLA1* 5′ UTR "primer 1" and reverse *TLA1* Exon-2 "primer 3" (Table 1S and Fig. 1S, supplementary material) were used in the PCR and RT-PCR reactions, the wild type produced a 519-bp genomic PCR product (Fig. 5c) and a 403 bp RT-PCR product (Fig. 5d), while the *tla1* mutant, *tla1*-comp1 and *tla1*-comp2 did not generate any product, consistent with the absence of the 5′ UTR of the *TLA1* gene in the latter strains. As a control, RT-PCR was employed to test for the presence of the intact full-length *TLA1* transcript in the two complemented strains, using forward *TLA1* Exon-1 "primer 2" and reverse *TLA1* Exon-2 "primer 4" (Table 1S and Fig. 1S, supplementary material). Figure 5e shows that all strains gave a product of 642 bp, as all strains contain the coding region of the *TLA1* gene. These results offer evidence of successful complementation of the *tla1* mutant by the pSL18-*TLA1* construct and expression of the *TLA1* gene in the two independent transformant lines. These results further corroborate the notion that *TLA1* is solely responsible for the functional complementation of the *tla1* mutant.

Western blot analysis of *tla1*-complemented strains

Total protein extracts from the *tla1*-comp1, *tla1*-comp2, *tla1* mutant and wild type strains were resolved on a 12.5% SDS-PAGE gel, lanes loaded on an equal chlorophyll basis (Fig. 6a). Western blot analysis of the total cell extract from these samples with specific polyclonal antibodies raised against the recombinant TLA1 and Lhcb proteins showed that the amount of the TLA1 protein and the Lhcb proteins in the two complements (Fig. 6b, c, lanes 1 and 2, see also Table 2) were comparable to those in the wild type (Fig. 6b, c, lane 4; Table 2), whereas the amount of the TLA1 protein and of the Lhcb proteins in the *tla1* mutant were substantially lower than those of the other three (Fig. 6b, c, lane 3; Table 2). Levels of Lhca paralleled those of the Lhcb in the wild type, *tla1* mutant, and *tla1*-complemented strains (not shown). These results are consistent with earlier findings from this lab (Polle et al. 2003; Tetali et al. 2007) and provide evidence that a limited translation of the *TLA1* gene takes place in the *tla1* mutant. However, this is in no way comparable to the levels of translation seen in the wild type or *tla1*-comp strains. The substantially reduced rate of translation and level of TLA1 protein in the *tla1* mutant was attributed to the absence of the native 5′ UTR of the *TLA1* gene in this strain, as exogenous plasmid insertion occurred immediately prior to the *TLA1* ATG codon (Tetali et al. 2007), thus interfering with the ability of this strain to carry out high rates of *TLA1* mRNA translation. The absence of the 5′ UTR from the native *TLA1* gene in the *tla1* mutant did not apparently affect the molecular weight of the TLA1 protein, which is the same in wild type and *tla1* mutant (Fig. 6b, lane 3).

TLA1 protein sequence analysis

The *C. reinhardtii TLA1* gene has homologs in many eukaryotic organisms, including algae, plants, *Drosophila* and even human (Tetali et al. 2007). The highest identity known to date, however, is between the *C. reinhardtii TLA1* gene and its counterpart in the colorless microalga *Polytomella parva* (Chlorophyceae). Borza et al. (2007) presented a "conceptual translation" of a cDNA from this microalga, yielding a polypeptide matching the TLA1 sequence from *C. reinhardtii*. Figure 7 shows a ClustalW amino acid sequence alignment of the two putative TLA1 proteins, revealing a 50% identity and a substantially higher degree of homology. *P. parva* is noteworthy in this respect as it retains a functional chloroplast, in spite of the

