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Construction and characterisations of Photosystem II mutants in *Chlamydomonas reinhardtii* for study of proton pathway

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Contents

Abstract

Oxygenic photosynthesis is a series of reactions that utilizing carbon dioxide and solar energy to form organic compounds meanwhile releasing oxygen which take place in cyanobacteria, plants, and green algae. Among all complex substances in charge of reactions, the enzyme photosystem Ⅱ (PS Ⅱ) plays a crucial role in water oxidation during light-driven energy transduction step. Water oxidation takes place in catalytic center of PS Ⅱ which undergoes a cycle of through five intermediate states(S0-S4) resulting in the release of an oxygen molecule and four protons. During S-state transitions, ineffective H+ transport can become a limiting factor. So far, to examine the proton pathway in PS Ⅱ catalytic center this study involves the construction of certain site-directed mutations in PS Ⅱ of *Chlamydomonas reinhardtii* (double mutations R334N/N335R,R334A/N335R, and single mutation N335L). With the mutants obtained, growth through selection media can be compared with computational data for testing further hypotheses.

Introduction

1.1Overview of Photosynthesis

Photosynthesis, the word comes from Greek words “light” and “putting together”. Literally, this process means “synthesis with light”. Indeed, the whole process takes light as energy source while inorganic substrates as raw materials to synthesize organic molecules in certain membranes or cell organelles. [1]

Photosynthesis mentioned in this project refers to oxygenic photosynthesis which is the most common type green plants and algae perform. Phototrophs are organisms that are able to accomplish photosynthesis. Among these, most phototrophs are photoautotrophs while they derive solar energy to produce organic molecules from simple carbon dioxide and water. Energy stored is usually in the form of organic molecules such as carbohydrates, fat and proteins providing cellular energy for its producer as well other heterotrophs like human and other animals. Since many photoautotrophs produce oxygen as a by-product, hence they are the founder of aerobic life and acts as a link between solar energy and all living forms on earth. [2]

1.2 Photosynthetic organelle——Chloroplast

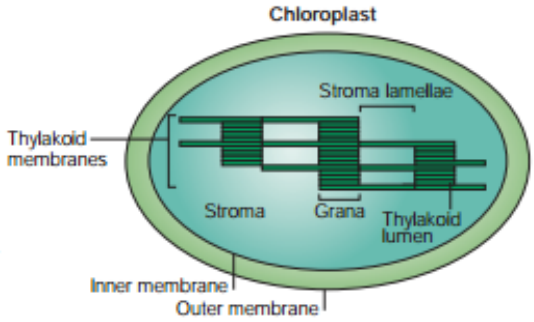
In eukaryotic photosynthetic organisms, organelle for photosynthesis is the chloroplast. In addition of emdosymbiosis mentioned above, chloroplasts are results of primary endosymbiosis which cyanobacterial-like cell becoming part of eukaryotic cell and introducing oxygenic-photosynthesis capability into host cell. [1]

The reasons why photosynthetic organisms are capable of photosynthesis basically depend on their pigments, if narrowly defined, chlorophylls. Photosynthetic pigments include certain type of chlorophylls, bacteriochlorophylls, even carotenoids and bilins. Pigments are always attached together with proteins in membranes to form functional units. Therefore accomplishment of the certain reactions photosynthesis is membrane-based, i.e. the core substances for photosynthesis are chlorophyll-associated membranes. [1]

Endosymbiosis results in chloroplasts becoming semiautonomous parts of eukaryotic photosynthetic organelles. Chloroplasts are about the size of a bacterium. Typical chloroplast (shown in Fig1.) is surrounded by two bilayer membranes while inside area is called stroma that is thought to be the cytoplasm of ancestor cyanobacterium. In the stroma, an extensive membrane called the thylakoid having function of initial light capture and storage while the inside of thylakoid known as thylakoid lumen. Furthermore, thylakoids are further condensed together to pack membranes system called grana which are connected by relax membranes called stroma lamellae. [1][4] Figure1a. displays a common chloroplast structure.

In this project, the experimental species *Chlamydomonas reinhardtii* have a single U-shaped chloroplast in each unicellular individual. Under electron microscopy *C. reinhardtii’s* chloroplast displays simple chloroplast structure, consists of packed and dense membranes

enclosed with granular materials and the starch grains. Besides, starch formed is stored in starch granules both are surrounded pyrenoid and scattered throughout the rest of chloroplast. The eyespot lies within the chloroplast at the edge is a carotenoid-containing body which color is deep orange under microscope. Figure1b. displays a simple sketch picture of *Chlamydomonas reinhardtii* structure. [3]



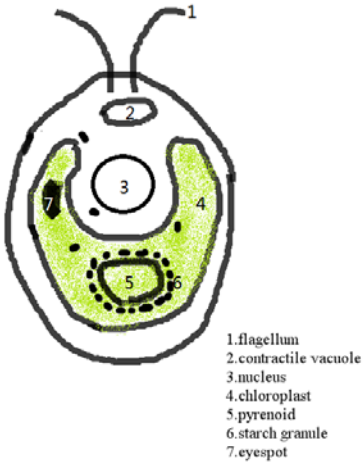


Figure 1a.Ordinary Chloroplast Structure [4] ; Figure 1b.Sketch of *Chlamydomonas reinhardtii* Structure

1.3 Photosynthesis Processes

Photosynthesis whole process can be seen as the opposite of cellular aerobic respiration. Often it can be divided into two main processes, light-driven energy transduction reactions and carbon assimilation reactions. Generally speaking, the reactions in energy transduction are light harvesting, electron transport to NADPH with simultaneous proton pumping, and ATP synthesis. With the ATP and NADPH synthesized from previous reactions together, CO_2 is involved in Calvin Cycles. Every six Calvin Cycle ends up with primary carbon-fixed product triose phosphates. Triose phosphates are later utilized to synthesize starch and sucrose. Since this project mainly focuses on one step in energy transduction reactions, only light-driven reactions are presented in details below.[2]

1.4 Light-driven energy transduction

Light has both properties of wave and particle, so light behaves as particles is called photons while each photon containing a quantum of energy. Chlorophyll absorbs photons and the energy brought by photons is transferred to excite electron. A photoexcited electron is unstable so the energy absorbed is transferred to other electron through resonance energy transfer——this process is called photochemical reduction. [2]

After photochemical reduction, light energy has been converted into chemical energy. Light-driven energy transduction comes to NADPH synthesis. Excited electrons from photochemical reduction are transported to oxidize NADP+ to NADPH——this process is called photoreduction. The reactions can be divided into four main parts. Photosystem Ⅱ transfers electrons from water to a plastoquinone, then the cytochrome b6/f complex transfers electrons from a plastoquinol to plastocyanin and photosystem Ⅰ transfers electrons from

plastocyanin to ferredoxin. Finally electrons are transferred to NADP+ by catalytic enzyme ferredoxin-NADP+ reductase forming one essential agent NADPH in the stroma.[2]

Energy transduction comes to the final step with the procedure of ATP synthase complex synthesizing ATP utilizing the energy stored in proton gradient. Protons move from low concentration in thylakoid lumen to high concentration across thylakoid membrane in stroma side.[2]

1.5 Photosystem Ⅱ (PS Ⅱ) structure and function

Photosystem is a kind of functional units which mainly comprises chlorophyll and pigments associated proteins. Chlorophyll binding proteins stabilize chlorophylls while pigments act as gathering light antenna. In photosynthesis process, there are two types of photosystems photosystem Ⅰ and Photosystem Ⅱ. Each PS has its own reaction center and distinguished functions. [2]Among all reaction centers photosystem Ⅱ (PS Ⅱ) is only one able to oxidize water.[1]

Located on thylakoid membrane in algae and plants, PS Ⅱ is a transmembrane enzyme which consists of intrinsic and extrinsic polypeptides, 2 out of 25 are the core reaction centers. They are two similar symmetrical subunits D1 and D2 which binds chlorophyll molecules. Because the maximum absorption wavelength of chlorophyll a molecules in PS Ⅱ is 680nm, so chlorophyll a of PS Ⅱ is named P_{680}. [2] Two kinds of plastoquinol Q_A and Q_B are tightly bound to D2 and D1 respectively and they have crucial role in electron transport. Aside of the reaction centers there are other intrinsic light-harvesting proteins CP43 and CP47 as well as extrinsic polypeptides, the magnesium-stabilizing protein (MSP) and light-harvesting complex II (LHCII) antenna complexes. [5] The structure of PS Ⅱ reaction center has already been determined through X-ray. [6]

