PROTEOMIC ANALYSIS OF A UNICELLULAR GREEN ALGA, *CHLAMYDOMONAS REINHARDTII*, IN RESPONSE TO HIGH-LIGHT STRESS

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ABSTRACT

Light is an important factor for growth and development of oxygenic photosynthetic organisms that produce carbohydrate from CO_2. However, high-light intensity can cause photo-oxidative stress to plants, which can eventually lead to death. For survival, plants and algae have evolved various mechanisms to protect themselves from excessive irradiance. Although the overall responses appear to be conserved among different species of photosynthetic organisms, detail mechanisms underlying such responses remain obscure. In this thesis, a proteomic approach was employed to identify proteins of a model unicellular green alga, *Chlamydomonas reinhardtii*, that are differentially expressed during the transition from low light (LL) to high light (HL) growth intensity. Proteins were separated on 2-dimentional gel electrophoresis and stained with colloidal Coomassie blue G. Protein spot patterns of samples collected at different time intervals after LL to HL shift were compared. The differentially expressed protein spots were subjected to further identification by tandem mass spectrometry. Results showed the typical landmark for HL responses, i.e. state transition of the light-harvesting complex and enhanced expression of HSP70B. Of particular interest was the observation that several heat-shock proteins were down-regulated in response to HL. This finding could probably explain the light-sensitive nature of this microalga.

KEY WORDS: 2–DE/ CHLAMYDOMONAS/ IRRADIANCE STRESS/ PHOTOINHIBITION/ PROTEOMICS

53 pages

การศึกษาโปรตีนทั้งหมดของสาหร่ายสีเขียว *Chlamydomonas reinhardtii* ในการตอบสนองต่อแสงที่มีปริมาณสูง

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บทคัดย่อ

แสงเป็นปัจจัยสำคัญต่อการเจริญเติบโตของพืชซึ่งเป็นผู้ผลิตคาร์โบไฮเดรตที่เป็นอาหารของสิ่งมีชีวิตอื่นๆ แต่มีสภาวะที่มีแสงในปริมาณมากเป็นสาเหตุให้เกิดอนุมูลอิสระที่อาจทำให้พืชตายได้ แต่เพื่อการอยู่รอด พืชและสาหร่ายได้สร้างกลไกลหลายอย่างเพื่อป้องกันตัวเองจากอันตรายที่เกิดจากปริมาณแสงที่มาก แม้กลไกดังกล่าวจะสอดคล้องและเหมือนกันในสิ่งมีชีวิตที่สังเคราะห์แสงหลายชนิด แต่รายละเอียดในเชิงลึกยังคลุมเคลือ ดังนั้นในวิทยานิพนธ์นี้จึงเลือกศึกษาโปรตีนทั้งหมด (proteomics) ของสาหร่าย *Chlamydomonas reinhardtii* ซึ่งเป็นสาหร่ายสีเขียวเซลล์เดียว โดยเปรียบเทียบโปรตีนที่เปลี่ยนแปลงที่เห็นความแตกต่างได้ใน Electrophoresis แบบ 2 มิติ ระหว่างที่เลี้ยงสาหร่ายในสภาวะแสงต่ำและแสงสูงมาก แล้วโปรตีนเหล่านั้นแต่ละตัวถูกไปวิเคราะห์จำแนกว่าเป็นโปรตีนอะไรด้วยวิธี tandem mass spectrometry จากการศึกษาพบว่าหลายโปรตีนเป็นที่ทราบกันทั่วอยู่แล้วว่ามีปริมาณเปลี่ยนแปลงสัมพันธ์กับปริมาณแสง เช่น โปรตีนใน state transition และ heat shock โปรตีน (HSP70B) อันเป็นการยืนยันผลทดลองว่าสอดคล้องกับนักวิจัยกลุ่มอื่น และนอกจากนั้นยังพบว่า heat shock protein หลายตัวถูกลดปริมาณลงอย่างมีนัยสำคัญ ซึ่งน่าจะเป็นเหตุผลที่อธิบายว่าทำไมสาหร่ายชนิดนี้จึงไม่ทนแสงที่มีปริมาณมาก

53 หน้า

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LIST OF ABBREVIATIONS

Abbreviation used throughout this thesis are listed below

LC-MS/MS	Liquid chromatography-mass spectrometry (tandem mass)
MALDI-TOF	Matrix-assisted laser desorption/ionization (time-of-flight)
LL	low light condition
HL	high light condition
2-DE	2-dimensional gel electrophoresis
Fv/Fm	optimal quantum yield
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
PSII	photosystem II
PSI	photosystem I
PQ	plastoquinone
PC	plastocyanin
Fd	ferredoxin
Cyt *b6f*	cytochrome b_6f complex
3-PGA	3-Phosphoglyceric acid
RuBP	ribulose-1,5-bisphosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
NPQ	Non-photochemical quenching
qE	ΔpH-dependent quenching
qT	state-transition

LIST OF ABBREVIATIONS (cont.)

qI	photoinhibitory quenching
V	violaxanthin
Z	zeaxanthin
A	antheraxanthin
VDE	violaxanthin de-epoxidase
LHC	light-harvesting protein complex
Chl	chlorophyll
TBP	Tris–Bicarbonate–Phosphate
PAM	pulse–amplitude modulated
DTT	dithiothreitol
HSP	heat-shock protein
NCBI	National Center for Biotechnology Information
CPN	chaperonin
$^1O_2$	Singlet oxygen

CHAPTER I
INTRODUCTION

1.1 Light and life

Light plays an important role for living organisms not only as energy source for driving photosynthesis, a biological reaction that uses solar energy to convert inorganic carbon in the form of CO_2 into organic compounds, but also as a regulatory factor for growth and development of some organisms. Difference wavelength of light is able to affect various activities inside the cell. For example, *C. elegans* can change behavior to accelerate its movement when exposed to blue or shorter wavelengths of light, possibly through two critical signaling pathways: cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG) (Edwards et al. 2008). For plants, light controls each state of growth and development, from seedling photomorphogenesis to flowering, via photoreceptors including phytochromes, cryptochromes, phototropins and unidentified ultraviolet B (UVB) photoreceptors (Jiao et al. 2007). Cryptochromes and phototropins perceive blue and ultraviolet A (UVA) wavelengths, whereas Phytochromes predominately absorbs the far-red and red wavelengths while the UVB photoreceptors, absorbs UVB. In fact certain photoreceptors have kinase activity that can phosphorylate their downstream regulatory proteins and have ability to be translocated between cytoplasm and nucleus for gene regulation in response to light stimulation (Jiao et al. 2007). There are some evidences suggesting that light signal could lead to modification of histone proteins, resulting in enhanced expression of several light-regulated genes (Guo et al. 2008). Cryptochromes can also function to optimize photosynthesis by helping absorb light energy under low-light growth condition, reduce rate of photodamage and supply CO_2 through physiological adaptation, chloroplast movement, leaf expansion, and stomatal expanding (Takemiya et al. 2005). In photosynthetic picoplankton, *Prochlorococcus* spp., it has been report that gene expression pattern are similar between treating the algae with high white light and blue light, suggesting that the sensing of high light might be mediated via the blue-light receptor (Steglich et al. 2006).

