PHOTOSYSTEM II DAMAGE AND REPAIR IN *CHLAMYDOMONAS REINHARDTII*; ROLE OF REP27

PHICHAYA KHAMAI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (BIOCHEMISTRY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2010

Thesis
entitled

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…….
Mr. Phichaya Khamai
Candidate

…….
Assist. Kittisak Yokthongwattana,
Ph.D. (Agricultural and environmental chemistry)
Major-advisor

…….
Prof. M.R. Jisnuson Svasti,
Ph.D. (Molecular Biology)
Co-advisor

…….
Lect. Jamorn Somana,
M.D., Ph.D. (Plant Biochemistry)
Co-advisor

…….
Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

…….
Assoc. Prof. Tuangporn Suthiphongchai,
Ph.D. (Biochemistry)
Program Director
Doctor of Philosophy Program in Biochemistry
Faculty of Science
Mahidol University

Thesis
entitled

PHOTOSYSTEM II DAMAGE AND REPAIR IN *CHLAMYDOMONAS REINHARDTII*; ROLE OF REP 27

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Biochemistry)
on
August 11, 2010

…….
Mr. Phichaya Khamai
Candidate

…….
Prof. Prapon Wilairat,
Ph.D. (Biochemistry)
Chair

…….
Prof. M.R. Jisnuson Svasti,
Ph.D. (Molecular Biology)
Member

…….
Assist. Kittisak Yokthongwattana,
Ph.D. (Agricultural and environmental chemistry)
Member

…….
Lect. Anchalee Sirikhachornkit
Ph.D. (Microbiology)
Member

…….
Lect. Jamorn Somana,
M.D., Ph.D. (Plant Biochemistry)
Member

…….
Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

……
Prof. Skorn Mongkolsuk,
Ph.D. (Biological Science)
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENTS

This thesis is succeeded by the contribution support from Dr. Kittisak Yokthongwattana, my major advisor and Prof. Anastasios Melis, Dr. Sungsoon Park. I would like to express my deepest appreciation and sincere gratitude to them for their encouragement, valuable advice, intelligent guidance and unlimited support. I am also most grateful to my co-advisor, Prof. M.R. Jisnuson Svasti and Dr. Jamorn Somana. I deeply thank them for their valuable advice and guidance in this research.

I would like to thank Prof. Prapon Wilairat and Dr. Anchalee Sirikhachornkit for their valuable advice and kind support.

I would like to thank B317 members and everyone in the department of Biochemistry, Faculty of Science, Mahidol University for their cheerfulness and kind support.

I would like to thank the Commission on Higher Education, Ministry of Education, Thailand, for financial support through a grant fund under the program Strategic Scholarship for Frontier Research Network for the Ph.D. Program Thai Doctoral degree.

Finally, I am gratefully thank my family especially my mother and my sister for their care and love. The usefulness of this thesis, I dedicate to my father, my mother and all the teachers who gave me amazing knowledge since my childhood.

Phichaya Khamai

PHOTOSYSTEM II DAMAGE AND REPAIR IN *CHLAMYDOMONAS REINHARDTII* ; ROLE OF REP 27

PHICHAYA KHAMAI 4736004 SCBC/D

Ph.D. (BIOCHEMISTRY)

THESIS ADVISORY COMMITTEE: KITTISAK YOKTHONGWATTANA, Ph.D. (AGRICULTURAL AND ENVIRONMENTAL CHEMISTRY), M.R. JISNUSON SVASTI, Ph.D. (MOLECULAR BIOLOGY), JAMORN SOMANA, M.D., Ph.D. (PLANT BIOCHEMISTRY)

ABSTRACT

The goal of this research is to elucidate the molecular mechanism for the unique photo system II (PSII) damage and repair cycle in chloroplast. A frequently occurring, irreversible photo-oxidative damage inhibits PSII charge separation reaction and stops photosynthesis. The chloroplast PSII repair process rectifies this adverse effect by selectively removing and replacing the photo-inactivated D1/32-kDa reaction center protein (encoded by *psbA* gene) located in the massive (>1,000 kDa) water-oxidizing and O_2-evolving PSII holocomplex. Elucidation of the PSII repair mechanism may reveal the occurrence of hitherto unknown regulatory and catalytic reactions for the selective in situ replacement of specific proteins from within multiprotein complexes. DNA insertional mutagenesis in the model organism *Chlamydomonas reinhardtii* was applied for the isolation and characterization of *rep27*, a repair-aberrant mutant. Gene cloning and biochemical analyses of this mutant resulted in the identification of *REP27*, a nuclear gene encoded putative chloroplast-targeted protein, which is specifically required for the completion of the D1 turnover process but is not essential for the *de novo* biogenesis and assembly of the PSII holocomplex in this model green alga. The REP27 protein contains two highly conserved tetratricopeptide repeats, postulated to facilitate the *psbA* mRNA cotranslational insertion of the nascent D1 protein into the existing PSII core complex.

KEY WORDS:REP27/CHLAMYDOMONAS/PHOTOSYSTEM II

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การสูญเสียและการซ่อมแซมของ PSII โปรตีนในสาหร่ายสีเขียว; บทบาทของ REP27โปรตีน
PHOTOSYSTEM II DAMAGE AND REPAIR IN *CHLAMYDOMONAS REINHARDTII*; ROLE OF REP 27

พิชญะ คำอ้าย 4736004 SCBC/D

ปร.ด. (ชีวเคมี)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : กิตติศักดิ์ หยกทองวัฒนา, Ph.D. (AGRICULTURAL AND ENVIRONMENTAL CHEMISTRY), ม.ร.ว. ชิษณุสรร สวัสดิวัตน์, Ph.D. (MOLECULAR BIOLOGY), จามร สมณะ, Ph.D. (PLANT BIOCHEMISTRY)

บทคัดย่อ

วัตถุประสงค์ของงานนี้คือการศึกษากลไกการสูญเสียและการซ่อมแซมโปรตีน PSII ที่เกิดขึ้นอยู่เป็นประจำโดยโปรตีนนี้เป็นโปรตีนองค์รวมมีขนาดมากกว่า 1000 kDa ทำหน้าที่ในการรับแสงและออกซิไดซ์น้ำให้ได้อิเลคตรอนเพื่อส่งต่อไปยังโปรตีนที่เกี่ยวกับการสังเคราะแสงตัวอื่นในขบวนการสังเคราะห์แสง ซึ่งหากโปรตีนนี้ถูกทำลายการสังเคราะแสงก็จะไม่สามารถเกิดขึ้นได้ โดยโปรตีนที่ถูกทำลายนั้นเป็นโปรตีน D1 มีขนาด 32kDa ที่อยู่ใจกลางของโปรตีนองค์รวม PSII นี้เมื่อ D1ถูกทำลายจะทำให้โปรตีนPSII นี้ทำหน้าที่ในการสังเคราะไม่ได้ การที่จะทำให้ PSII นี้กลับมาทำงานได้ต้องทำการย้ายโปรตีน D1ที่ถูกทำลายนั้นออกไปแล้วสร้างขึ้นมาใส่เข้าไปไว้ใน PSII เหมือนเดิมจึงจะสามารถทำให้ขบวนการสังเคราะแสงกลับมาทำงานได้ซึ่งขบวนการนี้มีขั้นตอนและโปรตีนที่เกี่ยวข้องมากมาย ในการศึกษากลไกการซ่อมแซมของโปรตีนองค์รวม PSII นี้อาจทำให้ทราบกลไกการควบคุมและปฏิกิริยาในการแทนที่ของโปรตีนที่จำเพาะของในโปรตีนองค์รวมที่มี โปรตีนองค์ประกอบจำนวนมากจากการศึกษาในสาหร่ายสีเขียวกลายพันธุ์ rep27 ที่ได้มาจากการทำให้บกพร่องใน การซ่อมแซมโปรตีนองค์รวม PSII จนทำให้ความสามารถในสังเคราะห์แสงลดลง การทำยีนโคลนนิ่งและศึกษาทางชีวเคมีพบว่า โปรตีน REP27ซึ่งเป็นโปรตีนที่สร้างขึ้นในไซโตพลาสซึมเพื่อที่ส่งไปทำงานในคลอโรพลาสต์ มีบทบาทกในการทำให้ D1กลับมาทำงานในโปรตีนองค์รวม PSII ได้แต่ไม่จำเป็นในการสังเคราะห์D1ใหม่และการรวมเข้าเป็นโปรตีนองค์รวม PSII โครงสร้างของโปรตีน REP27 นี้ ประกอบด้วย หน่วย สายเพบไทด์ที่ซ้ำๆกันที่เรียก Tetratricopeptide ซึ่งคาดว่าทำหน้าที่ในการช่วยอำนวยความสดวกให้สาย mRNA ของ psbA gene สร้างโปรตีน D1 ใส่เข้าไปในรวมเข้าโปรตีนองค์รวม PSII

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LIST OF ABBRIVIATIONS

ARG7	Argininosuccinate lyase gene
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Bp	Base pair
^{o}C	Degree Celsius
cDNA	Complementary DNA
Chl	Chlorophyll
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
DCMU	3-(3,4-Dichlorophenyl)-1,1-Dimethylurea
DNA	Deoxynucleic acid
ddH2O	Doubleidistilled water
DDT	Dithiothreitol
F_v/F_m	variable to maximal) fluorescence yield ratio
Pi	In organic phosphate
Pmax	light-saturated rate of photosynthesis
G3P	Glyceraldehyde 3-phosphate
HL	High- light intensity
IPTG	Isopropy-*β*-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
L	Liter
LB	Luria-bertani
LHC-II	Light-harvesting antenna complex of photosystem II
LL	Low-light intensity
M	Molarity
µg	Microgram
µl	Microliter

LIST OF ABBRIVIATIONS (CONT.)

µM	Micromole
mg	Miligram
Min	Minute
ml	Mililiter
mM	Milimolar
MW	Molecular weight
NADPH	Nicotinamide adeninedinucleotide phosphate, reduced form
ng	Nanogram
OEC	Oxygen evolving complex
PCR	Polymerase chain reaction
PSI	PhotosystemI
PSII	Photosystem II
5'RACE	Rapid amplification of 5' cDNA ends
RNA	Ribonucleic acid
rpm	Revolutions perminute
RT-PCR	Reverse transcriptase-polymerase chain reaction
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
TAP	Tris acetate phosphate
TAIL-PCR	Thermal asymmetric interlaced- polymerase chain reaction
TBP	Tris borate acetate
TPR	Tetratricopeptide repeat
QA	quinone A
QB	quinone B
μE	microeistein
WT	Wild type

CHAPTER I
INTRODUCTION

1.1 Photosynthesis

1.1.1 Photosynthetic organisms

Photosynthetic organism such as plants and green algae synthesize organic substance in the photosynthesis process and subsequently used it for their living. The process requires energy from light, carbon dioxide from atmosphere and water from the ground to generate simple sugar in their cells and release oxygen to the atmosphere. Sugar is then catabolized to generated energy for cellular activities. Oxygen which released as a byproduct is also very important for all other living organisms in the world for cellular respiration.

1.1.2 Photosynthetic organelles

Chloroplast is a unique organelle inside the plant cell. It acts as power plant that hosts the photosynthetic reactions. The chloroplast is composed of three lipid-bilayer membranes. Two layers of the lipid membrane signify the boundary of the chloroplast called the outer and inner envelope membranes, respectively. Between them is the inter-membrane space. The outer membrane is permeable to most bio-molecules whereas the inner membrane contains membrane proteins regulating the passage of substance in or out of the chloroplast. These two membranes enclose an aqueous phase called stroma. Stroma aqueous contains enzymes and other components for sugar synthesis as well as for DNA replication, transcription and translation. Floating in the stroma is the third membrane structure called the thylakoid membrane. It is lipid bilayer membrane engulfed free space called the thylakoid lumen. Thylakoid membrane forms stacking disc-like structures called granna and non stack membranes called stroma lamella. The complex thylakoid membrane building up with several granna which link together by stroma lamellae. Thylakoid membrane is the site where photosynthetic apparatus are located and

organized to function in light reaction of photosynthesis (Mustardy, 1996; Mustardy et al., 2008;)

1.2 Photosynthetic protein organized in thylakoid membrane

The proteins that coordinate function in the light reaction of photosynthesis in green plants include photosystem I (PSI), photosystem II (PSII), cytochrome b_6f (Cyt b_6f), and ATP synthase. The PSII functions to oxidize water producing H^+, electron and oxygen. Electron is subsequently transferred down to other photosynthetic components on the thylakoid membrane. Proton is used for ATP synthesis via proton gradient across the membrane. Oxygen is released as a by-product to atmosphere. The Cyt b_6f is a transmembrane protein complex functions to transfer electrons from plastoquinone (PQ) to plastocyanin (PC) along with the transport of H^+ across the thylakoid membrane. ATP synthase uses energy from proton concentration a cross thylakoid membrane to catalyze the substrates ADP and Pi to form ATP (Dekker and Boekema, 2005).

1.2.1 Photosystem II

PSII is the principal protein complex in the light-dependent reactions. It is composed of several proteins subunits including reaction center D1 and D2, oxygen evolution protein complex, light harvesting protein CP43 CP47, and other chlorophyll-binding protein. In the reaction center contain the special pair of chlorophyll P680 which absorb light energy and carry out electron transfer from water molecule to PSI via electron carrier in the electron transport chain (Anderson and Chow, 2002; Ferreira et al., 2004; Renger G and Renger R, 2008)

1.2.2 Photosystem I (PS I)

PSI is another important photosynthetic protein complex. It also contains several subunit proteins such as an antenna protein complex (LHCI), reaction center protein PsaA, PsaB and structural proteins PsaC and PsaF. Inside the reaction center contain chlorophyll a special pair of chlorophylls called P700. PSI functions to

catalyze electron transfer reaction from PC to ferredoxin (Fd) and then $NADP^+$. The resulting NADPH is subsequently used to reduce carbon dioxide to simple carbohydrate (Chitnis, 2001; Nelson and Charles, 2006).

As mentioned above there are many proteins involved in photosynthesis. However, the principal protein is the PSII complex that absorbs light energy and initiates electron transfer from water to $NADP^+$. As it is a proteins complex containing several subunits, its function is greatly affected by the associated proteins. Each protein in this complex may play important role in the response to environmental condition especially light regime. Several of them are already known and many others still needed to be verified.

1.3 Photosystem II damage and repair

Photosynthetic processes as described earlier require light as an energy source to initiate the transport of high energy electrons. In theory, increasing light intensities should result in proportional increase in the rate of electron transport reactions and, as a consequence, the rate of photosynthesis. However, such statement is true only for a narrow range of increasing irradiance. Light intensities in excess of the saturated capacity of photosynthesis often lead to phooxidative stress, which, if prolonged, may eventually cause plant death. The primary target of the photooxidative stress due to excessive irradiance is a *psbA* gene product (D1 protein) of the PSII. This protein undergoes frequent turnover of synthesis and degradation. The primary goal of this thesis is to investigate the molecular mechanism of this phenomenon using green microalga *Chlamydomonas reinhardtii* as a representative model for plant chloroplasts.