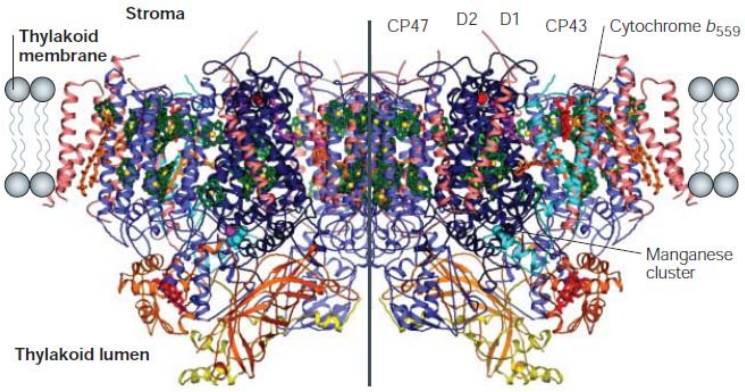


Figure 2. A view of structure of PS Ⅱ in thylakoid membrane. [4 P975]

D1 and D2 are quite similar to each other both in sizes (~40kDa), structure and function. They seem catalyze the reactions independently without much assistance between each other. In each subunit, there are 5 helices include one catalytic center (the oxygen evolving center-OEC) consists of $CaMn_4$ cluster with surrounding amino acid residues, which plays crucial role in water oxidation.[4]

The whole reaction process can be described as followed: Once a photon is absorbed, energy reaches PS Ⅱ and P_{680} obtains lower reduction potential then gives away an electron to *pheophytin*(Ph) and P_{680} is oxidized to P_{680}^{+} while at this point charge separation formed between P_{680}^{+} and Ph^{-}; Then the electron is passed to Q_B through Q_A, with the protons from the stroma, Q_B becomes Q_BH_2. In order to form Q_BH_2, two electrons and protons are needed hence after every two photoreactions one Q_BH_2 molecule is formed.[4]

Oxidized P_{680}^{+} is reduced by the oxygen evolving center-OEC which catalyzes water splitting to produce electrons, protons and oxygen molecule. Oxidized $P680^{+}$ oxidizes a nearby tyrosine (Y_Z in D1 or Y_D in D2), Y_Z/Y_D extracts electron or proton from the $CaMn_4$ cluster. After 4 photochemical cycles, the Mn_4Ca cluster acquires four oxidizing equivalents to oxidize two water molecules to product oxygen molecular. [4] The protons released have to be transported from the catalytic center into lumen to establish proton gradient between lumen and stroma so as to drive ATP synthesis afterwards. The net reactions happened in PS Ⅱ when two water molecules are oxidized can be shown as:

$$4\text{photons} + 2\ H_2O + 2Q_B + 4H^{+}_{\text{stroma}} \rightarrow 2\ Q_BH_2 + 4\ H^{+}_{\text{lumen}} + O_2$$

1.6 Water Oxidization and S-cycle mechanism

The oxygen-evolving complex (OEC) is the site of water oxidation, comprising four manganese ions and one divalent calcium ion. 3 out of 4 manganese ions form a cubic cluster with one calcium ion, in which the metal ions are bridged by four monooxygen atoms. The rest manganese Mn4 is special for binding water molecule as a substrate and extracts electrons from it. All these cluster structures are determined by X-ray absorption and electron paramagnetic resonance (EPR) experiments.[7] The oxygen-evolving photosystem II structure was resolved to a level of 1.9 angstroms revealing that five oxygen atoms serving as oxo bridges linking the five metal atoms and four water molecules bound to the Mn_4CaO_5 cluster.[6]

Once an oxygen molecule is produced, four-electrons must be extracted and four protons are released. The photochemical reaction chains take place one electron at a time, so how could the four electrons assemble together? One possible solution could be there are four reaction centers catalyze at the same time, four electrons are produced simultaneously or the four electrons are produced by turn but stored at one reaction center. The latter possibility was supported by Kok and co-workers through a series of experiments based on flashing light oxygen measurements. According to the experimental results, amount of oxygen produced reached the peak after the third flash. They proposed a model consists of five states: S_0-S_4. The cycle is displayed in the picture. [8] During the S-states cycle, OEC catalyze reactions by structural changes and physical changes.

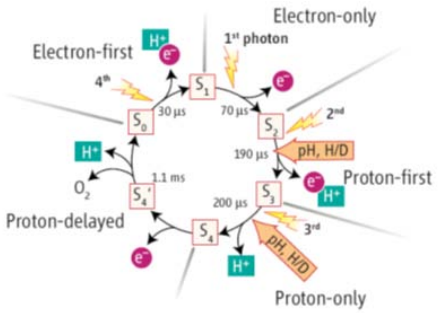


Figure 3. Show the S-cycle and the arrows are photons absorbed. During each state, time and products are also shown. [9]

The S-cycle in the Figure3 explains the experimental results very well. Only after the third photon absorbed in the reaction center, S_4 is reached and oxygen can be produced without any further light input. The move on to next S-state without any flash excitation or one flash activate twice are occasional situations, hence after dark adjustment, a steady state has been reached while there are the same possibilities to have S_0 to S_3 leading to the constant oxygen yield.

1.7 The Proton Pathway

The channels formed by residues of peptide chain in proteins are very common and necessary for certain reasons. One reason could be certain channels have promotion in molecules movement while another reason possibly is to avoid unwanted molecules passing through certain channels. Aquaporin is a kind of very well-studied protein which has water channels in order to permit water not other molecules to pass. The partly hydrophobic property and certain positive residues in the pore favors water molecules and avoid H^+ or H_3O^+.[10]. Then channels are key structural factors of proteins, both in property and function.

With the structure of PS Ⅱ in mind, the $CaMn_4$ cluster is buried in the middle of PS Ⅱ where charge separation takes place. No matter for releasing the molecular oxygen or transport the protons into lumen, there obviously must be exit channels for those molecules in the protein matrix, let alone S-cycle mechanism mentioned in previous part. From these two aspects, it is indeed proton pathway exists in PS Ⅱ catalytic center. [11]

Furthermore, proton pathway is necessary due to its particular property in transfer. When considering proton transfer, protons never exists as single H^+ because their transfer must be facilitated by water molecules or amino acid residues. According to the schematic diagram of Grotthuss mechanism, (seen Figure4.) proton is passed through a chain of hydrogen-bonded water molecule. Hydrated proton transferred till the end of chain is not the same proton transferred at the very beginning because proton is much easier for diffusion than other ions.[11] Similarly, amino acids which capable of holding water molecules could also form an efficient pathway to remove protons from PS Ⅱ catalytic center.

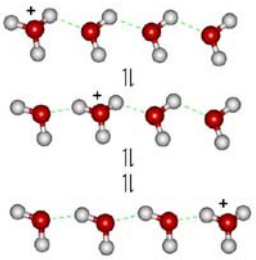


Figure 4. The Grotthuss mechanism of Proton transfer [11]

Proton pathway in PS Ⅱ is obligatory not only because reasons mentioned last paragraph, but also deal with energetic difference between steps of water oxidation in $CaMn_4$. [11]The proton gradient effects later ATP synthesis hence proton releases should enter luminal side and no reverse of protons are allowed.[2]

1.8 Mutants Design

How the proton being transferred through luminal greatly influences further catalysis efficiency. From the molecular dynamics study result, the area where channels have great chance to appear is occupied with hydrogen-bonded residues and water molecules from the $CaMn_4$ cluster to the lumen. Analysis of MD results reveals residues D61 and E65 of D1 subunit were intensely connected with water molecules around through water bonding. In agreement with these two residues were connected almost all the time in MD, the hydrogen bonds between D1-D61, D1-E65 and surrounding residues highly effected the stability of channels. [11]

Based on the MD results, there exists big possibility that N335 and R334 assist in holding the water molecules together with D61 and E65. With proposal of putative H+ transport pathway along D1-D61 and D1-E65, the titratable residues of N335 and R334 may play an important role in forming the proton channel. [11] Both D61 and E65 residues have the property of acidic amino acid which tends to show negative charge in solution. N335 residue is hydrophilic and R334 residue has character of basicity. [12] The putative function of N335 and R334 could be support in strengthen water bridges with D61 and E65 respectively.

