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REGULAR PAPER

Polyclonal antibodies against the TLA1 protein also recognize with high specificity the D2 reaction center protein of PSII in the green alga *Chlamydomonas reinhardtii*

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Abstract The *Chlamydomonas reinhardtii* DNA-insertional transformant *truncated light-harvesting antenna 1* (*tla1*) mutant, helped identify the novel *TLA1* gene (GenBank Accession # AF534570-71) as an important genetic determinant in the chlorophyll antenna size of photosynthesis. Down-regulation in the amount of the TLA1 23 kDa protein in the cell resulted in smaller chlorophyll antenna size for both photosystems (in Tetali et al. Planta 225:813–829, 2007). Specific polyclonal antibodies, raised against the recombinant TLA1 protein, showed a cross-reaction with the predicted 23 kDa TLA1 protein in *C. reinhardtii* protein extracts, but also showed a strong cross-reaction with a protein band migrating to 28.5 kDa. Questions of polymorphism, or posttranslational modification of the TLA1 protein were raised as a result of the unexpected 28.5 kDa cross-reaction. Work in this paper aimed to elucidate the nature of the unexpected 28.5 kDa cross-reaction, as this was deemed to be important in terms of the functional role of the TLA1 protein in the regulation of the chlorophyll antenna size of photosynthesis. Immunoprecipitation of the 28.5 kDa protein, followed by LC–mass spectrometry, showed amino acid sequences ascribed to the *psbD*/D2 reaction center protein of PSII. The common antigenic determinant between TLA1 and D2 was shown to be a stretch of nine conserved amino acids V-F—L(V)LP-GNAL in the C-terminus of the two proteins, constituting a high antigenicity "GNAL" domain. Antibodies raised against the TLA1 protein containing this domain recognized both the TLA1 and the D2 protein. Conversely, antibodies raised against the TLA1 protein *minus* the GNAL domain specifically recognized the 23 kDa TLA1 protein and failed to recognize the 28.5 kDa D2 protein. D2 antibodies raised against an oligopeptide containing this domain also cross-reacted with the TLA1 protein. It is concluded that the 28.5 kDa cross-reaction of *C. reinhardtii* protein extracts with antiTLA1 antibodies is due to antibody affinity for the GNAL domain of the D2 protein and has no bearing on the identity or function of the TLA1 protein.

Keywords Antigenic epitope · *Chlamydomonas reinhardtii* · Protein domain · TLA1 protein · D2 protein · NOC4 protein · Western blot analysis

Abbreviations

Chl	Chlorophyll
NOC4	Neighbor of cytochrome oxidase subunit 4
tla	Truncated light-harvesting chlorophyll antenna
UTR	Untranslated region
SDS	Sodium dodecyl sulfate
LDS	Lithium dodecyl sulfate
PVDF	Polyvinylidene fluoride
CGI-112	Comparative genome identification isolate 112

Introduction

The *truncated light-harvesting antenna 1* (*tla1*) mutant strain of *Chlamydomonas reinhardtii* was isolated from a

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DNA-insertional mutagenesis library and was shown to specifically possess a smaller than wild type chlorophyll antenna size in both photosystems (Polle et al. 2003). It showed down-regulated *Lhcb* and *CAO* gene expressions, diminished amounts of light-harvesting proteins, and lower levels of Chl *b* per cell relative to the wild type (Polle et al. 2003; Tetali et al. 2007; Fig. 1). Molecular and genetic analysis 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 (Tetali et al. 2007). The *TLA1* gene in the *tla1* mutant is transcribed with a new 5′ UTR sequence, derived from the 3′ end of the transforming plasmid. Western blot analysis with TLA1 specific polyclonal antibodies indicated that the TLA1 protein amount was substantially diminished in the *tla1* mutant (Tetali et al. 2007). Thus, replacement of the 5′ UTR resulted in down-regulation of the translation of the *tla1* mRNA. Transformation of the *tla1* mutant with wild type *TLA1* genomic DNA successfully rescued the mutation (Mitra and Melis 2010). These results established that polymorphism in the 5′ UTR of the *TLA1* transcripts resulted in the *tla1* phenotype and that expression of the *TLA1* gene is a prerequisite for the development/assembly of the Chl antenna size in *C. reinhardtii.*

Predicted molecular weight of the TLA1 protein, based on its amino acid sequence, is about 23 kDa, and this was confirmed in earlier western blot analyses upon heating of SDS-solubilized cellular proteins at 95 °C for 5 min (Tetali et al. 2007; Mitra and Melis 2010). Recent western blot analyses of total *C. reinhardtii* extracts, solubilized with SDS–urea buffer, incubated at room temperature for about 30 min, and probed with the same TLA1 specific polyclonal antibodies, showed the expected cross-reaction with the 23 kDa protein, but also showed a second more pronounced cross-reaction with a 28.5 kDa protein. The present work was undertaken to identify the origin of the second more pronounced cross-reaction of the TLA1 specific polyclonal antibodies with the 28.5 kDa protein band. A detailed investigation employing biochemical and molecular approaches showed that TLA1 and D2 share a common antigenic determinant of nine conserved amino acids V-F—L(V)LP-GNAL in the C-terminus of the two proteins, which is responsible for the cross-reaction of the two proteins with the same antibodies.