Chloroplast development and differentiation in the unicellular green alga *Chlamydomonas reinhardtii* can take place either under autotrophic or heterotrophic conditions. Biogenesis and assembly of functional PSII, and of the other thylakoid membrane complexes, including PSI, the cytochrome b6-f complex, and the ATP synthase (Wollman et al., 1999; Minai et al., 2006), can take place in the dark in *C. reinhardtii*, provided that organic carbon, e.g. acetate, is supplied to the cells (Guenther et al., 1990). Whether in the light or in the dark, assembled thylakoid

membrane complexes are fairly stable and do not turnover with physiologically relevant rates. An exception to this rule is the D1/ 32-kD PSII reaction center protein (the *psbA* gene product), which is known for its frequent turnover in the light (Mattoo and Edelman 1987; Vasilikiotis and Melis 1994). The PSII holocomplex performs the functions of light absorption and excitation energy transfer to P680, leading to water oxidation, the release of O_2 and protons, and the transport of electrons from water to reduce plastoquinone molecules in the thylakoid membrane. The transient formation of strong oxidants, the abundance of O_2, and the presence of excitation energy are conditions that may lead to photooxidative damage (Ohad et al., 1984; Barber 1994; Melis 1999), causing irreversible inactivation in the electron-transport function of the D1 protein and inhibiting the function of PSII (Kok 1956; Powles 1984; Melis 1991; Aro et al., 1993). An elaborate repair mechanism operates in organisms of oxygenic photosynthesis and restores the functional status of PSII.

The PSII damage and repair cycle, as the phenomenon has come to be known (Guenther and Melis 1990), operates in tandem with photosynthesis and appears to be conserved in cyanobacteria, algae, and crop plants. The rate constant of photodamage is proportional to the incident light intensity (Baroli and Melis 1996; Tyystjarvi and Aro 1996; Hakala et al., 2005; Ohnishi et al., 2005). Thus, photodamage can occur with a half time as slow as 24 h under very low light intensity conditions, or as fast as 30 min under bright sunlight (Kim et al., 1993; Yokthongwattana and Melis 2006). The rate-limiting step in the enzymatic repair of PSII occurs with a half time of about 2 h (Sundby et al. 1993; Vasilikiotis and Melis 1994; Neidhardt et al., 1998). This defines the overall rate constant for the repair process and is largely independent of the light intensity to which the photosynthetic organism is exposed. In PSII repair-aberrant mutants of *C. reinhardtii*, water oxidation and electron transport capacity depend on the balance between de novo biogenesis/ assembly of PSII complexes (presence of acetate) and the rate of photodamage. Therefore, in repair-aberrant mutants, the degree of photoinhibition of photosynthesis can be manipulated by the relative dominance of these two players, i.e. de novo biogenesis/assembly and photodamage of PSII. In the presence of acetate and under weak light intensities, when the rate of de novo biogenesis/assembly of PSII is comparable to that of photodamage, intermediate levels of photoinhibition will

manifest in relation to a wild-type control. Stronger light intensities, however, would make the rate of photodamage far exceed the rate of de novo biogenesis/assembly, causing a quantitative accumulation of photodamaged PSII and the end of detectable photosynthetic water oxidation activity.

In this thesis, DNA insertional mutagenesis of *C. reinhardtii* was employed to generate and isolate PSII repair mutants. The appropriate screening steps (Zhang et al., 1997) resulted in the isolation of *rep27*, a *C. reinhardtii* strain that is defective in photoautotrophic growth, which, however, greens normally in the presence of acetate and displays a lowered photosynthetic water oxidation and CO_2 conversion activity when grown under low irradiance conditions. Under moderate and high light intensity conditions, PSII activity is selectively abolished in this mutant, suggesting a defect in the repair from photodamage. Gene cloning and biochemical analyses in this PSII repair-aberrant mutant resulted in the identification of REP27, a nuclear gene encoding a putative chloroplast-targeted protein, which is specifically required for the completion of the D1 turnover process but is not essential for the de novo biogenesis/assembly of the PSII holocomplex in this model green alga. A working hypothesis model is presented, postulating a function of the REP27 protein in the psbA mRNA translation and/or cotranslational insertion of nascent D1 in existing PSII core templates.

CHAPTER II
OBJECTIVES

Since photosynthesis is very important for plant and subsequently for others living thing in the word. Investigation of the components relevant process is worth doing. From time to time plant keep produce food by photosynthesis in addition that they have to exposed to variety of environment stress such as light, salt, chill, drought causing plant to be stress and give rise to lower growth and production some might draft or dead. As a result of photosynthesis rely on dominant protein PSII complex that play a vital role in this process. PSII absorb light to split water molecule to generate electron flow along the photosysnthesis component, finally to PSI which will finally used it to generate sugar. Excessive light can cause stress and damage to PSII. The damage is to the PSII core, D1 protein. The damaged D1 is degraded, re-synthesized and reassembled into functional PSII complex. There are many mechanisms and proteins involve in this process of damage and repair which occurred normally in plant with some known and some that need to investigated. This work is to investigate REP27, the protein involved D1 Protein turnover and Photosystem II repair. So the objectives of this thesis were as following;

Part I: Isolation and Characterization of Repair Mutant

1.1 Isolation *rep27*, a Putative PSII Repair Mutant

1.2 Phenotypic characterization wild type and rep 27 strain

1.3 Biochemical Analysis of wild type and rep 27 strain

1.4 Molecular analysis of the putative PSII repair rep 27

Part II: Complement of REP27 Genomic DNA to rep27 mutant

2.1 Cloning the genomic DNA flanking the pJD67 insertion site

2.2 Identification of REP27 gene and structure

2.3 Physical and Biochemical analysis of rep27 complement

CHAPTER III
MATERIALS AND METHODS

3.1 Plant material and growth conditions

Chlamydomonas reinhardtii mutants were generated by transformation (Kindle, 1990 Tam and Lefebvre, 1993) of an Arg auxotroph, strain CC-425 (*arg7-8 cw15 mt + sr-u-2-60*; Chlamydomonas Center, Duke University), with pJD67 plasmid DNA (linearized with *Hin*dIII) containing the complementing argininosuccinate lyase gene (*ARG7*), as described by Davies and Grossman (1994) and Davies et al., (1996). Procedures for the screening, isolation, and maintenance of putative repair mutants, including the *rep27* strain, have also been described (Zhang et al., 1997). Wild type and *rep27* of the green alga *C. reinhardtii* were grown mixotrophically in a TAP medium (Gorman and Levine, 1965), either in liquid cultures or on 1% agar plates. To test photoautotrophic growth of wild-type and complemented strains, cells were grown on TBP minimal media, in which sodium bicarbonate (25 mM, pH 7.4) replaced the acetate as the growth carbon source (Polle et al., 2003). Culture density was measured by cell counting using a Neubauer ultraplane hemacytometer and a BH-2 light microscope (Olympus). Cells were grown to the early exponential growth phase (about 1–2 x 10^6 cells/mL) prior to harvesting and measurements of photosynthesis. Chlamydomonas BAC genomic DNA library filters and clones were acquired from the Clemson University Genomics Institute (https://www.genome.clemson.edu/).

3.2 Nucleic Acid Isolation and Analyses

To purify the genomic DNA of *C. reinhardtii*, cells were grown in liquid cultures, harvested by centrifugation, and resuspended in a cetyl-trimethyl-ammonium bromide buffer containing 2% cetyl-trimethyl-ammonium bromide, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 2% β-mercaptoethanol. The cell suspension was incubated at 65°C for 2 h, extracted twice with phenol-chloroform-isoamylalcohol

(25:24:1), and precipitated with isopropanol (Chen and Melis, 2004). Total RNA was isolated from the isopropanol pellet of the cell extract using Invitrogen's Trizol Reagent and by following the manufacturer's recommended procedure. 5' RACE by PCR was performed using FirstChoice RNA ligase mediated-RACE kit (Ambion) by following the manufacturer's recommended procedure. Southern- and northern-blot analyses were carried out according to standard protocol (Sambrook et al., 1989). The high light induction of *REP27* gene expression was tested upon shifting cultures from 50 to 1,000 μmol photons m^{-2} s^{-1} for 1 h prior to RNA isolation and by using the full size of the cDNA fragment as a probe in the measurement of transcript abundance. Northern-blot analysis with early light-inducible protein (ELIP)-specific DNA was performed with a radiolabeled, full-size *ELIP* cDNA probe, which was amplified from *C. reinhardtii* with primer sets

P02F = (GGGGGGATCCTTCGCTGCCTTTCGCGTTAC) and

P03R = (GGGGGGATCCGGAACTTGTGTGTCGTTTAG).

3.3 Cloning of the Flanking Regions of the pJD67 Insertion Site

C. reinhardtii genomic DNA flanking the plasmid insertion site was amplified using a TAIL-PCR procedure, optimized for *Chlamydomonas* genomic DNA, by a modification of the method described (Dent et al., 2005). The primers used for the TAIL-PCR are listed in Table II. Briefly, flanking genomic DNA was amplified by PCR from the region adjacent to the inserted pJD67 plasmid that was used for DNA insertional mutagenesis (Zhang et al., 1997). For *Hin*dIII-digested pJD67 transformants, specific primers for primary, secondary, and tertiary reactions were designed and named TPB1, TPB2, and TPB3, respectively (Table II). Two arbitrary degenerate primers were tested for amplification, TPdeg1 and TPdeg2, as previously described (Dent et al., 2005). The general TAIL-PCR protocol of Liu et al. (1995) was used with some minor modifications for the various PCR amplification reactions. Nucleotide sequences of the resulting PCR products were obtained via an ABI3100 sequencer. Chlamydomonas genomic sequence information was obtained

from the Chlamydomonas Genome Project Website (http://genome.jgisf.org/Chlre3/Chlre3.home.html).

3.4 Insertional Mutagenesis and Chlamydomonas Transformation

Generation of a *C. reinhardtii* insertional mutagenesis library and screening for the identification of specific PSII repair mutants was implemented as previously described (Zhang et al., 1997). Complementation of the *rep27* putative repair mutant was achieved by cotransformation of the mutant with BAC clone 10A5 and with subclones derived from this BAC with plasmid pSL18 (Depege et al., 2003), using the conventional glass bead transformation method (Kindle, 1990). pSL18 contains the paromomycin resistance gene (selectable marker) operated under the control of the *C. reinhardtii* Hsp70A and RbcS2 promoters (Sizova et al., 2001) and linked to the PsaD promoter and terminator that can be used to express ORFs in *C. reinhardtii* (Depege et al., 2003). *C. reinhardtii* transformants carrying the above-mentioned BAC clone(s) were selected on TAP (acetate-containing) or TBP (minimal media) plates containing 5 μg mL^{-1} paromomycin (Sigma Chemical).

3.5 Photosynthesis Measurements

Procedures for the measurement of the F_v/F_m variable-to-maximal fluorescence yield ratio and for the Pmax have been described (Zhang et al., 1997). To test for the effect of high light exposure on photosynthesis and PSII parameters, cw15, *rep27*, and *rep27*-comp strains were incubated under 1,000 μE for variable periods of time. The photosynthetic activity of strains cw15, *rep27*, and *rep27*-comp lines was estimated from the light-saturation curve of photosynthesis, measured as the oxygen evolution activity of the cells at different actinic intensities. Measurements of the light-saturation curve of photosynthesis commenced with the registration of dark respiration in the cell suspension and were followed by measurements of the rate of oxygen evolution at 500, 1,000, 1,500, 2,000, and 2,500 μE. Registration of the rate (slope) of oxygen evolution at each light intensity step was recorded for about 5 min. Therefore, in the 30-min duration of these measurements, samples were exposed to progressively higher light intensities (0–2,500 μE).

3.6 Protein Analysis

For the isolation of total cellular protein, C. reinhardtii strainswere grown in liquid TAP media under continuous illumination (50 mE). Cell biomass equivalent to 100 mg Chl were collected by centrifugation (5,000g) and resuspended in 400 mL of 0.1 M dithiothreitol and 0.1 M Na2CO3. Following incubation for 5 min, 400 mL of 23 sample solubilization buffer containing 10% SDS, 10% glycerol, and 10% b-mercaptoethanol was added and incubated for 30 min at room temperature. To enhance the solubilization efficiency of SDS, themix was incubated in boiling water for 2 min. Unsolubilized material was removed by centrifugation at 15,000g for 5 min prior to loading samples onto the SDS-PAGE. The Chl concentration of the various suspensions was measured as previously described (Lichtenthaler, 1987). Aliquots corresponding to an equal amount of Chl were loaded in thewells of the stacking gel and electrophoresed through 12.5% SDS-polyacrylamide running gels as described by Tetali et al. (2007). The electrophoretically separated proteins were transferred onto nitrocellulose (Immobilon-NC, Millipore) and probed sequentially with primary specific polyclonal antibodies and horseradish peroxidase conjugated secondary antibodies (Bio-Rad). Antibodies to D1, PetC, and AtpA subunit were described in Park and Rodermel (2004), and Hsp70b was described in Yokthongwattana et al. (2001). Antisera to PsaK, CytF, and PsbO were obtained as a kind gift from Dr. Paraq Chitnis. Specific polyclonal antibodies against D2 and CP47 were also described (Kim et al., 1993; Yokthongwattana et al., 2001). Cross-reactions between protein bands and antibodies were visualized by use of the Supersignal ECL (Pierce) detection kit following the manufacturer's specifications. Quantification of northern- and western-blot bands was made through application of the Imagej 1.37v software (http://rsb.info.nih.gov/ij/).

3.7 In Vivo (Pulse) Labeling with [^{35}S]-Sulfate

Radioactivity-labeling procedures were performed as previously described (Vasilikiotis and Melis, 1994; Preiss et al., 2001). Exponential growth phase cells of *C. reinhardtii*, equivalent to 60 μg Chl, were harvested by centrifugation at 5,000*g* for

5 min at room temperature. Pelleted cells were resuspended in 300 μL sulfur-deprived TAP to yield 0.2 μg Chl/μL and incubated for 2 h at room temperature under 50 μE. Cycloheximide (10 μg/mL) was added to block cytoplasmic protein synthesis and cells were incubated for 15 min prior to radioactive [^{35}S]-sulfate pulse treatment. The pulse was initiated upon addition of 100 μCi/mL [^{35}S]-sodium sulfate. At the end of a 10-min labeling period, 100 μg ml^{-1} lincomycin and cold sulfate (1 mM final concentration) were added to terminate the labeling reaction. Radioactivity-labeled samples were collected by centrifugation, solubilized (as described above), and subjected to SDS-PAGE. Polyacrylamide gels were fixed in 20% methanol, 7% acetic acid for 30 min, vacuum dried, exposed to a phosphor screen, and analyzed by a Storm PhosphorImager (Molecular Dynamics).

