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Employing functional genomics to study chlorophyll biosynthesis in the green micro alga *Chlamydomonas reinhardtii*

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Abstract

The green micro alga *Chlamydomonas reinhardtii* possesses photosynthetic apparatus very similar to higher plants, can grow photo autotrophically and heterotrophically (can metabolize exogenous acetate as a carbon source) and possesses a completely sequenced genome. These attributes make it an elegant model organism to study all aspects of oxygenic photosynthesis. The goal of this project is to identify molecular components that play a role in chlorophyll (Chl) biosynthesis in *Chlamydomonas*. Chlorophyll (Chl) and heme are major tetrapyrroles that play an essential role in energy metabolism in photosynthetic organisms. Chl and heme are synthesized via a common branched tetrapyrrole biosynthetic pathway. One of the enzymes in the pathway is Mg chelatase (MgChel) which inserts Mg^{2+} into protoporphyrin IX (PPIX, proto) to form magnesium-protoporphyrin IX (MgPPIX, MgProto), the first biosynthetic intermediate in the Chl branch. MgChel is a multimeric enzyme that consists of three subunits designated CHLD, CHLI and CHLH. Plants have two isozymes of CHLI1 (CHLI1 and CHLI2) which are 70%-81% identical in protein sequences. Although the functional role of CHLI1 is well characterized, that of CHLI2 is not.
We have generated a mutant, *5A7*, by random DNA insertional mutagenesis which is devoid of any detectable Chl. Steady state tetrapyrrole pool analyses by high performance liquid chromatography (HPLC) show that *5A7* over-accumulates proto. PCR based analyses shows that *5A7* is missing the *CHLI1* gene and at least four other functionally uncharacterized genes (*UP1*, *UP2*, *AMT* and *FDX3*). *5A7* has an intact *CHLI2* gene. Complementation of *5A7* with a functional copy of the *CHLII* gene restored Chl biosynthesis in *5A7*. Our results show for the first time that in *Chlamydomonas* that CHLI1 is the key enzyme in Chl biosynthesis unlike that in higher plants where CHLI2 does contribute in Chl biosynthesis. We are also investigating the functional role of the remaining four missing genes in *5A7*, if any, in photosynthesis and Chl biosynthesis. This paper focuses on the biochemical and molecular characterization of *5A7*.

Keywords: photosynthesis, tetrapyrrole biosynthesis, *Chlamydomonas*

1. Introduction

Chlamydomonas reinhardtii is a green micro alga which can be grown heterotrophically metabolizing exogenous acetate as its sole carbon source or photo autotrophically using atmospheric carbon. Its haplontic life cycle has a quick replication time of 8-10 hours and its genome is completely sequenced. In contrast to angiosperms, *Chlamydomonas* also has two pathways for chlorophyll (Chl) biosynthesis, which include the light-dependent pathway, which is commonly found in most photosynthetic organisms and a light-independent pathway, which is absent in angiosperms[6]. These attributes make it an ideal model system to study photosynthesis and regulation of Chl biosynthesis.

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In photosynthetic organisms, tetrapyrroles like Chl and heme are essential for energy metabolism (i.e. photosynthesis and respiration). Biosynthesis of Chl and heme occur via a common branched pathway that involves both nuclear- and chloroplast-encoded enzymes in most photosynthetic organisms[4] (Fig. 1). In photosynthetic eukaryotes 5-aminolevulinic acid (ALA) is synthesized from glutamine (Glu) through glutamyl-tRNA[18]. Conversion of ALA through several steps yields protoporphyrin IX (PPIX), the last common precursor for both heme and Chl biosynthesis[15]. Ferrochelatase inserts iron in the center of the PPIX thus committing it to the heme branch of the pathway. Insertion of Mg^{2+} by Mg chelatase (MgChel) leads to magnesium-protoporphyrin IX (MgProto), the first biosynthetic intermediate in the Chl branch[19]. Magnesium chelatase has three subunits, which are CHLD, CHLH and CHLI[23]. CHLI has two isozymes, *CHLI1* and *CHLI2*. CHLI which has ATP hydrolysis activity belongs to the AAA+ family of ATPases

