Identification and molecular characterization of a novel Chlamydomonas reinhardtii mutant defective in chlorophyll biosynthesis [v2; ref status: indexed, http://f1000r.es/1ic]

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Abstract
The green micro-alga Chlamydomonas reinhardtii is an elegant model organism to study all aspects of oxygenic photosynthesis. Chlorophyll (Chl) and heme are major tetrapyrroles that play an essential role in energy metabolism in photosynthetic organisms and 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 CHLI (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 isolated a non-photosynthetic light sensitive mutant 5A7 by random DNA insertional mutagenesis that is devoid of any detectable Chl. PCR based analyses show that 5A7 is missing the CHLI1 gene and at least eight additional functionally uncharacterized genes. 5A7 has an intact CHLI2 gene. Complementation with a functional copy of the CHLI1 gene restored Chl biosynthesis, photo-autotrophic growth and light tolerance in 5A7. We have identified the first chli1 (chli1-1) mutant of Chlamydomonas reinhardtii and in green algae. Our results show that in the wild type Chlamydomonas CHLI2 protein amount is lower than that of CHLI1 and the chli1-1 mutant has a drastic reduction in CHLI2 protein levels although it possesses the CHLI2 gene. Our chli1-1 mutant opens up new avenues to explore the functional roles of CHLI1 and CHLI2 in Chl biosynthesis in Chlamydomonas, which has never been studied before.
Introduction
The green micro-alga *Chlamydomonas reinhardtii* possesses a photosynthetic apparatus very similar to that of higher plants, can grow photo-autotrophically and heterotrophically (it can metabolize exogenous acetate as a carbon source) and possesses a completely sequenced genome. These attributes make it an elegant model organism to study oxygenic photosynthesis and chloroplast biogenesis. 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. In photosynthetic eukaryotes, 5-aminolevulinic acid (ALA) is synthesized from glutamine through glutamyl-tRNA. Conversion of ALA through several steps yields protoporphyrin IX (PPIX), the last common precursor for both heme and Chl biosynthesis. Ferrochelatase inserts iron in the center of PPIX thus committing it to the heme branch of the pathway. Insertion of Mg into PPIX by MgChel leads to Mgproto, the first biosynthetic intermediate in the Chl branch. Magnesium chelatase has three subunits, which are CHLD, CHLIH and CHLIJ. The ATP-dependent catalytic mechanism of the heterotrimeric MgChel complex includes at least two steps: an activation step, followed by the Mg insertion. Activation of MgChel with ATP involves CHLD and CHLI while CHLIH is required for the chelation step. CHLIJ belongs to the AAs family of ATPases. Plants have two isozymes of CHLI (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. Most of the data on CHLI comes from studies on Arabidopsis thaliana mutants and the functional significance of CHLI1 and CHLI2 has not been studied in green algae. In Arabidopsis, CHLI2 plays a limited role in Chl biosynthesis because of its lower expression level compared to that of CHLI1. In Arabidopsis the CHLI2 protein amount is lower than that of CHLI1. When overexpressed, CHLI2 can fully rescue an *Arabidopsis chil1 chil2* double mutant.

We have isolated the first (*chil1-1*) mutant of *Chlamydomonas reinhardtii* (5A7) which possesses an intact CHLI2 gene. Transformation of 5A7 with a functional copy of the *CHLI1* gene restored Chl biosynthesis. Western analyses show that the CHLI2 protein level is lower than that of CHLI1 in the wild type strain and CHLI2 protein is barely detectable in the mutant strain. In this study, we present our molecular data on the identification of the mutation locus in 5A7 and its complementation.

Materials and methods
Algal media and cultures
*Chlamydomonas* strains 4A+ (a gift from Dr. Krishna Niyogi (UC, Berkeley), 5A7/chil1-1 (generated by our laboratory) and chil1-1 rescued transformants (generated by our laboratory) were grown either in Tris-Acetate Phosphate (TAP) heterotrophic media or in Sueoka’s High Salt (HS) photo-autotrophic media. TAP and HS liquid media and agar plates were prepared in the lab using reagents from Fisher Scientific (Pittsburg, PA) according to the protocol given in Gorman and Levine (1965) and Sueoka (1960), respectively. The 4A+ strain and chil1-1 rescued transformants were maintained on TAP agar plates and TAP+zeocin (Sigma, St. Louis, MO) plates, respectively under dim light intensities (10–15 µmol photons m⁻² s⁻¹) at 25°C. The final zeocin concentration was 15 µg/ml. The *chil1-1* mutant (5A7) was maintained in the dark on TAP 1.5% agar plates containing 10 µg/ml of paromomycin (Sigma, St. Louis, MO). Liquid algal cultures used for RNA and genomic DNA extractions and protein analyses were grown in 100 ml flasks on the New Brunswick Scientific Excella E5 platform shaker (Enfield, CT) at 150 rpm in the dark or in the dim light.