CHAPTER IV
RESULTS

4.1 Isolation and Characterization of Repair Mutant

4.1.1 Isolation *rep27*, a Putative PSII Repair Mutant

After DNA insertion in *chlamydomonas reinhardii* strain was employed to generate and isolate PSII repair mutant (Zhang et al., 1997). A putative PSII repair mutant, termed *rep27*, was isolated following DNA insertional mutagenesis and on the basis of a stringent two-step screening protocol. Acetate requirement was the first screening step in this protocol. Whereas both wild-type and *rep27* mutant strains grew well on acetate-containing Tris-acetate phosphate (TAP) media, only the wild type grew on minimal Tris-bicarbonate-phosphate (TBP) media (Table I ; Fig. 1A).

4.1.2 Phenotypic characterizations

4.1.2.1 Growth media and condition

After the the PSII repair mutant strain was selected it' s phenotype was investigated in comparison with those of wild type under the same growth condition to lineate if PSII in rep 27 is malfunction in the irradiance growth. Cellular chlorophyll (Chl) content was similar in wild type and *rep27* mutant, about 3.4×10^{-15} mol/cell when grown under 10 μmol photons m^{-2} s^{-1} (10 μE) and about 1.7×10^{-15} mol/cell when grown under 150 μmol photons m^{-2} s^{-1} (150 μE). Moreover, wild type and *rep27* displayed similar Chl *a*/*b* ratios (data not shown). These results suggested that in the presence of acetate, growth, Chl accumulation, as well as the assembly and acclimation properties of the photosynthetic apparatus were not affected by the *rep27* mutation. When cultivated in TAP media under either 10- or 50-μE conditions, the wild type displayed a slightly faster rate of growth than the *rep27* mutant (Fig. 1B).

Pivotal in the successful screening of repair mutants in *C. reinhardtii* is the differential biogenesis/assembly of functional PSII units in the presence of acetate and the subsequent inability of the chloroplast to repair them once photodamage has occurred (Zhang et al., 1997). It is thus expected that, under low-light (10 μE) growth

in the presence of acetate, the rate of de novo biogenesis/assembly of the PSII holocomplex would be faster than the rate of PSII photodamage. Under these conditions, repair mutants will possess functional PSII reaction centers, the abundance of which will decline with growth irradiance faster than that in the wild type. This differential irradiance response can be monitored from the in vivo variable to maximal (F_v/F_m) fluorescence yield ratio and by the light-saturated rate of oxygen evolution in the two strains. The F_v/F_m ratio provides a convenient and direct method for the measurement of the photochemical charge separation efficiency of PSII reaction centers (Kitajima and Butler, 1975), while the light-saturated rate of oxygen evolution provides a measure of PSII electron transport capacity and, therefore, of the number of fully assembled/functional PSII units.

4.1.2.2 Efficiency of primary photochemistry

The F_v/F_m fluorescence yield ratio was measured in *C. reinhardtii* cells grown under different irradiance regimes (10–150 μE; Fig. 1C). The ratio was lower in the *rep27* mutant than in the wild type under all growth irradiance conditions, suggesting fewer functional PSII reaction centers in the former than in the latter. This was confirmed by sensitive absorbance difference spectrophotometry (ΔA_{320}; Melis, 1989), which revealed a substantially lower concentration of the functional primary quinone acceptor (Q_A) in the mutant than in the wild type. For cells grown at 150 μE, analysis showed Q_A/Chl (wild type) = 1:560 mol:mol; Q_A/Chl (*rep27*) = 1:1,800 mol:mol, whereas P700/Chl ratios were similar at about 1:650 mol:mol in each of these the two strains. Accordingly, the operational PSII/PSI ratio was lowered in the *rep27* mutant in an irradiance-dependent fashion (Table I). These results are consistent with the interpretation that the *rep27* strain is a putative PSII repair mutant.

4.1.2.3 Light-saturated rate of Oxygenevolution

To independently assess the functional capacity of PSII, the light-saturated rate of photosynthesis (Pmax) was measured (Fig. 1D). Wild type and *rep27* mutant were grown at three different irradiances (10, 50, and 150 μE) and Pmax was measured from the rate of oxygen evolution in a Clark-type oxygen electrode under saturating illumination conditions. Figure 1D shows that Pmax for the wild type

increased with growth irradiance, as is expected upon acclimation of the photosynthetic apparatus to the level of irradiance (Anderson, 1986; Baroli and Melis, 1996). On the contrary, Pmax for the *rep27* declined with growth irradiance and dropped below the isosbestic point when grown at 150 μE, consistent with the repair-aberrant phenotype of this mutant. Under higher than 150 μE growth irradiance conditions, photosynthetic O_2 evolution in the *rep27* mutant became negligible, and the only gas exchange reaction recorded was that of cellular respiration that consumed oxygen from the medium. These results are consistent with the notion that, at irradiance levels greater than 150 μE, the rate of photodamage was substantially greater than that of de novo PSII biogenesis/assembly, resulting in a quantitative PSII photoinhibition. Survival of the organism under these conditions is ensured by the presence of acetate, which supports oxidative phosphorylation by the cells. The F_v/F_m ratio of the *rep27* mutant did not exceed 0.4, irrespective of whether cells were grown under low light or in the dark.

At present, it is not clear why the *rep27* cells have a lower than wild type F_v/F_m ratio. The possibility cannot be excluded that, although PSII biogenesis/de novo assembly and the PSII repair processes occur in different regions of the chloroplast thylakoid membrane and likely involve substantially different mechanisms, they may nevertheless utilize at least some common enzymatic reactions, possibly including the one catalyzed by the REP27 protein. Further work is needed to delineate these points.

4.1.3 Biochemical analysis of wild-type and *rep27* strains

4.1.3.1 Determination of the steady state level of protein PSII subunit

To investigate if there is D1 or other protein in the PSII complex is defect in accordance to PSII function measurement above. The steady-state level of PSII subunits was determined from the intensity of the antibody cross-reaction in the western blots with specific polyclonal antibodies. Figure 2A shows that the *rep27* mutant has lower than wild-type steady-state levels of the D1/32-kD reaction center protein in its thylakoid membranes, consistent with earlier finding on this mutant (Zhang et al., 1997). Figure 2A also shows that subunits of the PSII holocomplex, other than the D1 reaction center (e.g. D2, CP47, and PsbO), occur in equivalent amounts in the wild type and *rep27* mutant. This observation offers additional

Table I Differential growth, irradiance screening, and functional characterization of the C. reinhardtii wild-type and rep27 mutant strains

Cell growth was tested in TBP or TAP as the sole carbon source. Chl and functional photosystem content was measured spectrophotometrically, as described (Melis, 1989). Wild type and rep27 mutant were grown in the light under either 10 (10 μE) or 150 μmol photons m^{-2} s^{-1} (150 μE) conditions

Parameter Measure	Wildtype 10 μE	Rep27 10 μE	Wildtype 150 μE	Rep27 150 μE
Growth in TBP (bicarbonate) media	Yes	No	Yes	No
Growth in TAP (Acetate) media	Yes	Yes	Yes	Yes
Chl/cell (mol x 10^{-15})	3.2	3.6	1.6	1.8
PSII/PSI ratio	1.3/1	0.6/1	1.16/1	0.36/1

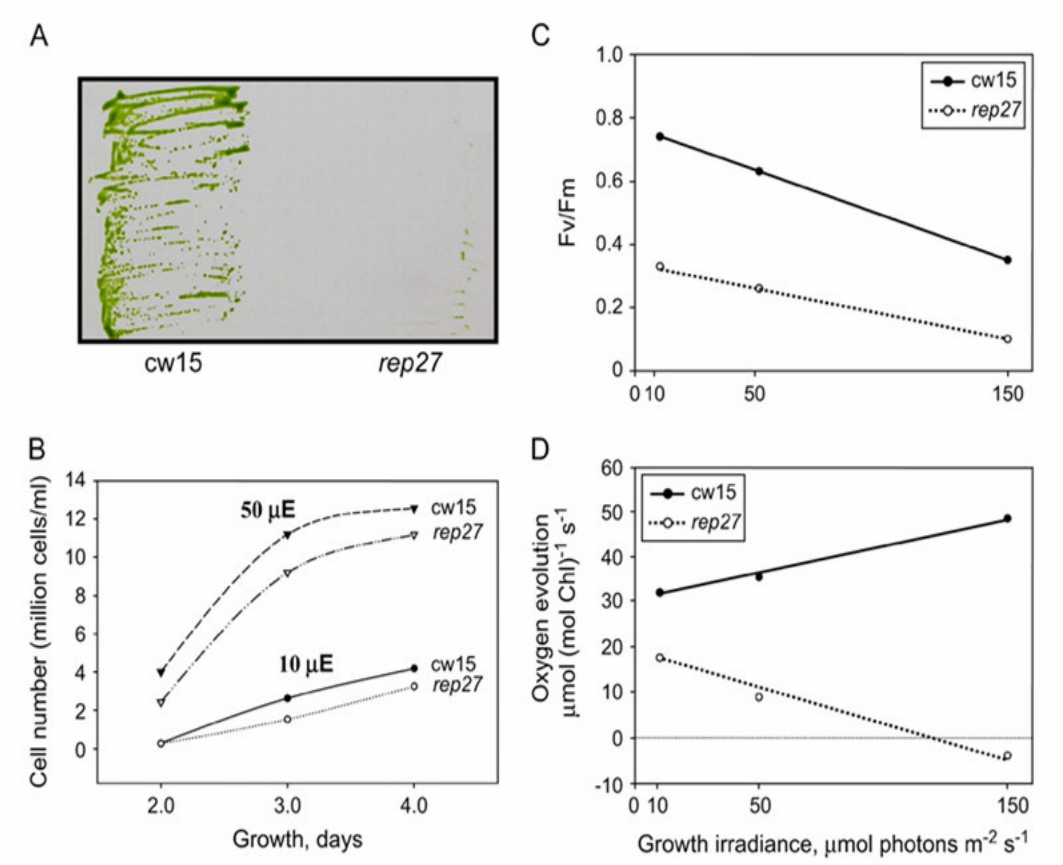

Figure 1 Phenotypic characterization of *C. reinhardtii* wild-type (cw15) and *rep27* mutant strains. A, Growth of cw15 wild type and lack of growth of the *rep27* mutant on TBP minimal media at 50 μmol photons m^{-2} s^{-1} (50 μE). B, Growth curves of wild type and *rep27* in TAP liquid media under approximately 10- or 50-μE conditions. The values shown are the average of the three independent experiments. C, Efficiency of PSII primary photochemistry (F_v/F_m) measured as a function of growth irradiance for wild type and *rep27* mutant. D, Light-saturated rate of oxygen evolution for wild type and rep27mutant as a function of growth irradiance. Cells were grown in TAP for 3 d prior to the measurement. Values shown are the average from the two independent experiments.

evidence that de novo biogenesis/assembly of the PSII holocomplex is not impaired in the *rep27* mutant. Rather, the effect of the mutation appears to be specific to the replacement (turnover) of the photodamaged D1. Figure 2B shows that subunits of non-PSII complexes in thylakoids, e.g. Cyt *b6-f*, PSI, and ATP synthase, also occur in equivalent amounts in wild type and *rep27* mutant. This observation strengthens the interpretation that biogenesis/assembly of the thylakoid membrane and the electron transport chain are not impaired in the *rep27* mutant. Somewhat enhanced HSP70B protein accumulation was observed in the *rep27* mutant relative to that in the wild type (Fig. 2B). This could be attributed to the apparently inhibited and, therefore, unfinished repair in the *rep27* mutant (accumulation of the PSII repair intermediate; Yokthongwattana et al., 2001).

4.1.3.2 Uncoupled *psbA* Transcription and D1 Translation in the *rep27*

One possible scenario for the presence of lower steady-state levels of the D1 protein in the *rep27* mutant might be a defect in the incorporation of nascent D1 in the photodamaged PSII core complex. This hypothesis requires that *psbA* gene transcription and mRNA levels are about the same in wild type and *rep27* mutant. Steady-state levels of *psbA* and *psbD* mRNA were measured by northern-blot analysis (Fig. 3A), showing that *psbA* mRNA levels in mutant and wild type were indistinguishable from each other. Interestingly, *rep27* showed somewhat enhanced steady-state levels of *psbD* mRNA relative to the wild type, underlining the greater relative amounts of D2 protein in the mutant (Fig. 3A) relative to that in the wild type The preceding suggested that a lower level of the D1 protein in the *rep27* mutant, relative to that in the wild type, may be a consequence of the unimpeded degradation of photodamaged D1, coupled with the chloroplast inability to replace the lost D1 in the PSII-core complex. It should thus be possible to delineate between the de novo biogenesis/assembly of PSII from the selective D1 turnover in the chloroplast of the *rep27* mutant.

4.1.3.3 Chloroplast Protein de novo biosynthesis/assembly and accumulation in Chloroplast is not affect in rep27

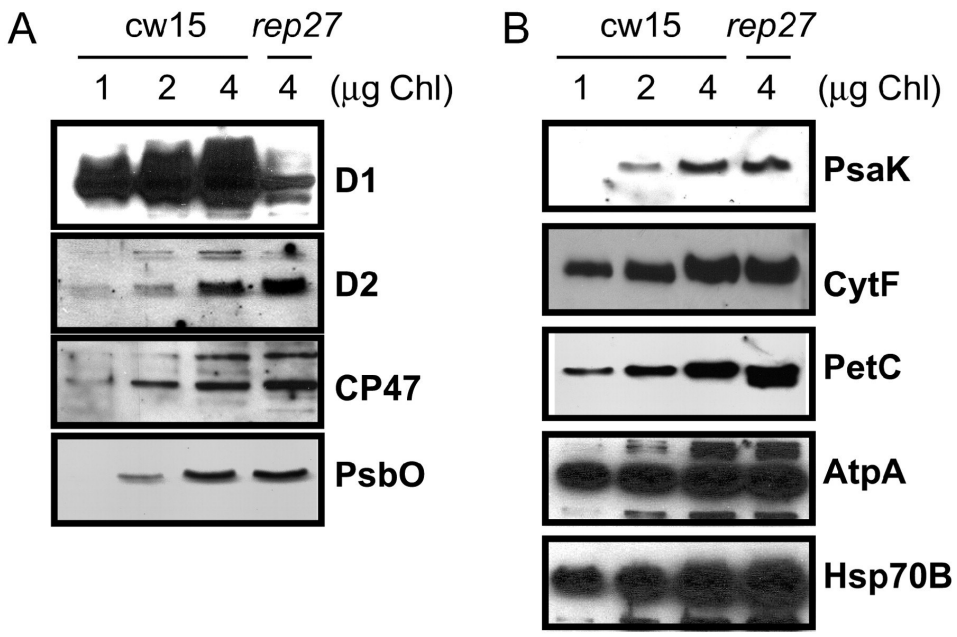

Figure 2 A, Western-blot analysis of 50-μE grown *C. reinhardtii* wild type and *rep27* total protein cell extract. The steady-state level of PSII subunits was determined from the intensity of the antibody cross-reaction in the western blots with specific polyclonal antibodies generated against D1, D2, CP47, and PsbO, respectively. B, Western-blot analysis of 50-μE grown wild type and *rep27* total protein cell extract. The steady-state level of these thylakoid membrane proteins was determined from the intensity of the antibody cross-reaction in the western blots with specific polyclonal antibodies generated against PsaK, Cyt f, PetC, AtpA, and Hsp70B.