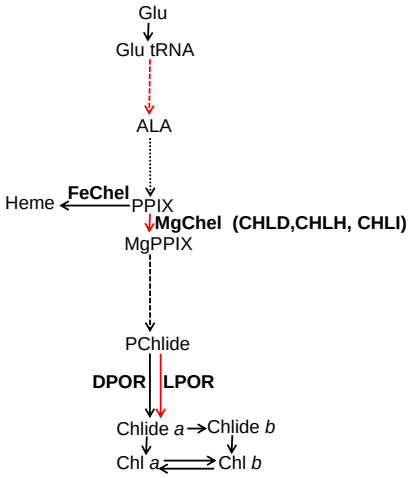

Figure 1. A simplified tetrapyrrole biosynthetic pathway in *Chlamydomonas.*

Figure 1. Tetrapyrrole intermediate precursors are shown as non-bold letters while the enzymes responsible for the biosynthesis of the intermediates are shown in bold. Glu tRNA: glutamyl-tRNA; ALA:5-aminolevulinic acid; PPIX: protoporphyrin IX; MgPPIX: magnesium-protoporphyrin IX; PChlide:protochlorophyllide; Chlide: chlorophyllide.

(ATPase associated with various cellular activities) and forms a homo-hexameric ring[8]. The N-terminal half of CHLD is homologous to CHLI suggesting that CHLD is also an AAA+ protein although no ATPase activity has been detected (Ikegami et al., 2007). The ATP-dependent catalytic mechanism of the hetero-trimeric MgChel complex includes at least two steps[23,12]; an activation step, and a Mg^{2+} chelation step[22]. First, ATP activates the formation of a doubled hexameric ring structure of CHLI and CHLD subunits[24,5] followed by ATP hydrolysis by CHLI which drives the chelation of proto bound to CHLH[10]. The relative contribution of CHLI1 and CHLI2 in Chl biosynthesis is controversial in green plants and has not been studied in green algae. Stringent control of tetrapyrrole biosynthesis is necessary for oxygenic photosynthetic organisms because of their sensitivity to oxidative stress. Free Chl, heme, and their immediate precursor, being highly photo-toxic molecules, generate reactive oxygen species (ROS) under aerobic conditions in presence of light[15]. Therefore, cellular Chl and heme and their precursors are bound to apoproteins. Chl and heme biosynthesis in plants is under transcriptional, translational and post-translational control at multi-levels and is accomplished by a complex regulatory network among the chloroplasts, mitochondria and nucleus[16].

The broad goal of our research project is to identify molecular components that play a role in Chl biosynthesis and photosynthesis under different irradiance conditions in the model organism *Chlamydomonas* utilizing a forward genetics approach. We have generated a *Chlamydomonas* random DNA insertional mutant library. Mutants were screened both in heterotrophic and in photoautotrophic media under different light conditions. Mutants which could not grow photo autotrophically and /or were pigment-deficient in the screen, were isolated. *5A7* is one of the twenty-one mutants that were identified during the screening. *5A7* is devoid of Chl. Our manuscript focuses on biochemical and molecular characterization of *5A7*. Our analyses of steady state tetrapyrrole intermediate accumulation by high performance liquid chromatography (HPLC) show that *5A7* is over-accumulating PPIX. Molecular analyses show

that *5A7* is missing the *CHLI1* gene but has an intact CHLI2 gene. Additionally, *5A7* lacks four other functionally uncharacterized genes: *UP1*, *UP2*, *AMT* and a chloroplast-localized ferredoxin, *FDX3*. *5A7* is the first known *chli1* and *fdx3* mutant in green algae and plants, respectively. We have restored Chl biosynthesis in *5A7* by complementing it with a functional *CHLI1* gene.