Generation of the 5A7 mutant
The purified pBC1 plasmid from the DH5α *Escherichia coli* clone harboring the pBC1 plasmid (obtained from Dr. Krishna Niyogi’s laboratory at UC, Berkeley) was used for random DNA insertional mutagenesis. This plasmid contains two antibiotic resistance genes: APHVIll and Ampβ (Figure 1). APHVIll confers resistance against the antibiotic paromomycin (Sigma, St. Louis MO) and was used as a selection marker for screening of *Chlamydomonas* transformants. Ampβ was used as a selection marker for screening of *E. coli* clones harboring the pBC1 plasmid. *E. coli* was grown in LB broth containing 1% tryptone, 0.5% of yeast extract, 1% NaCl and ampicillin [final concentration of ampicillin:100 µg/ml]. LB reagent was prepared in the laboratory using reagents purchased from Fisher (Pittsburgh, PA). Ampicillin was purchased from Fisher (Pittsburgh, PA). The culture was incubated at 37°C overnight. Plasmid purification from *E. coli* cells was facilitated by Qiagen plasmid mega kit according to the protocol given in the technical manual (Qiagen, Valencia, CA). Once purified from *E. coli*, the circular pBC1 vector was linearized with the restriction enzyme KpnI (NEB, Beverly, MA) according to the protocol given in the technical manual. The linearized DNA was purified using a QIAEX II gel extraction kit (Qiagen, Valencia, CA) according to the protocol given in the technical manual. All agarose DNA gel electrophoresis was visualized by BioRad Molecular Imager Gel Doc XR+ (BioRad, Hercules, CA). Transformation of parental strain 4A+ by the linearized pBC1 vector was performed utilizing the glass bead transformation technique described by Kindle et al. (1989) and Dent et al. (2005). Transformants were plated onto fresh TAP agar plates containing 10 µg/ml paromomycin (TAP+P) in the dark. Single colonies of mutants were picked and transferred onto fresh TAP+P plates using a numbered grid layout. Screening of photosynthetic and pigment deficient mutants was done by visual inspection and monitoring of growth under different light intensities in heterotrophic, mixotrophic and photo-autotrophic conditions.

Genomic DNA and RNA extraction
4A+, *chil1-1* rescued transformants complements and 5A7/chil1-1 were grown in TAP liquid media in the dark to a cell density of...
Figure 1. Linearized pBC1 plasmid used for random insertional mutagenesis. The cleavage site of the Kpn1 restriction enzyme, used for linearization of the vector is shown. APHVIII is under the control of combo promoters consisting of the promoter of the gene encoding the small subunit of Rubisco (RbcS2) and the promoter of the gene encoding the heat shock protein 70A (Hsp70A). pBC1 is a phagemid and its F1 origin (F1 ori) and pUC origin (pUC ori) are shown. The size of the plasmid is 4763 bp.

about 5 × 10⁶ cells/ml of the culture. Genomic DNA was purified using a phenol-chloroform extraction method⁶. RNA extraction was facilitated by TRIzol reagent from Invitrogen (Carlsbad, CA) following the protocol in the technical manual. DNA and RNA concentrations were measured using a Nanodrop 1000 spectrophotometer from Thermo Fisher Scientific (Wilmington, DE). DNase treatment was performed using Ambion’s TURBO DNA-free kit from Invitrogen (Carlsbad, CA) following the protocol in the technical manual to remove genomic DNA from the RNA preparation. Generation of cDNA was performed using Life Technologies Superscript III First-Strand Synthesis System from Invitrogen (Carlsbad, CA) following the protocol in the technical manual. The purified TAIL2 PCR product was sequenced at the UC, Berkeley DNA Sequencing Facility (Berkeley, CA).

Genomic and reverse transcription PCR

Primers were designed based on genomic DNA sequences available in the Chlamydomonas genome database in Phytozome. Amplifications of genomic DNA and cDNA were executed using MJ Research PTC-200 Peltier Thermal Cycler (Watertown, MA). HotStar Taq Plus DNA polymerase kit (Qiagen, Valencia, CA) was used for PCR following the cycling conditions given in the Qiagen protocol booklet. Annealing temperature was between 55 and 60°C depending on the Tₚ of the primers. Extension time was varied according to the size of the PCR product amplified. Final extension was set at 72°C for ten minutes. All genomic and reverse transcription PCR products were amplified for a total of thirty-five cycles. 50–150 ng of genomic DNA or cDNA templates were used for PCR reactions. For semi-quantitative RT-PCR using CHLI1 and CHLI2 gene specific primers, 3 µg of total RNA was converted into cDNA and then 150 ng of cDNA templates were used for RT-PCR. Sequences of primers used for genomic and RT-PCR are shown in Table 2, Table 3 and Table 4.

Cloning of the CHLI1 cDNA in the pDBle vector

The pDBle vector (obtained from Dr. Saul Purton, University College London, UK) was double-digested with restriction enzymes EcoRI and Ndel (NEB, Beverly, MA) according to the protocol given in the technical manual. The CHLI1 cDNA template was amplified using primers given in Table 4. Ligation of the double digested (Ndel and EcoRI digested) CHLI1 cDNA and the Ndel/EcoRI double-digested pDBle vector was done using the T4 ligase and 1 mM ATP (NEB, Beverly, MA). Chemically competent (CaCl₂ treated) E. coli cells were used for transformation. After transformation, E. coli cells were plated on LB+ampicillin (100 µg/ml) plates and incubated at 37°C overnight. Single colonies were picked the next day.

Table 1. List of primers used for Thermal Asymmetric InterLaced (TAIL) PCR, verification of TAIL-PCR product and DNA sequencing. These primers were used to generate the data in Figure 3 and Figure 4.

| Primer name | Sequence of primer | Location |
|-------------|--------------------|----------|
| AD          | 5'-NC GGG TTS CNA GC-3' | Random degenerate primer |
| AD2         | 5'-ATCGTGTTCCCAGC-3' | Non-degenerate primer derived from the primer AD |
| 1F          | 5'-AAA GAC TGA TCA GCA CGA ACG GGG-3' | APHVIII 3’UTR |
| 2F          | 5'-TAA GGC TAC GGT TCA GCA CTT GAG-3' | APHVIII 3’UTR |
| 2R          | 5'-CTC AAG TGC TGT AGC GGT AGC TTA-3' | APHVIII 3’UTR |
| 3R          | 5'-CCT GTG GCT GGA CCT GAT GGT TTA-3' | APHVIII 3’UTR |
| 4R          | 5'-GGG CCG TAT CCG AGG AAA AGC TG-3' | APHVIII 3’UTR |
day and plasmids were isolated from these clones. Isolated plasmids were double-digested with EcoRI and NdeI to verify the cloning of the CHLI1 cDNA. The CHLI1-pDBle construct from the selected clone was sequenced by the UC, Berkeley DNA Sequencing Facility (Berkeley, CA). Chromas Lite (http://technelysium.com.au/) and BLAST were used to analyze DNA sequences.