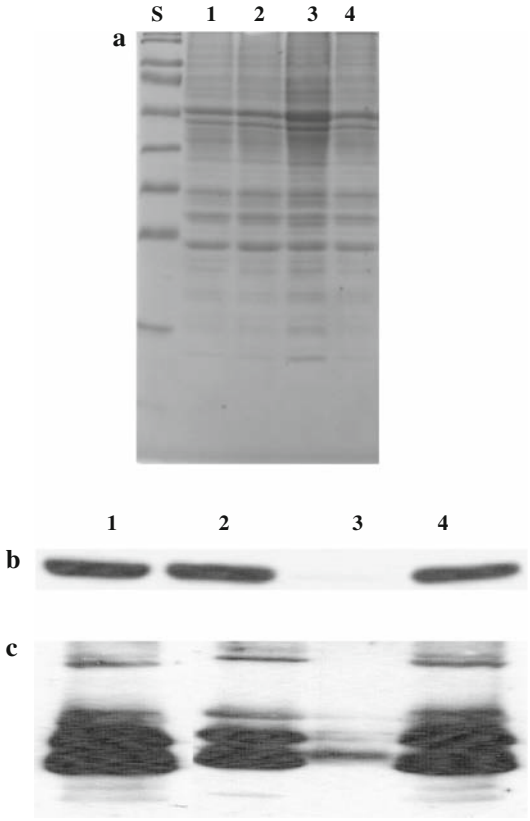

Fig. 6 SDS-PAGE and western blot analysis of *Chlamydomonas reinhardtii* proteins. *Lane 1 tla1*-comp1, *lane 2 tla1*-comp2, *lane 3 tla1* mutant, *lane 4* wild type. **a** SDS-PAGE of total cell protein extracts. Lanes were loaded on an equal Chl basis (1.5 nmol Chl per lane). *Lane S* shows the pre-stained low molecular weight markers. **b**, **c** Western blot analysis of *C. reinhardtii* total cell protein extracts. Lanes, loaded as in **a**, were probed with TLA1-specific **(b)** and Lhcb-specific polyclonal antibodies **(c)**. Note the substantially lower levels of the TLA1 and Lhcb proteins in *lane 3* (*tla1* mutant) relative to that in the other samples

apparent absence of thylakoids and chlorophyll. Retention and expression of the *TLA1* gene homolog in a colorless microalga supports the hypothesis that TLA1 is defining the relationship between nucleus and organelle, and probably playing a role in the control of organelle development (Tetali et al. 2007; see also discussion below).

Discussion

Chloroplasts have the ability to regulate the size of the functional Chl antenna in response to the level of irradiance (Bjorkman et al. 1972; Anderson 1986). For example, when high-light acclimated plants and algae are shifted to low-light, enlargement of the Chl antenna size occurs with a concomitant increase in cellular Chl, LHC apoproteins and a decrease in the Chl *a*/Chl *b* ratio, as Chl *b* accumulates in the photosystems (Sukenik et al. 1988; Webb and Melis 1995; Tanaka and Melis 1997; Neidhardt et al. 1998). At the molecular level, these changes are preceded by a rapid up-regulation of the transcription of *CAO* and *Lhcb* genes (LaRoche et al. 1991; Webb and Melis 1995; Masuda et al. 2003), suggesting that increase in the Chl antenna size occurs by coordinate induction in Chl biosynthesis and expression of *Lhcb* and *CAO* genes. In an alternative approach, application of powerful gene silencing RNA interference technologies in the model microalga *C. reinhardtii* resulted in the generation of a viable mutant, termed *stm3LR3,* which had a significantly reduced content of Lhcb and Lhca proteins (Mussgnug et al. 2007).