2. Materials and Methods

2.1 Growth of Various *Chlamydomonas* strains

The wild type strain of *Chlamydomonas reinhardtii*, *4A+*, and chlorophyll deficient mutant, *5A7* were maintained heterotrophically on a Tris-acetate phosphate (TAP) medium, in the dim light (10-15 µmol $m^{-2}s^{-1}$) and in the dark, respectively. Mutant *5A7* was also grown for experimental purpose in the dark on TAP agar plates containing the antibiotic paromomycin (TAP+P), in TAP liquid cultures in dim light and in the photosynthetic HS agar media (high salt media) under different light conditions. Since *5A7* was generated by random insertional mutagenesis using the plasmid pBC1, which contains a gene conferring paromomycin resistance, it can grow on TAP+P plates.

2.2 Cell Density And Pigment Concentration Measurements

Liquid TAP cultures were used to determine the cell density and Chl concentration. Estimations of cell density were done by counting the number of cells per mL using a Neubauer ultraplane hemacytometer. Photosynthetic pigments were extracted from intact cells using 80% acetone. The pigments were separated from the cell debris via centrifugation in a microcentrifuge at 17,000 x g for 5 minutes. The absorbance of the pigment-containing supernatant was then measured using a Beckman Coulter (Beckman Coulter, Brea, CA) DU730 Life Science UV/Vis spectrophotometer. Measurements of Chl *a* and *b* concentrations were then determined using Arnon[3] formulas as described by Melis et al[14].

2.3 Purification And Linearization Of Pbc1 Vector

A *Eschericia coli* clone, containing the pBC1 plasmid, which carries an ampicillin resistance gene, was grown overnight in 1 L Luria-Bertini (LB) + ampicillin (100 µg/mL) media at 37°C. The plasmid purification was done by Qiagen (Qiagen, Valencia, CA) plasmid mega kit according to the protocol given in the technical manual. The purified plasmid was linearized by the restriction enzyme Kpn1 (NEB, Beverly, MA) according to the instructions in the product manual.

3. Results

3.1. generation of the mutant *5A7* and comparative growth studies of the wild type strain *4A+* and *5A7*

3.1.1 the pBC1 plasmid

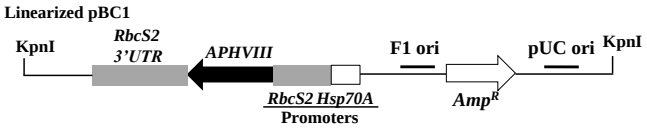

Figure 2. A schematic diagram of the linearized pBC1 plasmid used for insertional mutagenesis. The cleavage site of Kpn1 restriction enzyme, used for linearization of the vector is shown.

Figure 2. pBC1 plasmid was used as the vector for random DNA insertional mutagenesis to generate the *Chlamydomonas* mutant library. This plasmid contains both a paromoyocin resistance gene (*APHVIII*) and an

ampicillin resistance gene (*Amp*^{R}). Paromomycin is an antibiotic which affects eukaryotic translation while ampicillin is an antibiotic which affects prokaryotic cell wall synthesis. The pBC1 plasmid was amplified in *E. coli.* Ampicillin was used as a selection marker for *E. coli* clones containing the plasmid. The purified pBC1 plasmid was then linearized by the restriction enzyme Kpn1. *Chlamydomonas* was transformed using the linearized plasmid using the protocol followed by Kindle et al[11]. *Chlamydomonas* transformants were plated on TAP +P solid media plates in the dark to isolate mutants containing the *APHVIII* gene.