As shown in figure 2A amount of D1 in rep27 is lower than that of wild type but the psbA mRNA is in equivalent amount, so that it might defect in the de novo biogenesis/assembly of PSII from the selective D1 turnover in the chloroplast of the *rep27* mutant. This was tested by comparative [^{35}S]-sulfate pulse-labeling experiments with the cw15 wild type and *rep27* mutant (Fig. 3B). In such experiments, cycloheximide was added just prior to the application of the radioactivity to inhibit synthesis of cytosolic proteins. Figure 3B shows about equivalent amounts of large subunit of Rubisco, ATP synthase α-subunit (CF1-α), and D2 protein that were labeled in wild type and mutant. These provide a measure of the de novo biogenesis/assembly and accumulation of chloroplast components as a function of cell growth in the presence of acetate. Interestingly, in the *rep27*, but not in the wild type, radiolabeled D1 and D2 accumulated in about equal amounts, evidence that de novo biogenesis/assembly of D1 is not affected in the mutant. However, the wild type showed a much greater accumulation of radiolabeled D1 than the mutant, consistent with the active turnover of this PSII reaction center protein. These results are evidence of selective inhibition of the D1 turnover/PSII repair and lack of impairment in the de novo biogenesis/assembly of the PSII complex during cell growth in the mutant. In sum, except for the remarkable disparity of D1 radiolabeling in wild type and *rep27*, the labeling of other PSII and thylakoid membrane proteins was found to occur in equivalent amounts in the two strains.

4.1.4 Molecular analysis of the putative PSII repaire mutant

4.1.4.1 Determination of pJD67 fragment in *rep27* genomic DNA

After the rep 27 mutant was achieved following the assessment above. It was used to analyzed for the insertion gene by crossing to wild type strain cc1068. Backcrosses of *rep27* (mt+) and strain CC1068 (mt–) revealed that the repair-aberrant *rep27* phenotype cosegregated with the *ARG7* tag that was employed to generate the insertional mutagenesis library (data not shown). To determine the copy number of pJD67 plasmid insertions in *rep27*, Southern-blot analysis with the wild type and *rep27* genomic DNA was carried out, as follows. *ARG7-Nde*I primer set (Table II) was used to PCR amplify a 0.75-kb *Nde*I/*Nde*I DNA fragment, derived from the 3' end of the *ARG7* plasmid, as indicated in Figure 4 . This DNA fragment of

the *Arg-7* gene was employed as a probe in Southern-blot analysis of wild-type and *rep27* genomic DNA, digested with *Nco*I or *Hpa*I restriction enzymes. Figure 4A shows a single hybridization band between the probe and the wild-type genomic DNA corresponding to the endogenous inactive *ARG7* gene. The *rep27* showed polymorphism with two distinct hybridization bands between probe and mutant genomic DNA. In addition to the endogenous *ARG7* gene, a single extra DNA band was observed, confirming a single pJD67 plasmid insertion in the *rep27* mutant genome (Fig. 4A; see also Yokthongwattana and Melis, 2006).

4.1.4.2 Located the DNA insertional site in the rep 27 genomic DNA

Following the assessment of the insertional DNA coppy in the rep27 mutant genomic DNA , locating of the position in which it interrupted in the rep 27 genomic DNA is performed. Southern hybridization and PCR were conducted to test for the presence of the pBluescript origin of replication and ampicillin resistance, to be used in a plasmid rescue effort for the cloning of the genomic DNA flanking the pJD67 insertion site (Gumpel and Purton, 1994). Such analysis revealed that a significant portion of the 5' end of the plasmid was deleted, including the important ori and amp sites (Fig. 4B). Deletion of the 5' end of the transforming linearized plasmid is a common occurrence in DNA insertional mutagenesis (Tetali et al., 2007). As an alternative approach by which to clone the genomic DNA flanking the pJD67 insertion site, a thermal asymmetric interlaced (TAIL)-PCR was employed with *rep27* genomic DNA (Liu et al., 1995; Dent et al., 2005). TAIL-amplified PCR products were blasted against the Ver. 3.0 annotated *C. reinhardtii* genomic database (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). We found that the 3' end of the pJD67 plasmid interrupted the coding sequence of an exostosin-like gene (*open reading frame* [*ORF*]*0* in Fig. 4B) at contig 25 on scaffold 8. To define the opposite (5' end) site of the pJD67 insertion locus, we designed six marker primer sets for PCR-based diagnostic analysis (RT0–RT5; Fig. 4B; Table II). Reverse transcription (RT)-PCR results with the RT0 to RT5 set of primers revealed that a 25-kb fragment of the *C. reinhardtii* genomic DNA, encompassing the RT1-RT4 loci, was missing and apparently deleted during the pJD67 insertional event (Fig. 4B). Four *ORF*s were annotated in the missing fragment of the *Chlamydomonas* genomic DNA. Of those,

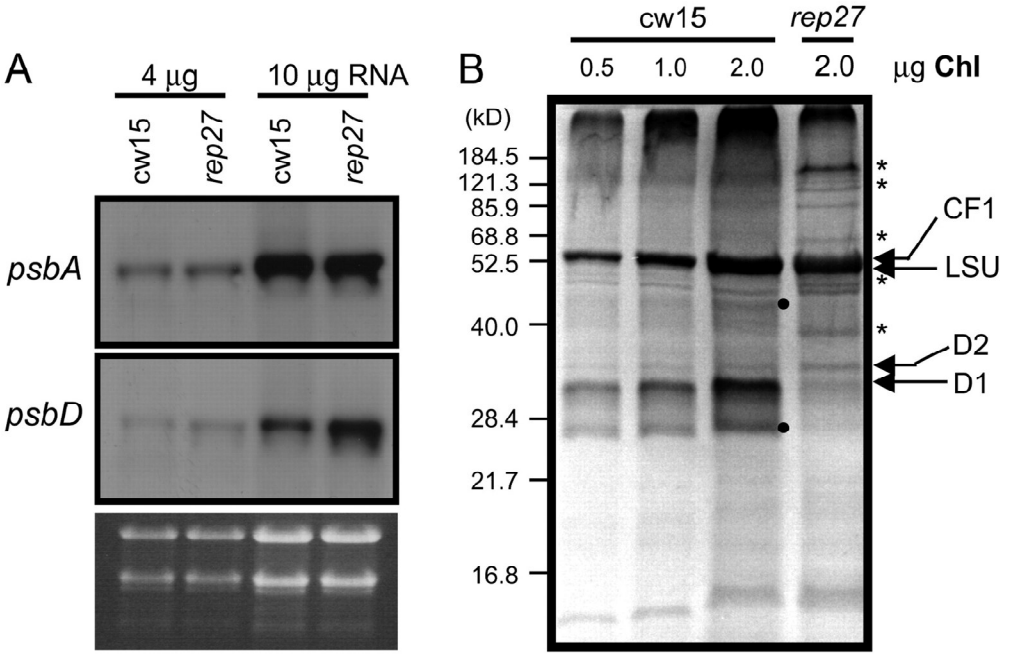

Figure 3 A, Northern-blot analysis of total RNA from 50-μE grown wild-type and *rep27* mutant strains separated onto 1.5% agarose gel. Following transfer to a nylon membrane, samples were treated with radiolabeled *psbA* and *psbD* cDNA. The *C. reinhardtii* rRNA loading control is shown at the bottom. B, Autoradiogram of [^{35}S]-radiolabeled proteins in *C. reinhardtii.* Chloroplast protein biosynthesis was conducted in vivo under 50 μE illumination for a period of 10 min (pulse) in the presence of [^{35}S]-Na_2SO_4. [^{35}S]-radiolabeled proteins were visualized upon exposure of the SDS-PAGE onto a radioactivity detector. The protein positions corresponding to D1, D2, Rubisco large subunit (LSU), and ATP synthase α-subunit (CF1) are denoted. Minor *rep27*-specific protein bands are denoted by asterisk, while black circles denote wild-type-specific bands.

ORF1 (Fig. 4B,shaded) turned out to be the gene necessary and sufficient to alleviate the acetate-requiring phenotype of the *rep27* strain (please see below). The TAIL-PCR-amplified genomic DNA flanking the insertion site was subsequently used as a probe for the screening of a *C. reinhardtii* bacterial artificial chromosome (BAC) genomic library, obtained from the Clemson University Genomics Institute (https://www.genome.clemson.edu/). Four BAC clones (27N13, 10C5, 10A5, and 10K12) hybridized to the TAIL-PCR-amplified genomic DNA fragment (data not shown). Restriction patterns of these BAC clones showed that they were identical to each other and contained the missing 25-kb genomic DNA fragment of *rep27* (data not shown).

4.2 Complement of REP27 Genomic DNA to rep27 mutant

4.2.1 Cloning of the genomic DNA flanking the pJD67 insertion site

After the position of interrupted gene including it's 5' and 3' end flanking were verified. It was subsequently used as a probe for the screening of a *C. reinhardii* bacterial artificial chromosome (BAC) genomic DNA Library. To find the clone that contain the genomic DNA fragment that can rescue rep27 mutant. The result shown that four subclones (1A, 2A, 4A, and 8) isolated from BAC clone 10A5 were used for the *rep27* complementation experiments. 10A5 was used to complement the *rep27* acetate-requiring phenotype. Cotransformation of the *rep27* mutant with the BAC clone 10A5 DNA was implemented with the pSL18 plasmid, conferring paramomycin resistance (Depege et al., 2003). Transformants were selected on TAP-agar plates containing 5 μg/mL paramomycin. To test whether BAC clone 10A5 complements the acetate-requiring phenotype of *rep27*, each BAC transformant (approximately 200 colonies in total) was replica plated onto paramomycin containing TAP-agar and TBP-agar media. One such transformant,
Which showed robust and unequivocally photoautotropic growth in TBP media (Fig. 5A , BAC-T), was recovered and further analyzed by PCR (Fig. 5B). A randomly selected BAC clone 10A5 transformant (BAC-C), which did not grow on TBP media, was chosen as a negative control for the molecular and PCR analysis. Genomic DNA from BAC-T and BAC-C were used as templates in these PCR amplifications.

Table 2 Primers used for *rep27* cloning

1	2	3	4	5
TAIL PCR	TPB1	GGATGACGATGAGCGCATTGTTAGATTTC		
	TPB2	CACGGTGCCTGACTGCGTTAGC		
	TPB3	CTGCGTTAGCAATTTAACTGTG		
	TPDeg1	NTCGWGWTSCNAGC		
	TPDeg2	WGNTCWGNCANGCG		
Deleted genes check	RT0	GAGCCGCAGTGCAAGGAG	CAGGTGTTCGCCACCGGTG	198
	RT1	AAGTTTGTACATGCACAGAAGGAC	GTTGCAGCTATTGAGGCAGAG	605
	RT2	TTCCAGACTTCAAAGCACTCGTG	TTCAAGGTTTTAGCTGGAATGCG	362
	RT3	TGTCGCTGTCATCCAAGTTAGTG	AGACACGTTGACTGCAGTCGAAAG	254
	RT4	GGATACGGCAAGTTATGTCGAG	GTGTGTATAACTACACTTGGCATC	280
	RT5	GCAACAAGACGCACACTCTGTG	GTTACATTACTCCGCTTATCCAAG	856
Full size REP27 cDNA cloning	27P-F3	CTAGGTCAAGCAGACAGATG		
	27E4-F	GAACGAATCGCTGACGAACCTG		
	27E5-R	GAACTTGAGCAGGGGCAGCACAC		
	27E11-R1X	GCCTTCTAGAGTCTACTCCTGCGGCCCCAC		
Probe prep	ARG7-NdeI	GTTTGTGCAGGAGTGTTGGGAG	AACGTTCGATAGCTCTCACAAC	672
	PSBA	ATGACAGCAATTTTAGAACGTCG	TTAGTTGTTTGAGCTAGAGTTAG	1059
	PSBD	ATGACAATTGCGATCGGTACATA	TTATAGAGCGTTACCACGTGTAAT	1057
RACE PCR	RACE-A	GCATTTCCAAGCCAACACG		
	RACE-B	CGTTGGGCTGCATTTCCAAG		
	RACE-C	CACGAGCCTCATCGTCGTTG		

1. Purpose of primers, **2**. Primer names, **3**. PCR primer sequence 1, **4**. PCR primer sequence 2, **5**. Estimated amplification product size.