Regulation of the Chl antenna size is of practical importance as it defines the quantum yield and productivity of photosynthesis, especially under mass culture conditions (Melis 2007, 2009; Mitra and Melis 2008). In spite of a substantial number of physiological and biochemical studies on this phenomenon (Anderson 1986; Melis 1991, 1996), genetic determinants of the Chl antenna size and the signal transduction pathway for the regulation of this phenomenon are mostly unknown. To date, only two genes, namely *TLA1* (Polle et al. 2003; Tetali et al. 2007) and *NAB1* (Mussgnug et al. 2005), have been identified as players in the regulation of the photosynthetic Chl antenna size. *TLA1,* the first gene to be identified in the regulation of the chlorophyll antenna size of photosynthesis, encodes a protein of 213 amino acids that has homologs in diverse groups of eukaryotic organisms, ranging from microalgae to vascular plants, insects and mammals. Although all TLA1-like proteins from the different organisms show highly conserved domains in their amino acid sequence, these

Fig. 7 ClustalW generated amino acid sequence alignment of the TLA1 protein from *Chlamydomonas reinhardtii* and *Polytomella parva*. The sequence alignment revealed a 50% identity and 67% homology between the two proteins

```
Chlamydomonas  MTFSCSADQTALLKILAHAAKYPSNSVNGVLVGTAKEGGSVEILDAIPLCHTTLTLAPAL 60
Polytomella    ------------------------------IGEILSSEEIIVKDAIPLCHTNLSLAPAI 29
                                             :*   .. .: : ********.*:****:

Chlamydomonas  EIGLAQVESYTHITGSVAIVGYYQSDARFGPGDLPPLGRKIADKVSEHQAQAVVLVLDNK 120
Polytomella    EIGLAQIQSYIDLEAKLKIVGYYHSDSKYESGDLPPVGRRIADKIQEKQNAAVSIVLDNK 89
               ******::** .: ..: *****:**::: .*****:**:****:.*:*  ** :*****

Chlamydomonas  RLEQFCKAQADNPFELFSKDGSKGWKRASADGGELALKNADWKKLREEFFVMFKQLKHRT 180
Polytomella    KLSGFNLGEVDTPLDLFIKEGSSRGWKR----GGTLQLSEGSWNEEKLKFHESYKVQKYKE 145
               :*. *  .:.*.*::** *:**:****    ** * *.:..*:: : :*.  :*  *::

Chlamydomonas  LHDFEEHLDDAGKDWLNKGFASSVKFLLPGNAL 213
Polytomella    LDDFESHLDDIRKDYLNKSFN------------ 166
               *.***.****  **:***.*
```

domains do not match any known protein motifs (Tetali et al. 2007). Conserved domain architecture retrieval tool (CDART) analysis categorized the family of TLA1 protein as a novel protein family, i.e., uncharacterized protein family (UPF0172). On the other hand, the NAB1 protein is a cytosolic putative nucleic acid-binding protein that binds RNA, thought to play a role in controlling the expression of the light-harvesting antenna proteins of PSII at the posttranscriptional level (Mussgnug et al. 2005). Unlike *TLA1*, down-regulation of the *NAB1* gene leads to enlargement of the Chl antenna size in *C. reinhardtii,* presumably due to an increase in the amount of Lhcb proteins in the cell (Mussgnug et al. 2005).

The mechanism by which the *TLA1* gene regulates the Chl antenna size of photosynthesis is not presently understood. A current hypothesis, under investigation in this lab, postulates that *TLA1* defines the relationship between nucleus and organelle in eukaryotes. According to this hypothesis, *TLA1* regulates chloroplast development in *C. reinhardtii*, directly or indirectly affecting rates of Chl biosynthesis and the Chl antenna size of the photosystems. This hypothesis of a role in the relationship between nucleus and organelle explains the ubiquitous presence of this gene in eukaryotes only, including non-photosynthetic tissues (e.g. *Drosophila* and human). In the latter, TLA1-like genes may regulate the relationship between nucleus and mitochondria in these systems. In the *tla1* mutant, lower levels of the TLA1 protein resulted in less total Chl (Chl *a* and Chl *b*) per cell, lower levels of *Lhcb* and *CAO* gene expression, and diminished abundance of LHC polypeptides, translating into a slow-down in the development of the photosynthetic apparatus, apparently as a consequence of down-regulation of translation of the *TLA1* mRNA. It is tempting to postulate that expression of the *TLA1* gene promotes the development of organelles (plastids and mitochondria) in eukaryotic cells via a currently unknown signal transduction pathway that entails communication with and/or control of organelles. This contention is supported by the fact that *P. parva*, a colorless alga, belonging to the Chlorophyceae family possesses the *TLA1* gene, although it lacks thylakoids and chlorophyll (Borza et al. 2007). Signal peptide analysis of the TLA1 protein sequence failed to indicate a transit peptide, suggesting that TLA1 is localized in the nucleus or cytoplasm (Tetali et al. 2007). Conversely, analysis of COX4NB, a TLA1 homolog from human, also indicated localization to the cytoplasm, mitochondria, and/or nucleus (Bachman et al. 1999). Biochemical investigations leading to the localization of the TLA1 protein in *Chlamydomonas* are currently under way in this laboratory.