Fig. 1 Liquid cultures of *Chlamydomonas reinhardtii* wild type strain CC-425 and the *tla1* mutant. Cell density was 3×10^6 cells/mL for the wild type and 6×10^6 cells/mL for the *tla1* mutant. Note the different pigmentation of the two samples, where the *tla1* mutant appears pale green compared to CC-425

Materials and methods

Growth of the algae

The following *C. reinhardtii* strains were employed in this work: the arginine-requiring CC-425, the chlorophyll-deficient mutant *tla1* strain, the cytochrome *f* deficient mutant (Δ*petA*); the D1-less mutant (*Fud7*), the D2-less mutant (Δ*PsbD*), and the PSII repair-deficient mutant (*rep27*). These strains were grown to the mid-exponential growth phase in TAP + Arg media (Tris Acetate Phosphate + Arg, pH 7.4) (Sueoka 1960; Harris 1989), in flat 1-L Roux bottles at 25 °C under continuous illumination of 100 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps. The cultures were stirred slowly but continuously to ensure a uniform illumination of the cells and to prevent settling.

Cell count and chlorophyll concentration determinations

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 supernatants 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).

Amino acid sequencing of the purified His-tagged full-length TLA1 recombinant protein and the generation of polyclonal TLA1-specific antibodies

Overexpression, purification, and concentration of the His-tagged TLA1 protein from *E. coli* clone of *TLA1* was done following the protocol established in published work (Tetali et al. 2007). Purification of the recombinant protein was tested

upon SDS-PAGE using the Page Ruler prestained protein ladder (Fermentas, Glen Burnie, Maryland). The purified recombinant TLA1 protein was sequenced by the Protein Sequencing Facility at UC San Diego (http://proteinsequencer.ucsd.edu/). The sequenced, purified recombinant TLA1 protein was used for the generation of specific polyclonal antibodies (Covance, Princeton, NJ) following a Melis-lab 118-day protocol.

Generation of TLA1 antibodies against a TLA1 oligopeptide sequence lacking the "GNAL" C-terminal domain

Polyclonal antibodies were raised in rabbits against the synthetic peptide "KHRTLHDFEEHLDDAGKDWLNK" (TLA1 amino acids residues 177–198) corresponding to hydrophilic region of the *C. reinhardtii* TLA1 protein (Covance Immunological Services). Peptides were coupled to keyhole limpet hemocyanin (KLH) with a tyrosine residue added to the N-terminal position. The same immunization protocol as above was employed.

Immunopurification of the new TLA1 antibodies from the immunoblots

A total of 2 mg of antigen was loaded into the wells of an SDS-PAGE gel. The blots were run as described below but after the incubation with the primary TLA1 antibody and washing, the location of the antibodies bound to the TLA1 antigen was determined by cutting the strips of the blot from the sides. These side strips were processed as normal for antigen detection while the main body of the blot was stored in 1× PBS buffer (9.1 mM $Na_2HPO_4{\cdot}7H_2O$; 1.7 mM $NaH_2PO_4{\cdot}H_2O$ and 150 mM NaCl, pH 7.4) at 4 °C. After the localization of the antigen–primary antibody complexes on the side strips, the strips were aligned with the untreated portion of the blot. The area of the blot that contained the antibodies bound to the TLA1 antigen was excised using a sharp scalpel. This piece of PVDF membrane was chopped-up into small pieces and transferred to a 1.5 mL Eppendorf tube. The pieces of the PVDF membrane were incubated with 100 mM glycine (pH 2.5) for 15 min. After the end of the incubation period, the buffer was neutralized with 1 M Tris (pH 8) and the purified antibodies were stored at −80 °C for the subsequent western blot analyses.

Preparation of total membrane fractions from *C. reinhardtii* cells

Chlamydomonas cells from the wild type strain CC-425 were harvested and resuspended in TEN buffer. The resuspended cells were sonicated in TEN buffer (10 mM Tris–HCl, 10 mM EDTA, and 150 mM NaCl; pH 8) and then spun at a maximum speed of 20,000×*g* in a microcentrifuge for 2 h at 4 °C. The supernatant was used as the soluble fraction. The viscous pellet obtained after the spin was resuspended in the solubilization buffer (SDS–urea buffer minus the bromophenol blue and 10 % β-mercaptoethanol) and used as the membrane fraction. Isolated membrane fractions were solubilized by incubating in 1× PBS buffer (9.1 mM $Na_2HPO_4{\cdot}7H_2O$; 1.7 mM $NaH_2PO_4{\cdot}H_2O$ and 150 mM NaCl, pH 7.4) containing 4 % w/v Triton X-100 (Sigma, St. Louis, MO) and Halt protease inhibitor single use cocktail (Pierce protein research products, Thermo Fisher Scientific, Rockford, IL) at 4 °C with gentle shaking. The Triton X-100, solubilized membranes were used for immunoprecipitations.