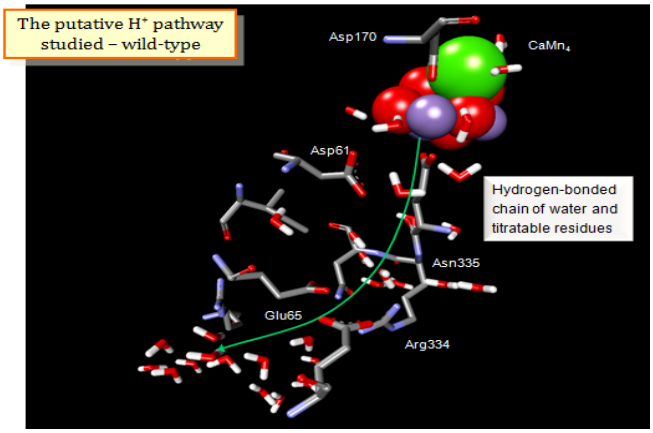


Figure 5. The putative H+ pathway studied by MD stimulations in D1 subunit of PS Ⅱ in thylakoid membrane. The green arrow points to the assumed proton transfer direction. [13]

During S-state transitions, ineffective H+ transport can become a limiting factor. To understand why and how the residues surrounding $CaMn_4$ cluster function the proton transfer

chain, it is reasonable by combination molecular dynamics with *in vivo* mutagenesis.[11] The project aims to mutate these two amino acids of D1 subunit and measure the growth parameters of *C. reinhardtii*.

Table 1. Mutants Details

Name	Mutant	Details
Construct 1	D1-N335L	Asparagine→Leucine
Construct 2	D1-R334N/N335R	Arginine→Asparagine/Asparagine→Arginine
Construct 3	D1-R334A/N335R	Arginine→Alanine/Asparagine→Arginine

In the MD stimulation, D1-D61 and D1-E65 cooperate in holding chain of water molecules which may construct the proton channel. [11] For construct 1, it is a single mutation which replacing asparagine with leucine at position 335. The structure and size of asparagine and leucine are very similar, however among them leucine is hydrophobic while asparagine is hydrophilic. [12] In the MD stimulation, attraction to water molecules of N335 could explain whether N335 is important or not and may further helps determining proton channel.[11] Construct 1 aims to use a very similar residue but objects to water molecules to test if the attraction force between D1-D61 and N335 works out. For construct 2, the computational dynamics data showed these two amino acids were “hot-spot” for putative proton channel.[11] Due to this, Dr. Linke constructed the mutants R334N and N335R which both shed light on the proton pathway study. [13] The connections among D1-D61&N335 and D1-E65&R334 are quite close.[11] The size of asparagine is much smaller than size of arginine.[12] Therefore, for mutant R334N, the residue might be too small to connect with D1-E65; for mutant N335R, the residue might be so big that it may block the pathway. The double mutation of R334 to an arginine and N335 to an asparagine might give chance to repair the interactions with D1-D61 and D1-E65. For construct 3, alanine has the smallest residue among all amino acids, but it is hydrophobic same as arginine.[12] Mutant N335R brought about almost deadly consequence to photosynthesis of *C. reinhardtii*[13], the double mutation of R334 to an alanine and N335 to an asparagine aim to replace R334 with the smallest residue and compare with mutants R334N/N335R. This mutant might also give us milepost results.

In the method of measuring mutants’ growth and oxygen evolution, it would be really promising for revealing the key feature whether certain residues are functional in the proton pathway.

1.9 *Chlamydomonas reinhardtii* as host

Chlamydomonas reinhardtii is a kind of single cell green algae which diameter is around 10μm and swims in water with two flagella. [14]The structure of *C. reinhardtii* is not complicated and the main organelles are nucleus, chloroplast and a large pyrenoid.[3] Figure1b. displays a simple sketch picture of *C.reinhardtii* structure. For quite a long time, *C.reinhardtii* has been used as a model organism in research of basic cell topics. There are also many mutants of *C. reinhardtii* act as tools for various biological processes.[14]

The *psbA* gene is located on the chloroplast genome and it codes for the D1 subunit of the PS Ⅱ reaction center. The *C.reinhardtii* chloroplast must have the *psbA* gene to acquire D1 and the ability of light-driven energy transduction. [1]

1.10 Experimental Plan

The project includes firstly obtaining an overview of reaction process in green algae,proton transfer and structural study of potential mutation site in PS Ⅱ by reviewing related literature. Secondly, sketch out a project plan for the experimental work on the basis of the constructs. Thirdly, carry out the experiments by construction of site-directed mutants and further determination and growth selection of mutants obtained.

Materials and Methods

2.1 Strains

Plasmids were propagated in *Escherichia coli* strain XL1-Blue (Stratagene).
C. reinhardtii strains were bought from the *Chlamydomonas* genetic centre. *C. reinhardtii* FUD7 is a strain which both copies of *psbA* gene were knocked-out and it is not capable of photosynthesis by itself.[15]

2.2 Media and Growth conditions

All bacterial strains with plasmids transformed were cultured in LB (Lysogeny broth) medium [16] with ampicillin to the concentration of 100μg/ml. XL1-Blue cells were grown under 37℃ incubator overnight with the plate inverted. As for plasmid transformation period, competent cells were grown in SOB (Super Optimal Broth) medium with 1.1M glucose in order to reach higher transformation efficiencies. [17]

C. reinhardtii mutants strains were maintained and grown on Tris-acetate-phosphate (TAP) medium and Minimal medium (without acetate), as a complete and minimal medium respectively [18]. For a selection of mutation construct strains, spectinomycin was added to reach a final concentration of 100μg/ml in TAP medium.

C. reinhardtii FUD7 cells were being maintained on TAP medium plate under room temperature with a growth cycle of one month. *C. reinhardtii* FUD7 cells in TAP medium then later used as the host for mutated *psbA* gene through homologous recombination were grown in 25℃ shaker under low light. *C. reinhardtii* strains with constructs introduced in genome were incubated in similar conditions with FUD7 except under normal light. Mutation *psbA* gene transformed strains were grown under same conditions with additional same concentration of spectinomycin with TAP plates.

2.3 Plasmids

pBA157 is a plasmid generated by Jun Minagawa and Antony R. Crofts. [19] which contains intron-free *psbA* gene followed by a bacterial aadA gene conferring spetinomyicin resistance as well as additional restriction sites for generations of mutations. The original vector antibiotic resistance is ampicillin.

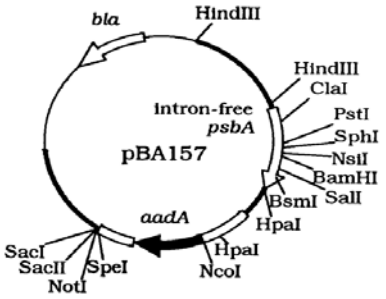


Figure 6. The pBA 157 Vector [19]

2.4 Primer Design

The primers used for site-directed mutagenesis PCR were designed according to the QuikChange® Site-Directed Mutagenesis Kit's primer design guidelines.[20] Both mutagenic primers anneal the same sequence on opposite strands while each desired mutation site was relatively located in the center of the primer. Refer to GC content, each primer was designed to terminate with G/C residues and GC percentage in total should at least 40%. Total length of primers varies from 25bp to 45bp. Tech notes from premierbiosoft [21] were complement guide for checking after primer design. Presence of possible secondary structure of primers could lead to the poor yield of product; therefore the secondary structures were examined by OligoAnalyzer tools before ordering primers. [22]

Based on H. Naya's paper [23], translational selection strongly influences codon usage in *C. reinhardtii*; hence different codons translating same amino acid display various expression frequencies. When designing the mutation primers, the most frequent codons were chosen if those did not give strong secondary structure. If so, a compromise was considered to give way for achievement of the best quality primers. Take the single mutation N335L as an example, there are 6 codons to choose from, apply them all in mutagenic primers and check their secondary structures, potential secondary structures results reveal that UUA, the most frequently expressed codon, give best quality. Then converting from RNA to DNA, the mutation sequence should be TTA. The design of the other two mutation primers followed the same procedure. [24]

However, except construct 1 the other two constructs both contained double mutations. Thank to Dr. Linke‘s previous work, there already existed plasmids with mutation R334N and with mutation N335R.[13] Primers for R334N/N335R could be designed whether based on templates pBA157_R334N or pBA157_N335R. After taking codon frequency and primers' secondary structure into consideration, pBA157_R334N and CGC (DNA: CGC) were chosen as template and codon respectively.