1.2 Light-driven photosynthetic electron transport reactions

Photosynthesis is biochemical reaction that occurs in plants, algae, and many species of bacteria. Photosynthetic organisms are crucially important for all life on earth, since it maintains the normal level of oxygen in the atmosphere, and provide energy source for herbivore in form of organic compounds. The electron transport reactions of oxygenic photosynthesis, from water oxidation to the generation of NADPH, are driven by light. Therefore, they are called the light reactions. The light reactions occur within the thylakoid membrane structures inside the chloroplast. Several multisubunit protein complexes that perform the photosynthetic electron transport, such as photosystem II (PSII), cytochrome b_6f complex (Cyt b_6f) and photosystem I (PSI), are all embedded in the lipid-bilayer of the thylakoid membranes. Other mobile electron carriers, i.e. a lipid-soluble quinone (plastoquinone, PQ) and the water-soluble plastocyanin (PC) and ferredoxin (Fd), are also involved in the light-driven electron transport from water to $NADP^+$. Hence, the photosynthetic electron transport involves oxidation and reduction of various electron and/or proton carriers.

Photosynthesis is initiated when light energy in the form of photons is absorbed by chlorophyll molecules bound within light-harvesting protein complex (LHC) associated with the PSII. Energy from the absorbed photons is transferred among chlorophylls as resonance energy transfer toward a special chlorophyll pair (P680) in the PSII reaction center, where charge separation between the P680 and the primary electron acceptor, pheophytin (Pheo) occurs. The resulting $P680^+$, a state of the special chlorophyll pair that loses an electron, is one of the most powerful oxidizing agents in any known biological system capable of oxidizing water molecules. Water oxidation occurs at the Mn cluster of the peripheral O_2 evolution complex of the PSII. The oxidation of two water molecules generates molecular oxygen, which is subsequently released to the atmosphere. At the acceptor side, Pheo- transfers its electron to the first immobilized quinine acceptor (Q_A) and subsequently to a mobile plastoquinone (PQ) bound at the Q_B-binding site. After receiving 2 electrons, the PQ picks up two protons from the stroma side to become PQH_2 leaving the PSII to the lipid phase of the thylakoid membranes. Therefore, the actually pathway of electron transfer at the PSII is generally summarized to be as follows:

$H_2O \rightarrow [Mn_4CaCl] \rightarrow Yz/Y_Z^{\bullet} \rightarrow P680/P680^{+} \rightarrow Pheo_a/Pheo_a^{-} \rightarrow Q_A/Q_A^{-} \rightarrow Q_B/Q_B^{-}$

When the PQH_2 docks at the Cyt b_6f complex, it carries out the electron transfer reactions in two routes: one of the electrons is transferred toward the Rieske iron-sulfur protein and another to cytochrome *f* that subsequently reduces plastocyanin (PC). During the electron transfer process, 2 protons are deposited into the thylakoid lumen. The electron at the Rieske protein is transferred through the *b*-hemes to a bound quinone on the stromal side of the Cyt b_6f complex. After 2 turns of PQH_2 oxidations, a molecule of PQH_2 is formed in return. In the next step, reduced PC is oxidized by a special chlorophyll pair (P700) inside the reaction center of PSI. At the PSI, light energy is independently absorbed by its peripheral chlorophyll antenna and funneled toward the reaction center where charge separation between P700 and another chlorophyll *a* acceptor (A_0) occurs. Electron from A_0 is passed through a phylloquinone (A_1), several Fe-S clusters and finally to ferredoxin. Ferredoxin, which is an intermediate electron carrier localized in the chloroplast stroma consequently reduces $NADP^+$ to NADPH via ferredoxin-NADP reductase. In summary, the light reaction of photosynthesis generates NADPH and ATP to be used in the CO_2 assimilation pathway. Moreover, alternatively, reduced ferredoxin can also reduce thioredoxin, by ferredoxin-thioredoxin reductase, that acts as redox mediator molecule to reduce various downstream proteins both inside- and outside-chloroplast.

1.3 Photosynthetic CO_2 assimilation

Enzymes involved in the reductive assimilation of CO_2 are localized in the chloroplast stroma. To reach this compartment, CO_2 has to pass through several barriers, from cell wall outside of plant cell to chloroplast envelope membranes. Thus, any slowdown in diffusion rate of CO_2 reaching the chloroplast stroma can limit photosynthesis. Many plants and algae do possess carbon-concentrating mechanisms that effectively elevate the concentration of CO_2 around the primary carbon-fixing enzyme, the Rubisco (Taiz and Zeiger 1991). C_4 photosynthetic carbon assimilation is one example of such CO_2-concentrating mechanisms. Most plant species possess C_3-type of photosynthesis. Every photosynthetic plastid in the C_3 plants functions in

the same way: all of the reactions from photon absorption to the synthesis of reduced carbon compounds take place in the same chloroplast. CO_2 fixation reaction performed by the Rubisco enzyme primarily yields two molecules of 3-PGA. The 3-PGA is subsequently reduced and phosphorylated by NADH and ATP generated by the light reactions to become triose phosphate (TP). TP can either be transported outside the chloroplast for the synthesis of sucrose, or it can be used for transitory starch synthesis within the chloroplast, or else it can be used to regenerate RuBP, the substrate of the Rubisco. Beside carboxylase activity, the Rubisco enzyme can also catalyze an oxygenase reaction. Under low CO_2 partial pressure, Rubisco in C_3 photosynthesis can catalyze the reaction of O_2 with the same substrate (RuBP) to generate 3-phosphoglycolate that cannot be used for the synthesis of TP (Furbank and Taylor 1995). This oxygenation reaction catalyzed by Rubisco enzyme, or so-called photorespiration, is competing with the carboxylation reaction for ATP and NADPH. Hence, increasing level of photorespiration can also undermine the efficiency of photosynthesis in C_3 plants (Woodrow and Berry 1988).

C_4, CAM, and other types of CO_2-concentrating mechanisms, can effectively suppress photorespiration by increasing the CO_2 partial pressure around the vicinity of the Rubisco enzyme. Typically, C_4 plants have two types of chloroplasts, each found in different cell types inside the leaf. The mesophyll-cell chloroplasts contain all the protein complexes required for the light reactions but lack the CO_2 fixing enzymes. Instead, the primary CO_2 fixation is performed by PEPC enzyme localized in the cytosol of the mesophyll cells. The C_4 intermediate then diffuses to the bundle sheath-cell chloroplasts, which are abundant in enzymes necessary for CO_2 assimilation and generation of TP. In addition, the bundle-sheath-cell chloroplasts also contain very minor amount of the proteins of the photosynthetic electron transport reactions to avoid the O_2 formation by the PSII. The initial C_4 acid generated by PEPC is oxaloacetate commonly found in all C_4 plants. The subsequent steps in different types of C_4 photosynthetic plants, however, differ in the decarboxylation reaction. Maize, sugarcane and sorghum use NADP-dependent malic enzyme (NADP-ME), residing in the bundle sheath-cell chloroplast, to decarboxylate the 4-carbon intermediate (malate). Some plants can also use the mitochondrial NAD-dependent malic enzyme (NAD-ME) or the cytosolic phosphoenolpyruvate

carboxykinase (PEP-CK) for the release of CO_2 from the aspartate (another kind of C_4 acid intermediate).