Cellular protein analysis

Chlamydomonas cells from different strains were harvested, washed twice with fresh medium and resuspended in TEN buffer (10 mM Tris–HCl, 10 mM EDTA, and 150 mM NaCl; pH 8). Following sonication, the crude cell extract was mixed in a 1:1 ratio with either the SDS–urea (150 mM Tris–HCl, pH 6.8; 7 % w/v SDS; 10 % w/v glycerol; 2 M urea, bromophenol blue and 10 % β-mercaptoethanol) or SDS (SDS–urea buffer minus 2 M urea) solubilization buffer (Smith et al. 1990). The solubilization mixtures were incubated differently, according to the type of solubilization buffers used. SDS-solubilized samples were heated at 95 °C for 5 min and SDS–urea solubilized samples were incubated at room temperature for about 30 min, with intermittent vortexing. After incubation, the solubilized protein samples were vortexed and spun at a maximum speed of 20,000×*g* in a microcentrifuge for 5 min at 4 °C. The soluble fraction was loaded on a 12.5 % or on a 10 % SDS-PAGE gel.

Protein concentrations of samples were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Gel lanes were either loaded with an equal amount of Chl, in the range of 3–6 nmol Chl or with equal amount of proteins. SDS-PAGE analysis was performed according to Laemmli (1970) on a 12.5 or 10 % gel, using Page Ruler prestained protein ladder (Fermentas, Glen Burnie, Maryland) at a constant current of 30 mA for 2 h. Gels were either stained with 1 % Coomassie brilliant Blue R or with colloidal Coomassie (Gel code blue stain reagent from Pierce protein research product, Thermo Fisher Scientific, Rockford, IL) for protein visualization.

Western blot analysis

Electrophoretic transfer of the SDS-PAGE resolved proteins onto Immobilon P–PVDF membrane (Millipore, Billerica, MA) 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). All primary antibodies used for immunoanalysis were diluted with buffer [Tris-buffered saline, 0.005 % Tween 20 and 1 % bovine serum albumin (pH 7.4)] to attain the desired dilution. The TLA1 immune serum (Covance, Princeton, NJ) was diluted to a ratio of 1:4,000 before being used as a primary probe. The secondary antibodies used for western blotting were conjugated to horseradish peroxidase (Pierce protein research product, Thermo Fisher Scientific, Rockford, IL) and diluted to a ratio of 1: 30,000 with the antibody buffer. Western blots were developed by using the Supersignal West chemiluminescent substrate kit (Pierce protein research product, Thermo Fisher Scientific, Rockford, IL).

Immunoprecipitation

Magnetic Dynabeads Protein A (Invitrogen, Carlsbad, CA) was used for immunoprecipitation of the 28.5 kDa protein from the detergent solubilized membrane fraction of *Chlamydomonas*. 150 μL of well-resuspended Dynabeads protein-A were transferred to a 1.5-mL Eppendorf tube and placed on the magnetic stand. The supernatant was removed and the beads were washed five times with the 0.1 M sodium monobasic phosphate buffer (pH 8, containing 0.01 % Tween-20). The washed Dynabeads were resuspended in the 0.1 M sodium monobasic phosphate buffer and incubated with 10 μL of TLA1 primary immune serum at room temperature with rotation for 30 min. After incubation, the beads were washed twice with 0.1 sodium monobasic phosphate buffer and then three times with the PBS buffer (pH 7.4) containing 4 % Triton X-100 to remove unbound IgGs and bound nonspecific IgGs. The beads were then mixed with either solubilized membrane (experimental sample) or with only PBS buffer containing 4 % Triton X-100 (negative control) and incubated overnight at 2–8 °C with tilting and rotation. Washed dynabeads (with no bound IgG), incubated only in the solubilized membrane, were used as a second negative control in the experiment. Beads are washed five times with PBS buffer (pH 7.4) containing 4 % Triton X-100 to remove nonspecific antigen binding. The washed Dynabeads from different samples were then incubated directly at room temperature in the solubilization SDS–urea buffer for extraction of proteins from the beads. Extracted protein samples were used for SDS-PAGE and western analysis to identify the immunopreciptated 28.5 kDa protein from the SDS-PAGE gel.

LC–mass spectrometry

The SDS-PAGE gel [containing the immune-precipitated samples, positive control (solubilized membrane) and the two negative controls] was stained with colloidal Coomassie. The desired protein band was excised from the gel and chopped-up into small pieces with a clean razor blade. "In-gel" digestion of the proteins in SDS-PAGE gel, de-salting of the protein mixture and loading of the protein mixture on a nanoscale HPLC column employing reverse phase (one-dimensional) was performed at the Mass spectrometry facility of UC Berkeley Cancer research laboratory: http://biology.berkeley.edu/crl/mass_spec/information.htm#Identification_of_proteins_from_gel. The eluant from the LC column was subjected directly to tandem mass spectroscopy, and the mass and fragmentation spectrum of each major ion was recorded. The final output was a file listing each gene product for which peptides were found in the data.

Bioinformatic analysis

Webserver BLAST-P (http://www.ncbi.nlm.nih.gov/) was used for the identification of TLA1 homologs in *Homo sapiens* and *Arabidopsis thaliana* using the non-redundant protein sequence database. ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used for alignment of TLA1 and D2 protein sequences from *C. reinhardtii*.