3.2. comparative growth analysis of *5A7* and 4A+

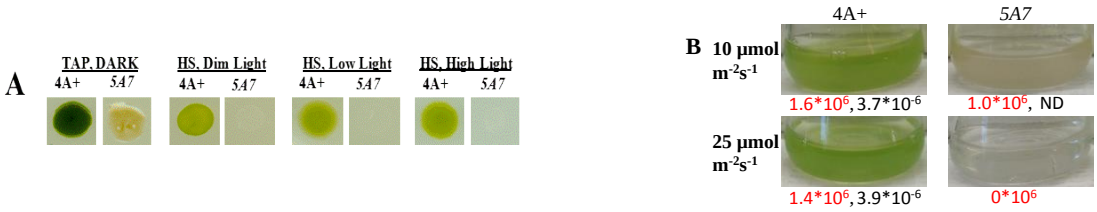

Figure 3.Comparative growth studies of 5A7 and 4A+.

(**A)** Heterotrophic and photo autotrophic growth of 5A7 and the wild type. (**B)** Mixotrophic growth of 5A7 and 4A+. The red and black numbers denote cell density in cells/mL and Chl concentration in nmol Chl/cell, respectively. Statistical error (+/- SD) was <10% of the values shown. ND: not detected.

Figure 3. When grown in solid TAP media in the dark *5A7* has a brown phenotype. It is not able to grow photosynthetically in the HS media. Liquid cultures were used to compare the mixotrophic growth of *5A7* and *4A+* under two different light intensities (10-15 µmol $m^{-2}s^{-1}$ and 25 µmol $m^{-2}s^{-1}$). Figure 3 shows that *5A7* is able to grow in 10-15 µmol $m^{-2}s^{-1}$ and cannot grow in 25 µmol $m^{-2}s^{-1}$.

3.3. HPLC analysis of steady state tetrapyrrole precursors

Table 1. High Performance Liquid Chromatography Analysis

Table 4 Tetrapyrrole HPLC analysis for *5A7*. HPLC analyses of steady state levels of tetrapyrrole pools and chlorophyll content in wild type (WT) and *5A7* mutants in dark and dim light. Statistical error (+/- SD) was <10% of the values shown.

Parameter	Ratio of *5A7* to WT in dark	Ratio of *5A7* to WT in light
PPIX	52.66	87.5
MgPPIX	0.66	0.05
MgPME	1.32	0.10
Heme	0.08	0.03
Pchlide	0	0
Chlide	0	0
Chl *a*	0.0004	0.0004
Chl *b*	ND	ND
Chl *a*/chl *b* ratio	0	0

ND: not detected

Table 1. High performance liquid chromatography (HPLC) analyses of steady state tetrapyrrole pools in dark and dim light grown *5A7* and 4A+ cells was performed by our supervisor in collaboration with lab members of Dr. Bernhard Grimm's laboratory (Humboldt University, Germany). HPLC analyses show that in both the dark and in the light, mutant *5A7* was over-accumulating proto. There was no over-accumulation of any intermediates downstream of MgPPIX in *5A7* compared to that in the wild-type. Chl *a* was barely detected, while Chl *b* was not detected at all. Based on the result it was hypothesized that MgChel, the enzyme which commits proto into the Chl branch, was not functioning properly.

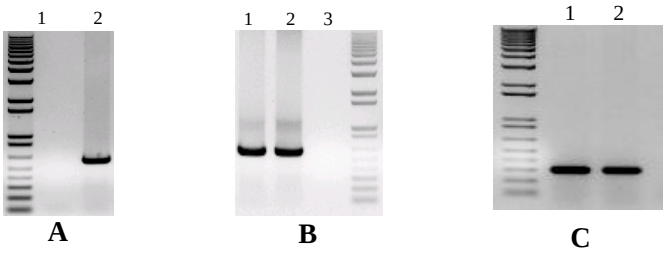

Figure 4 Molecular analyses to check for the presence or absence of the CHLI1, CHLI2 and GUN4 transcript. RT-PCR to check for the absence/presence of the: (**A)** CHLI1 transcript (**B**) CHLI2 transcript (**C)** GUN4 transcript in 5A7 and 4A+. Lane1: 5A7; Lane 2: 4A+; Lane 3: blank lane