Furthermore, for construct 3 (R334A/N335R) pBA157_N335R was selected as the template and primers were firstly designed using codon GCU (DNA: GCT). After adjusting PCR by diversifying the reaction mix and conditions, they still did t not work. To achieve construct 3, design a second pair of primers choosing codon GCC (DNA: GCC) to run SDM PCR.

All primers were ordered from Finnzymes, they were after standard purification, when arrived they were dissolved in dH_2O to certain concentration and stored in -20°C freezer.

2.5 Site-Directed Mutagenesis PCR

The SDM PCRs for different constructs endured several tries and optimizations of reaction mixes and thermal cycler conditions. Thus, different constructs were obtained through distinct conditions.

For construct 1 (N335L) and construct 2(R334N/N335R), PrimeSTAR HS DNA Polymerase

(Takara) was used for site directed mutagenesis. The reaction mix contains 10µl 5x PrimeSTAR Buffer (Takara), 1µl 100mM dNTP Mix (Fermentas), 1µl 12.5 µM primers, 1.5µl 100% DMSO(3%), 0,025 U/µl PrimeSTAR DNA Polymerase (Takara), 60ng plasmid and dH_2O added to 50µl. The thermal cycler program was 10 seconds at 98°C, 5 sec at 55°C, 8 minutes 30seconds at 72°C (1min/kb). Thermal cycles were run 30 times. 6 µl of product was then checked on gel and the rest was digested with 1 µl FastDigest Enzyme DpnI (Fermentas) and 5µl 10 x FastDigest Buffer (Fermentas) under 37°C water bath for 1 hour.

For construct 3(R334A/N335R), the reaction mix contains 10µl 5x PrimeSTAR Buffer (Takara), 1µl 100mM dNTP Mix (Fermentas), 1µl 12.5 µM primers (codon GCU (DNA: GCT)), 1µl 100% DMSO (2%), 0,025 U/µl PrimeSTAR DNA Polymerase (Takara), 60ng plasmid and dH_2O added to 50µl. The thermal cycler program was 10 seconds at 98°C, 5 sec at 58.5°C, 8 minutes 30seconds at 72°C (1min/kb). Thermal cycles were run 30 times. 6 µl of product was then checked on gel and the rest was digested with 1 µl FastDigest Enzyme DpnI (Fermentas) and 5µl 10 x FastDigest Buffer (Fermentas) under 37°C water bath for 1 hour.

Upon all three constructs, various combinations of PCR mixtures and thermal cyclers had been screened to reach specific and high yield of PCR product. For construct 1 (N335L) and 2 (R334N/N335R), SDM-PCRs were done using Phusion High Fidelity DNA Polymerase (Finnzymes) at the beginning. As for reaction mixture, different percentage of DMSO (0%, 2%, 4%, and 6%), amount of template (30ng, 60ng) and primers concentration (5µM, 12.5µM) had been tried for running PCR. As for thermal cycler, annealing temperature low from 50°C till high up 72°C together with two speeds for extension (15sec/kb, 30sec/kb) had been run. For construct 3 (R334A/N335R), SDM-PCRs were done both using Phusion High Fidelity DNA Polymerase (Phusion) and PrimeSTAR DNA Polymerase (Takara) upon primers with codon GCU (DNA: GCT). With the Phusion High Fidelity DNA Polymerase, different percentage of DMSO (0%, 2%, and 3%) and annealing temperature gradient for thermal cycler range from 55°C to 71°C had been tried for running PCR. With PrimeSTAR DNA Polymerase (Takara), reaction mix and conditions successful for construct 1 and 2 were tested.

2.6 Colony PCR for *C. reinhardtii* Mutants

Colony PCRs for *psbA* gene isolation from the genome of transformed FUD7 were done using DreamTaq DNA Polymerase (Fermentas). A standard 50 µl reaction consisted of 5µl 10x DreamTaq Buffer, 1µl 100mM dNTP (Fermentas), 5µl 5µM primers (psbA_01_Forward&psbA_02_Reverse), 0.5µl DreamTaq DNA Polymerase and 1.5µl 100% DMSO (3%), 1µl colony solution (pick with tip dissolving in TAP medium), and 31µl dH_2O. Thermal cycler program was 30 seconds at 95°C, 30 seconds 50°C, 1minute 15seconds at 72°C(1min/kb) and run for 30 cycles.

2.7 Cloning PCR for *C. reinhardtii* Mutants

PCRs for gene isolations were done using Phusion High Fidelity DNA Polymerase (Finnzymes). A standard 20 µl reaction consisted of 4µl 5x Phusion HF Buffer, 0.4µl 100mM

dNTP (Fermentas), 2µl 5µM primers (psbA_01_Forward& psbA_02_Reverse), 0.2µl Phusion DNA Polymerase and 0.2µl 100% DMSO, 1µl template, and 10.3µl H_2O. Thermal cycler program was 30 seconds at 98°C, 15 seconds 57°C to 65°C (57°C, 57.6°C, 60.2°C, 62°C, 63.4°C, 65°C) temperature gradient, 1minute at 72°C (15sec/kb) and run for 30 cycles.

2.8 Check PCR product

In order to check whether there is PCR product and the size of the product, 5 µl sample was run on a 1% agarose (Sigma) gel with 1x gel buffer 10 mM Lithium Hydroxide, 29 mM Boric Acid, 0,5 mM EDTA, dH_2O and Thiazole Orange for 25 minutes at 140-160 volts. Meanwhile, GeneRuler 1kbp DNA Ladder (Fermentas) was applied on the gel for comparison of PCR product sizes.

2.9 Cloning

For colony PCR for *C. reinhardtii* mutants, the products were firstly checked following above steps then whole volume were loaded in the gel to purify specific size product. The potential gel bands were cut under UV light and put into ethanol sterilized 1.5mL centrifuge tube freezing under -80°C overnight. To get the product in the gel, squeeze it slowly in sterilized parafilm (Heathrow Scientific) and collecting through pipetteing.

For the cloning reaction, add following components on ice: 1µl PCR product, 10µl 2x Reaction buffer, 1µl DNA Blunting enzyme (Thermo Scientific Clone JET PCR Cloning Kit) vortex briefly and spin down. Incubate the mixture at 70°C for 5 minutes. Then chill the mixture on ice add 1µl (50ng/µl) pJET1.2/blunt Cloning Vector and 1µl T4 DNA ligase, vortex briefly and spin down. Incubate the ligation mixture at room temperature (20-22°C) for 5 minutes. The ligation mixture could be latterly used for transformation into XL1-Blue cells.

2.10 Transformation

Competent cells XL1-Blue were home-made and kept under -80°C freezer. Before utilizing it, it is necessary to test its competency. The plasmid pUC18 from QuickChange® Site-Directed Mutagenesis Kit was used and XL1-Blue showed relatively good competency.

Digested PCR products were applied for transformation into competent cells. 100ng DNA was added into 60µl ice-thawed competent cells (XL1-Blue) by gently mixing and then incubating on ice for 30 minutes. Cells with DNA were heat-shocked at 42°C for 1 minute, put back on ice for 5 minutes. Next, 500µl SOB medium with 1.1M glucose were added to the cell tubes, cells were grown under 37°C 250rpm shaker for 1 hour. Cells were then concentrated by centrifuge in 200µL medium and spread on LB plates with 100µg/ml ampicillin. Plates were inverted under 37℃ incubator overnight. For negative control plates, competent cells without adding any DNA was treated the same.

2.11 Plasmid Extraction

In order to prepare plasmids from competent cells, colonies got after transformation were

picked and grown in 5 ml LB medium with 100µg/ml Ampicillin under 37°C in 250rpm shaker overnight. To collect pellet, cell cultures were centrifuged 4500 g for 10 minute. Apply GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich)'s protocol for standard plasmid preparation. In last step, use 50µl dH_2O instead of 100µl elution buffer to dissolve isolated plasmids. To determine the concentration of plasmids prepared, micro-volume UV-Vis spectrophotometer (Nanodrop from Thermo) was used to measure the concentration of DNA.

2.12 DNA sequencing

Sequencing of amino acid mutations in plasmid was done according to Macrogen Europe EZ-Seq user guide. Samples were prepared by combining 5µL of 100ng/µL of plasmid with 5 µL of 5mM of primer together. The plasmids were sequenced from both forward and reverse directions. Samples were then labeled with Macrogen's tag and sent to Macrogen Europe lab. Original names of samples as well as corresponding Macrogen number were recorded carefully. The results were downloaded from the Macrogen website [21] and analyzed using online Sequence Alignment with word processing.