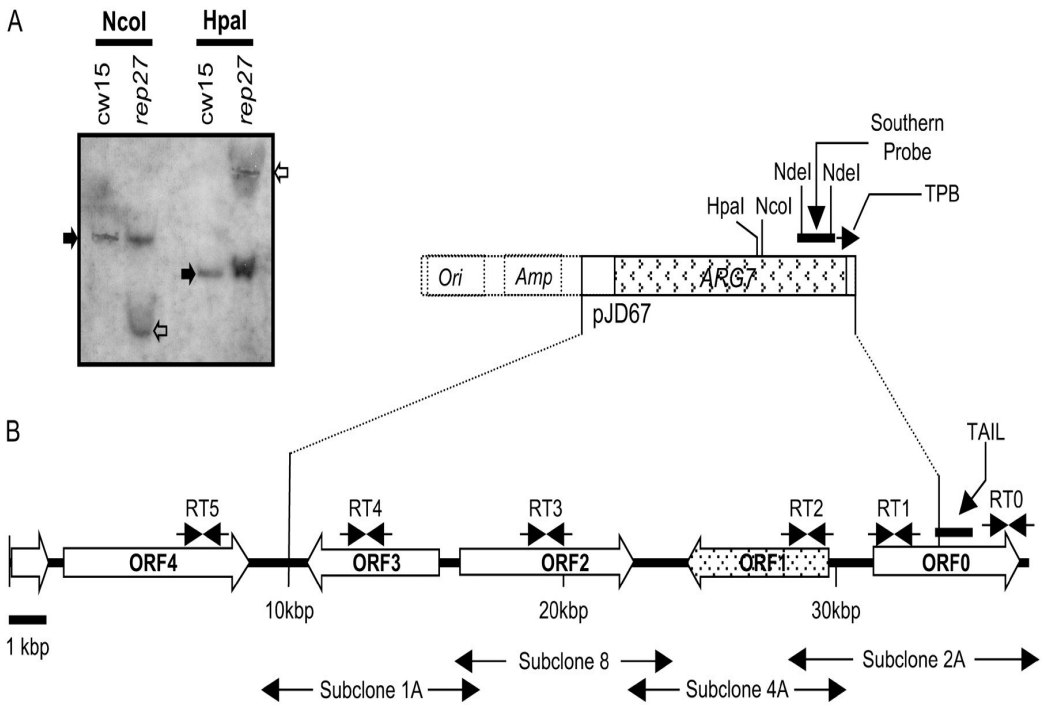

Figure 4 A, Southern-blot analysis of *C. reinhardtii* genomic DNA using the *Nde*I/*Nde*I 3' end of the *Arg-7* gene on *Nco*I or *Hpa*I digested wild-type and *rep27* genomic DNA. Black arrows indicate the position of the intrinsic inactivated *ARG7* genes in the wild-type and *rep27* DNA and white arrows show the position of a single additional band resulting from the insert DNA in the *rep27* mutant. B, *C. reinhardtii* genomic DNA map, derived from the JGI *Chlamydomonas* Genome Project, showing the relative position of five ORFs (scaffold 8, contigs 25 and 26) and the insertion locus of pJD67. Restriction enzyme sites (*Nco*I and *Hpa*I) used in the digestion of genomic DNA and the position of probes for Southern-blot analysis (*Nde*I/*Nde*I) are indicated on the pJD67 plasmid. The dotted rectangle in the 5' end of the pJD67 shows the missing pBluescript portion, apparently deleted during the pJD67 insertion. Also indicated by opposite-facing arrows is the location of six primer sets (RT0–RT5). Four subclones (1A, 2A, 4A, and 8) isolated from BAC clone 10A5 were used for the *rep27* complementation experiments. TAIL-PCR amplified fragment (TAIL) and the position of the TPB primer for the TAIL-PCR also shown.

Figure 5B shows that RT0 and RT5 primer sets, probing a DNA region outside the deleted 25 kb in the *rep27* strain, generated PCR products, consistent with the presence of this genomic DNA in the mutant. The RT1 primer set failed to generate PCR products in either BAC-T or BAC-C transformants, suggesting that *ORF0* may not be responsible for the autotrophic growth properties of the BAC-T strain. RT2 primer set successfully generated PCR products from the BAC-T genomic DNA only, but not from the BAC-C DNA, suggesting that *ORF1* is present in the BAC-T and might encode a protein that confers autotrophic growth properties of the BAC-T strain (Fig. 5B). RT3 primer set successfully generated PCR products in both BAC-T and BAC-C transformants. Conversely, RT4 primer set generated PCR products in the BAC-C but not in the BAC-T transformant, suggesting that *ORF3* may not be responsible for the autotrophic growth properties of the BAC-T strain. These results point to *ORF1* as the likely gene candidate that confers autotrophic growth properties to the *rep27* mutant. The results also provide testimony to the complex nature and unpredictable outcome of *C. reinhardtii* complementation by BAC clone DNA. DNA. PCR amplification of BAC-T and BAC-C was performed with a variety of different marker primer sets (RT0–RT5), as indicated.

Because the mutant phenotype of *rep27* was successfully complemented by BAC clone 10A5, *Pst*I partially digested 10A5 DNA fragments were ligated onto the pBR322/*Pst*I vector to isolate subclones, which contained the various deleted *ORF*s (*ORF0–ORF3*). Four different subclones, each containing ORFs from the deleted 25-kb region, were identified by DNA hybridization and direct sequencing analysis (Fig. 4B, subclones 1A, 8, 4A, and 2A). Transformants of the *rep27* mutant with each of these subclones were tested for autotrophic growth on TBP minimal media. Only subclone 4A, containing a 6.2-kb *Pst*I partial-genomic DNA fragment that includes *ORF1*, successfully rescued the acetate-requiring phenotype of the *rep27* mutant. Transformation of the *rep27* mutant with any other subclone (1A, 8, and 2A) failed to rescue the acetate-requiring phenotype of *rep27* (data not shown). Accordingly, *ORF1* was designated as the putative *REP27* gene.

4.2.2 Physical and Biochemical analysis of rep27-complented strain

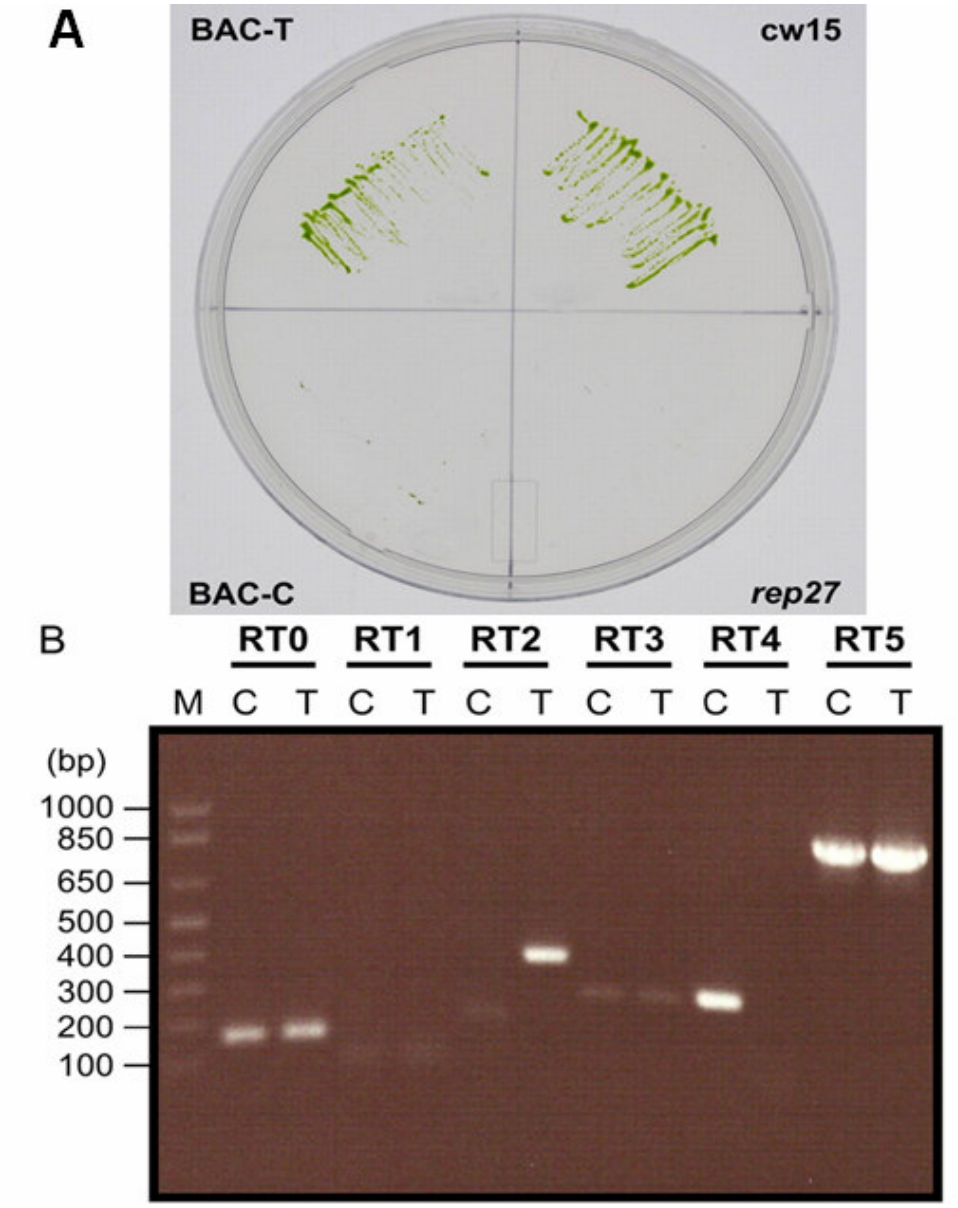

Figure 5 A, Growth of wild type, *rep27* mutant, and BAC clone 10A5 complemented *rep27* strains on TBP minimal media under 50-μE irradiance. BAC clone 10A5, which contains the 25-kb deleted genomic DNA fragment, successfully rescued the acetate-requiring mutant phenotype in *rep27* (BAC-T). BAC-C was randomly selected as a negative control for BAC-T. B, PCR characterization of BAC-T and BAC-C genomic DNA. PCR amplification of BAC-T and BAC-C was performed with avariety of different marker primer sets (RT0–RT5), as indicated.

For further molecular and biochemical analysis, three independent transformant lines with subclone 4A were selected (*rep27*-complemented [*rep27*-comp] strains). In the subsequent physiological and biochemical analyses, and to alleviate crowding and enhance clarity of the presentation, results from only one line are reported. However, all three lines displayed similar properties to those of the *rep27*-comp strain shown. RT-PCR with the RT2 primer set confirmed the presence of the *REP27* transcript in the *rep27*-comp strains (data not shown). Because the *rep27*-comp strains were selected on the basis of growth on TBP-agar minimal media, which lack acetate, it was initially assumed that transcription of *REP27* is necessary and sufficient to rescue the acetate-requiring phenotype of the *rep27* mutant. To further test this hypothesis, the F_v/F_m fluorescence yield ratio and photosynthetic activity in wild-type, *rep27* mutant, and *rep27*-comp strains were measured under a variety of growth conditions.

A phenotype of the *rep27* mutation is the lower-than-wild-type F_v/F_m ratio under all growth irradiance conditions (Fig. 1C), consistent with the interpretation that the *rep27* strain is a PSII repair-aberrant mutant (Zhang et al., 1997). The effect of light-shift experiments on the F_v/F_m value of the *rep27*-comp strains was compared to that of the wild type and *rep27* mutant (Fig. 6A). Cells grown under 10 μE were transferred to approximately 1,000 μE. Chl fluorescence yield parameters were measured as a function of exposure time to 1,000 μE (Fig. 6A). Prior to the light shift, F_v/F_m for the wild type and *rep27*-comp strain was high (about 0.78), whereas it was considerably lower for the *rep27* mutant (0.37). F_v/F_m for *rep27* decreased to nearly zero within 1 h under a 1,000-μE irradiance, whereas the F_v/F_m for the wild type and *rep27*-comp strains declined to about 0.45 in the same period of time. These results are consistent with the notion that *rep27* is a PSII repair-aberrant mutant and that complementation of this strain with a wild-type copy of the *REP27* gene rescues the mutation. The activity of photosynthesis and the response of wild-type, *rep27*, and *rep27*-comp strains to irradiance were further evaluated from the measurement of the light-saturation curve of photosynthesis (Fig. 6B). In the wild-type and *rep27*-comp strains, photosynthetic activity increased as a function of light intensity with identical slopes (quantum yield of photosynthesis) and reached a Pmax of approximately 40 mmol O_2 (mol Chl)$^{-1}$ s^{-1} at about 1,000-μE light intensity. This pattern was nearly

indistinguishable among wild-type and *rep27*-comp strains and independent of growth irradiance in the 10 to 50 μE region. Contrary to the wild-type and *rep27*-comp strains, the *rep27* mutant showed a quantum yield of photosynthesis (initial slope in the photosynthesis versus irradiance curve) that was only about 50% of that in the wild-type and the *rep27*-comp strains (Fig. 6B), consistent with the approximately 50% lower F_v/F_m and with the notion of a state of chronic photoinhibition in the mutant. It is also of interest to observe that a lower Pmax of approximately 15 mmol O_2 (mol Chl)$^{-1}$ s^{-1} in the *rep27* mutant was reached at about 1,000-μE light intensity. Upon exposure to progressively higher light intensities, Pmax declined in the *rep27*, a consequence of the progressively faster rates of PSII photodamage coupled with the mutant's inability to complete the repair reactions. It is also of interest to note that this photoinhibitory effect was more pronounced with the mutant strain grown at 50 rather than 10 μE (Fig. 6B, white versus black triangles).

The preceding analysis suggested that *rep27* undergoes a normal biogenesis/assembly of functional PSII units when grown in the presence of acetate, but cannot repair them when PSII photodamage occurs. There is thus a competition between the de novo biogenesis/assembly of functional PSII units in the presence of acetate and the irreversible inhibition of PSII due to photodamage in the *rep27* mutant. This interpretation requires that either photodamaged and inactive D1 accumulates in the thylakoid membrane or, if the lesion is in a step subsequent to inactive D1 degradation, a depletion of the D1 protein in the mutant relative to that in the wild type. To delineate between these two different mechanistic alternatives, western-blot analysis was applied to assay steady-state levels of D1 protein in wild-type, *rep27*, and *rep27*-comp strains (Fig. 6C). It is seen that *rep27* has substantially lower steady-state amounts of the D1 protein relative to the wild type, whereas the *rep27*-comp strain displayed levels of D1 that were comparable to those of the wild type. These results strengthened the notion that the *rep27* lesion does not prevent degradation of the photodamaged and inactive D1, but it does interfere with the next step, i.e. the biosynthesis and/or cotranslational insertion of the nascent D1, thereby impeding the completion of the D1 protein turnover and the PSII repair process.