Table 2. List of primers used for amplifying CHLI1 and four neighboring genes downstream of CHLI1. These primers were used to generate the data in Figure 6A. The gene loci in Phytozome (http://www.phytozome.net/) are: CHLI1 (Cre06.g306300), UP1 (Cre06.g306250), UP2 (Cre06.g306200), UP3 (Cre06.g306150) and UP4 (Cre06.g306100).

| Primer name | Sequence of primer | Gene |
|-------------|--------------------|------|
| CHLI1AF     | 5’-ACTACGACTTCCGCATCGATCA-3’ | CHLI1 |
| CHLI1BR     | 5’-CATGCCGAACACCTGCTGAGAT-3’ | CHLI1 |
| PB138       | 5’-ATGCCGATACGGCTGAGTC-3’ | UP1   |
| PB139       | 5’-CTGAAGCAGAATGTTAGC-3’ | UP1   |
| PB132       | 5’-AAGGGCAATCAGCTCAGGTC-3’ | UP2   |
| PB133       | 5’-GGGATCGGATGTTAGTGTA-3’ | UP2   |
| UP3F        | 5’-GAC ACA CAA GCC TGA TTT TCT GG-3’ | UP3   |
| UP3R        | 5’-TGG CAC GTC GAA GAC AAA CT-3’ | UP3   |
| UP4F        | 5’-CTT TGA CCT GCA AAG AGA GAC C-3’ | UP4   |
| UP4R        | 5’-CAC CAC CCT GAT GCC CTT GAG-3’ | UP4   |

Table 3. List of primers used for amplifying CHLI1, four neighboring genes upstream of CHLI1 and the actin gene and transcript. These primers were used to generate the data in Figure 6B and 6C. The gene loci in Phytozome (http://www.phytozome.net/) are: CHLI1 (Cre06.g306300), FDX3 (Cre06.g306350), AMT (g7098), UP5 (Cre06.g306450) and UP6 (Cre06.g306500) and Actin (Cre13.g603700).

| Primer name | Sequence of primer | Gene |
|-------------|--------------------|------|
| CHLI1AF     | 5’-ACTACGACTTCCGCATCGATCA-3’ | CHLI1 |
| CHLI1BR     | 5’-CATGCCGAACACCTGCTGAGAT-3’ | CHLI1 |
| PB134       | 5’-CTGGAAGCAGAATGTTAGC-3’ | AMT   |
| PB135       | 5’-AGGGCAATCAGCTCAGGTC-3’ | FDX3  |
| PB132       | 5’-AAGGGCAATCAGCTCAGGTC-3’ | FDX3  |
| UP5F        | 5’-GGG CAA CTG GAG CTT TGG C-3’ | UP5   |
| UP5R        | 5’-CTT CTA TGT GGC CCA CGT C-3’ | UP5   |
| UP6F        | 5’-GCA ACT GGA GCT TCG GCC C-3’ | UP6   |
| UP6R        | 5’-CTG GAC CGC ACA AAG ACC C-3’ | UP6   |
| F2          | 5’-ACGACACACACTCCAATCCTCAG-3’ | Actin |
| R2          | 5’-TGGTACTTTATGCATCGAT-3’ | Actin |

Table 4. List of primers for amplifying CHLI1 transcripts and complement testing. These primers were used in the experiments that generated the data in Figure 7 and Figure 10 and also used for CHLI1 cDNA amplification for cloning.

| Primer name | Sequence of primer | Gene/purpose |
|-------------|--------------------|--------------|
| CHLI1CR     | 5’-TGACCTTTGACGAGAACACACCAC-3’ | CHLI1        |
| CHLI1BR     | 5’-CATGCCGAACACCTGCTGAGAT-3’ | CHLI1        |
| CHLI1AF     | 5’-ACTACGACTTCCGCATCGATCA-3’ | CHLI1        |
| CHLI2BF     | 5’-TGACCGATTTGAGTGGACTGTCATGCA-3’ | CHLI2        |
| CHLI2BR     | 5’-CACACTTACACGTCCGCCAGACAC-3’ | CHLI2        |
| CHLI1XF     | 5’-GGAATCTCAATTGACGATAGGTCGTTG-3’ | CHLI1 cDNA amplification for cloning |
| CHLI1XR     | 5’-CCGGAATCTTCATCCATCGCAGAACACCTGCTGCA-3’ | CHLI1 cDNA amplification for cloning |
| PsaDF1      | 5’-CCACTGTACTACAAACAAAGCACA-3’ | Complementation testing |
Generation and screening of chli1-1 rescued transformants

Complementation of the chli1-1 was performed utilizing the glass bead transformation technique described by Kindle et al. 198926. 2 µg of the linearized CHLI1-pDBle was used to complement chli1-1. Transformed cells were plated onto fresh TAP plates containing 15 µg/ml zeocin (Z) and placed in the dark at 25°C. Single colonies were picked and transferred onto fresh TAP-Za plates using a numbered grid template for screening of potential chli1-1 rescued transformants. Screening of chli1-1 rescued transformants was done by visual inspection of green coloration and monitoring growth of light adapted complement strain cells either on TAP in the dark or in the dim light or HS plates under medium light (300 µmol photons m−2 s−1).