Acknowledgments The work was supported by the US Department of Energy Hydrogen Program grant # DE-FG36-05GO15041.

References

Anderson JM (1986) Photoregulation of the composition, function and structure of thylakoid membranes. Annu Rev Plant Physiol 37:93–136

Arnon D (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Auchincloss AH, Zerges W, Perron K, Girars-Bascou J, Rochaix JD (2002) Characterization of Tbc2, a nucleus encoded factor specifically required for translation of the chloroplast psbC mRNA in *Chlamydomonas reinhardtii*. J Cell Biol 157:953–962

Bachman NJ, Wu W, Schmidt TR, Grossman LI, Lomax MI (1999) The 5′ region of the COX4 gene contains a novel overlapping gene NOC4. Mamm Genome 10:506–512

Bjorkman O, Boardman NK, Anderson JM, Thorne SW, Goodchild DJ, Pyliotis NA (1972) Effect of light intensity during growth *of Atriplex patula* on the capacity of photosynthetic reactions, chloroplast components and structure. Carnegie Inst Yearbook 71:115–135

Borza T, Rajeswaran A, Lee RW (2007) The colorless plastid of the green alga *Polytomella parva*: a repertoire of its functions. GenBank Accession No. ABH10987

Boudreau E, Nickelsen J, Lemaire SD, Ossenbuhl F, Rochaix JD (2000) The Nac2 gene of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. EMBO J 19:3366–3376

Capili AD, Schultz DC, Rauscher IF, Borden KLB (2001) Solution structure of the PHD domain from the KAP-1 co-repressor: structural determinants for PHD, RING and LIM zinc-binding domains. EMBO J 20:165–177

Debuchy R, Purton S, Rochaix JD (1989) The arginosuccinate lyase gene of *Chlamydomonas reinhardtii:* an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. EMBO J 8:2803–2809

Dominguez C, Folkers GE, Boelens R (2004) Biological introduction: RING domain protein. Contributions to Handbook of Metalloproteins 3:338–351

Escoubas JM, Lomas M, LaRoche J, Falkowski PG (1995) Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. Proc Natl Acad Sci USA 92:10237–10241

Faux NG, Bottomley SP, Lesk AM, Irving JA, Morrison JR, Garcia de la Banda MG, Whisstock JC (2007) Functional insights from the distribution and role of homopeptide repeat- containing proteins. Genome Res 15:537–551

Fischer N, Rochaix JD (2001) The flanking regions of *PsaD* drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii*. Mol Genet Genomics 265:888–894

Harris EH (1989) The *Chlamydomonas* source book: a comprehensive guide to biology and laboratory use. Academic Press, San Diego

Huner NPA, Oquist G, Sarhan F (1998) Energy balance and acclimation to light and cold. Trends Plant Sci 3:224–230

Inoue K, Keegstra K (2003) A polyglycine stretch is necessary for proper targeting of the protein translocation channel precursor to the outer envelope membrane of chloroplasts. Plant J 34:661–669