4.2.3 *REP27* Gene and protein structure identification

To identify the transcription start site of the *REP27* gene, 5′ RNA ligase mediated-RACE was employed and resulted in the identification of 455 coding region nucleotides, in addition to those reported by the U.S. Department of Energy Joint Genome Institute (JGI) for ORF1 (Fig. 4). These occurred in the upstream region of the JGI-reported ORF1 translation initiation site. A full-length REP27 cDNA was subsequently isolated by RTPCR amplification using 27P-F3 and 27E11-R1X primer set (Table II), which confirmed the extended REP27 coding sequence. The REP27 cDNA nucleotide sequence was deposited in the GenBank with accession number EF127650, predicted to encode a 449-amino acid putative precursor protein (Fig. 7A), Amino acid sequence analysis by ChloroP1.1 software (Emanuelsson et al., 1999) predicted that REP27 contains a chloroplast transit peptide. Similarly, application of the HMMTOP v2.0 software (Tusnady and Simon, 2001) pointed to the existence of two putative transmembrane helices in REP27 (amino acid regions TMH1 and TMH2 including amino acids 180–202 and 217–239, respectively; Fig.7A), which may suggest that REP27 is a thylakoid membrane integral protein. Application of Inter-ProScan 13.1 software analysis (Quevillon et al., 2005) identified two distinct *TRP* motifs in REP27 (TPR1A/TPR1B and TPR2A/TPR2B; Fig. 7A). The helix-rich TPR motifs have been described in the literature to form a compact unit of two helices interacting with each other in the antiparallel direction. It is thought that such conserved TPR motifs are involved in a variety of critical protein-protein interactions in the living cell (for review, see Lamb et al., 1995) and are consistent with the putative role of the REP27 in the D1 turnover and PSII repair. A search of the National Center for Biotechnology Information database revealed the occurrence of six *REP27* homologs, including two copies each in rice (*Oryza sativa*), Arabidopsis (*Arabidopsis thaliana*), and Ostreococcus, a small unicellular green alga whose genomic DNA sequence and annotation recently became available (Derelle et al., 2006). Moreover, a search of the *C. reinhardtii* genomic DNA database revealed the occurrence of a *REP27* homolog in this green alga. Accordingly, it appears that two copies of *REP27* homologs are present in each of these photosynthetic organisms. These can be divided into two distinct groups: *REP27* orthologs (Os01g0358300, rice; At1g02910, Arabidopsis; and CAL55849, Ostreococcus) contain the chloroplast transit peptide and two TPR motifs (Fig. 7A), while *REP27* paralogs (Os04g0507100 for rice;

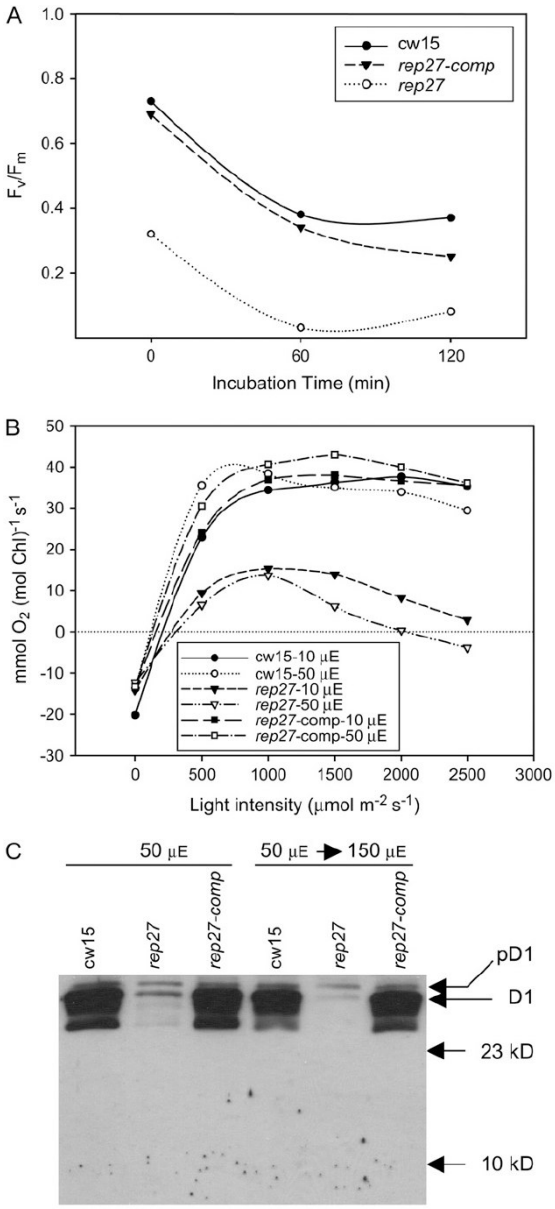

Figure 6 Physiological and biochemical analyses of *C. reinhardtii* wild-type, *rep27*, and *rep27*-comp strains. A, Strains were grown on TAP under 10 μE and transferred to 1,000 μE for different periods of time. F_v/F_m was plotted as a function of incubation time in 1,000 μE. Values shown are the average of two independent experiments. B, The light-saturation curves of photosynthesis in wild-type, *rep27*, and *rep27*-comp strains grown in TAP under 10- or 50-μE conditions. Registration of the dark respiration in the cell suspension was followed by 5-min measurements of the rate of oxygen evolution at 500, 1,000, 1,500, 2,000, and 2,500 μE. The values shown are the average of two independent experiments. C, Western-blot analysis of total cell protein extracts from wild-type, *rep27*, and *rep27*-comp strains loaded on an equal Chl basis. Proteins were probed with specific anti-D1 polyclonal antibodies.

At2g28740 for Arabidopsis; CAL58275 for Ostreococcus; and C_142189 for *Chlamydomonas*) are devoid of both a transit peptide and the TPR domains (data not shown). REP27 had about a 35% amino acid sequence identity with its orthologs (CAL55849, Os01g0358300, and At1g02910). However, it had limited similarity and That was only in the C-terminal half of the protein with the paralogs, which were devoid of transit peptide and TPR motifs.

The REP27 protein appears to exist only in grana-containing organisms of oxygenic photosynthesis. The phylogenetic comparison diagram in Figure 7B shows that *REP27*, CAL55849, Os01g0358300, and At1g02910 are grouped together and, therefore, closely related. Likewise, REP27 paralog proteins Os04g0507100, At2g28740, CAL58275, and C_142189 are closely related among them and distantly related to the former. This may suggest a distant phylogenetic relationship between the two groups of genes and proteins and points to the possibility that *REP27*-like genes in photosynthetic eukaryotes exist in pairs, one copy encoding a putative chloroplast-targeted protein, the other encoding a nonplastid counterpart.

4.2.3 *REP27* Gene Expression Requirement for D1 Protein Turnover But Not for de Novo PSII Biogenesis/Assembly

To test whether expression of the *REP27* gene is constitutive or induced by irradiance, steady-state *REP27* mRNA levels were measured before and after a shift in light intensity. Northern-blot analysis with a probe specific for the *REP27* gene showed that 1,000 μE-treated wild-type cells had about 5 times more transcript accumulation than that of 50 μE-acclimated control cells (Fig. 8A). This observation is consistent with the well-documented, irradiance-induced increase in repair gene expression, including *ELIP*/*Cbr* (Fig. 8B) and *HSP70B* (Yokthongwattana et al., 2001). Such increase in gene expression is though to be a meaningful response of the cells to the increased rates of photodamage and the need for enhanced rates of repair with increasing irradiance (Melis, 1999; Yokthongwattana and Melis, 2006).

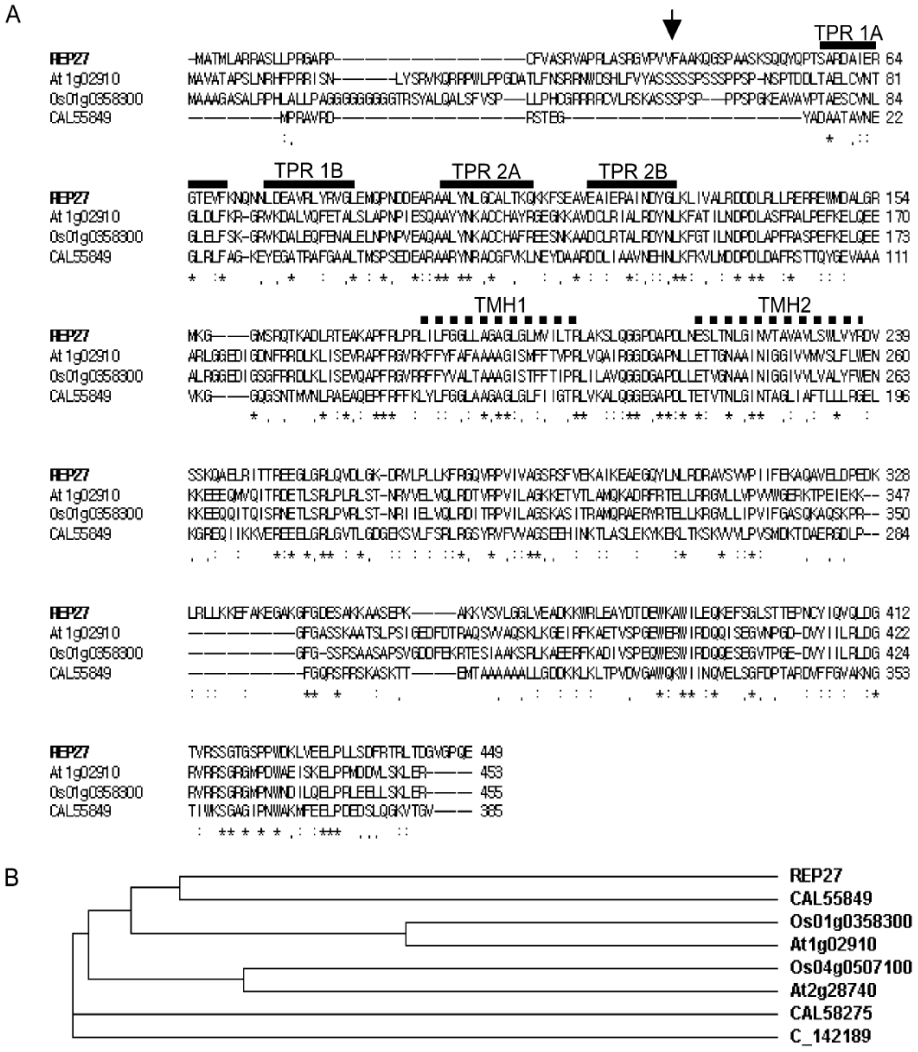

Figure 7 Deduced amino acid sequence alignments (ClustalW analysis; Higgins et al., 1994) of REP27 and its homologs. A, Database searches revealed that the deduced amino acid sequence of rice (Os01g0358300), Arabidopsis (At1g02910), and Ostreococcus (CAL55849) gene products are orthologs to that of *REP27*. Asterisks mark absolutely conserved residues among the four protein sequences. High and low similarity residues are marked by double or single dots, respectively. Vertical arrow indicates the predicted cleavage site of the chloroplast transit peptide targeting sequence. Solid lines and dotted lines over the amino acid sequences indicate the TPR motifs and predicted transmembrane helices, respectively. B, Phylogenetic tree showing the relative evolutionary proximity of the REP27 ortholog (Os01g0358300, At1g02910, and CAL55849) and paralog proteins (Os04g0507100 [rice], At2g28740 [Arabidopsis], CAL58275 [Ostreococcus], and C_142189 [Chlamydomonas]) based on the amino acid sequence comparisons.

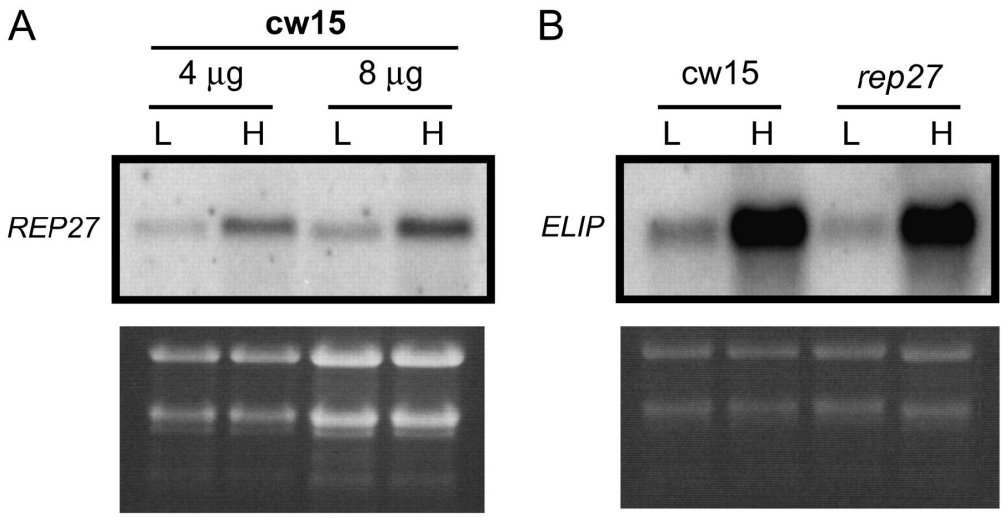

Figure 8 A, Northern-blot analysis probed with *C. reinhardtii REP27*-specific DNA. Wild-type cells, grown under 50-μE conditions (L), were incubated under high light (1,000 μE, H) for 1 h. Agarose gel lanes were loaded with 4 or 8 μg of total RNA from the 50 μE-grown (L) and 1,000 μE-exposed (H) cells, transferred to nylon membrane, and probed with a radiolabeled full-size *REP27* cDNA probe. A (bottom), rRNA loading control. B, Northern-blot analysis probed with *C. reinhardtii* ELIP-specific DNA. Wild-type and *rep27* mutant grown under 50-μE conditions were incubated under high light (1,000 μE) for 1 h. Agarose gel lanes were loaded with 4 μg of total RNA from the 50 μE-grown (L) and 1,000 μE-exposed (H) cells, transferred to nylon membrane, and probed with a radiolabeled full-size *ELIP* cDNA probe. Ribosomal RNA loading controls are shown at the bottom.

CHAPTER V
DISCUSSION

rep27, a Putative PSII of Repair Mutant

It is known that specific D1-less (Δ*psbA*) or D2-less (Δ*psbD*) mutants fail to assemble the entire PSII core complex (Bennoun et al., 1986; Erickson, 1986; Cohen et al., 2001; Baena-Gonzalez et al., 2003). The *rep27* is distinct from such mutants, as it undergoes biogenesis/assembly of a functional PSII holocomplex but fails to perform the D1 reaction center protein turnover. Therefore, under weak growth irradiance, *rep27* displays a limited water oxidation and O_2 evolution capacity. Under moderate to high rates of photodamage, PSII activity ceases to exist.

REP 27 Structure

Due to incomplete annotation of the *C. reinhardtii* genomic DNA sequencing in the *REP27* locus, the true transcription initiation site for this gene was not available. 5' RACE PCR was performed in this work, resulting in the proper identification of the transcription initiation site and translation start locus of *REP27*, which is 261 bp upstream from the tentative translation start site reported by the JGI. A search of the GenBank database identified a pair of REP27 homologs in photoautotrophic eukaryotes, including Chlamydomonas, Arabidopsis, rice, and Ostreococcus (Fig. 7). REP27 orthologs contained both the transit peptide for chloroplast targeting of the protein, the two apparently conserved TPR motifs, and the two transmembrane domains. REP27 paralogs lacked both the transit peptide for chloroplast targeting of the protein and the two apparently conserved TPR motifs and are apparently cytosolic proteins.

An Arabidopsis T-DNA mutant was isolated in which the *REP27* ortholog gene was interrupted by the T-DNA insertion. This mutant, termed *lpa1* for low PSII accumulation (Peng et al., 2006), had many characteristics similar to those reported in this work for the *rep27* mutant. However, contrary to the conclusions reached in this work, Peng et al. (2006) assigned a PSII biogenesis function to the LPA1 protein in

Arabidopsis, as opposed to the specific D1 turnover function of the REP27 gene in this work.