Cellular protein analysis

Chlamydomonas cells from different strains grown in TAP in the dark 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). Protein concentrations of samples were determined by the method of Lowry et al. (1951)21 with bovine serum albumin as standard. Gel lanes were either loaded with an equal amount of Chl (4 µg Chl) or with 40 µg of protein. Resuspended cell suspension was mixed in a 1:1 ratio with the sample solubilization buffer SDS-urea buffer (150 mM Tris-HCl, pH 6.8; 7% w/v SDS; 10% w/v glycerol; 2 M urea, bromophenol blue and 10% β-mercaptoethanol) and were incubated at room temperature for about thirty minutes, with intermittent vortexing. The sample solubilization buffer was prepared according to the protocol of Smith et al. (1990)22 using reagents from Fisher (Pittsburgh, PA). After incubation, the solubilized protein samples were vortexed and spun at a maximum speed of 20,000 g in a microcentrifuge for five minutes at 4°C. The soluble fraction was loaded on a “any kD” Mini-PROTEAN® TGX™ Precast Gel (BioRad, Hercules, CA) and SDS-PAGE analysis was performed according to Laemmli (1970)24 using a Page Ruler prestained or unstained protein ladder (Fermentas, Glen Burnie, Maryland) at a constant current of 80 V for 2 hours. Gels were stained with colloidal Coomassie Gel code blue stain reagent (Thermo Fisher Scientific, Rockford, IL) for protein visualization.

Western analysis

Electrophoretic transfer of the SDS-PAGE resolved proteins onto an Immobilon P–PVDF membrane (Millipore, Billerica, MA) was carried out for 2 hours at a constant current of 400 mA in the transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). The CHLI1 polyclonal antibody was raised in rabbit against the full predicted transit peptide CHLI1 primary antibodies were diluted to a ratio of 1:20,000 with the antibody buffer. Western blots were developed using the Supersignal West Pico chemiluminescent substrate kit (Pierce protein research product, Thermo Fisher Scientific, Rockford, IL). and diluted to a ratio of 1:20,000 with the antibody buffer. Western blots were developed by using the Supersignal West Pico chemiluminescent substrate kit (Pierce protein research product, Thermo Fisher Scientific, Rockford, IL).

Cell counts and chlorophyll extraction

Cell density (number of cells per ml of the culture) was calculated by counting the cells using a Neubauer ultraplane hemacytometer (Hausser Scientific, Horsham, PA). Pigments from intact cells were extracted in 80% acetone and cell debris was removed by centrifugation at 10,000 g for 5 minutes. The absorbance of the supernatant was measured with a Beckman Coulter DU 730 Life science UV/Vis spectrophotometer (Brea, CA). Chl a and b concentrations were determined by Arnon (1949)25 equations, with corrections as described by Melis et al. (1987)28.

Results

Generation and identification of the mutant 5A7

Mutant 5A7 was generated by random insertional mutagenesis of the Chlamydomonas reinhardtii wild type strain 4A+ (137c genetic background). 5A7 lacks detectable chlorophyll, appears yellowish-brown in color and grows only under heterotrophic conditions in the dark or in the dim light in the presence of acetate in the growth media (Figure 2). It is incapable of photosynthesis and is sensitive to light intensities higher than 20 µmol photons m−2 s−1 (Figure 2A).

Molecular characterization of the mutation in 5A7

The linearized plasmid pBc1 was used to generate 5A7 (Figure 1). To find the insertion of the APHVIII end of the plasmid in 5A7, a modified TAIL (Thermal Asymmetric InterLaced) PCR method was used. Figure 3A shows the position of the vector specific TAIL PCR primers and also shows the arbitrary position of the random non-degenerate primer. A 850 bp DNA product from TAIL2 PCR was purified from the agarose gel (Figure 3B, Table 1). This purified DNA product was used for PCR using internal primers specific to the 3´UTR (UnTranslated Region) of the gene that the end of the plasmid has been inserted in the fourth exon of a hypothetical gene which we have named as UP6 (Figure 4). UP6 (Cre06.g306500) is located on chromosome 6.

Figure 5 shows a schematic map of the UP6 locus with its eight neighboring genes UP4 (Cre06.g306100), UP3 (Cre06.g306150), UP1 (Cre06.g306250), UP2 (Cre06.g306200) CHLI1 (Cre06.g306300), FDX3 (Cre06.g306350), AMT (g7098) and UP5 (Cre06.g306450). It is to be noted that we have named all of these genes arbitrarily for our study except for the CHLI1 and FDX3 genes, which were annotated in the Chlamydomonas genome database. Readers are requested to identify these unknown genes by the gene locus number (Cre or g number) in the Phytozome database, PCR analyses with the genomic DNA of 4A+ and 5A7 were performed using primers specific to four neighboring genes upstream of the CHLI1 (including UP6) and four neighboring genes downstream of the CHLI1 locus (Table 2 and Table 3; Figure 6A and 6B). PCR analyses revealed that all eight genes neighboring the CHLI1 locus were deleted or displaced from their native location (Figure 6A and 6B). UP5 primers gave nonspecific multiple products in 5A7 (Figure 6B). The first two exons of UP6 are present in the 5A7 genome as the UP6 primers spanning the first and the second exon, gave similar genomic DNA PCR product of the expected size as in the 4A+ lane (Figure 6B). Reverse transcription (RT-PCR) analyses using the same UP6 primers on 5A7 and 4A+ cDNA did not yield a PCR product in 5A7 unlike that in 4A+ (Figure 6C; Table 3). This shows that the insertion of the plasmid in the fourth exon of UP6 in 5A7 has hampered the transcription of the UP6 gene.
Figure 2. Growth phenotype of 5A7. (A) This figure shows the phenotypic difference of 5A7 compared to the parental strain, 4A+ on heterotrophic/mixotrophic agar media (TAP) plates under five different light conditions: dark + paromomycin (P), dark, very dim light (VDL, 2–4 μmol photons m⁻² s⁻¹), dim light A (DLA, 10–15 μmol photons m⁻² s⁻¹) and dim light B (DLB, 20–25 μmol photons m⁻² s⁻¹). (B) This figure shows the growth phenotype of 5A7 in liquid photo-autotrophic media (HS) under dim light (DL = 10–15 μmol photons m⁻² s⁻¹).