Results

Western blot analysis of *C. reinhardtii* total protein extracts

Immunopurified TLA1-specific polyclonal antibodies, raised against the full-length recombinant protein, recognized the 23 kDa TLA1 protein, but also cross-reacted, with apparently high specificity, with a 28.5 kDa protein (Fig. 2). In total protein extracts from wild type *C. reinhardtii,* the apparent 28.5 kDa/23 kDa band intensity ratio was greater than 2:1. In wild type samples, an additional cross-reaction was noted at about 66 kDa (Fig. 2, WT). By way of comparison, Western blot analysis of total protein extracts from a Δ*petA* deletion mutant of *C. reinhardtii*, lacking the cytochrome *f* protein, had a 28.5 kDa/23 kDa band intensity ratio was about 1:1, attributed to a diminished abundance of the 28.5 kDa cross-reaction (Fig. 2, Δ*petA*). The intensity of the 66 kDa cross-reaction was also substantially diminished in the latter. Also by way of comparison, Western blot analysis of total protein extracts from the *tla1* mutant of *C. reinhardtii*, had a 28.5 kDa/23 kDa band intensity ratio was about 10:1, attributed to a substantially diminished abundance of the 23 kDa TLA1 protein (Fig. 2, *tla1*).

Immuno-precipitation and identification of the 28.5 kDa protein

Effort was made to identify the cross-reacting 28.5 protein, and to elucidate the origin of this unexpected cross-reaction.

Immuno-precipitation and isolation of the cross-reacting 28.5 kDa protein, followed by mass spec analysis yielded the peptide sequence R.TWFDDADDWLR.Q, which is specific for the *psbD*/D2 reaction center protein of photosystem-II (Fig. 3; Table 1). This finding was surprising, as the two proteins (TLA1 and D2) were not expected to have any similarities, the TLA1 being a predicted soluble cytosolic protein (Tetali et al. 2007), while the D2 is a hydrophobic transmembrane thylakoid membrane protein (Zouni et al. 2001). Indeed, a ClustalW 2.0.10 multiple sequence amino acid alignment of D2 and TLA1 revealed no obvious similarity between the two proteins (Fig. 4).

To ensure a more thorough comparison of the two proteins, a systematic epitope analysis between the two amino acid sequences was undertaken. This analysis suggested a common epitope at the C-terminal of the two polypeptides. A ClustalW 2.0.10 partial sequence amino acid alignment of the C-termini of D2 and TLA1 revealed essential identity among nine consecutive amino acids comprising the "GNAL" domain (Fig. 5). This nine amino acid domain consists of amino acids V-F—L(V)LP-GNAL. Results from this partial sequence alignment (Fig. 5) suggested that amino acids V-F—L(V)LP-GNAL form an identical epitope and serve as common antigenic determinants, thus explaining how TLA1-specific antibodies also recognize the D2 protein.

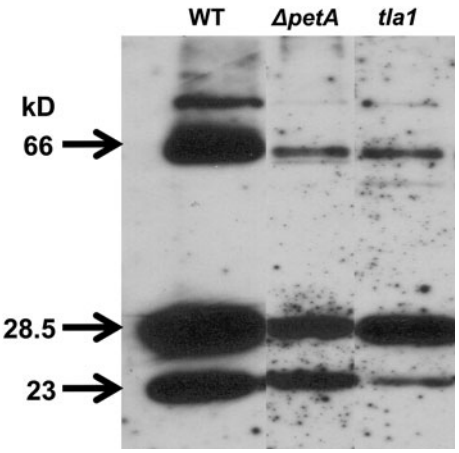

Fig. 2 Western blot analysis of total protein extracts from *Chlamydomonas reinhardtii* probed with TLA1-specific polyclonal antibodies. *Lanes* were loaded with protein extracts from the wild type (WT), a cytochrome *f* deletion mutant (Δ*petA*), and the *truncated light-harvesting antenna 1* mutant (*tla1*) strain. Note the substantially diminished amounts of the 23 kDa TLA1 protein in the *tla1,* relative to the 28.5 kDa cross-reaction

The 28.5 kDa cross-reaction is absent in the D1-less, D2-less, and *rep27* mutants of *C. reinhardtii*

Experimental evidence in support of the D2 origin of the 28.5 kDa cross-reaction was obtained in western blot analyses of *C. reinhardtii* total protein extracts from D1-less, D2-less, and *rep27* mutants. These strains fail to assemble the PSII reaction center proteins, although they

```
  1 mtiaigtyqe krtwfddadd wlrqdrfvfv gwsglllfpc ayfalggwlt gttfvtswyt
 61 hglatsyleg cnfltaavst pansmahsll fvwgpeaqgd ftrwcqlggl wafvalhgaf
121 gligfmlrqf eiarsvnlrp ynaiafsapi avfvsvfliy plgqsgwffa psfgvaaifr
181 filffqgfhn wtlnpfhmmg vagvlgaall caihgatven tlfedgdgan tfrafnptqa
241 eetysmvtan rfwsqifgva fsnkrwlhff mllvpvtglw msaigvvgla lnlraydfvs
301 qeiraaedpe fetfytknil lnegirawma aqdqpherlv fpeevlprgn al
```