TPR motif-containing proteins are widely encountered among a variety of organisms, including nonphotosynthetic bacteria, cyanobacteria, yeast (*Saccharomyces cerevisiae*), fungi, plants, animals, and humans (Blatch and Lassle, 1999). The number of TPR motifs is also variable, and no position preference exists with respect to their location along the primary polypeptide sequence. TPR domains are highly degenerate 34-amino acid repeats that are often arranged in tandem arrays, known to mediate protein-protein interactions (Lamb et al., 1995). Since the discovery of a TPR motif in soybean (*Glycine max*), several cases of TPR-containing proteins in eukaryotic chloroplasts have been reported and characterized (Hernandez Torres et al., 1995). For example, the subunits of the translocation machinery in the chloroplast envelope (Tic40 and Toc64) contain a single and three TPR motifs, respectively (Sohrt and Soll, 2000; Chou et al., 2003). Recent studies revealed that TPR domains in Toc64 are responsible for the recognition of precursor proteins targeted to the chloroplast (Qbadou et al., 2006). Moreover, a TPR motif in Tic40 is believed to form a cochaperone with HSP70B and/or ClpC (Chou et al., 2003), possibly helping in the translocation of proteins into the chloroplast and/or cleavage of the transit peptide sequence from the imported precursor protein. The work presented in this article suggests that the REP27 protein is essential for the D1 reaction center protein turnover, probably facilitating translation and/or insertion of the nascent D1 in the vacated PSII reaction center template. It is of interest to speculate about the mechanistic role of TPR motifs in this process. TPR motifs are thought to be important factors in both transcription and translation processes. For example, Chlamydomonas Mbb1 and its Arabidopsis ortholog, HCF107, contains 10 and 11 TPR motifs, respectively, thought to be responsible for the mRNA stability and translation initiation of the plastidic *psbH* (Vaistij et al., 2000; Sane et al., 2005). NAC2, which has nine TPR motifs, is required for stable accumulation of the *psbD* mRNA in Chlamydomonas (Boudreau et al., 2000). A regulator of Chl biosynthesis, FLU, harbors two TPR motifs in its C terminus, which are required for interaction with glutamyl-tRNA reductase (Meskauskiene et al., 2001; Meskauskiene and Apel, 2002). A novel chloroplast protein, TCP34, with three TPR motifs, was localized in the fraction of

transcriptionally active chloroplast chromosome, indicating that TPR motifs possessed the ability to bind plastid DNA (Weber et al., 2006). The Ycf3 mutant analysis also showed that TPR motifs are involved in the PSI stability via a direct interaction with PsaA and PsaD (Naver et al., 2001).

It is well known that molecular chaperones, such as the cytoplasmic HSP70 and HSP90, interact with a number of cochaperones that contain TPR motifs (for review, see Blatch and Lassle, 1999). In the chloroplast, Tic40 may bind to chloroplast ClpC and HSP70B (Chou et al., 2003). In the green algae *Dunaliella salina*, the presence of chloroplast-localized HSP70B was detected as a component of the transiently forming 320-kD PSII repair intermediate (Kim et al., 1993; Melis and Nemson, 1995; Yokthongwattana et al., 2001).

REP27 function

It is possible (currently under investigation) that REP27 is also a component of this PSII repair intermediate, in a structure that may serve to preserve the conformation of the disassembled PSII core complex, from which the D1 protein has been temporarily vacated. Therefore, a working hypothesis is that the TPR motifs contained by the REP27 protein act as a receptor of the nascent D1 and are required for the proper guidance and insertion of the de novo-synthesized D1 protein in the existing PSII template. The REP27 and HSP70 proteins may act coordinately in the scaffold of the PSII repair intermediate in a process that facilitates the stability of the D1-less PSII complex and guides *psbA* cotranslational insertion of the de novo-synthesized D1 protein in the PSII template. Figure 9 shows a preliminary schematic, aiming to illustrate the step(s) in the PSII repair process and the possible mechanism of action for the REP27 protein. Consistent with the evidence presented in this work, de novo biogenesis/assembly of PSII in the presence of acetate is possible in *C. reinhardtii*, leading to the formation of functional PSII. Photodamage by excess irradiance leads to irreversible inhibition in the function of the D1 protein, occurring in the appressed thylakoid membranes of the chloroplast. PSII disassembly, followed by degradation of the inactive D1, takes place in stroma-exposed thylakoids (Melis, 1991, 1999; Ossenbuhl et al., 2002), leading to a D1 vacancy from the PSII reaction center complex. The latter serves as a template for the insertion of a de novo-synthesized

nascent D1 protein, a process catalyzed by the REP27 protein. According to this working hypothesis, the REP27 protein is essential for the incorporation of the nascent D1 but not for the de novo biogenesis/assembly of the PSII holocomplex. This model is consistent with the *psbA* mRNA association with thylakoid membranes, which is accentuated under conditions of enhanced photodamage and repair (Kettunen et al., 1997), underlining a cotranslational assembly of the D1 protein during the PSII repair (Zhang et al., 1999, 2000). In the *rep27* mutant, incorporation of the nascent D1 in the PSII template during the D1 turnover process is inhibited, leading to the phenomenology described in this work.

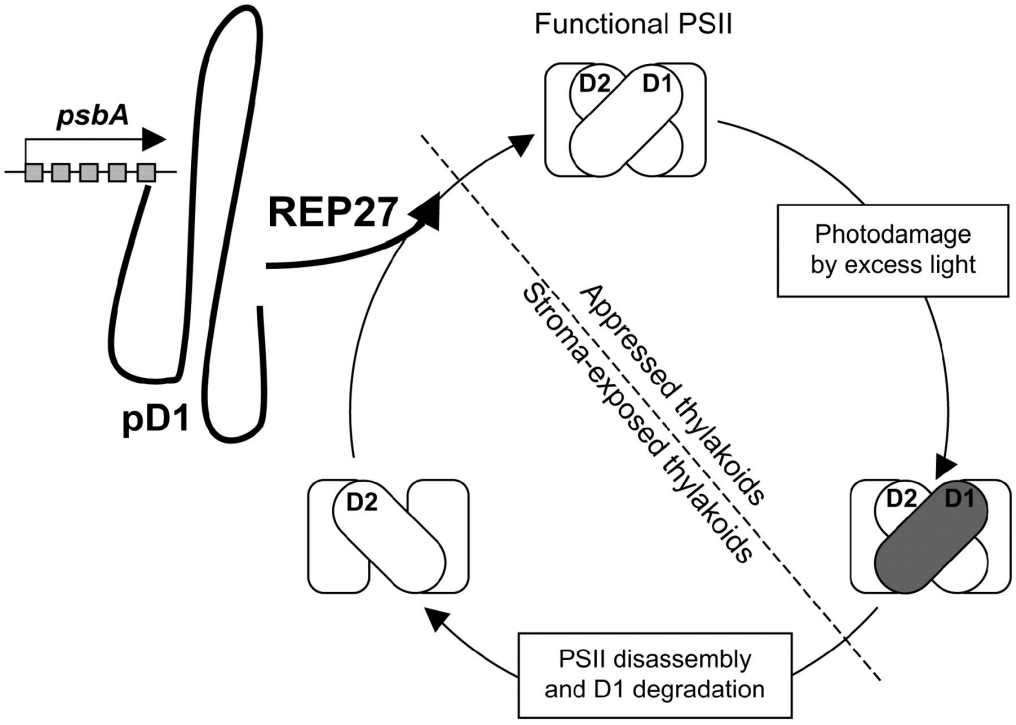

Figure 9 Schematic of the putative function of the REP27 protein in the D1 protein turnover and PSII repair process. REP27 is shown to play a role in the cotranslational insertion of a nascent D1 (pD1) in the D1-less PSII template during the PSII repair cycle. This step follows the PSII disassembly and specific degradation of photodamaged D1 in the thylakoid membrane.

CHAPTER VI
CONCLUSION

Since photosynthesis is very important for plant and subsequently for others living thing in the word. Investigation of the components relevant process is worth doing. Photosynthesis process is a result of the coordinated function of the photosynthetic membrane protein in the thylakoid membrane. The dominant protein that is very vital to this function is PSII protein complex. In the stress such as light stress can cause this protein to damage and the damage is to the PSII reaction center, D1 protein which carry many component that important for the photosynthesis reaction. If this protein damage, so that the photosynthesis is stopped, and plant is in critical living status. In order to stay alive, plant has developed the system to repair those protein by degradation, re-synthesize and reassembly into PSII complex which then can perform photosynthesis again. There are many proteins and many steps involving this process to be investigated. To reveal this, the photosynthesis organism that defect photosynthesis process have been generated and analyzed for protein that respond for those defection.

In this study the PSII repair aberrant rep 27 mutant *chlamydomonas reinhardtii* was generated by DNA insertional mutagenesis fallowed by molecular and biochemical analysis for the protein that missing and cause PSII not function. The result had shown that the foreigner DNA from plasmid that use in the process of mutagenesis interrupted the *chlamydomonas reinhardii* genomic DNA at the coding sequence of an exostosin-like gene (*open reading frame* [*ORF*]*0* Fig. 4 at contig 25 on scaffold 8. After the position of interrupted gene including it's 5' and 3' end flanking were verified. It was subsequently used as a probe for the screening of a *C. reinhardii* bacterial artificial chromosome (BAC) genomic DNA Library to find the clone that contain the genomic DNA fragment that can rescue rep27 mutant from acetate requiring. BAC clone 10A5 were used for the *rep27* complementation experiments. Co-transformation of the *rep27* mutant with the BAC clone 10A5 DNA was implemented with the pSL18 plasmid, conferring paramomycin resistance. After

screening for the transformant that can rescued rep27 mutant from the acetate requiring successfully. The structure of gene and protein was verified. The result shown that this gene is encoded for the protein REP27 which is synthesized in the cytosol to function in the thylakoid membrane as it structure contain the tetratricopeptide repeat which from the literature review shown that this part of protein is responded for protein binding. Suggesting that this protein play a role in the cotranslational insertion of a nascent D1 (pD1) in the D1-less PSII template during the PSII repair cycle (Fig. 9). This was shown by numbers of experiment, western blot of total protein (Fig. 2A) shown that only D1 amount of rep27 mutant lower than that of WT others protein is the same, this mean the defect of rep27 is to PSII reaction center protein D1. Northern blot (Fig. 3A) shown that psbA in WT and rep27 is the same level, this mean the defect of lower D1 is not a result of transcription step. Further analysis is to see the level of newly synthesis of chloroplast protein to see if lower D1 in rep27 than WT is resulted from defect in protein synthesis (Fig.2B). The result has shown that the synthesis of D1 is not defect as the protein intensity of others protein is a bout the same in WT and rep27 only amount D1 is grater in WT than rep27. Moreover, the newly synthesized of D1 and D2 in rep27 is the same but D1 is greater amount than D2 in WT which can confirm that there is more active turn over of D1 in WT than rep27.

From the evidences of lower D1 than WT, transcription is not defect, denovo synthesis of newly protein is also not defect, this mean rep27 mutant defect in PSII repair process and specific to the turnover of D1 not biosynthesis of D1. So that we can conclude that this mutant lack of REP 27 protein in which is specifically required for the completion of the D1 turnover process but is not essential for the de novo biogenesis and assembly of the PSII holocomplex in this model green alga.

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APPENDIX

Light inducible protein (ELIP) in *Chlamydomonas reinhardtii*

INTRODUCTION

ELIP is a nuclear-encoded chloroplast-targeted protein (Adamska, 2001). Its expression is dramatically increased in response to shifting of plants from dark to light. Originally it was proposed to function in the greening process of the etiolated plants (Meyer and Kloppstech 1984; Adamska 1995). Furthermore, stress protective role of Elip has been long been proposed and studied. In *Arabidopsis*, ELIP was expressed in response to high light intensities in which photoinhibition has occurred along with reduction of D1 protein of the PSII core protein (Adamska 1992; Hutin 2000). However, the protective function of ELIP is under controversy after knock-out mutants in *Arabidopsis* were studied and characterized. In the absence of ELIP protein the ability to recover from photooxidative stress or photoinhibition was indistinguishable from wild type (Rossini et al., 2006). In *Chlamydomonas* ELIP was previously reported as an Lhl1 protein, of which the transcription rate is induced in response to high light intensity (Teramoto H et al, 2002). However, the function of this protein was not investigated. In this thesis I have cloned and expressed *Chlamydomonas reinhardtii Lhl1 (elip*) in *E. coli* and generated polyclonal antibodies for further investigation. To elucidate the function of ELIP in *C. reinhardtii* first cloned *elip* cDNA in pSL18 vector aiming to overexpress in the algal cells. I also constructed *elip* downregulating vector using inverted repeat RNAi method (Pawloski 2005), aiming for post-transcriptional suppression of the *elip* transcripts in *C. reinhardtii*.

RESULTS

Expression of Elip in *E.coli* to generate antibodies for ELIP

C-terminal fragment (120bp) corresponds to about 40 amino acid residues was cloned and ligated to pGEX linking to 3'GST gene (Fig. A1). The plasmid was

transformed in to *E .coli* XL-blue strain for plasmid amplification. Plasmids were extracted from putative bacterial transformants and were subsequently cut with restriction enzyme to check for the right clone (Fig.A2a). Plasmids from the right clones were then transformed into *E. coli* BL21 (DE3) (Novagen). However, after induced gene expressed in *E.coli* by 0.1mM IPTG for 3hr at 25 °C, the protein was not express (Fig. A2b). After checking for codon usage in *E.coli*, I found that *elip* gene contains several rare codons for *E. coli* expression scattering throughout the coding sequence (Fig. A3). To solve this problem, I opted to find the fragment that contain fewest rare codons and performed site direct mutagenesis using Quik Change II (Stratagene) using following pair of primers

F 3T3- AGCTGTGGGCCGGCCGCCTGGCCATGCTGGGCTTCGC
R3C3- GCGAAGCCCAGCATGGCCAGGCGGCCGGCCCACAGCT

After mutagensis, the ELIP-GST construct could be expressed and purified by GST-agarose affinity (Sigma). The purified protein was used to generate ELIP polyclonal antibodies (Fig. A4).

Over-Expression of ELIP in *Chlamydomonas reinhardtii*

Full length (525bp) cDNA of elip gene linked to HA tag was cloned into pSL18 vector between Ndel and XbaI (Fig. A5) and transformed in to *E. coli* XLblue. The amplified plasmids were isolated and subjected to restriction cut check for the right clone (Fig. A6). Plasmid from the right clone was sequenced to verify the construct before it was linearized and transformed in to *C. reinhardtii* strain cc503 by glass bead method for overexpression.