Figure 4. As stated before, MgChel has three functional subunits, CHLD, CHLH, and CHLI. Based on the HPLC analysis, it was hypothesized that a mutation could be in any one of the subunits of MgChel or the regulatory protein, GUN4 (genomes uncoupled mutant 4) which regulates MgChel. There are known mutants in CHLD and CHLH subunits of MgChel and GUN4 which do not show the same phenotype observed in *5A7*. Therefore, we decided to check for the presence /absence of the transcript of two *CHLI1* genes namely *CHLI1* and *CHLI2* and *GUN4*. RT-PCR shows *CHLI2* and GUN4 transcripts are present in *5A7* but the CHLI1 transcript is absent. Genomic DNA PCR with the *CHLI1* specific primers show that the *CHLI1* gene is missing. This result shows *5A7* is defective in the *CHLI1* gene which is essential for Chl biosynthesis.

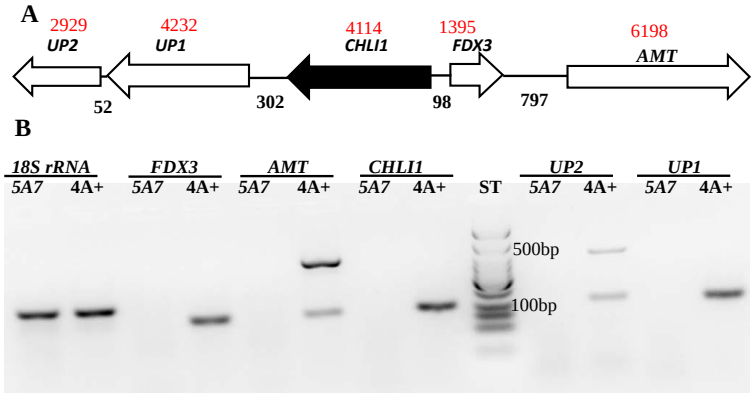

Figure 5. **(A)** A schematic map showing a 20 kb genomic DNA region that contains the CHLI1 locus. The red numbers denote size of gene (bp) while black numbers denote distance between genes (bp). **(B)** RT-PCR with CHLI1 and neighboring gene specific primers. All primers used spanned an intron. 18S rRNA primers were used as a control. UP2 and UP1: hypothetical genes; AMT: class I aminotransferase; ST: low molecular weight markers.

Figure 5. Current analyses show that *5A7* is missing at least five genes including the *CHLI1* gene. The four other genes *UP1*,*UP2*, *AMT* and a chloroplast localized ferredoxin, *FDX3*, are functionally uncharacterized.

3.4. complementation of the mutant

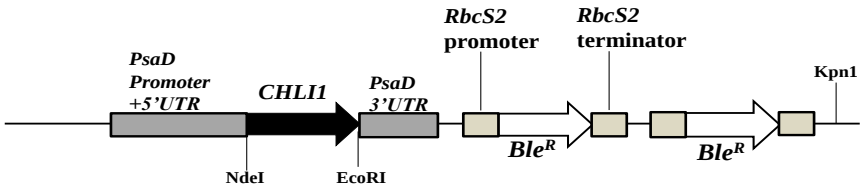

Figure 6. A schematic figure of the pDBle-CHLI1 construct used for complementation of 5A7.

Figure 6. The cDNA of *CHLI1* was cloned in the Ndel and EcoR1 double digested pDBle vector. *CHLI1* was expressed under the constitutive *PsaD* (*PsaD* codes for a photosystem I protein) promoter in the *pDBle* vector. The vector contains Ble^R gene driven by the *RbcS2* promoter. The Ble^R gene confers resistance against zeocin and was used for selection of *chlil* complements.