Fig. 3 Amino acid sequence of a partial peptide from the immuno-precipitated 28.5 kDa protein (*red font*) and amino acid sequence of the *psbD*/D2 reaction center protein of PSII. Immuno-precipitation and isolation of the cross-reacting 28.5 kDa protein, followed by mass spec analysis yielded the peptide sequence R.TWFDDADDWLR.Q, which is specific for the *psbD*/D2 photosystem-II reaction center protein

Table 1 Liquid chromatography (LC) with electrospray ionization MS data. The detected peptide sequences, positions of the amino acids of the peptides in the full-length protein sequence, the charge state of the measured ion (*z*), the calculated deviation of the experimentally determined mass from the theoretical average mass of the peptide (ΔM) and the X_{corr} calculated by using the Sequest algorithm are shown. All peptide sequences reported produced X_{corr} values equal to or above 1.5, 2.25, or 3.5 for singly, doubly, or triply charged precursor ions, respectively

No.	Protein	Peptide	Position	*z*	ΔM	X_{corr}	# of peptides
1	D2	RTWFDDADDWLR	12–23	1	−0.6	4.97	8
2	Lhcbm3	QAPASSGIEFYGPNR	31–45	2	0.5	3.43	2
3	Lhcbm6	GPVQNLDDHLANPTVNNAFAFATK	222–245	3	0	5.41	2
4	Lhcb4 (CP29)	GSVEAIVQATPDEVSSENR	102–120	2	0.7	4.67	2
5	Lhcb5 (CP26)	GWLGGQGGAADLDK	38–51	2	0.9	4.23	2

```
D2     MTIAIGTYQEKRTWFDDADDWLRQDRFVFVGWSGLLLFPCAYFALGGWLTGTTFVTSWYT 60
Tla1   ----------------------------------MTFSCSADQT-ALLKILAHAAKYPS 24
                                          : *.*:     . *.  :..:.: :

D2     HGLATSYLEGCNFLTAAVSTPANSMAHSLLFVWGPEAQGDFTRWCQLGGLWAFVALHGAF 120
Tla1   NSVNGVLVGTAKEGGSVEILDAIPLCHTTLTLAPALEIG-------LAQVESYTHITGSV 77
       :.:    :  .:   :.    * .:.*: * :  .   *       *. : ::. : *:.

D2     GLIGFMLRQFEIARSVNLRPYNAIAFSAPIAVFVSVFLIYPLGQSGWFFAPSFGVAAIFR 180
Tla1   AIVGYYQSDARFGPGD----------------------LPPLGR---------------- 99
       .::*:   : .:. .                        : ***:

D2     FILFFQGFHNWTLNPFHMMGVAGVLGAALLCAIHGATVENTLFEDGDGANTFRAFNPTQA 240
Tla1   ------------------------KIADKVSEHQAQAVVLVLDN----KRLEQFCKAQA 130
                                *   : * * .   ::::    : :. *  :**

D2     EETYSMVTANRFWSQIFGVAFSNKRWLHFFMLLVPVTGLWMSAIGVVGLALNLRAYDFVS 300
Tla1   DNPFELFSKD-----------GSKGWKR-----------ASADGGELALKNADWKKLRE 167
       ::.:.:.: :           ..* * :             ** *      *    .: .

D2     QEIRAAEDPEFETFYTKNILLNEGIRAWMAAQDQPHERLVFPEEVLPRGNAL 352
Tla1   EFFVMFKQLKHRTLHDFEEHLDDAGKDWLNKGFASSVKFLLPGNAL------ 213
       : :   :: :..*::  :  *::. : *:     .  ::::* :.*
```

Fig. 4 ClustalW amino acid sequence alignment of the full D2 and TLA1 proteins from *C. reinhardtii*. *Asterisk* denotes conserved amino acids. *Colon* denotes conserved similar amino acid substitutions. *Period* denotes semi-conserved similar amino acid substitutions. Note the overall lack of similarity between these two proteins

```
D2     V-FPEEVLPRGNAL 13
Tla1   VKF---LLP-GNAL 10
       * *    :** ****
```

Fig. 5 ClustalW amino acid sequence alignment of the C-terminal portion of the D2 and TLA1 proteins from *C. reinhardtii*. Partial amino acid sequence alignment of the last 12 amino acids from the C-terminal ends of D2 and TLA1 protein revealed essential identity among 9 consecutive amino acids comprising the "GNAL" domain (Fig. 5). This nine amino acid domain consists of amino acids V-F—L(V)LP-GNAL. Results from this partial sequence alignment suggested that nine amino acids from the C-terminus form a common epitope and serve as common antigenic determinants in D2 and TLA1. *Asterisk* denotes conserved amino acids. *Colon* denotes conserved similar amino acid substitutions. *Period* denotes semi conserved similar amino acid substitutions. Note the overall lack of similarity between these two proteins

are green and they contain the full complement of the peripheral LHC of PSII and PSI (Park et al. 2007; Dewez et al. 2009). Figure 6 compares the total protein cross-reaction profile of the D1-less, D2-less, and *rep27* mutants with that of the wild type and Δ*petA* deletion mutant of *C. reinhardtii,* probed with TLA1-specific polyclonal antibodies. All samples showed the cross-reaction with the TLA1 23 kDa protein. Striking was the absence of a cross-reaction with a 28.5 kDa protein specifically in the D1-less, D2-less, and *rep27* mutants. Also absent from the latter was the cross-reaction with a 66 kDa complex (Fig. 6). The 66 kDa cross-reaction has been attributed to a D1/D2 heterodimer complex that apparently fails to fully solubilize in SDS-PAGE or SDS–urea-PAGE analyses (Smith et al. 1990; Kim et al. 1993). These results provided strong support for the assertion that a specific cross-reaction is generated between TLA1 polyclonal antibodies and the D2 reaction center protein of PSII.