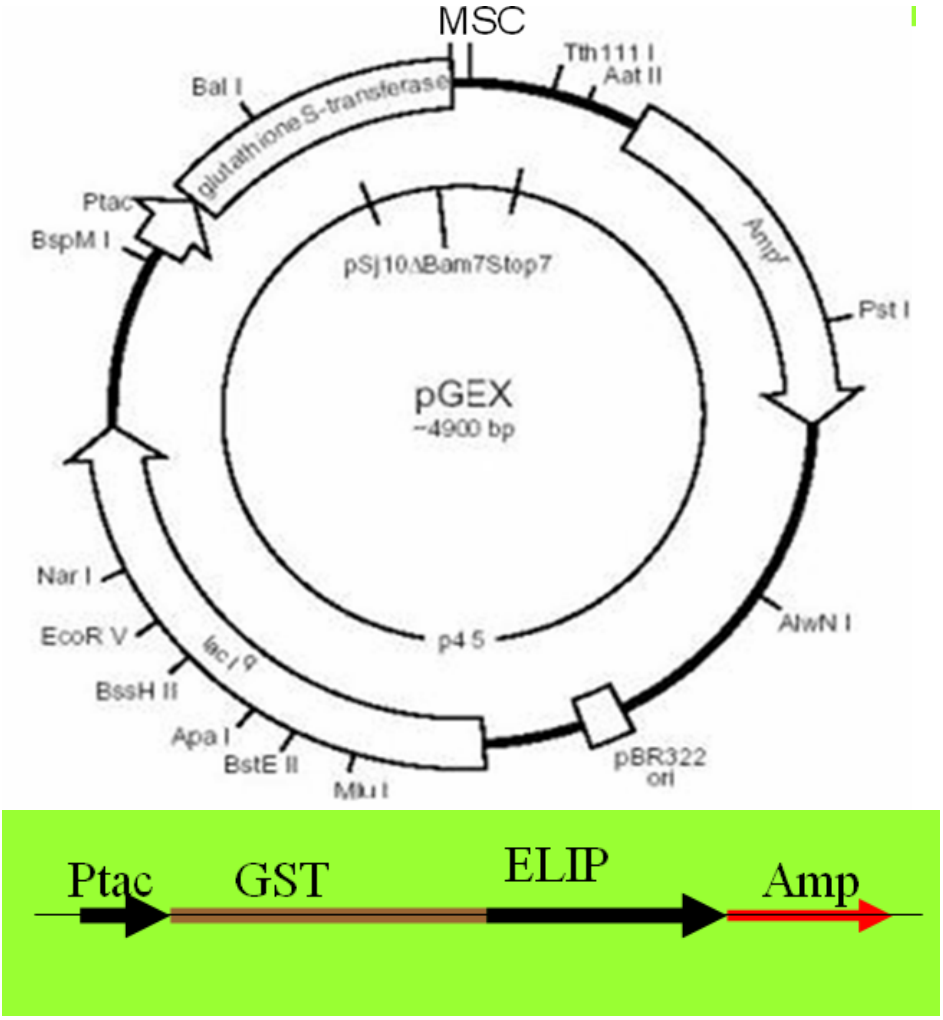
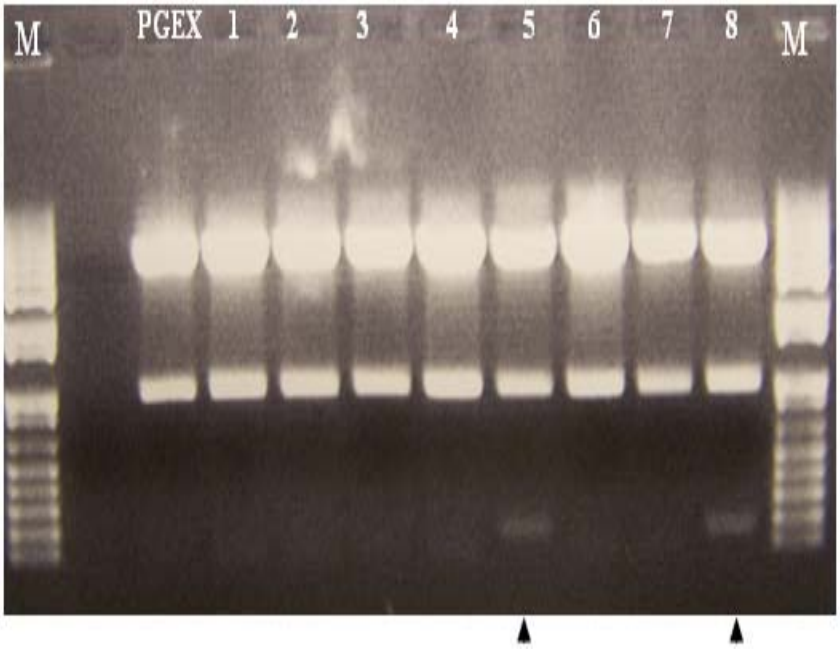


Figure A1 The plasmid construct for express ELIP protein in *E.coli*. Elip gene C-terminal fragment(120bp) 405-525 correspond for 40 amino acid 135-175 was constructed in MSC of pGEX linking to 3'GST gene under the control of Ptac promoter

(a)



(b)

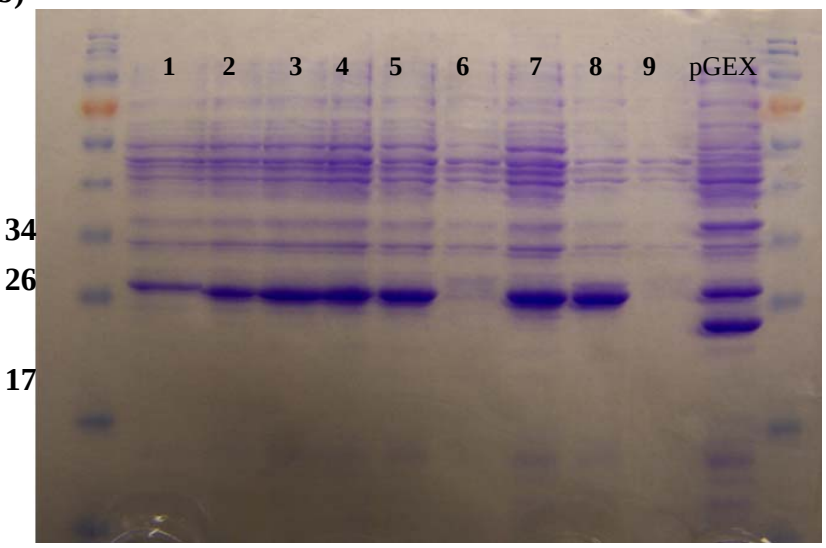
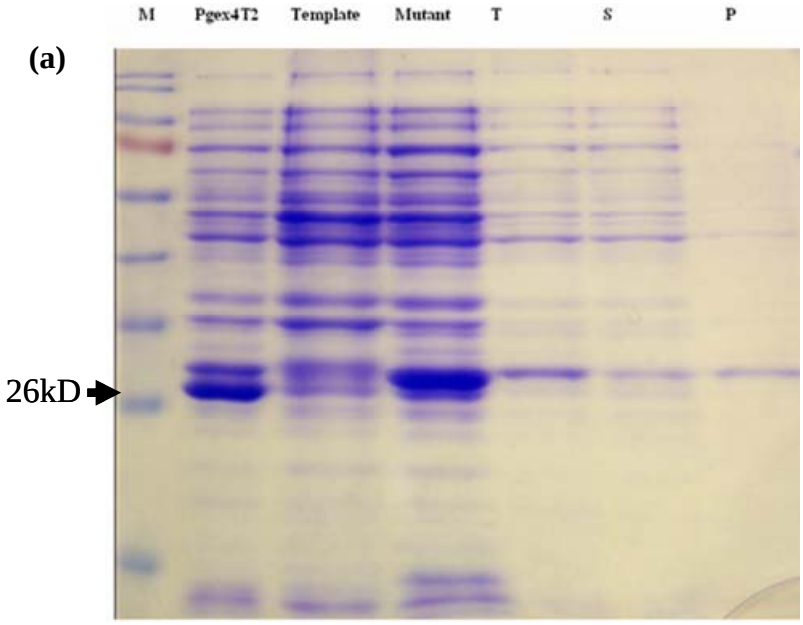
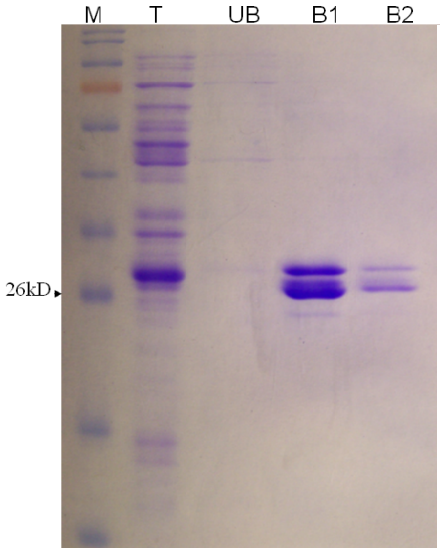


Figure A2 (a) Restriction cut check for the right clone (indicated by arrowheads)
(b) In duce express by IPTG

Figure A3 Elip sequence showing rare codon usage. Underline is the selected fragment containing fewest rare condons. The asterisk is the target of site-directed mutagenesis.





(b)

Figure A4 Expression of ELIP fragment

(a) Expression of *elip* after induction by IPTG M: Marker, T=Total fraction, S=soluble fraction , P=Pellet fraction

(b)) SDS-PAGE showing ELIP after purification by GST-Agarose affinity;

M: Marker, T=Total fraction, UB=Unbound , B1,B3=Bound Fraction; Arrow indicate the ELIP-GST fusion fragment.

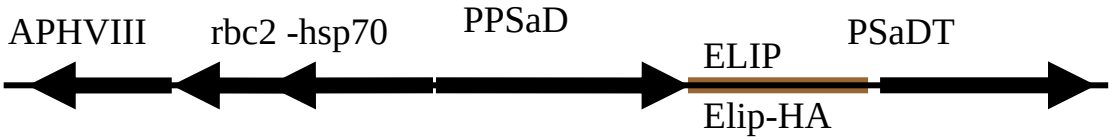


Figure A5 ELip over expression construct Elip or Elip link to HA tag was construct on pSL18 under pSAD promoter and pSAD terminator. The selective marker is paromomycin (APHVIII).

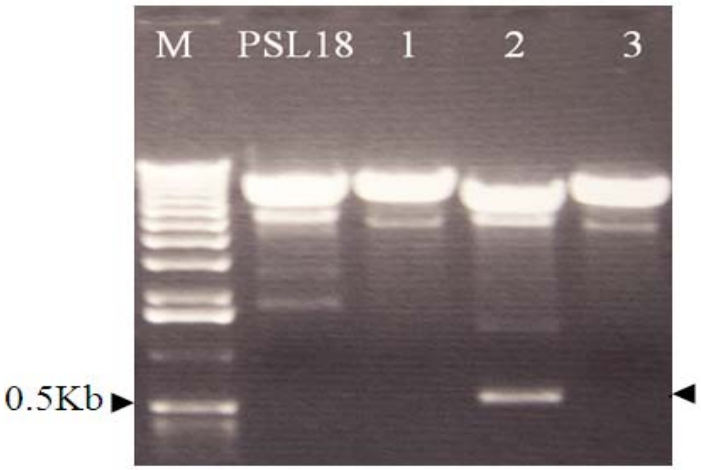


Figure A6 Agarose gel electrophoresis of restriction cut plasmids for the right clone. The DNA plasmid pSL18 and plasmids from 3 transformants (1-3) were isolated and restriction cut by Ndel and Xbal. The expected DNA insertion sized is 525 bp. Only clone number 2 contains the right product of 525 bp; M =1kb ladder marker, pSL18 = negative control.

Post-transcriptional suppression of ELIP in *Chlamydomonas reinhardtii*

Suppression of *elip* expression was performed by inverted repeat RNAi method with two-step cloning procedure. Sense (S) and anti-sense (AS) strands of partial *elip* coding sequence were cloned flanking Cytrocrome C6 intron (CyC6) downstream to paromomycin resistant gene (Par) under the control of *psaD* promoter and terminator of the psL72 vector (Fig. A7). The plasmid containing *elip* sense and antisense fragments was then cut and ligated into pSL18 plasmid. The psL18 carrying elip RNAi construct was extracted, linearized, and used for transformation into *C. reinhardtii* strain cc503 by glass bead method.

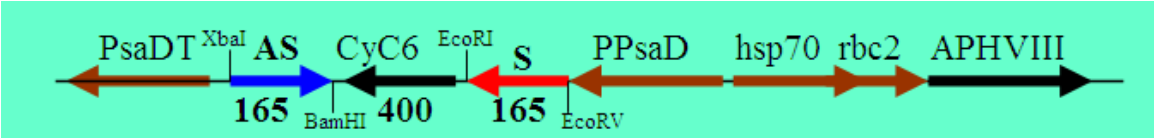


Figure A7 Elip RNAi construction for post transcriptional suppression of ELIP in *C. reinhardtii* and restriction cut for transformant analysis.
ELIP sense (S) and anti-sense (AS) strand were cloned flanking to Cytrocrome C6 intron (CyC6) under control of psaD promoter (PpsaD) and psaD terminator (psaDT) whereas paromycin resistant gene(APHVII), the selective marker was controlled by hsp70-rbc2 chimerical promoter.

After transformation of overexpression and downregulation vectors into *C. reinhardtii* and subsequent screening, I could not obtain the algal transformants that exhibit the desire phenotype.

CONCLUSIONS

ELIP from *C. reinhardtii* was successfully cloned an Expressed in *E.coli.* Polyclonal ELIP antibody was then generated. The full length ELIP was successfully

constructed on pSL18 and transformed into *C. reinhardtii* for ELIP overexpression. Vector for elip downregulatin was also successfully constructed in pSL18 and transformed into *C. reinhardtii*.

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BIOGRAPHY

NAME	Mr. Phichaya Khamai
DATE OF BIRTH	04 Febuary 1972
PLACE OF BIRTH	Uttaradit, Thailand
INSTITUTIONS ATTENED	Mahidol University , 1995-1996 Bachelor of Science (Technology in animal Production) Mahidol University, 1997-2001 Master of Science (Biochemistry) Mahidol University, 2004-2010 Doctor of Philosophy (Biochemistry)
SCHORLARSHIP	Strategic Scholarship for Frontier Research Network for the Ph.D. Program Thai Doctoral Degree
HOME ADDRESS	283 M2, Maenaruae District, Nampad Amphur, Uttaradit Province , Thailand,56000 E-mail: phichayak@gmail.com Tel. 087-0002043