3.5. molecular and phenotypic analysis of complements

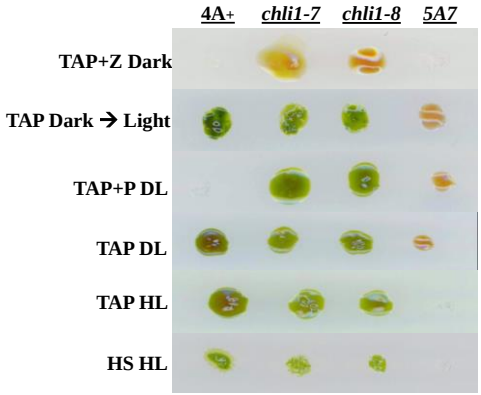

Figure 7. Phenotype analysis of two chli1 complements. Two chli1 complements were grown with 5A7 and 4A+ under six different growth conditions: TAP+ Z (zeocin) in the dark, TAP dark to light shift, DL: dim light (25 µmol photons m^{-2} s^{-1}) TAP and DL, TAP+P (paromomycin), TAP HL (high light) and HS HL (300µmol photons m^{-2} s^{-1}).

Figure 7. The *chli1* complements (*chli1-7* and *chli1-8*) appeared chlorophyll deficient like *5A7* in the dark. However, after they were exposed to dim light (10-15 µmol photons $m^{-2}s^{-1}$) for five days, the complements started to make Chl. The complements were able to make Chl under all light conditions in both heterotrophic and photo autotrophic media.

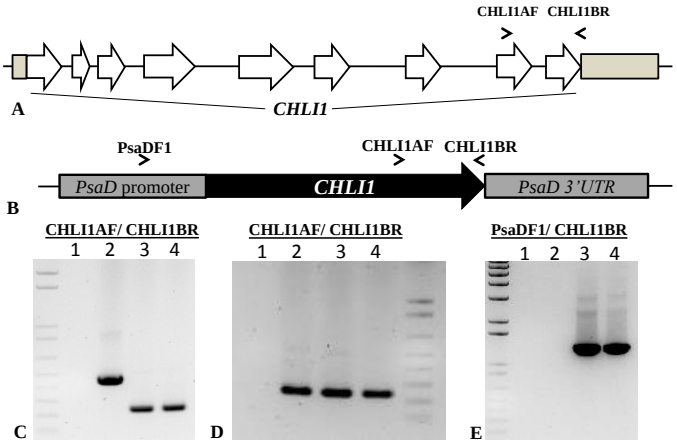

Figure 8. Molecular analysis of chli1 complements. **(A)** A schematic of the CHLI1 gene. The tan bars denote UTRs, the white arrows represent exons, and the black lines denote introns. Labeled primers span an intron. **(B)** A schematic figure of the pDBle-CHLI1 complementation vector containing CHLI1 cDNA **(C)** Genomic DNA PCR using CHLI1 specific primers (product size: 459 bp). (D) RT-PCR using CHLI1 specific primers (product size: 249 bp). (E) Genomic DNA PCR using PsaD specific with a CHLI1 specific primer (product size: 1272 bp). Lane1: 5A7; Lane 2: 4A+; Lane 3: chli1-7; Lane 4: chli1-8.

Figure 8. Molecular analyses of the *chli1* complements were conducted in order to verify if they contained the pDBle-*CHLI1* construct. Both *chli1* complements show the presence of the *CHLI1* gene and transcript. Genomic DNA PCR using a *PsaD* specific primer in the *PsaD* promoter region with a *CHLI1* specific primer yielded a DNA product (1272 bp) in the complements that was absent in 4A+. Taken together these results confirmed that *chli1* complements are harboring the trans-*CHLI1* gene driven by the *PsaD* promoter and we have successfully complemented *5A7*.