2.13 Chloroplast transformation

Transformation of *psbA* gene knock-out strain FUD7 was performed by particles inflow gun. Tungsten particles coated with plasmid DNA were shot on the TAP plates containing concentrated early-logarithmic phase FUD7 cells. Tungsten particles, macrocarriers were sterilized in 95% ethanol before use. Coating particles were mixed as follows: 25µL (60µg/µL) tungsten, 2µL or 3µL plasmid DNA(ca. 1µg/µL) , 25µL (2.5M) $CaCl_2$, 10µL (0.1M) spermidine. While adding each ingredient the mixture was kept vortexing and afterwards need 3 more minutes' vortex. Spin down the tungsten particles to remove supernatant, then used 200µL (70%) ethanol to wash particles, repeat previous spin down procedure. Finally particles were resuspended in 18µL (70%) ethanol and they were ready for shooting. Apply half of the amount onto macrocarriers let it air dry, set the shooting machine, and shooting plates were incubated at 25°C under dim light with coverage of paper. The gene gun was set according to the standard instruction manual, vacuum: 25 inches Hg; pressure: 1000-1100psi (rapture disk 1100 psi, tank pressure 1300 psi); macrocarrier assembly: second slot below the one that carries the macrocarrier holder. After 14days transformed colonies were visible and isolated onto fresh TAP plates for several cycles that later segregations were performed every 7 days. The colonies later were the templates for PCR to sequence the insertion.

Results

3.1 Site-Directed Mutagenesis PCR

The three constructs of mutations were achieved after many tries of SDM PCR. Table 2 includes the summary of three constructs SDM PCR templates and primers.

Table 2. Summary of the constructs, primers and templates

Construct	Primers		Templates	Size
	Forward	Reverse		
1-N335L	1_psbA_N335L_w/o_F	2_psbA_N335L_w/o_R	pBA157	8.2kb
2-R334N/N335R	3_psbA_N335R_w/_F	4_psbA_N335R_w/_R	pBA157_R334N	8.2kb
3-R334A/N335R	5_psbA_R334A_w/_F	6_psbA_R334A_w/_R	pBA157_N335R	8.2kb
3-R334A/N335R	7_psbA_R334A_w/_F2	8_psbA_R334A_w_R2	pBA157_N335R	8.2kb

Figure 7 shows the products for all three constructs. Lane 1 and 2 were construct 1 N335L products; lane 3 and 4 were construct 2 R334N/N335R products; lane 5 and 6 were construct 3 R334A/N335R products; lane 7 was 1kbp ladder. For all constructs, the former one was reaction with 3% DMSO and latter one was reaction without DMSO. For construct 1, the reaction with DMSO had brighter band than one without DMSO but they all showed clear bands at correct size. For construct 2, only one with DMSO showed clear bands which were around 8kb and no band except primer-dimers band on the gel picture. For construct 3, there were quite strong bands hang up high and mere bands around 8kb position; it's not ideal product compared with other two constructs. In this reaction, primer 5&6 were used to conduct the mutation.

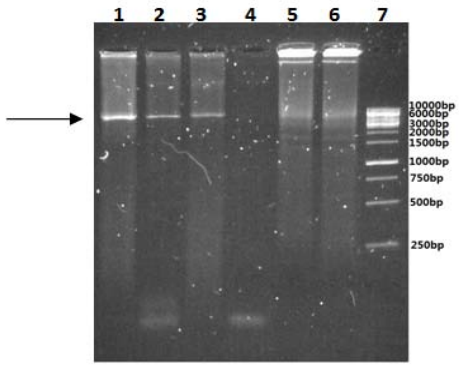


Figure 7. SDM PCR gel checking picture for all three constructs.

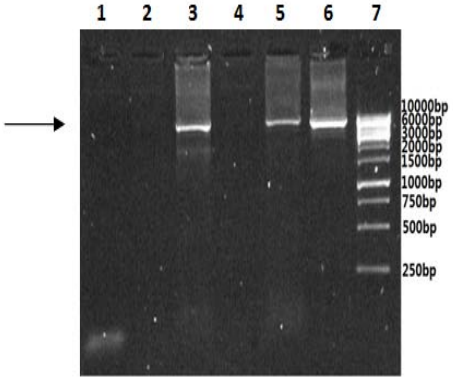


Figure 8. SDM PCR gel checking picture for Construct3 (with primer7&8)

Figure 8 shows the SDM PCR products with primer 7&8 used to generate construct 3. Lane 1 and 6 were negative control and positive control respectively while lane 7 is 1kbp ladder for size comparison. Negative control was also construct 3 SDM PCR mixture without polymerase. Positive control was construct 2 SDM PCR product. In lane 3&5 reaction products which annealing temperature was 55°C were loaded while in lane 4&6 annealing

temperature was 58.4°C. The two lanes (4&6) where clear bright bands could be seen were reaction with 2% DMSO whereas the rest two (3&5) blank lanes were reaction without DMSO. Products' sizes were almost the same with positive control and only one mere band in lane 3 appeared.

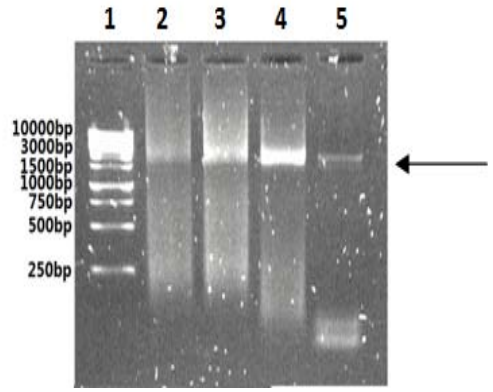


Figure 9. SDM PCR gel checking picture for Construct3R334A/N335R(primer5&6). Temperature gradient from 55°C to 61.3°C

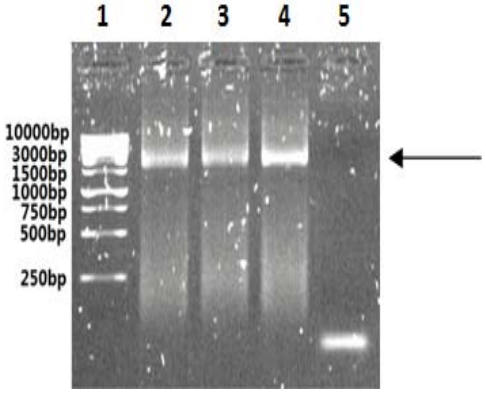


Figure 10. SDM PCR gel checking picture for Construct3R334A/N335R(primer5&6). Temperature gradient from 65°C to 71°C

Figure 9 and 10 both were from the same SDM PCR for construct3. Lane 1 in both figures was 1kbp ladder and the band sizes were marked on the left. Lane2-5 in figure 3 were reaction products annealing temperature range 55°C, 56.2°C, 58.4°C, 61.3°C. Lane 2-4 in figure 4 were reaction products annealing temperature from 65°C, 67.8°C, and 71°C; lane 5 was negative control (same SDM PCR mixture except no DNA polymerase). Even there were quite bright bands in both gel pictures, but compared to the ladder, the products size were around 3kbp. Moreover, if compared with bands in figure 1 and 2, there were vague bands like the background in almost every lane. Obviously in lane 5 figure 4, the negative control had only the primer-dimer band at bottom.

Important to point out here is that figure 7 and 8 results were SDM PCR run by PrimeSTAR DNA Polymerase from Takara. Figure 9 and 10 results were SDM PCR run by Phusion High Fidelity DNA Polymerase from Phusion. Further details and possible explanations would include in discussion part.

In Figure 8, only under annealing temperatures 57.6°C, 62°C, 63.4°C, there were bright bands around 2000bp. In figure 9, products appeared under annealing temperatures 57.6°C, 60.2°C, 62°C, 63.4°C.In both templates, 57°C and 65°C were not efficient temperatures.

3.2 Cloning PCR

In order to sequence the transformation result in FUD7, the *psbA* gene was cloned by doing colony PCR and then ligated into pJET2.1/blunting Cloning Vector. Followed table xx displays templates, primers and potential product size of colony PCRs.