Figure 3. Locating the APHVIII flanking genomic sequence in 5A7. (A) A diagram showing a truncated pBC1 illustrating the APHVIII end of the linearized pBC1 vector. Primers used for PCR are shown by numbered black arrows. Thermal Asymmetric InterLaced 1 (TAIL1) PCR was performed using primer 4R and AD2. (B) TAIL2 PCR was performed using primer 3R and AD2. Lanes 1 and 4 are zero DNA lanes; in lane 2, a 10-fold diluted TAIL1 PCR product was used for TAIL2 PCR; in lane 5, a 25-fold diluted TAIL1 PCR product was used for TAIL2 PCR; lanes 3 and 6 are blank lanes. The 850 bp product used for DNA sequencing is highlighted. (C) Gel purified DNA product (850 bp) from the TAIL2 PCR was used to verify if the product was specific to the APHVIII gene. F and R stand for forward and reverse primers, respectively. AD2 is a non-degenerate primer. PCR primer names are labeled on the top of the gel. In lanes A and B, where triple primers were used for PCR, PCR products are labeled by the corresponding primer combinations that gave rise to the specific product. PCR product sizes are shown beside the primer combinations. All primer sequences are shown in Table 1. ST stands for 1 kb plus ladder (Invitrogen, Carlsbad, CA). DNA samples were run on a 1% agarose gel.
Taken together the data shows that at least a 35,715 bp genomic region has been deleted and/or displaced when the plasmid got inserted in the 5A7 genome. Except for the CHLI1 gene, the functions of the remaining eight genes (including UP6) are not known. We do not yet know the exact location of the pUC origin (pUC ori) end of the plasmid (Figure 1) in the 5A7 genome.

Checking for the absence/presence of the CHLI1 transcript and the CHLI2 gene and transcript

As CHLI plays a role in Chl biosynthesis, we checked for the presence/absence of the CHLI1 and CHLI2 in 5A7. RT-PCR results show that CHLI1 transcript is absent and CHLI2 transcript is present in 5A7 (Figure 7A, Table 4). Figure 7B shows the presence of the CHLI2 gene in 5A7.

Complementation of 5A7

We will be referring to strain 5A7 as chil1-1 from here onward. As our chil1-1 lacks Chl and CHLI1 is involved in Chl biosynthesis, we cloned the CHLI1 cDNA in the pDBle vector to transform chil1-1 (Figure 8, Table 4). CHLI1 expression is driven by the constitutive PsAD promoter in the CHLI1-pDBle construct (Figure 8). pDBle has two Ble genes that confer resistance to the antibiotic zeocin.

Figure 9 shows growth phenotypes of two chil1-1 rescued transformants (chil1-7 and chil1-8); chil1-1 and 4A+. chil1-1 rescued transformants are able to synthesize Chl, are not light sensitive and are capable of photosynthesis (Figure 9). As the chil1-1 rescued transformants harbor the Ble gene (from the pDBle vector) and APHVIII gene (derived from the parental strain chil1-1), they can grow on both zeocin and paromomycin media plates unlike chil1-1 and 4A+ (Figure 9).

Chl analyses show that both chil1-1 rescued transformants are about 33–46% Chl deficient. chil1-1 rescued transformants have a similar Chl a/b ratio as that of the wild type (Table 5, Data File below). Figure 10A and 10B show a schematic figure of the native Chlamydomonas CHLI1 gene and the trans CHLI1 gene used for complementation, respectively. PCR analyses using the genomic DNA show that the chil1-1 rescued transformants have the trans CHLI1 gene (Figure 10C and 10D). In Figure 10D the genomic DNA PCR product sizes in the two chil1-1 rescued transformant lanes are smaller than that in the 4A+ lane as we have cloned the CHLI1 cDNA for complementation. The Chlamydomonas CHLI1 protein has about 71% sequence identity to the Arabidopsis CHLI1 protein. Figure 11A shows a stained protein gel. The two chil1-1 rescued transformants and the 4A+ were loaded on an equal Chl basis in each lane in the protein gel (Figure 11A). As chil1-1 lacks Chl, the maximum amount of protein (40 µg) that can be loaded in a mini protein gel, was used (Figure 11A). Light harvesting complex proteins (LHCs) can barely be detected in the chil1-1 mutant (Figure 11A).

Figure 4. The APHVIII flanking genomic DNA sequence in 5A7. Primer 2R (Table 1), specific to the 3’ UTR of the APHVIII gene was used for sequencing the 850 bp Thermal Asymmetric InterLaced 2 (TAIL2) PCR product. 3’UTR sequence of APHVIII is in bold black, extra nucleotide additions are in bold blue. The flanking Chlamydomonas UP6 genomic sequence is denoted in red. The APHVIII end of the plasmid has been inserted after the eighth nucleotide in the fourth exon of UP6 gene.