4. Discussion

When *5A7* was first isolated, it was identified as a "brown" non-photosynthetic mutant. Spectrophotometric and HPLC results have shown that *5A7* lacks detectable Chl (Fig. 3; Table 1). Collaborative work with Dr. Bernhard Grimm of Humboldt University (Berlin, Germany), has revealed that *5A7* over-accumulates PPIX under steady state conditions in the dark and the light. (Table 1). Molecular results show *5A7* has a deletion in the *CHLI1* gene which codes for one of the three subunits of MgChel (Fig. 5) MgChel has three subunits, CHLD, CHLH and CHLI. CHLD and CHLH transcripts are expressed after an hour of light exposure, while *CHLI1*and *CHLI2* are expressed after two hours of light exposure[21]. This explains the Chl-less phenotype of the *chli1* complements when grown under constant darkness and the green phenotype of the *chli1* complements when the dark adapted cells were shifted to light (Fig. 7). Although the function of CHLI1 is well characterized, the functional significance of *CHLI2* is still controversial. In higher plants like *Arabidopsis*, it has been shown that CHLI2 does play a limited role in Chl biosynthesis and recessive *chli1* mutants possessing a functional CHLI2 are pale green in color. *5A7* possesses a functional CHLI2 gene but is devoid of Chl. Researchers have found that in *Arabidopsis* the transcript level of *CHLI2* is much lower than that of *CHLI1*[13,7]. Over-expression of *CHLI2* can substitute for the function of CHLI1 in *Arabidopsis*[6]. *5A7* is not able to produce Chl even in the presence of *CHLI2*, which shows that *CHLI2* may not be able to replace the function of *CHLI1* in *C. reinhardtii*, unlike that in *Arabidopsis*[7, 12, 17]. We are currently quantifying transcript levels of *CHLI1* and *CHLI2* in the wild type and in *5A7* by semi-quantitative RT-PCR. We will be also over-expressing *CHLI2* in *5A7* to see if over-expression of *CHLI2* can substitute CHLI1 to some extent. Additionally, *5A7* is missing four other functionally uncharacterized genes namely *UP1*, *UP2*, *AMT* and *FDX3* (Fig. 5). *AMT* encodes aminocyclopropane-1-carboxylic acid which is an enzyme that catalyzes the synthesis of 1-aminocyclopropane-1-carboxylic acid, a precursor in ethylene biosynthetic pathway, a pathway which is absent in *Chlamydomonas*. UP1 and UP2 code for hypothetical proteins. FDX3 codes for a chloroplast localized ferredoxin[20].

Ferredoxins are small iron sulfur proteins that act as electron donors in various metabolic pathways. It has been shown in *Arabidopsis* that CHLI1 and CHLI2 activities are modulated by the thioredoxin-ferredoxin (TRX/FDX) mediated reduction of a specific disulfide bond involving two conserved cysteines in the C-terminal end of the CHLI protein[8,12]. As *Chlamydomonas* has these conserved cysteines in the CHLI1 and CHLI2 protein, it raises the question if TRX/FDX regulates activities of CHLI1/CHLI2. *5A7* is the first known *Chlamydomonas chli1* and plant *fdx3* mutant. Although complementation of *5A7* has restored Chl biosynthesis, the complements have a lower Chl/cell compared to that in the wild-type and also grow slowly, especially under high light (data not shown). These observations raise three questions: 1) If the difference in Chl/cell and growth rate between the *chli1* complements and the wild type is due to a low amount of CHLI1 protein in the complements. 2) If any of the four missing genes in *5A7*, particularly FDX3, play a role in Chl biosynthesis and photosynthesis and 3) if the severe Chl deficiency in *5A7* is due to FDX3-induced inactivity of CHLI2. We are currently checking the CHLI1 protein level with a CHLI1 specific antibody by western blotting. The results will show the protein level in the *chli1* complements compared to that in the wild type. We will be transforming the *chli1* complement *chli1-8* and the mutant *5A7* with a functional *FDX3* gene to answer these question number 2 and 3. If FDX3 is necessary for the activity of CHLI1 and CHLI2, then transforming *5A7* and *chli1-8* with FDX3 should increase the amount of Chl/cell in these strains and probably improve the growth rate of the *chli1-8* complement. In summary, a thorough characterization of *5A7* can clarify the functional roles of FDX3 and CHLI2 in green plants and algae.

5. References

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