Table 3. Summary of the colony PCR

No.	Construct	Primers		Templates	Size
		Forward	Reverse		
1	1-N335L	psbA_01_Forward	psbA_02_Reverse	Mutated FUD7 Colony1	Not unique
2	1-N335L	psbA_01_Forward	psbA_02_Reverse	Mutated FUD7 Colony2	Not unique
3	1-N335L	psbA_01_Forward	psbA_06_Reverse	Mutated FUD7 Colony2	4324bp
4	2-R334N/N335R	psbA_01_Forward	2_psbA_N335L_w/_R	pBA157_N335L	1218bp
5	2-R334N/N335R	psbA_01_Forward	psbA_02_Reverse	Mutated FUD7 Colony1	Not unique
6	2-R334N/N335R	psbA_01_Forward	psbA_02_Reverse	Mutated FUD7 Colony2	Not unique
7	FUD7	psbA_01_Forward	psbA_02_Reverse	FUD7 cDNA	Not unique

All colonies PCR used sonic-break cells as template, so despite of DNA there were a lot of other cellular components like proteins and membranes. The condition 4 mentioned in table 3 was a positive control, so pBA157_ N335L acted as template.

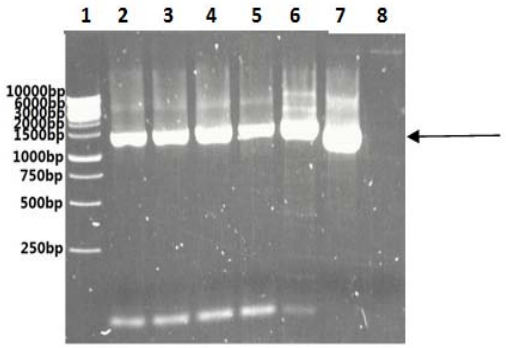


Figure 11. Colony PCR (Dream Taq Polymerase) gel checking picture for Construct1 N335L and Construct2 R334N/N335R. 6,7 and 8 were different controls.

Figure 11 shows the colony PCR products for construct1 and 2. Lane 1 was 1kbp ladder. Lane 2 and 3 were construct 2 R334N/N335R products No5. &No.6 in table3; lane 4 and 5 were construct 1 N335L products No1. & No.2 in table 3. Lane 6 was No.3 in table 3. Lane 7 and 8 were positive control and negative control, both No.4 mixtures in table 3. Negative control was run without addition of polymerase.

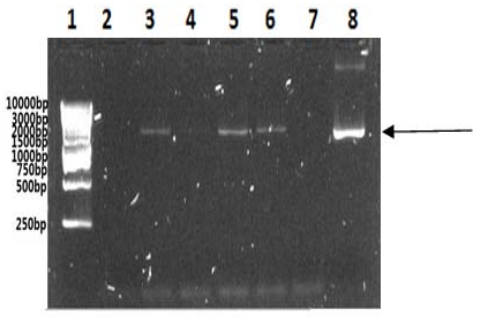


Figure 12. Colony PCR (Phusion High Fidelity DNA Polymerase) gel checking picture for FUD7. Temperature gradient from 57°C to 65°C

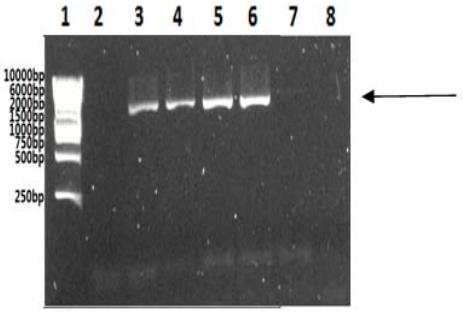


Figure 13. Colony PCR (Phusion High Fidelity DNA Polymerase) gel checking picture for Construct2 (R334N/N335R).

Pointed by the arrow, for constructs PCR products, there were all enough bright bands just below the band of 1500bp which were expected size. There were also weak and diffuse shape bands higher than 1500bp. Bands in lane 6 and 7 had small differences in size, lane 7 surely had lower band which was also as expected. For negative control, the thin and weak band hang very highly could be templates. The product size of lane 6 with psbA_06_Reverse should be 3000bp bigger than lane 2-5, however, it showed similar size.

Figure 12 and Figure 13 were colony PCR run using Phusion High Fidelity DNA Polymerase. DNA templates in Figure 6 used were freshly prepared FUD7 cells. In figure 9 mutation-contained FUD7 were applied. Lane 1 in both figures was 1kbp ladder. Lane 2-7 in both were annealing temperature gradient PCR products, 57°C, 57.6°C, 60.2°C, 62°C, 63.4°C, 65°C were temperatures in subsequence. Lane 8 in Figure 8 was the positive control which condition was No.4 in Table 3; lane 8 in Figure 9 was the negative control condition also No.4 but without polymerase. The bands got on construct2 were stronger than the bands on FUD7.

One crucial thing to bring about here is that after aligning FUD7 sequence with primer psbA_02_Reverse, there were surprising results. Despite of the whole length annealing period, many places could also anneal with most parts of the primers. Therefore, the PCR products sizes were not specific. However, according to the alignment results, because FUD7 is a *psbA* knock-out strain, the products got from mutated-*psbA* contained strains could still be distinguished from products got from FUD7.

3.2 Transformation Results

3.2.1 Transformation into XL1-Blue

In table 4, only correct SDM PCR products are shown. They were checked on the gel first and then transformed into XL1-Blue cells.

Table 4. Transformation into XL1-Blue

	Construct1 N335L	Construct 2 R334N/N335R	Construct R334A/N335R
SDM PCR (w/o DMSO)	3	/	0
SDM PCR (w/ DMSO)	5	5	1
Negative Control	0	0	0

Results for different constructs in Table4 were the number of colonies appeared on transformation plates after growing overnight. For all constructs, SDM PCR with adding DMSO transformation showed colonies on every plate. For construct 1&2 there were more colonies than construct3 while construct 3 took several trails of optimization on SDM PCR condition. Among all colonies grown on each plate, whether the construct contained the right mutation needs to be determined through further sequencing. Negative control plates had no colony growing at all.

3.2.2 Transformation into FUD7

Constructs 1 and 2‘s transformation into FUD7 strain were finished. Construct 3

transformation plates were still under growth during the time this thesis was written. Here showed the transformation results of construct 1 and 2.

Table 5. Transformation into FUD7

Construct	FUD7 culture	Plasmid coating [Con]1μg/μL	Contamination	Colony
1N335L	Low [O.D.]	2μL	bacteria	1
1N335L	Low [O.D.]	3μL	No	⩾10
1N335L	High[O.D.]	2μL	No	0
1N335L	High[O.D.]	3μL	No	⩾20
2R334N/N335R	Low [O.D.]	2μL	fungi	≈8
2R334N/N335R	Low [O.D.]	3μL	no	0
2R334N/N335R	High[O.D.]	2μL	fungi	⩾10
2R334N/N335R	High [O.D.]	3μL	Severe fungi	/
Negative Control	Low [O.D.]	/	No	2
Negative Control	High [O.D.]	/	No	0

Due to the transformation process, the FUD7 culture liquid were spread on empty plates firstly and the mutated plasmid were coated onto tungsten particles. There were two flasks of FUD7 culture growing in the shaker before transformation. The starting [O.D.] was slightly different while after the growth turned into early exponential phase; the measured [O.D.] were also slightly different. Low [O.D.] culture was around 0.1 less than high [O.D.] culture. In addition, the plasmid volume used for coating varied from 2μL -3μL. These results were observed and recorded 7 days after the transformation. Usually the FUD7 culture liquid turned yellow when they started dying. Following were the photos of transformation plates taken one month after the transformation. On the photos shown, we could see there were more colonies formed than before. Also, the colonies were segregated onto fresh TAP plates to for second round selection and purification.