1.4 Parameters influencing the rate of photosynthesis

1.4.1 Photo-oxidative damage to the PSII reaction center

In oxygenic photosynthetic organisms, light absorption, rate of electron transport and carbon metabolism are coordinated to provide optimal photosynthesis. Under limiting irradiance, light energy is captured and utilized with high efficiency. With increasing light intensities, more photons are absorbed resulting in enhanced rates of CO_2 assimilation. However, at the light intensity where the rate of CO_2 fixation becomes a limiting factor, photosynthesis is saturated (Stitt 1986). Absorption of light in excess of that required for the saturation of photosynthesis can bring about photo-oxidative damage to proteins, lipids, and pigments in photosynthetic apparatuses. Excess absorption of photons by chlorophyll molecules in the light-harvesting complex proteins (LHC) of PSII increases the lifetime of the singlet excited-state chlorophyll (^{1}Chl), which can be converted into triplet chlorophyll (^{3}Chl) that is more stable and has longer lifetime. ^{3}Chl property has an ability to transfer its excitation energy to ground-state triplet O_2 molecules, converting to singlet oxygen ($^1O_2$) which is the most destructive reactive oxygen species, capable of destroying the proteins, lipids, and pigments located in the nearby vicinity (Niyogi 1999). In addition the other type of ROS molecules can also be generated under high irradiance. When the amount of $NADP^+$ is limited, i.e. when the CO_2 fixation is slower than the rate of electron transport, ferridoxin can transfer its electron to molecular O_2, resulting in the formation of superoxide anion (O_2^-). Detoxifiation of O_2^- by the enzyme superoxide dismutase (SOD) generates H_2O_2, another type of ROS. H_2O_2 can be neutralized to water by an activity of ascorbate peroxidase enzyme. Under certain circumstances, H_2O_2 can be converted to a reactive hydroxyl radical ($OH^{\cdot}$). These mentioned ROS can attack membrane lipids, resulting in lipid hydroperoxide, a product that decreases

the fluidity of the membrane causing membrane leakage, and causes of secondary damage to membrane proteins (Møller et al. 2007).

In addition to lipids, proteins can also be a target of ROS attack, especially the D1 reaction center protein of the PSII. Light-induced irreversible inactivation of the PSII reaction center is a well-known phenomenon that entails a permanent damage to the D1 protein (Powles 1984; Krause 1988; Aro et al. 1993; Melis 1999). The rate of PSII photoinhibitory damage was found to be linearly dependent on the growth light intensity (Baroli and Melis 1996; Tyystjärvi and Aro 1996). Through the process of 2-3 billion years of evolution, organisms of oxygenic photosynthesis have not been able to either prevent or avoid this photo-oxidative adverse effect from occurring (Payton et al. 1998). Thus, to date, every oxygen-evolving photosynthetic organism known, from cyanobacteria to C_4 plants, is subject to this irreversible D1 photodamage. Nature, however, has evolved photoprotective mechanisms, ranging from the molecular level to the whole plant level, which serve to mitigate or alleviate the adverse effect of the excess irradiance. Additionally, plants have also evolved a repair mechanism by which they rectify the apparently irreversible PSII photo-oxidative damage. There is interplay between the processes of photoprotection, photodamage and repair. When rates of photodamage exceed the capacity of photoprotection and of the repair mechanism, then photodamaged PSII reaction centers accumulate in the thylakoid membrane. This condition is known as 'photoinhibition' of photosynthesis (Powles 1984). When photoinhibition is manifested (Ohad et al. 1984; Greer et al. 1986), it entails an overall decline in the rate of photosynthesis (Kok 1956; Long et al. 1994). In addition to light, other environmental stress factors that lead to an imbalance in the photosynthetic reactions can also elicit photoinhibition, even under moderate light intensities. Such conditions include drought, chilling or freezing temperature and heat stresses (Powles 1984; Havaux 1992; Król et al. 1997).

1.4.2 Photoprotection

Photoprotection entails various mechanisms employed by photosynthetic organisms to minimize the harmful effects of excess photon absorption. These mechanisms include physiological responses to lower the level of incident light such

as movement of leaves and chloroplasts. At the molecular level, scavenging of reactive oxygen species by antioxidant molecules, non-photochemical dissipation of excess excitation energy as heat and modification of photosynthetic machineries to enhance photon usage and reduce light absorption can also help diminish the photo-oxidative damage (Niyogi 1999). A variety of carotenoid molecules have been suggested to play important roles in photoprotection. For example, β-carotene associated with the reaction center proteins of PSII can help scavenge $^1O_2$ (Telfer et al. 1994) while xanthophylls, and especially zeaxanthin, are thought to be involved in the process of non-photochemical quenching (Demmig-Adams 1990). Non-photochemical quenching (NPQ) is one of the photoprotective mechanisms by which excess energy from singlet-excited chlorophyll is quenched and safely dissipated as heat (Niyogi 1999; Niyogi 2000). Generally, NPQ can be observed by a decrease in Chl fluorescence emission. Three different types of NPQ can be distinguished by their relaxation kinetics following dark incubation as well as their responses to various inhibitors (Müller et al. 2001). Temporally, energy- or ΔpH-dependent quenching (qE) relaxes within a few minutes, followed by state-transitions (qT) in many minutes and then by photoinhibitory quenching (qI), which relaxes on a time scale of hours.

The energy-dependent mechanism of NPQ is initiated by a buildup of trans-thylakoid ΔpH (Niyogi 1999; Niyogi 2000). In general, a slowdown in ATP synthase activity by excessive irradiance results in a rapid decrease in lumenal pH, which in turn triggers protonation of the PSII proteins and activates a reversible xanthophyll cycle (Müller et al. 2001). In plants and some algae, the xanthophyll cycle involves a conversion of violaxanthin (V), which is associated with the inner light-harvesting antenna under normal growth conditions, to antheraxanthin (A) and eventually to zeaxanthin (Z). Lowering of the pH in the lumen space is thought to activate the enzyme violaxanthin de-epoxidase (VDE) that is responsible for the V→Z conversion (Yamamoto and Bassi 1996). Upon transition to normal growth irradiance, Z is converted back to V via the enzyme zeaxanthin epoxidase. In addition, one of the PSII subunits, PsbS, has been shown to be essential for the qE-type of non-photochemical quenching (Li et al. 2000). Over-reduction of the PQ pool due to excessive light also triggers a protein kinase that phosphorylates the light

harvesting complex (LHC) and other protein subunits of the PSII holocomplex (Bennett 1979). Phosphorylated LHC detaches from the core antenna of PSII and is separated from the PSII-core complex. This phenomenon is known as a transition from state 1 to state 2 (Williams and Allen 1987). State transition results in quenching of Chl fluorescence known as qT. However, the quenching extent of qT is usually much less than that of qE and qI (Krause and Weis 1991), and is not considered to play a key role in photoprotection (Niyogi 1999; Müller et al. 2001). The third component of NPQ is related to photoinhibition of photosynthesis. It has been observed that photodamaged PSII reaction centers cannot perform a stable charge separation but are efficient in dissipation of absorbed energy (Cleland et al. 1986). This type of quenching is normally observed as a decrease in the yield of Chl fluorescence recorded after a long dark incubation period (Krause and Weis 1991). At present, there is no clear hypothesis regarding the mechanism of qI. However, it has been suggested that photoinhibition-type of non-photochemical dissipation of excitation energy can involve both reversible and permanent photoinactivation of the PSII (Krause 1988). Moreover, there have also been suggestions in support of a relationship between qI and Z accumulation (Verhoeven et al. 1996; Niyogi 2000). In fact, it has been proposed that qE and qI follow a similar mechanism regarding the accumulation of Z, with the only difference being their formation and relaxation kinetics (Demmig-Adams 1990).