Jansson S, Pichersky E, Bassi R, Green BR, Ikeuchi M, Melis A, Simpson DJ, Spangfort M, Staehelin LA, Thornber JP (1992) A nomenclature for the genes encoding the chlorophyll a/b-binding proteins of higher plants. Plant Mol Biol Rep 10:242–253

Katti MV, Sami-Subbu Ranjekar PK, Gupta VS (2000) Amino acid repeat patterns in protein sequences: their diversity and structural–functional implications. Protein Sci 9:1203–1209

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 87:1228–1232

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

LaRoche J, Mortain-Bertrand A, Falkowski PG (1991) Light-intensity-induced changes in *cab* mRNA and light-harvesting complex II apoprotein levels in the unicellular chlorophyte *Dunaliella tertiolecta*. Plant Physiol 97:147–153

Lown FJ, Watson AT, Purton S (2001) *Chlamydomonas* nuclear mutants that fail to assemble respiratory or photosynthetic electron transfer complexes. Biochem Soc Trans 29:452–455

Masuda T, Polle JEW, Melis A (2002) Biosynthesis and distribution of chlorophyll among the photosystems during recovery of the green alga *Dunaliella salina* from irradiance stress. Plant Physiol 128:603–614

Masuda T, Tanaka A, Melis A (2003) Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll *a* oxygenase (*CAO*) and *Lhcb* gene expression. Plant Mol Biol 51:757–771

Maxwell DP, Falk S, Huner NPA (1995) Photosystem II excitation pressure and development of resistance to photoinhibition. 1. Light harvesting complex II abundance and zeaxanthin content in *Chlorella vulgaris*. Plant Physiol 107:687–694

Melis A (1991) Dynamics of photosynthetic membrane composition and function. Biochim Biophys Acta 1058:87–106

Melis A (1996) Excitation energy transfer: functional and dynamic aspects of *Lhc* (*cab*) proteins. In: Ort DR, Yocum CF (eds) Oxygenic photosynthesis: the light reactions. Kluwer Academic Publishers, Dordrecht, pp 523–538

Melis A (2002) Green alga hydrogen production: progress, challenges and prospects. Int J Hydrogen Energy 27:1217–1228

Melis A (2005) Bioengineering of green algae to enhance photosynthesis and hydrogen production. In: Collins AF, Critchley C (eds) Artificial photosynthesis: from basic biology to industrial application. Wiley-VCH, Weinheim, pp 229–240

Melis A (2007) Photosynthetic H_2 metabolism in *Chlamydomonas reinhardtii* (unicellular green algae). Planta 226:1075–1086

Melis A (2009) Solar energy conversion efficiencies in photosynthesis: Minimizing the chlorophyll antennae to maximize efficiency. Plant Sci 177:272–280

Melis A, Spangfort M, Andersson B (1987) Light-absorption and electron-transport balance between PSII and PSI in spinach chloroplasts. Photochem Photobiol 45:129–136

Melis A, Neidhardt J, Benemann JR (1999) *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. J Appl Phycol 10:515–552

Merendino L, Perron K, Rahire M, Howald I, Rochaix JD, Goldschmidt-Clermont M (2006) A novel multifunctional factor involved in trans-splicing of chloroplast introns in *Chlamydomonas*. Nucleic Acids Res 34:262–274

Mitra M, Melis A (2008) Optical properties of microalgae for enhanced biofuels production. Opt Express 16:21807–21820

Mladek C, Guger K, Hauser M (2003) Identification and characterization of the ARIADNE gene family in *Arabidopsis*. A group of putative E3 ligases. Plant Physiol 131:27–40

Mussgnug JH, Wobbe L, Elles I, Claus C, Hamilton M, Fink A, Kahmann U, Kapazoglou A, Mullineaux CW, Hippler M, Nickelsen J, Nixon PJ, Kruse O (2005) NAB1 is an RNA binding protein involved in the light-regulated differential expression of the light-harvesting antenna of *Chlamydomonas reinhardtii*. Plant Cell 17:3409–3421