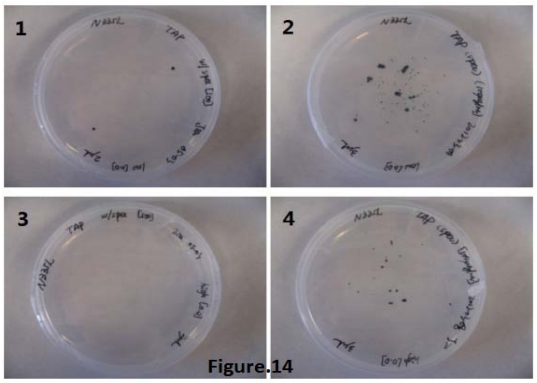


Figure 14. Construct 1N335L transformation plates.
1-N335L-low[O.D.]-2μLplasmid coating
2- N335L-low[O.D.]-3μLplasmid coating
3- N335L-high [O.D.]-2μLplasmid coating
4- N335L-high[O.D.]-3μLplasmid coating

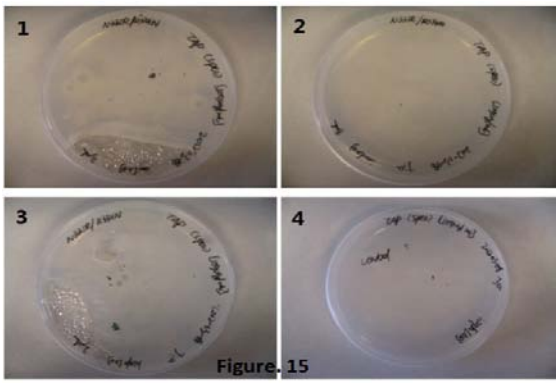


Figure15. Construct2 R334N/N335R transformation plates.
1- R334N/N335R -low [O.D.]-2μLplasmid coating
2- R334N/N335R -low [O.D.]-3μLplasmid coating
3- R334N/N335R -high [O.D.]-2μLplasmid coating
4- Negative Control-high [O.D.]-3μLplasmid coating

In figure 14, all four construct 1 transformation plates are shown. On No.2 and 4 plates, colonies could be easily seen and they were quite dense in the certain cycle which was about

the shooting area. The colonies got on the photo was much more than recorded in the table 5 due to between these two was 3 weeks time difference.

In figure 15, construct 2 transformation and negative plates are shown. The gap in No.1 and 3 were fungi contamination area which was cut off as long as it appeared. No.4 was negative control with high [O.D.] FUD7 culture liquid. It was really strange that two big colonies appeared on negative control plate. The reason was unknown and further steps were mentioned in discussion part.

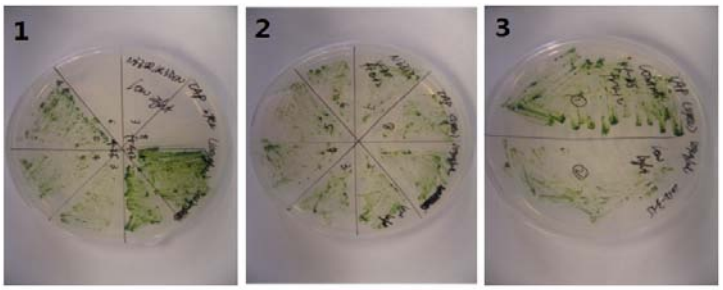


Figure 16. Consturct 1 N335L; Construct2 R334N/N335R; Negative Control Segregation plates.
1-Construct 2 R334N/N335R-second segregation plate
2- Construct1 N335L-second segregation plate
3-Negative Control-second segregation plate

Figure 16 shows a comparison between two constructs and negative control segregations. All three plates were growing under low light and they were the second-run of segregation having grown for around 14 days. The results were not that convincing since negative results grew really well on TAP. FUD7 didn't have any antiobiotic resistance, truly they should not grow. But now that they still grew after two-cycle segregation, the reason could be contamination of original FUD7 culture or other unknown reasons.

3.3 Sequencing Results

3.3.1 Check Mutants in pBA157

The mutants were firstly constructed on pBA157 hence sequencing the mutated pBA157 was the first step of checking mutants. In table 6,7,8, sequence No. was the tag reference number when samples sent to Macrogen Europe center (in following paragraphs, the tag reference is represented by last two digits, e.g.1246ZAA016 short for 16); primer was the primer name used for sequencing corresponding sample; sequencing quality reflects whether the base pair scanning showed reliable results or not; target mutation literally was whether the sequenced plasmid contained the correct mutation(s).

Table 6. Construct 1 N335L

Sequence No.	Primer	Sequencing Quality	Target Mutation
1246ZAA016	psbA_01_Forward	good	/
1246ZAA017	psbA_02_Reverse	good	yes
1246ZAA018	psbA_02_Reverse	Not good	yes

Primer psbA_01_forward and primer psbA_02_reverse are two primers used for sequencing the mutated *psbA* gene in pBA 157 vector. The former primer is to the upstream of *psbA* gene

and the latter is to the downstream of *psbA* gene reversely. Hence with sequencing from forward and reverse directions, the whole *psbA* gene is determined. Because the mutation sites were almost till the end of *psbA* gene, sequence result worked out by reverse primer included the mutation.

Table 6 displays sequencing result of construct 1. Sample 16 and 17 were sequencing results of colony 1 on the plate; colony 2 only had reverse primer result numbered 18. For colony 1, after aligned sequencing result with the non-mutated pBA157 vector sequence, alignment revealed the gene was complete and mutation included. The sequencing result for colony 2 which was not as reliable as that of colony1. Construct 1 N335L was confirmed.

Table 7. Construct 2 R334N/N335R

Sequence No.	Primer	Sequencing Quality	Target Mutation
1246ZAA019	psbA_01_Forward	good	/
1246ZAA020	psbA_02_Reverse	good	yes
1246ZAA021	psbA_02_Reverse	good	yes

In table 7, construct 2's sequencing result is shown. All three results were good enough to be reliable. The sequence was compared with pBA_157_R334N vector gene because it was used as the template. Sample 19 and 20 were both from colony 1 on transformation plates. Sequence alignment report revealed the plasmid was successfully mutated without any error. Sample 21 was from colony 2 and only the part containing target mutation was sequenced and result was positive. Construct 2 R334N/N335R in pBA_157 vector was confirmed.

Table 8. Construct R334A/N335R

Sequence No.	Primer	Sequencing Quality	Target Mutation
1246ZAA029	psbA_01_Forward	good	/
1246ZAA030	psbA_02_Reverse	good	yes
1246ZAA031	psbA_02_Reverse	good	yes

In table 8, construct 3's sequencing result is shown. All three results were in good quality to be reliable. The sequence was compared with pBA_157_N335R vector gene because it was used as the template. All three samples were from the only colony on transformation plates. Sequence alignment report revealed the plasmid contained target mutation and mutated without any error. Construct 2 R334N/N335R in pBA_157 vector was confirmed.

3.3.2 Check Mutants in *C. reinhardtii*

In table 9, sequencing results of *psbA* gene in *C. reinhardtii* is shown. All samples were extracted from colony PCR of *C. reinhardtii* mutants. Samples 32 to 35 were of construct 2 R334N/N335R, and samples 36 to 39 were of construct 1 N335L. Every clone of mutated *psbA* gene from *C. reinhardtii* was sequenced from forward and reverse directions. For construct 2, 3 out of 4 results were reliable and left one gene was in disorder. For construct 1, half of the results were reliable and the rest were unreadable. All the 8 sequencing gene were

incomparable with *psbA* gene and it was impossible to find target mutation gene, hence the results were really incomprehensible. Till the degree project was finished, if the mutants were confirmed in *C. reinhardtii* were not known yet.

Table 9. Sequencing mutants in *C. reinhardtii*

Sequence No.	Primer	Sequencing Quality	Target Mutation
1246ZAA032	psbA_01_Forward	good	not know
1246ZAA033	psbA_02_Reverse	good	not know
1246ZAA034	psbA_01_Forward	bad	not know
1246ZAA035	psbA_02_Reverse	good	not know
1246ZAA036	psbA_01_Forward	very bad	not know
1246ZAA037	psbA_02_Reverse	good	not know
1246ZAA038	psbA_01_Forward	bad	not know
1246ZAA039	psbA_02_Reverse	good	not know

Discussion

The overall goal of this master degree project is to identify and study the role of the amino acid residues involved in the proton pathway in Photosystem Ⅱ subunit D1 in *C.reinhardtii* by construction of sited-directed mutants. The project has significance for understanding how these residues affect proton transport from the PS Ⅱ catalytic center, and is a foundation for explaining the transitions mechanism more clearly.

Based on the experiments, results can be divided into two parts. One part is the construction of the designed mutants and another is characterization of the mutants. Most part of the construction of the designed mutants had been achieved in this degree project. Site-directed mutation PCR, transformation into *E.coli* and *C.reinhardtii,* and sequencing check of mutants strain were about to discussed in following paragraphs.