1.4.3 Signaling by reactive oxygen species

Basically, ROS in chloroplast are promoted by an imbalance between rate of photon absorption and utilization. As mentioned earlier, predominantly $^1O_2$ is formed in the vicinity of PSII, H_2O_2 and O_2^- are generated around the acceptor side of PSI. At high level, the generated ROSs may become toxic leading to cell death. However, lower amount of the ROS than the lethal level may serve as a signal that triggers the photoprotection through both nuclear and chloroplast gene regulation.the *flu* mutant in *A.thaliana* that accumulates chlorophyll biosynthesis intermediate protochlorophyllide (Pchlide) has been reported to have elevated level of $^1O_2$. Numerous nuclear stress responsive proteins were found to be up-regulated in this mutant. On the other hand, mutation in the chloroplast genes encoded for

EXECUTER1 and EXECUTER2 protein leads to reduced expression of stress responsive protein levels. These evidences may suggest that both Flu and EXECUTERs proteins may be protein involved in the signaling cascade of $^1O_2$ (Lee et al. 2007). Signaling by H_2O_2 has been suggested to be transmitted by mitogen activated protein kinase (MAPK) that subsequently activates gene expression in the nucleus (Pfannschmidt et al. 2008). Moreover, other factors may also stimulate gene expression such as glutathione or ROS and/or ROS-scavenging products.

1.4.4 Photoacclimation

Long-term acclimation to irradiance stress entails the assembly of a truncated light-harvesting antenna size for the photosystems (Melis 1996) and adjustments in the stoichiometry between PSI and PSII (Smith et al. 1990; Webb and Melis 1995; Melis 1998). Such acclimation mechanisms are manifested as an overall reduced content of cellular Chl and the thylakoid membrane space. These adjustments help attenuate light absorption, regulate and better balance the relative activities of the light versus the carbon reactions. Photoacclimation processes optimize photosynthetic reactions and, thus, bring about attenuation of the rate of PSII photodamage (Park et al. 1997; Baroli and Melis 1998; Huner et al. 1998; Melis 1999).

Although the aforementioned HL responses seem to be conserved among various plant species, different organisms exhibit different level of tolerance toward irradiance stress. Some organisms can sustain extreme irradiance whereas some others are shade obligated. This suggests a possibility that regulation and the level of responses may be different among individual plants; or else there are other unidentified responses that promote or attenuate tolerance toward irradiance-stress conditions.

In this study, to dissect high light response in irradiance-intolerant plant, study of the changes in protein expression using a proteomic approach was undertaken in a shade obligated unicellular green alga, *Chlamydomonas reinhardtii*. Due to the fact that under natural field-grown condition plants are rarely exposed to high light for more than 6 h, the analysis was performed during the initial 6 h of transition to LL to HL growth intensity. Samples were collected at time 0 (LL), and

after 1.5, 3, and 6 hours interval after high light exposure. The unicellular green alga *C. reinhardtii* was employed in this research because it offers several significant advantages including the fact that its photosynthetic apparatus are closely related to that of vascular plants, ease of cultivation in the laboratory as well as having a short life cycle. More importantly, its genome has been completely sequence, which should aid the protein identification by mass spectrometry.

CHAPTER II
OBJECTIVE

Due to the fact that detail molecular mechanisms underlying plant responses to irradiance stress are far from complete, my objective is to investigate such response in a model unicellular green alga, *Chlamydomonas reinhardtii*, using a proteomic approach as following:

1. to follow changes in overall protein expression profile during the shift of *C. reinhardtii* culture from low light to high light.
2. to elucidate the identities of proteins that showed differential expression during the experimental course.

CHAPTER III
MATERIALS AND METHODS

3.1 Algal strain and growth conditions

Chlamydomonas reinhardtii strain CC–503, obtained from the *Chlamydomonas* Culture Collection (http://www.chlamy.org), was grown photoautotrophically in a Tris–Bicarbonate–Phosphate (TBP) medium (Polle et al. 2001). Initially the algal cultures were grown under LL (50 µmol photons m^{-2} s^{-1}). When the cultures reached mid–logarithmic phase, they were shifted to HL (1,200 photons m^{-2} s^{-1}). Cells were collected at time 0 (LL), 1.5, 3, and 6 h after the LL→HL shift for further analyses.

3.2 Fv/Fm measurement

Maximum quantum efficiency of the PSII of intact cells was measured as the F_v/F_m ratio using a standard pulse–amplitude modulated (PAM) fluorometer model FMS–2 (Hansatech Instruments, UK).

3.3 Protein isolation and separation by 2–DE

Cell aliquots were subjected to centrifugation at 2,000 *g* for 2 min at room temperature and the supernatant was discarded. The pellet was washed twice with distilled water before lysis with buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% thiourea. Cell debris and unsolubilized materials were separated by centrifugation at 10,000 *g* for 5 min, the resulting green supernatant was transferred to a new microcentrifuge tube while the pellet was discarded. To eliminate photosynthetic pigments and other

hydrophobic compounds that may interfere with 2–DE, the supernatant was added with 3–4 volumes of ice–cold acetone and kept at –20 °C overnight. The precipitated material was harvested by centrifugation at 10,000 *g* for 5 min and was resuspended in rehydration buffer containing 8 M urea, 4% CHAPS and 2% thiourea, 2% IPG buffer, 40 mM Dithiothreitol, 0.002% bromophenol blue. Protein concentration of the extraction was determined using Bradford protein assay kit (Bio–Rad Laboratory). Approximately 500 µg of protein samples were subjected to 2–DE using Immobiline dry strip pH 4–7 (GE Healthcare) with running condition set according to the protocol recommended by the manufacturer (GE Healthcare). Protein spots were visualized upon staining the resolved 2–DE gel with colloidal Coomassie blue G. Proteins from at least 3 independent biological replicates of each time interval of LL→HL shift were isolated. For each of the biological replicate, the isolated proteins were resolved on at least 3 or 4 2–DE gels, called sample replicates.