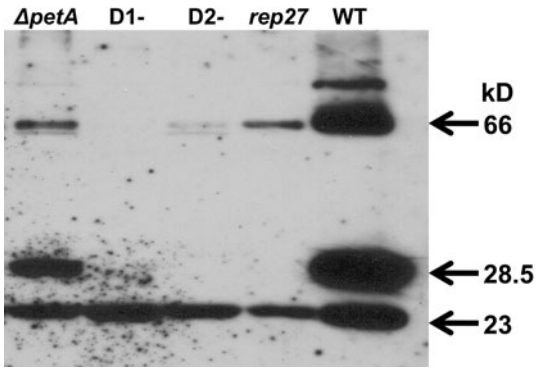

Fig. 6 Western blot analysis of total protein extracts from *Chlamydomonas reinhardtii* probed with TLA1-specific polyclonal antibodies. *Lanes* were loaded with protein extracts from the wild type (WT), a cytochrome *f* deletion (Δ*petA*), a D1-less (*FUD7*), a D2-less (Δ*psbD),* and a PSII repair-aberrant mutant (*rep27*). Note the positive cross-reaction of antiTLA1 antibodies with a 23 kDa protein and the total absence of a cross-reaction between antiTLA1 antibodies and a protein at 28.5 kDa in the D1-less, D2-less, and *rep27* extracts. Absence of a cross-reaction between the TLA1 antibodies and the D1/D2 heterodimer migrating to 66 kDa was also noted

Role of the "GNAL" domain in the cross-reactivity of D2 and TLA1 proteins

Specific polyclonal antibodies were raised against the recombinant TLA1 protein, modified to remove the nine amino acid domain of the C-terminus, consisting of the amino acid V-F—L(V)LP-GNAL epitope (TLA1-GNAL protein). Independently, polyclonal antibodies were raised against the synthetic oligopeptide "KHRTLHDFEEHLDDAGKDWLNK" (TLA1 amino acids residues 177–198) corresponding to a hydrophilic region of the *C. reinhardtii* TLA1 protein (TLA1-GNAL antibodies). We hypothesized

that such antibodies ought to be specific to the TLA1 protein and should not cross-react with the PSII-D2 reaction center protein. The differential sensitivities of specific polyclonal antibodies generated against the full-length TLA1 protein (antiTLA1) and, separately, against the TLA1 protein lacking the GNAL domain (antiTLA1-GNAL) were demonstrated in Western blot analyses (Fig. 7). Both antiTLA1 and antiTLA1-GNAL polyclonal antibodies cross-reacted with the recombinant TLA1 protein, migrating to 25 kDa because of the presence of the His-tag (Fig. 7, lanes 1 and 3). In lanes loaded with total protein extract from *C. reinhardtii* strain CC-425, the antiTLA1-GNAL antibodies cross-reacted with a 23 kDa protein only (Fig. 7, lane 2), whereas the antiTLA1 antibodies showed a primary cross-reaction with a 28.5 kDa protein (Fig. 7, lane 4). These results provided further support for the assertion that the GNAL epitope of the TLA1 and D2 proteins serves a strong antigen, causing these antibodies to recognize both proteins.

The antiTLA1-GNAL antibodies were further tested for their cross-reactivity with proteins from different *C. reinhardtii* strains. Figure 8 shows a western blot analysis of recombinant TLA1 protein (lane 1), and total protein cell extracts from CC-425 (lane 2), the D2-less Δ*PsbD* strain (lane 3) and the *tla1* mutant (lane 4), probed with anti-TLA1-GNAL immune serum. It is evident from the results that the antiTLA1-GNAL immune serum cross-reacted with the TLA1 protein at 23 kDa (25 kDa for the recombinant protein) while, at the same time, there was lack of cross-reactivity with a protein at 28.5 kDa.

Further evidence in support of the above contentions was provided upon examination of the cross-reactivity of the TLA1 recombinant protein with antiTLA1, antiD2, and antiD1 polyclonal antibodies. Both antiTLA1 and antiD2 immune sera cross-reacted with the TLA1 recombinant protein (Fig. 9, TLA1 and D2). However, antiD1 immune sear failed to yield such cross-reaction (Fig. 9, D1).