Talking about SDM-PCR and clone for all three constructs, several aspects should be paid attention to: the polymerase, the annealing temperature, the extension speed and the DMSO concentration. Set designing the ideal primers for mutations aside, the SDM-PCR is not a cutting-age technology and it seems easy and fast to get certain points mutated in process of amplification DNA. Yet, on my way to reach three mutants, PCR conditions optimization took quite long time. Different polymerases have their own properties and suitable reactions, in this project; a high extension-speed enzyme did not work out the product, instead a slower enzyme made the aim. The next key factor very influential is the annealing temperature. Various formulas to calculate the melting temperatures for primers can be found, but to certain companies' primer, it's better to follow the recommendation and try temperature gradient when calculated Tm was not successful. At last, addition of DMSO or not could make a difference.

As showed in results, the negative control plates got strong colonies even after second-round segregation. The phenomena might result from FUD7 got contaminated by other mutants; one spot of non FUD7 culture could lead the growth on TAP plates with antibiotic. Several solutions were carried out to test the FUD7. Clone the *psbA* gene from construct strain and send for sequencing; check FUD7 strain purity by use same culture onto fresh TAP plates with antibiotic; start all over from the transformation into FUD 7 for parallel experiments.

In order to confirm the mutation in pBA157 plasmid and mutated insertion in FUD7, sequencing was carried out through trial and error. Checking mutations in pBA157 plasmids underwent several tries for construct 3 but worked out smoothly for construct 1 and 2. Among three constructs, construct 3 was the most difficult one to get through SDM PCR. This reason could lie in SDM PCR conditions and codon usage for this mutation. The codon selected for alanine cause relatively close secondary structure in primers.[22] To sequence mutated insertion *psbA* gene in *C. reinhardtii*, the ligation pJET1.2/blunt cloning vector was used. The clone of insertion part in target FUD7 dealt with several issues. After transformation, primers provided in that kit should be added for further colony PCR and sequencing. The sequenced gene should include the period of *psbA*. For all constructs, none of it showed reliable

alignment. For construct 3, the SDM PCR was quite hard to achieve, then transformation efficiency into FUD7 was also not high hence the insertion might not successfully recombined into FUD7. For construct 1 and2, low transformation efficiency into FUD7 was possible also. Indeed, these also could due to problem with sequencing itself.

So far, all three constructs have been transformed but none of them are guaranteed to be correct mutants. The main purpose of construction of these mutants is to prove computational study results and discover the possible proton pathway in D1 in *C. reinhardtii*. The sequencing results display constructs have been achieved in psbA gene but insertion into FUD7 genome has not been completed.

Moreover, the characteristic of mutants are key steps for understanding how the light-driven reactions are so efficient. However, more experiments could only be carried out when sequence is confirmed.

Turning to the constructs I did during this project, the chance that SDM-PCR and cloning would fail was still high. That is the reason why I did not have time enough to finish sequencing the constructs. All these little setbacks required me to repeat the experiments and adjust the conditions. Let alone it takes *C. reinhardtii* usually a week to grow for once segregation. All these small setbacks meant that experiments had to be repeated, sometimes several times. But that does not mean the effort in designing and performing the experiments were with mistakes, just like the sentence says, ‘the road is tortuous, but the future is bright’.

In conclusion, the results presented here demonstrate a start of getting these three mutants. This master thesis ends there, but much work is still to be made in this project. Furthermore, after acquire the mutants, sequencing these three mutants, measurement of the growth curve, selection through autotrophic/heterotrophic growth media, steady-O2 evolution measurment, EPR spectroscopy measurement for specific S-state transition.etc.[5] The results can be compared with computational data for testing further hypotheses.

Acknowledgements

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Appendix

1. LB Medium formula

Table 1. LB media

LB (for 1liter)	
10.0g	Tryptone
5.0g	Yeast extract
10.0 g	NaCl
15.0 g	Agar if for plates

2. TAP Medium formula

Table 2. Salts Solution for TAP

Salts Solution (1Liter)
15.0g NH_4Cl
4.0g $MgSO_4 \cdot 7H_2O$
2.0g $CaCl_2 \cdot 2H_2O$

Table 3. Phosphate Solution for TAP

Phosphate Solution (100mL)
28.8g K_2HPO4
14.4g KH_2PO4

Hutner's Trace (Hutner et al. (1950) Proc. Am. Philos. Soc. 94, 152-170) (Merchant et al. (2006) Biochim. Biophys. Acta 1763, 578-594.)

Table 4. Hutner's Trace for 1 Liter

Compound	Amount	Demineralized Water
EDTA disodium salt	50.0g	250mL
$ZnSO_4 \cdot 7 H_2O$	22.0g	100mL
H_3BO_3	11.4g	200mL
$MnCl_2 \cdot 4 H_2O$	5.06g	50mL
$CoCl_2 \cdot 6 H_2O$	1.61g	50mL
$CuSO_4$ $5H_2O$	1.57g	50mL
$(NH_4)_6Mo_7O_{24} \cdot 4 H_2O$	1.10g	50mL
$FeSO_4 \cdot 7 H_2O$	4.99g	50mL

$FeSO_4$ should be prepared last to avoid oxidation. Boil the mixtures of all solutions except EDTA and then add EDTA. When fully dissolved, cool down the solution to 70°C add 85mL hot 20 %(M/V)KOH solution. Adjust the total volume to 1 Liter, solution should look green. Let the flask stand for 1-2 weeks, shaking once a day. Finally the solution should look purple and have brown precipitate. Get rid of the precipitate by filtration until the solution is clear. If there is no precipitate, check pH before use. Use KOH or HCl to adjust pH to 7. Store the liquid in 4°C fridge or frozen aliquots.

Table 5. TAP Media

TAP for 1 Liter	
2.42g	TRIS
25mL	Salts Solution (Table 2.)
0.375mL	Phosphate Solution (Table 3.)
1.0mL	1 x Hutner's Trace (Table 4.)
1.0mL	Glacial acetic acid
To 1 Liter	Demineralized Water
15.0g	Agar if for plates

For Tris-minimal medium, do not add the acetic acid to the final solution and adjust pH to 7.0 with HCl.

3. Primers List

Name	Sequence	Length	Tm	Statement
1_psbA_N335L_w/o_F	5'GTAATGCACGAGCGTTTAGCTCACAACTTCCCTCTAG3'	37	65	pBA157 as template
2_psbA_N335L_w/o_R	5'CTAGAGGGAAGTTGTGAGCTAAACGCTCGTGCATTAC3'	37	65	pBA157 as template
3_psbA_N335R_w/_F	5'GTAATGCACGAGAATCGCGCTCACAACTTCCCTCTAG3'	37	66	Mutants pBA157_R334N as template
4_psbA_N335R_w/_R	5'CTAGAGGGAAGTTGTGAGCGCGATTCTCGTGCATTAC3'	37	66	Mutants pBA157_R334N as template
5_psbA_R334A_w/_F	5'GTAATGCACGAGGCTCGCGCTCACAACTTCCC3'	32	68	Mutants pBA157_N335R as template
6_psbA_R334A_w/_R	5'GGGAAGTTGTGAGCGCGAGCCTCGTGCATTAC3'	32	68	Mutants pBA157_N335R as template
7_psbA_R334A_w/_F2	5'GTAATGCACGAGGCCCGCGCTCACAACTTCCC3'	32	70	Mutants pBA157_N335R as template
8_psbA_R334A_w_R2	5'GGGAAGTTGTGAGCGCGGGCCTCGTGCATTAC3'	32	70	Mutants pBA157_N335R as template
psbA_01_Forward	5'GGGAAGGGGACGTAGGTACA3'	20	58	158 bp upstream of *psbA* start
psbA_02_Reverse	5'CCTGCCAACTGCCTATGGTA3'	20	57	106 bp downstream of *psbA* end
psbA_06_Reverse	5'GTTGTGAAAAAGCTGAGGGGAC3'	22	55	3000 pb downstream of *psbA* end
psbA_10_Reverse	5'CGAAGTGATTATTGACCACTAGGAAGG3'	27	57	1474 bp downstream of BamHI for sequencing PCR

4. Plasmid list

Name	Antibiotic	Statement
pBA157	Ampicillin	*psbA* intronless & recombination flanks for FUD7 transformation
pUC18	Ampicillin	Control Plasmid from Quickchange Kit
pBA157_R334N	Ampicillin	pBA157 with Mutation R334N
pBA157_N335R	Ampicillin	pBA157 with Mutation N335R