3.4 Image analysis and spot comparison

Gel images were scanned and analyzed electronically with computer software PDQuest (Bio–Rad Laboratory). The best 2–DE image from each of the sample replicates was selected as a representative of the corresponding biological sample. Protein spot patterns from independent biological replicates of the same time point were compared. The spots that consistently appear on every biological replicates of each time were marked by the software for construction of a master image and the spot intensities were averaged. Any protein spot inconsistently appears in different biological samples was not included in the master image and was also ignored from the subsequent cross–comparison with the spot patterns from other time points. Master images from different time intervals were compared and statistically analyzed by two–way student *t*–test for significant difference (P value < 0.05). In each spot was normalized by total intensity in each gel. The spots that showed significant up– or down–regulation were subjected for subsequent tryptic digestion and protein identification by mass spectrometry.

3.5 In-gel digestion

After protein spots were excised, the gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Jaresitthikunchai et al., 2009). The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

3.6 HCT Ultra LC-MS analysis

The digested peptides were injected into Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 80% 0.1% formic acid in 80% acetonitrile was run in 40 min.

3.7 Protein identification

All retrieved MS/MS mass spectra were reached via online mascot tool by several following parameters; Data base NCBI, MSDB, and Swiss Prot (mostly achieved protein searching by NCBI), allowing up to 1 missed cleavages, fixed carbamidomethyl variable, variable methionene oxidation modification, peptide tolerance ±1.2 Da, MS/MS tolerance ± 0.6 Da, peptide charge 1+,2+, and 3+. Result searching were agreed that mass spectrum from a spot protein correlate hit protein from data bases if probability based Mowse score is more over indicate identity value or easily interpret by red bar which represent candidate protein appearance out site green area.

CHAPTER IV
RESULT

4.1 Irradiance stress in *C. reinhardtii*

Irradiance stress was imposed on the cells of *C. reinhardtii* by shifting the cultures from LL growth intensity to HL. Under this experimental condition, photo–oxidative damage was manifested as the lowering of the PSII photochemical efficiency (F_v/F_m) determined by PAM fluorometer. The F_v/F_m ratio declined from ~0.85, which is the typical value for healthy plants, to a low value of ~0.50 within 2 h and was retained at this number during the subsequent 2 to 6 h of HL exposure (Fig. 1). Such lowering in the F_v/F_m value is a common parameter indicating photo-oxidative stress in plants. This result, therefore, affirms that our HL condition was sufficient to elicit irradiance–stress responses for the subsequent proteomic analysis.

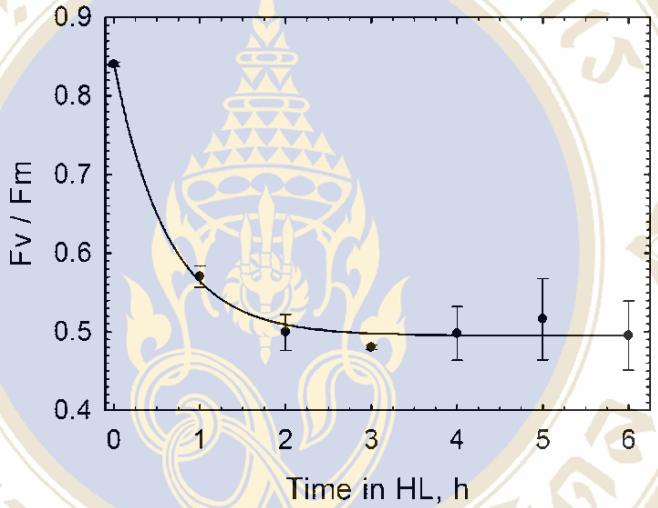


Figure 1. Fv/Fm value of *C. reinhardtii* during the shift from LL to HL for 6 hours. data points are average of 3 independent experiments ± SE.

3.2 Proteomic analysis

Total proteins extracted from cell aliquots of *C. reinhardtii*, which include both soluble and membrane polypeptides, were resolved on 2–DE and stained with Commassie Brilliant Blue G. We opted for the use of Coomassie staining because the intensity of the protein spots stained by this type of dye is more consistent and is quantifiable. Although many of the low–abundant proteins might be undetectable by the Coomassie staining, we could already discern a large number of polypeptide spots in this study. Initially a broad range of pH gradient (3–10) was employed for the 1st dimension. However, as most of the proteins scattered between pH 4–7 (data not shown), we therefore used the latter narrow gradient for better resolution. Fig. 2 shows representatives of the 2–DE–resolved gels of the samples collected at time 0 (LL), 1.5, 3 and 6 h after HL exposure. In the 4–7 pH range, we could detect approximately 514 protein spots consistently present in all 3 independent biological replicates of the LL samples (Fig. 2a). Using the same comparative criteria, proteome of *C. reinhardtii* cultures exposed to photoinhibitory condition for 1.5 h contained ~526 protein spots (Fig. 2b). At 3 h after the HL transition, ~530 proteins could be detected (Fig. 2c). After *C. reinhardtii* was exposed to irradiance stress for 6 h, about 527 total protein spots were observed (Fig. 2D). By normalization spot proteins, they were used total intensity of itself.