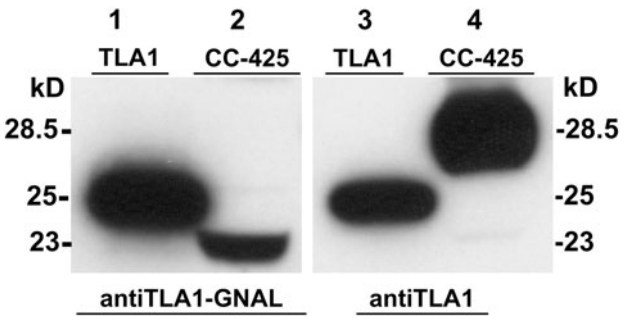

Fig. 7 Comparative western blot analysis of the recombinant His-tagged TLA1 protein (*lanes 1* and *3*) with total protein extracts from *Chlamydomonas reinhardtii* strain CC-425 (*lanes 2* and *4*) probed with TLA1-specific polyclonal antibodies. The latter were raised either against a synthetic peptide that excluded the last 12 amino acids of the C-terminus (antiTLA1-GNAL), or against the full size recombinant protein (antiTLA1). The His-tagged recombinant TLA1 protein migrated to 25 kDa (*lanes 1* and *3*). AntiTLA1-GNAL antibodies specifically recognized a 23 kDa protein in the extracts from CC-425, but failed to recognize the 28.5 kDa protein in the extracts from CC-425 (*lane 2*). AntiTLA1 antibodies strongly recognized a 28.5 kDa protein in the extracts from CC-425, and faintly recognized the 23 kDa protein in the extracts from CC-425 (*lane 4*)

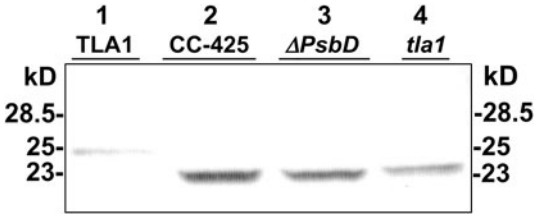

Fig. 8 Comparative western blot analysis of the recombinant His-tagged TLA1 protein (*lane 1*) with total cell protein extracts from *Chlamydomonas reinhardtii* strain CC-425 (*lane 2*), a D2-less Δ*psbD* (*lane 3*), and the *truncated light-harvesting antenna 1* mutant (*tla1*) probed with antiTLA1-GNAL specific polyclonal antibodies. Loadings were as follows: 100 pg of the TLA1 recombinant protein was loaded in *lane 1*, 20 µg of protein in *lane 2* (CC-425) and *lane 3* (D2-less Δ*psbD),* and 40 µg protein was loaded in lane 4 (*tla1 mutant*). Noted is the antibody's cross-reactions with the 23 kDa protein and the absence of a cross-reaction with the 28.5 kDa protein. Also noted is the lower relative amount of the TLA1 protein present in the *tla1* mutant. The latter is less than half the amount of that present in the wild type strain, although the total loading of protein in the *tla1* mutant lane is twice that in the wild type lane

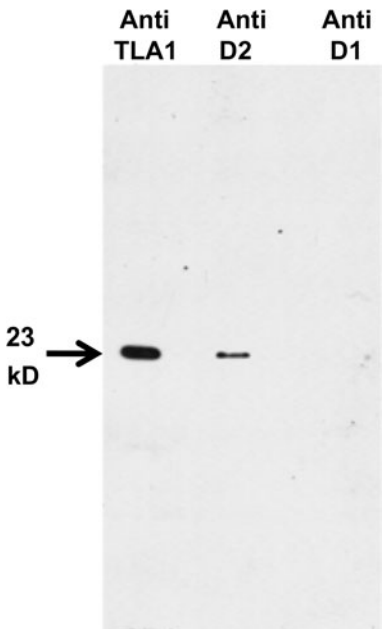

Fig. 9 Comparative western blot analysis of the recombinant TLA1 protein (23 kDa) with specific polyclonal antibodies raised against the full-length TLA1 protein itself (*lane 1*), against the D2 reaction center protein of PSII (*lane 2*), or against the D1 reaction center protein of PSII (*lane 3*). 0.5 ng of recombinant TLA1 protein was loaded in each of the *three lanes*. Note the cross-reactivity of the recombinant TLA1 protein in *lane 1* with antiTLA1 and *lane 2* with antiD2 antibodies, and the absence of cross-reaction in *lane 3* with antiD1 antibodies

Discussion

Results presented in this work conclusively show that the 28.5 kDa protein that cross-reacts with antiTLA1 antibodies is the *psbD*/D2 reaction center protein of PSII and not a novel isoform of the TLA1 protein, or a posttranslational modification of the TLA1 protein. The TLA1 and D2 proteins have a common nine amino acid V-F—L(V)LP-GNAL epitope in their C-terminus (GNAL domain), which is antigenic enough to generate a strong immunity response against either protein. BLAST-P analysis showed that the GNAL domain is conserved in the D2 reaction protein from all non-vascular and vascular plants, whereas the heterodimeric D1 reaction center protein lacks this domain. This information is important to the field, as we are now beginning to examine the functional role of the highly conserved TLA1 protein. The unexpected property of the D2 protein cross-reaction with antiTLA1 antibodies complicated the analysis of the TLA1 function, but this complication is now resolved.