Figure 5. A schematic map of the UP6 locus on chromosome 6. The map shows a 35.7 kb genomic DNA region that harbors the UP6 and eight genes located upstream of it. Each arrow represents a gene. The name of the gene is given on top of the arrow. The black numbers on the top of the arrows denote sizes of genes (bp) while black numbers below denote distances in between genes (bp).
Semi-quantitative RT-PCR analyses of chli1-1. Ndel/EcoRI double digested CHLI1 cDNA (1260 bp) was cloned into the Ndel/EcoRI double digested pDBle plasmid. Primers used for amplification of CHLI1 cDNA are shown in Table 4. CHLI1 expression is driven by the constitutive PsAD promoter. Ndel and EcoRI restriction sites are labeled. pDBle contains two copies of Ble\(^\beta\) genes driven by the Rubisco (RbcS2) promoter. The size of the CHLI1-pDBle construct is 7957 bp. Black arrow and white arrow denotes CHLI1 cDNA and Ble\(^\beta\) gene, respectively. Grey boxes denote UnTranslated regions (UTR).

Western analyses of the two chli1-1 rescued transformants with a CHLI1 antibody show that the CHLI1 protein is absent in the chli1-1 mutant but present in the chli1-1 rescued transformants (Figure 11B). Western analyses also show that the Arabidopsis CHLI1 antibody detects both the CHLI1 (40 kDa) and CHLI2 (42 kDa) protein in Chlamydomonas as the Chlamydomonas CHLI2 has about 62% sequence identity to the Arabidopsis CHLI1 (Figure 11B). In the wild type the CHLI2 protein amount is much lower than that of CHLI1. As the chli1-1 rescued transformants are Chl deficient.
compared to the wild type, the two rescued transformant lanes show higher amount of protein loadings (Figure 11A). Although more protein was loaded in the chli1-1 lane in the protein gel compared to that in the 4A+ and the chli1-1 rescued transformant lanes, the CHLII2 protein was barely detectable in 5A7 (Figure 11B).

**Discussion**

5A7 is the first chli1-1 mutant to be identified in *C. reinhardtii* and in green algae. CHLII1 deletion has affected Chl biosynthesis and photosynthetic growth in the chli1-1 mutant (Figure 2). 

Over-accumulation of photo-excitatable PPIX leads to photo-oxidative damage to the cells in presence of light and oxygen\(^{56}\). The light sensitivity of the chli1-1 is most probably due to an over-accumulation of PPIX which occurs due to the inactivity of MgChel enzyme which converts PPIX to MgPPIX. Future HPLC (High Performance Liquid Chromatography) analyses of steady state tetrapyrrole intermediates will confirm this hypothesis.

Based on the current molecular analyses, our chli1-1 mutant has a deletion of at least nine genes (including the CHLII1 gene). Currently we are investigating the exact insertion point of the pUC ori end of the plasmid in the chli1-1 genome (Figure 1). This will provide us with a precise estimate of the number of gene deletions in chli1-1. Although complementation of chli1-1 with the CHLII1 gene restored Chl biosynthesis, tolerance to high light levels and photo-autotrophic growth, chli1-1 rescued transformants are still Chl deficient to some extent (Table 5). This is probably due to a lower expression of the CHLII1 protein in these chli1-1 rescued transformants (Figure 11). 

**Table 5. Spectrophotometric analyses of chlorophyll in 4A+, chli1-1, chli1-7 and chli1-8 strains.** Chlorophyll analyses were done on three biological replicates for each strain. Strains were grown mixotrophically in TAP under 15–20 μmol photons m\(^{-2}\)s\(^{-1}\). Mean values are shown in the table. Statistical error (± SD) was ≤10% of the values shown. ND: not detected.

| Parameter | 4A+ | Strains | chli1-1 | chli1-7 | chli1-8 |
|-----------|-----|---------|---------|---------|---------|
| Chl/cell (nmol/cell) | 3.6 × 10\(^{-6}\) | ND | 2.0 × 10\(^{-6}\) | 2.5 × 10\(^{-6}\) |
| Chl a/b ratio | 2.8 | – | 2.7 | 2.8 |

In Arabidopsis, it has been shown that CHLII2 does play a limited role in Chl biosynthesis in the absence of CHLII1\(^{15–15}\). chli1-1 possesses an intact CHLII2 gene but the CHLII2 protein is barely detectable in the mutant. This raises two questions:

1) Is the low abundance of the CHLII2 protein a general effect or is it a specific effect of the CHLII1 mutation?

2) Is the total absence of Chl in strain chli1-1 due to the specific absence of CHLII1 or due to the absence of both CHLII1 and the near absence of CHLII2 protein?

**Table 5. Spectrophotometric analyses of chlorophyll in 4A+, chli1-1, chli1-7 and chli1-8 strains.** Chlorophyll analyses were done on three biological replicates for each strain. Strains were grown mixotrophically in TAP under 15–20 μmol photons m\(^{-2}\)s\(^{-1}\). Mean values are shown in the table. Statistical error (± SD) was ≤10% of the values shown. ND: not detected.

**Figure 10. Molecular analysis of chli1-1 rescued transformants.** (A) A schematic of the native CHLII1 gene. The tan bars denote UnTranslated Regions (UTRs), the white arrows represent exons and the black lines denote introns. (B) A schematic of the CHLII1-pDBIe complementation vector containing the CHLII1 cDNA. PsaD promoter, 5' UTR, 3' UTR and CHLII1 specific primers are labeled. (C) Genomic DNA PCR using a PsaD 5' UTR specific primer and a CHLII1 specific primer. Product size: 1272 bp. Lane 1: chli1-1; Lane 2: 4A+; Lane 3: chli1-7; Lane 4: chli1-8. ST represents 1 kb plus DNA ladder. (D) Genomic DNA PCR using CHLII1 specific primers. Genomic DNA product size: 459 bp; cDNA product size: 249 bp. Lanes 3 and 4 show smaller PCR products compared to that in lane 2 as cDNA was used for complementation. Lane 1: chli1-1; Lane 2: 4A+; Lane 3: chli1-7; Lane 4: chli1-8. ST represents 1 kb plus DNA ladder. All primer sequences are shown in Table 4.