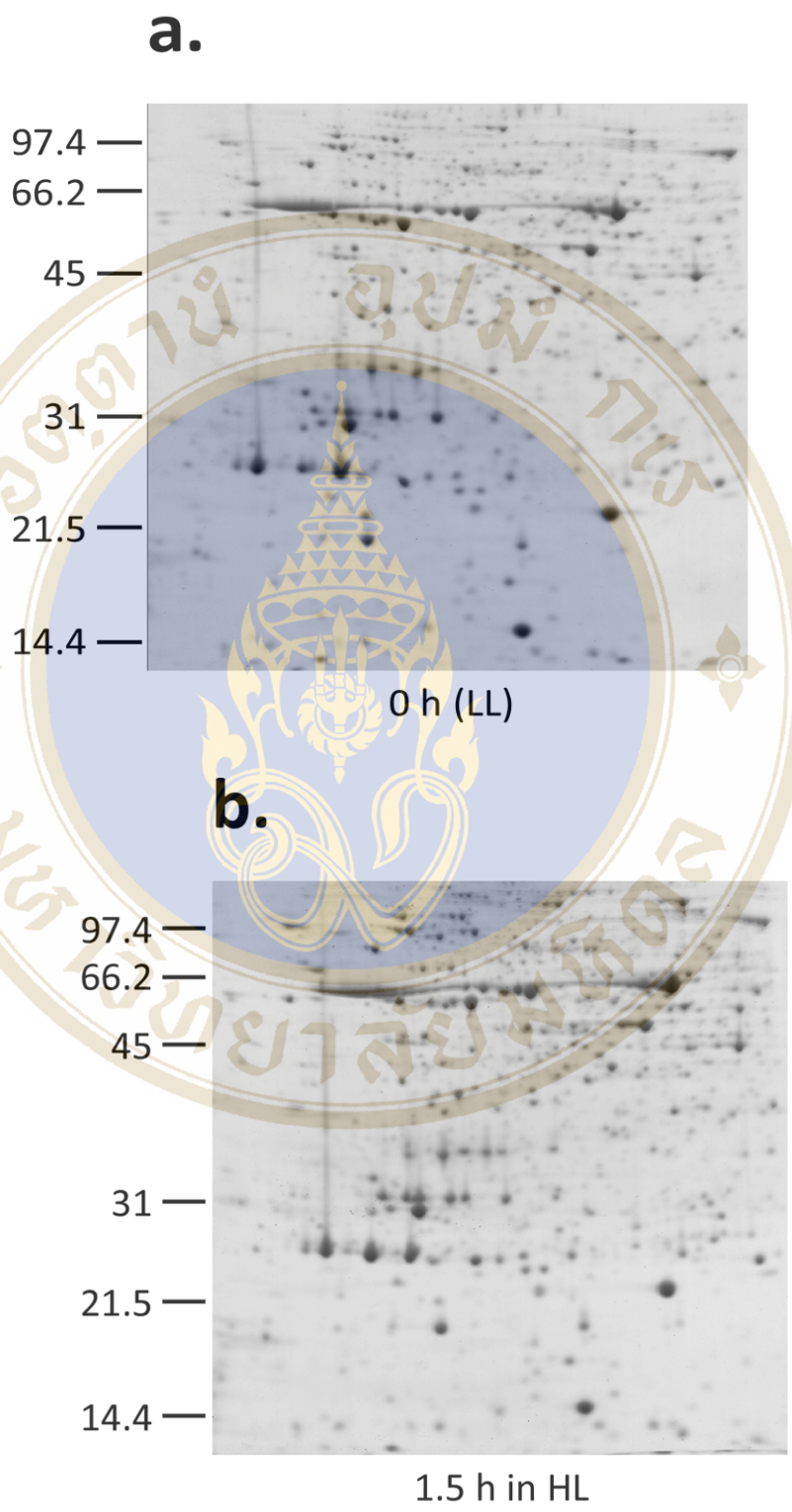
Cross comparison of the proteome profiles between LL–grown alga and the cells at 1.5 h after the LL→HL shift revealed that 19 proteins were down–regulated while the intensities of 18 spots were enhanced. It must be noted that the number of up-or down regulated protein spots are counted based on polypeptides that show statistically significant change in spot intensity. Therefore, the difference in total number of protein spots between sample at LL and 1.5 h after HL exposure may not always consistent with the number of proteins spots that are up- or down-regulated. These protein spots were subjected to identification by LC–MS/MS and their identities are listed in Table 1. Among the proteins that their expression deviates from the pattern under LL, we could detect a state transition of the LHC-II and enhanced expression of the oxygen evolution enhancer (OEE) protein, which are the typical landmarks for irradiance-stress response. Transition from state 1 to state 2 is initiated upon phosphorylation of the LHC-II subunits by STN7 kinase under

excessive irradiance (Bellafiore et al. 2005). Such phosphorylated LHC-II then detaches from the PSII complex to attenuate the ensuing photon flux reaching the reaction center core. In Table 1, the protein spot # 2130 and 2132 were identified as the same protein with the NCBI accession number gi|20269804 (major light-harvesting complex II protein M1). The spot number 2132, whose apparent pI is 5.1, showed a decrease in spot intensity after 1.5 h of the transition from LL→HL. On the other hand, expression of the spot number 2130, which has a lower pI of 4.8, was enhanced under the same condition. Moreover, there are 5 other up-regulated LHC-II proteins that their observed pI is lower than the theoretical value (Table 1). These results suggested that the pI of the LHC-II was decrease in response to the excessive irradiance. Such lowering in pI of the same protein could originate from the a phosphate group added to the polypeptides. We also detected an enhanced expression of phytoene desaturase (PDS, Table 1). PDS is one of the important enzymes in carotenoid biosynthesis pathway catalyzing a conversion of phytoene to ζ-carotene via 2 successive dehydrogenation reactions. As carotenoid accumulation is one of the typical plant responses to HL, the elevation of PDS is foreseeable and is commonly observed (Steinbrenner and Linden 2003; Schäfer et al. 2006). We also saw changes in the expression, both up and down, of proteins related to cellular metabolisms. Of particular interest are the down regulations of several amino acid biosynthesis enzymes in response to HL whereas expressions of proteins involved in carbohydrate metabolism are enhanced.

When the alga was exposed to HL for 3 h, the expressions of 9 additional proteins were decrease and 10 were elevated (Table 2). These additional protein changes were non–redundant with the spots identified at 1.5 h, which is already excluded from this comparison. Under this condition, we could still observe an ongoing of the state transition of the Chl antenna proteins, particularly the minor LHC, as well as the increase in the abundance of the OEE proteins. Another up-regulated protein observed after 3 h of HL exposure is the chloroplast-localized heat-shock protein 70 (HSP70B). Expression of HSP70B, at both transcript and protein levels, has been reported to be enhanced under HL (Drzymalla et al. 1996; Schroda et al. 1999). It has been suggested that HSP70B facilitates the repair of photodamaged PSII, of which the rate is accelerated under irradiance stress (Schroda et al. 2001;

Yokthongwattana et al. 2001). Therefore, the increase in HSP70B abundance in this work is consistent with its postulated function. However, to our surprise was the observation that level of the chaperonin 60 B1 subunit (CPN60) decreases after 3 h of the LL→HL shift. Due to normally chaperone proteins have to protect the cell by increasing level.

After prolonged exposure of *C. reinhardtii* cultures to irradiance stress for 6 h, a large number of proteins are underexpressed whereas the level of only 2 proteins were elevated (Table 3). Of those, most are proteins involved in wide range of general cellular processes, such as amino acid and carbohydrate metabolisms, cytoskeleton and cell movement, etc. We also noticed the decline in the amount of the antenna proteins without concomitant increase of the phosphorylated forms. This observation suggested that at 6 h of HL exposure, the Chl antenna size is being truncated rather than being phosphorylated. Furthermore, our proteomic analysis also revealed a remarkable down regulation of several other molecular chaperones beside the CPN60. Such chaperones include HSP70A, HSP70E, and ClpC (HSP100 family).