A thorough search of the database for proteins containing "GNAL" domain at or near their C-terminus resulted in a "best match" with a hypothetical protein in Volvox (putative TLA1 homolog). In addition, the PSII PsbM and PsbQ proteins were found to contain a GNAL domain very close to the C-terminal end, but not at the very end of the protein. Moreover, the PSII PsbH and PsbS proteins possess a GNAL domain closer to the middle/N-terminal end of the protein sequence. Several other hypothetical/predicted proteins from photosynthetic and non-photosynthetic organisms contain the GNAL domain but not at the C-terminal end. At present, there is no indication about the potential structural or functional role that is played by the GNAL domain in these proteins. More work is needed, possibly with transformants from which the GNAL domain has been altered or deleted, to identify its function.

The intensity of the cross-reaction of the antiTLA1 antibodies with the 28.5 kDa (D2) protein was always stronger than that with the 23 kDa (TLA1) protein (e.g., Figs. 2, 6, 7). This is attributed to the much greater abundance of the D2 protein, relative to the TLA1 protein, in extracts from *C. reinhardtii*. The low levels of TLA1 accumulation in the cells are consistent with its proposed regulatory rather than catalytic role in the cell (Mitra and Melis 2010).

TLA1 is the first gene to be identified that plays a role in the regulation of the chlorophyll antenna size of photosynthesis. It was isolated from a DNA-insertional mutagenesis library as one of few transformants having a truncated light-harvesting chlorophyll antenna size (*tla1*) for both photosystems (Polle et al. 2003). In the *tla1* mutant, both the Chl antenna size of photosystems I and II, and the total chlorophyll per cell were lowered relative to the wild type. Further molecular insights for the role of the *TLA1* gene in the regulation of the Chl antenna size of photosynthesis were obtained from the mating and linkage studies of *C. reinhardtii* and from *tla1* mutant complementation experiments (Tetali et al. 2007). The work here confirms that a down-regulation in the expression of the *TLA1* gene in the *tla1* mutant is accompanied by the Chl-deficient phenotype of the mutant strain, in a process that is independent of the cross-reaction with the 28.5 kDa D2 protein.

There are homologs of the TLA1 protein in the higher plant *Arabidopsis thaliana* (AT5G55940 and AT5G51620) and also in *Homo sapiens* (CGI-112 [Fam158A] and NOC4 [FAM158B]) (Bachman et al. 1999; Mitra et al. 2012). In *Arabidopsis*, one of the TLA1 homologs (TAIR: AT5G55940) has been localized to the endoplasmic reticulum (ER) (Dunkley et al. 2006). Localization of the other *Arabidopsis* TLA1 homolog (TAIR: AT5G51620) is not known at present. Homozygous mutants (EMBRYO DEFECTIVE 2731) of the *Arabidopsis thaliana TLA1* gene (AT5G55940) are defective in embryo development resulting in seed dormancy (Meinke 1995). The functional role of AT5G51620 is not known. It is of interest that both of these *Arabidopsis* TLA1 homologs lack the GNAL domain.

Heating of protein samples solubilized in SDS buffer to 95 °C for 5 min causes aggregation and precipitation of hydrophobic thylakoid membrane proteins, such as D1 and D2, making them insoluble in SDS buffer, than if they were incubated in the SDS-buffer at a moderate temperature range of 30–50 °C (Mizuno and Kageyama 1979; Sagné et al. 1996). D2 is present in the PSII reaction center in the granal thylakoid membranes and it can only be solubilized with strong detergents, as it is an integral membrane protein. When both nonionic detergent-like Triton X-100 and anionic detergent-like SDS are used to solubilize proteins, a shift in the electrophoretic mobility of proteins is observed in SDS-PAGE (Helenius and Simons 1977; Ossipow et al. 1993). Mild non-ionic detergents like Triton X-100, if used in excess (concentrations greater than 0.5 %) remove SDS from SDS-hydrophobic protein complexes and sequester them into micelles. Thus, these proteins usually retain their native conformations and migrate more slowly on SDS-PAGE compared to when they are solubilized in anionic SDS buffer only (Helenius and Simons 1977; Ossipow et al. 1993).

The D1 and D2 reaction center proteins of PSII show electrophoretic mobility on SDS-PAGE that depends on the solubilization conditions employed. The calculated molecular mass of the D1 and D2 proteins from *C. reinhardtii* was estimated to be 39 and 39.4 kDa, respectively. When protein samples are solubilized in LDS buffer and subjected to LDS-PAGE at 4 °C, the D2 protein migrates

more slowly to about 34 kDa than the D1 protein, which migrates to 32 kDa position on the gel (Fleischmann and Rochaix 1999). When thylakoid membrane proteins are solubilized in SDS–urea buffer and subjected to PAGE on a gel containing 8 M urea at 4 °C, the D2 protein migrates more slowly than the D1 protein on the gel (de Vitry et al. 1987, 1989). On SDS-PAGE, *C. reinhardtii* D1 and D2 proteins migrate to about 32 and 28 kDa, respectively (Kuchka et al. 1988; Mayfield et al. 1987, 1994; Yohn et al. 1996; Mayfield and Taylor 1984), although the calculated molecular weight of D2 is greater than that of D1 protein.

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