**Figure 11. SDS-PAGE and Western analyses.** (A) A stained protein gel. Lanes 1, 2, 3 and 4 represent chli1-8, chli1-7, 4A+ and chli1-1, respectively. Light harvesting complex (LHC) protein bands are labeled. PS and US denote pre stained and unstained molecular weight protein ladders, respectively. Total cell extract of different strains were loaded on equal Chl basis (4 μg of Chl) in lanes 1, 2 and 3. In lane 4, 40 μg of protein (the maximum amount of protein that can be loaded on a mini protein gel) was loaded as chli1-1 lacks Chl. (B) Western analyses using a CHLII1 antibody generated against the Arabidopsis CHLII1 protein. Lanes 1, 2, 3 and 4 represent chli1-8, chli1-7, 4A+ and chli1-1, respectively. CHLII1 (40 kDa) and CHLII2 (42 kDa) proteins detected by the antibody are labeled.
The first question can be addressed by performing Western analyses of chl1-1 with antibodies raised against any non-photosynthetic and/or photosynthetic protein. If the low abundance of the CHL2 protein is due to a general effect of the mutation, there will be an overall reduction of different cellular proteins. The second question can be addressed by overexpressing CHL2 in chl1-1 to see if Chl biosynthesis can occur in the absence of CHL1 or by silencing CHL2 in the wild type strain using RNA interference or micro RNA based techniques.

Nonflurazon (NF) causes photo-oxidative damage to the chloroplast by inhibiting carotenoid biosynthesis,24-27,31. In Arabidopsis MgPPIX is hypothesized to be a retrograde signal from the chloroplast to the nucleus on the basis of data obtained with mutants that are defective in the NF, induced down-regulation of the transcription of the light harvesting complex protein B(LHCB) expression [gun (genomes uncoupled) phenotype]27,28. In Arabidopsis, there are controversies regarding whether chl1 mutants are gun mutants29-31. To date in Arabidopsis, MgPPIX mediated regulation of genes encoding only photosynthetic or chloroplastic proteins, have been documented27-31. In Chlamydomonas, hemin and MgPPIX has been shown to induce global changes in nuclear gene expression in Chlamydomonas, unlike that in Arabidopsis27-30,32-34. In Chlamydomonas, the above mentioned tetrapyrroles altered expressions of genes encoding TCA cycle enzymes, heme binding proteins and stress response proteins as well as proteins involved in protein folding and degradation (eg. heat shock proteins)31-33. Hence the roles of tetrapyrroles in retrograde signaling appears to be distinct in green algae and in higher plants. In summary, in the future our chl1-1 mutant can be used to clarify the functional role of CHL1 and CHL2 in Chl biosynthesis in C. reinhardtii.

Author contributions
MM and PG conceived the study, designed the experiments and took the lead role in preparing the manuscript. DW generated the mutant chl1-1 and isolated genomic DNA from the strains. PG did the PCR analyses, isolated genomic DNA, performed growth and Chl analyses of different strains, prepared cDNA, cloned the CHL11 gene and complemented the mutant chl1-1. JS maintained all the strains and was involved in the mutagenesis experiment. MM, PG, CL, KS, JM, KL, TH and AC maintained cultures, performed the TAIL-PCR analyses, extracted TAIL-PCR product from agarose gels, prepared DNA samples for sequencing and analyzed the DNA sequencing data. MM performed the protein and Western analyses. All authors were involved in the revision of the manuscript draft and have agreed to the final content.

Competing interests
No competing interests were disclosed.

Grant information
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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We would like to thank Dr. Krishna K. Niyogi (UC, Berkeley) for providing the 4A+ strain and the pBC1 plasmid that were used for mutagenesis, Dr. Saul Purton (University College London, UK) for providing us the FDX3, UP1, UP2, and AMT gene specific primers for PCR analyses. We would also like to thank Dr. Bernhard Grimm and Dr. Pawel Brzezowski (Humboldt University, Berlin, Germany) for providing us the FDX3, UP1, UP2, and AMT gene specific primers for PCR analyses. We would also like to thank Dr. Leos Kral (University of West Georgia) for allowing us to use his nano spectrophotometer and Dr. Anastasios Melis (UC, Berkeley) for allowing us to perform the protein and Western analyses at his laboratory.

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Referee Responses for Version 2

David Oppenheimer
Department of Botany, University of Florida, Gainesville, FL, USA

Approved: 30 August 2013

Referee Report: 30 August 2013
In this version of the manuscript, the authors have made all of the suggested revisions that I requested. I have no further requests from the authors.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Tatsuru Masuda
Environmental Sciences, University of Tokyo, Tokyo, Japan

Approved: 28 August 2013

Referee Report: 28 August 2013
I have reviewed the manuscript by Grovenstein et al. In this manuscript, the authors identified a Chl deficient mutant of Chlamydomonas reinhardtii by insertional mutagenesis.

As described in the manuscript, Arabidopsis possesses two CHLI isoforms, CHLI1 and CHLI2, like Chlamydomonas. However, it is not clear whether these isoforms are paralogous or not among these two photosynthetic organisms. The authors should perform phylogenetic analysis to show whether these isoforms are truly phylogenetically related or not.

Considering the chl1-1 mutant is Chl deficient, it is reasonable to assume that CHLI2 is not functional in Chlamydomonas. In my understanding, the expression of CHLI2 is not affected by the mutation of CHLI1 gene in Arabidopsis. The upper band of Fig.11B could be a non-specific band of CHLI1 protein that hides the original band of CHLI2 in Western blot analysis. In this sense, the expression analysis of CHLI2, as well as functional complementation with CHLI2 in the chl1-1 mutant, are apparently necessary for further understanding.