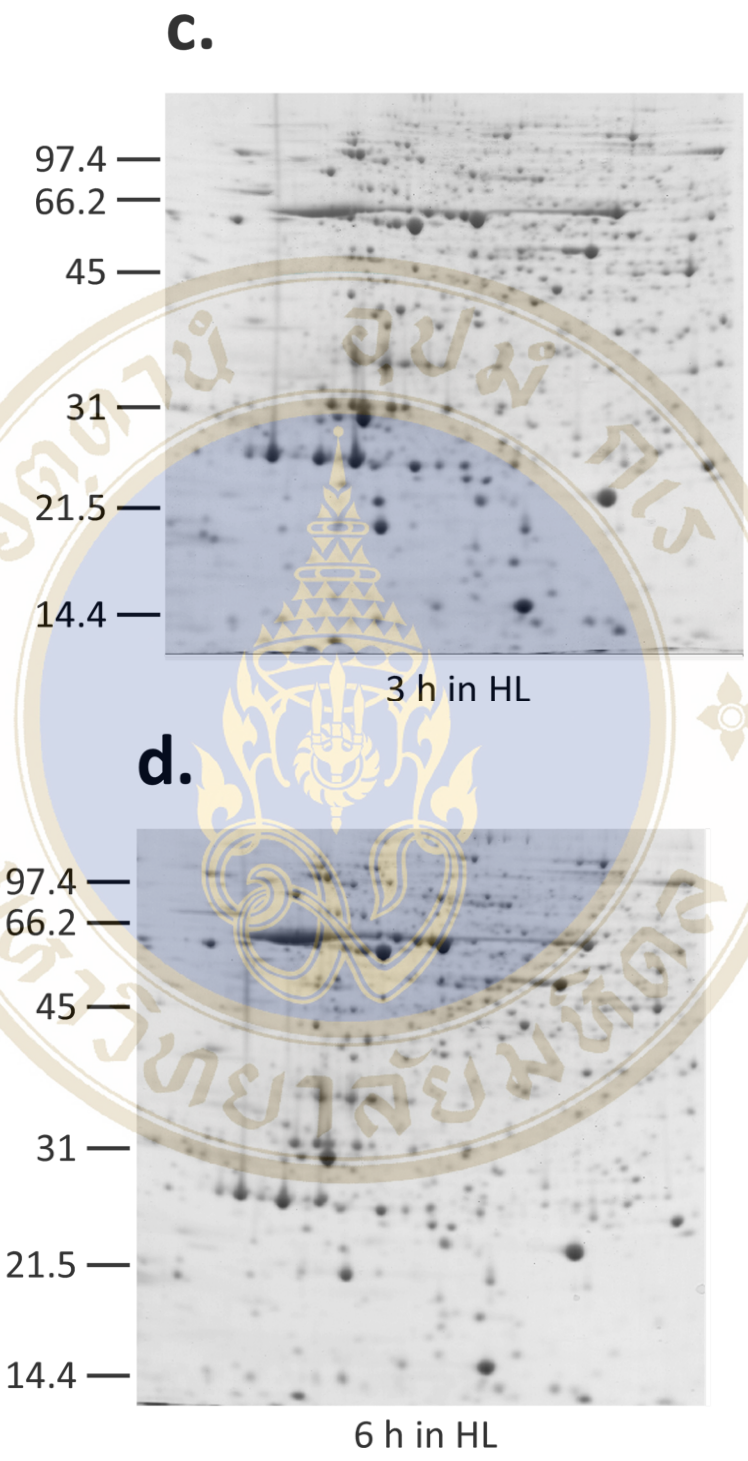


Figure 2. Represenatives of two-dimensional gel electrophoresis protein profiles of *C. reinhardtii* samples collected at different time intervals during the LL to HL shift for 0 (a), 1.5 h (b), 3 h (c), and 6 h (d). Proteins were stained with colloidal Commassie Blue G.

Table 1. list of identified *C. reinhardtii* proteins with considerable significantly difference between LL to exposed 1.5h in HL.

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	1.5 h (HL)
Down-regulated proteins								
Photosynthetic proteins								
2132	Major light-harvesting complex II protein M1	*C. reinhardtii*	gi|20269804	25.4 / 5.1	27.6 / 5.9	252	15071	11437
6334	Chlorophyll *a-b* binding protein of photosystem II	*C. reinhardtii*	gi|159478202	34.5 / 5.9	30.1 / 6.2	93	452	214
7331	Chlorophyll *a-b* binding protein of photosystem II	*C. reinhardtii*	gi|159478202	35.5 / 6.3	30.1 / 6.2	219	1856	1127
8627	RuBisCO large subunit	*C. reinhardtii*	gi|41179049	65.9 / 6.5	53.2 / 6.1	471	1631	996
Carbohydrate metabolism								
4229	Ribose-5-phosphate isomerase	*C. reinhardtii*	gi|159467673	30.1 / 5.5	29.0 / 7.6	95	5402	3230
8731	6-Phosphogluconate dehydrogenase	*C. reinhardtii*	gi|159477567	73.1 / 6.6	61.3 / 8.4	516	631	516
Amino acid metabolism								
7738	Acetohydroxyacid dehydratase	*C. reinhardtii*	gi|159470063	72.9 / 6.4	64.8 / 7.5	335	1632	1241
5328	Diaminopimelate epimerase	*C. reinhardtii*	gi|159479426	33.8 / 5.6	34.8 / 6.9	366	903	742
8628	Isopropylmalate dehydratase large subunit	*C. reinhardtii*	gi|159488260	60.0 / 6.5	53.6 / 7.0	385	1192	743
7627	Isopropylmalate dehydratase large subunit	*C. reinhardtii*	gi|159488260	59.3 / 6.3	43.1 / 6.0	416	1159	880

Table1. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	1.5 h (HL)
Down-regulated proteins Protein translation								
4527	Eukaryotic initiation factor 4A-like protein	*C. reinhardtii*	gi|159466510	53.4 / 5.5	47.3 / 5.5	703	1209	618
6737	Aspartyl-tRNA synthetase	*C. reinhardtii*	gi|159474374	70.2 / 5.8	60.7 / 5.6	385	469	353
Down-regulated proteins Energy metabolism								
6744	Vacuolar ATP synthase subunit A	*C. reinhardtii*	gi|159480680	76.9 / 6.0	69.0 / 5.7	198	770	551
7735	Succinate dehydrogenase subunitA	*C. reinhardtii*	gi|159463224	77.8 / 6.3	69.8 / 7.5	160	927	759
Proteins with other function								
3629	Selenium binding protein	*C. reinhardtii*	gi|159490794	61.1 / 5.3	52.5 / 5.2	504	1930	1392
5917	Hypothetical protein CHLREDRAFT_120875	*C. reinhardtii*	gi|159481287	93.8 / 5.6	101.8 / 5.7	80	748	653
6538	Sugar nucleotide epimerase	*C. reinhardtii*	gi|159462534	52.4 / 6.0	43.8 / 5.8	186	891	752
7329	26S Proteasome regulatory subunit	*C. reinhardtii*	gi|159479806	41.5 / 6.2	37.1 / 5.7	143	1311	872
8630	Ascorbate peroxidase	*C. reinhardtii*	gi|159487873	68.6 / 6.5	36.0 / 8.67	174	648	401

Table1. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	1.5 h (HL)
Up-regulated proteins Photosynthetic proteins								
2130	Major light-harvesting complex II protein M1	*C. reinhardtii*	gi|20269804	25.8 / 4.8	27.6 / 5.9	271	5510	14242
1236	Light-harvesting complex II chlorophyll *a-b* binding protein M3	*C. reinhardtii*	gi|159491492	31.9 / 4.8	27.4 / 5.7	152	79	1099
242	Stress-related chlorophyll *a/b* binding protein 2	*C. reinhardtii*	gi|159475924	31.1 / 4.4	28.2 / 4.8	227	n/d	795
1230	Stress-related chlorophyll *a/b* binding protein 1	*C. reinhardtii*	gi|159476046	28.9 / 4.9	27.5 / 4.9	108	n/d	1030
2224	Light-harvesting complex II chlorophyll *a-b* binding protein M3	*C. reinhardtii*	gi|159491492	30.9 / 4.9	27.4 / 5.7	103	2241	4208
2228	Chlorophyll *a-b* binding protein of LHCII type I	*C. reinhardtii*	gi|115827	31.1 / 5.0	27.0 / 6.0	73	2619	4347
3223	Chlorophyll *a-b* binding protein of LHCII type I	*C. reinhardtii*	gi|115827	30.5 / 5.3	27.0 / 6.0	301	2254	4081
3126	Oxygen-evolving enhancer protein 2	*C. reinhardtii*	gi|131389	19.6 / 5.3	25.9 / 9.1	361	463	1400
2730	RuBisCO large subunit-binding protein alpha subunit	*C. reinhardtii*	gi|2493647	76.1 / 5.0	62.0 / 5.6	760	n/d	688