Mussgnug JH, Thomas-Hall S, Rupprecht J, Foo A, Klassen V, McDowall A, Schenk PM, Kruse O, Hankamer B (2007) Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. Plant Biotech J 5:802–814

Nakada E, Asada Y, Arai T, Miyake J (1995) Light penetration into cell suspensions of photosynthetic bacteria and relation to hydrogen production. J Ferment Bioeng 80:53–57

Neidhardt J, Benemann JR, Zhang L, Melis A (1998) Photosystem-II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae). Photosynth Res 56:175–184

Ohtsuka T, Ito H, Tanaka A (1997) Conversion of chlorophyll *b* to chlorophyll *a* and the assembly of chlorophyll with apoproteins by isolated chloroplasts. Plant Physiol 113:137–147

Polle JEW, Benemann JR, Tanaka A, Melis A (2000) Photosynthetic apparatus organization and function in wild type and a Chl *b*-less mutant of *Chlamydomonas reinhardtii*. Dependence on carbon source. Planta 211:335–344

Polle JEW, Niyogi KK, Melis A (2001) Absence of lutein, violaxanthin and neoxanthin affects the functional chlorophyll antenna size of photosystem-II but not that of photosystem-I in the green alga *Chlamydomonas reinhardtii*. Plant Cell Physiol 42:482–491

Polle JEW, Kanakagiri S, Melis A (2003) *tla1,* a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size. Planta 217:49–59

Pollock SV, Colombo SL, Prout DL Jr, Godfrey AC, Moroney JV (2003) Rubisco activase is required for optimal photosynthesis in the green alga *Chlamydomonas reinhardtii* in a low-CO_2 atmosphere. Plant Physiol 133:1854–1861

Rivier C, Goldschmidt-Clermont M, Rochaix JD (2001) Identification of an RNA-protein complex involved in chloroplast group II intron trans-splicing in *Chlamydomonas reinhardtii*. EMBO J 20:1765–1773

Sizova I, Fuhrmann M, Hegemann P (2001) A *Streptomyces rimosus* aphVIII gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. Gene 277:221–229

Smith BM, Morrissey PJ, Guenther JE, Nemson JA, Harrison MA, Allen JF, Melis A (1990) Response of the photosynthetic apparatus in *Dunaliella salina* (green algae) to irradiance stress. Plant Physiol 93:1433–1440

Sueoka N (1960) Mitotic replication of deoxyribonucleic acids in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 46:83–91

Sukenik A, Bennett J, Falkowski PG (1988) Changes in the abundance of individual apoproteins of light-harvesting chlorophyll *a/b*-protein complexes of photosystem I and II with growth irradiance in the marine chlorophyte *Dunaliella tertiolecta*. Biochim Biophys Acta 932:206–215

Tanaka A, Melis A (1997) Irradiance-dependent changes in the size and composition of the chlorophyll *a–b* light-harvesting complex in the green alga *Dunaliella salina*. Plant Cell Physiol 38:17–24

Tetali SD, Mitra M, Melis A (2007) A Development of the light-harvesting chlorophyll antenna in the green alga *Chlamydomonas reinhardtii* is regulated by the novel *TLA1* gene. Planta 225:813–829

Webb MR, Melis A (1995) Chloroplast response in *Dunaliella salina* to irradiance stress. Effect on thylakoid membrane assembly and function. Plant Physiol 107:885–893

Wilson KE, Huner NPA (2000) The role of growth rates, redox-state of the plastoquinone pool and the trans-thylakoid pH in photoacclimation of *Chlorella vulgaris* to growth irradiance and temperature. Planta 212:93–102

Yakovlev AG, Taisova AS, Fetisova ZG (2002) Light control over the size of an antenna unit building block as an efficient strategy for light harvesting in photosynthesis. FEBS Lett 512:129–132