Specific comments:
In Fig. 7, the authors performed semi-quantitative RT-PCR analysis of CHLI1 and CHLI2. As the authors discussed quantities of these transcripts in the Discussion section (p.10), it is necessary to show the levels of housekeeping genes such as ACTIN8 or Ubiquitin10 as loading control.
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.

Ruby A Ynalvez  
Department of Biology and Chemistry, Texas A&M International University, Laredo, TX, USA

Approved: 13 August 2013

**Referee Report:** 13 August 2013  
The authors did incorporate the suggestions from version 1 into version 2 of the article. On my end, version 2 is already approved with no more revisions required.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.

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**Referee Responses for Version 1**

Ruby A Ynalvez  
Department of Biology and Chemistry, Texas A&M International University, Laredo, TX, USA

Approved: 26 June 2013

**Referee Report:** 26 June 2013  
The title is appropriate for the content of the article. In addition, the abstract represents a suitable summary of this interesting work. The authors were able to successfully isolate a novel *Chlamydomonas reinhardtii* mutant defective in chlorophyll biosynthesis. Using molecular biology techniques they have provided sufficient evidence that the novel mutant is missing a CHLI1 gene and having an intact CHLI2 gene. Their results also provided evidence that CHLI1 in *C. reinhardtii* is important in chlorophyll biosynthesis and that the presence of its homolog CHLI2 is not sufficient to make up for the function of CHLI1 in chlorophyll biosynthesis. The growth phenotype analysis of the rescued transformants has shown clearly the role of CHLI1 in chlorophyll biosynthesis. It would be interesting to know what percentage of rescued transformants was in their complementation analysis. Overall this is an interesting article. More importantly, this work reports the first *chli1* mutant to be identified in *C. reinhardtii*, as well as in green algae. The *chli1* mutant will open up new avenues to further explore the functional roles of CHLI1 and CHLI2 in chlorophyll biosynthesis in particular in *C. reinhardtii*.

**Suggested revisions:**

On page 6, sentence 2 “5A7 lacks detectable chlorophyll, appears yellowish-brown in color and grows only under heterotrophic conditions in the dark or in the dim light in the presence of acetate in the growth media (Figure 1).” Figure 1 should be Figure 2. On page 6, sentence 3 “It is incapable of photosynthesis
and is sensitive to light intensities higher than 20 μmol photons m-2s-1 (Figure 2),” ---“can be more clearly referred to as Figure 2A instead of Figure 2 only. On page 8, Figures 5A, 5B and 5C mentioned in text actually refers to Figures 6A, 6B and 6C.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.

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**Tatsuru Masuda**  
Environmental Sciences, University of Tokyo, Tokyo, Japan

**Approved: 24 June 2013**

**Referee Report: 24 June 2013**

In this paper, the authors have successfully identified a *Chlamydomonas* mutant deficient in chlorophyll biosynthesis using insertional mutagenesis. Through molecular genetic analyses, the authors demonstrated that the deficiency of the *CHLI1* gene, which encodes a subunit of Mg-chelatase, caused the chlorophyll-deficient phenotype. Although *Chlamydomonas* has another homologous *CHLI2* gene, this mutant failed to accumulate chlorophylls, suggesting the limited function of CHLI2 on chlorophyll biosynthesis. To clarify whether the reduced expression of CHLI2 caused chlorophyll deficiency, overexpression of CHLI2 in the mutant should give further insights into the functionality of CHLI2. In addition, it is desirable to determine the levels of chlorophyll intermediates that are correlated to the photosensitivity of the mutant.

**Suggested revisions:**

Considering the distinct regulation of chlorophyll biosynthesis and nuclear gene expression between *Chlamydomonas* and *Arabidopsis*, I am not sure whether this mutant is useful for analysis of the retrograde signaling. Actually, a number of previous papers suggested distinct roles of tetrapyrroles on nuclear gene expression in *Chlamydomonas* (Kropat *et al*. 1997, Kropat *et al*. 2000, Chekounova *et al*. 2001 and Vos *et al*. 2011). In this sense, the last paragraph of the Discussion section should be largely revised including already known mechanisms in *Chlamydomonas* cells and not be so focused on *Arabidopsis*.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.

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**David Oppenheimer**  
Department of Botany, University of Florida, Gainesville, FL, USA

**Approved: 20 June 2013**

**Referee Report: 20 June 2013**
In this paper, the authors describe the isolation and characterization of a mutant of *Chlamydomonas reinhardtii* deficient in chlorophyll biosynthesis.

The authors screened a population of Chlamydomonas that was transformed with an insertional mutagenesis vector. They found a chlorophyll deficient mutant that only grew under heterotrophic conditions. The authors provided convincing evidence that this mutant lacked the CHLI1 gene as well as several flanking genes. The CHLI1 gene encodes a subunit of the enzyme responsible for inserting Mg$^{2+}$ into protoporphyrin IX. Interestingly, the chli1 mutant also showed decreased expression of a homolog of CHLI1 and CHLI2. The authors also rescued the chli1 mutant phenotype using a wildtype CHLI1 cDNA transgene.

This work is important because it is the first report of a *chli1* mutant from a green alga. This mutant provides a starting point for additional genetic and biochemical analyses of chlorophyll biosynthesis in *Chlamydomonas*.

**Suggested revisions:**
The authors should change their mutant name to *chli1*-1, since it is the first mutant allele isolated. This sets a precedent for naming new alleles as they are discovered.

Instead of calling the rescued transformants that contain the wildtype CHLI1 cDNA "complements", the authors should consider calling them "rescued transformants". Some geneticists adhere to a more strict use of the term "complementation" and its derivatives where complementation is done by crossing two mutants, and not by genetic transformation of a mutant using a wildtype gene.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Competing Interests:** No competing interests were disclosed.