Table1. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	1.5 h (HL)
Up-regulated proteins								
Carbohydrate metabolism								
6428	Phosphoribulokinase	*C. reinhardtii*	gi|159471788	46.4 / 5.9	42.1 / 8.1	310	n/d	605
5225	Triose phosphate isomerase	*C. reinhardtii*	gi|159463610	28.5 / 5.7	30.4 / 7.6	272	n/d	751
6223	Triose phosphate isomerase	*C. reinhardtii*	gi|159463610	28.5 / 6.0	30.4 / 7.6	540	1441	2455
Pigment biosynthesis								
3424	Magnesium-chelatase subunit ChlI	*C. reinhardtii*	gi|20137882	47.6 / 5.2	45.5 / 6.2	411	n/d	392
8632	Phytoene desaturase	*C. reinhardtii*	gi|159465297	66.1 / 6.6	63.0 / 7.7	106	n/d	344
Protein translation								
8422	Plastid-specific ribosomal protein 1	*C. reinhardtii*	gi|159479306	47.4 / 6.5	31.9 / 9.2	412	n/d	431
Unknown proteins								
238	Putative membrane protein	*C. reinhardtii*	gi|159488214	26.6 / 4.3	30.0 / 5.0	288	n/d	833
6544	Hypothetical protein CHLREDRAFT_132041	*C. reinhardtii*	gi|159482705	52.5 / 6.0	47.0 / 6.4	144	n/d	687

Table 2. list of identified *C. reinhardtii* proteins with considerable significantly difference between LL to exposed 3h in HL.

Spot	**Matched Protein**	**Organism**	**NCBI Accession**	**Observed MW (kDa) / pI**	**Theoretical MW (kDa) / pI**	**MOWSE Search Score**	**Spot Density at Time (arbitrary unit)**	
							0 h (LL)	**3 h (HL)**
Down-regulated proteins Photosynthetic proteins								
4328	Light-harvesting chlorophyll-*a*/*b* binding protein Lhcb5	*C. incerta*	gi|87313239	35.2 / 5.4	27.6 / 4.7	54	3590	1873
8116	Light-harvesting chlorophyll-*a*/*b* binding protein Lhca3	*C. reinhardtii*	gi|87313217	26.0 / 6.6	27.1 / 8.1	117	1919	1378
Molecular chaperone								
4622	Chaperonin 60B1	*C. reinhardtii*	gi|159486163	70.7 / 5.5	62.3 / 6.3	583	548	367
Protein translation								
6920	Elongation factor 2	*C. reinhardtii*	gi|159490505	91.9 / 5.8	95.0 / 5.6	139	457	342
Amino acid metabolism								
7626	Acetolactate synthase small subunit	*C. reinhardtii*	gi|159484278	62.7 / 6.3	52.8 / 8.9	412	2112	1202
8626	Acetohydroxy acid isomeroreductase	*C. reinhardtii*	gi|159489328	67.6 / 6.5	60.6 / 8.3	75	1942	1713
Proteins with other function								
4826	Hypothetical protein CHLREDRAFT_192147	*C. reinhardtii*	gi|15947745a7	88.1 / 5.4	73.7 / 5.3	67	662	472
6826	Arsenite translocating ATPase-like protein	*C. reinhardtii*	gi|159488560	83.1 / 5.8	54.4 / 8.7	333	863	616

Table2. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	3 h (HL)
Down-regulated proteins								
Proteins with other function								
7623	S-Adenosylmethionine synthetase	*C. reinhardtii*	gi|159477124	62.4 / 6.1	43.1 / 6.0	271	915	560
Up-regulated proteins								
Photosynthetic proteins								
2326	Minor chlorophyll *a-b* binding protein of photosystem II	*C. reinhardtii*	gi|159475641	35.2 / 5.2	30.7 / 5.4	182	4575	5895
2328	Minor chlorophyll *a-b* binding protein of photosystem II	*C. reinhardtii*	gi|159475641	35.9 / 5.1	30.7 / 5.4	245	1919	4789
240	Stress-related chlorophyll *a/b* binding protein 2	*C. reinhardtii*	gi|159475924	33.2 / 4.3	28.2 / 4.8	58	n/d	746
4117	Oxygen-evolving enhancer protein 2	*C. reinhardtii*	gi|131389	21.7 / 5.5	25.9 / 9.3	437	n/d	946
5029	Oxygen-evolving enhancer protein 3	*C. reinhardtii*	gi|159486609	13.5 / 5.9	21.8 / 9.6	312	n/d	1821
Molecular chaperone								
2828	Heat shock protein 70B	*C. reinhardtii*	gi|159476666	83.2 / 5.0	72.1 / 5.3	99	731	1288
Carbohydrate metabolism								
6335	Phosphoglycolate phosphatase	*C. reinhardtii*	gi|159464681	36.4 / 5.9	33.5 / 5.4	48	182	290

Table2. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	3 h (HL)
Up-regulated proteins Proteins with other function								
616	Calreticulin 2 (calcium-binding protein)	*C. reinhardtii*	gi|159462862	63.6 / 4.5	47.4 / 4.5	384	1777	2722
245	Hypothetical protein	*C. reinhardtii*	gi|159475228	29.6 / 4.5	27.9 / 4.9	356	n/d	968
6331	Predicted protein	*C. reinhardtii*	gi|159463656	36.1 / 6.0	40.1 / 9.3	201	n/d	630

Table 3. list of identified *C. reinhardtii* proteins with considerable significantly difference between LL to exposed 6h in HL.

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	6 h (HL)
Down-regulated proteins Photosynthetic proteins								
6121	Chlorophyll *a*/*b*-binding protein	*C. reinhardtii*	gi|19421770	19.1 / 5.9	23.9 / 9.4	249	3631	1868
6328	Chlorophyll *a*/*b*-binding protein of photosystem II	*C. reinhardtii*	gi|159478202	35.0 / 5.8	30.1 / 6.2	294	2374	834
8219	Chlorophyll *a*-*b* binding protein of LHCII	*C. reinhardtii*	gi|159478875	29.0 / 6.5	28.7 / 7.8	69	1043	639
7529	RuBisCO large subunit	*C. reinhardtii*	gi|41179049	55.7 / 6.4	53.2 / 6.1	420	1116	699
Molecular chaperone								
6824	Chaperone Hsp100 family, ClpC-type	*O. lucimarinus*	gi|145356586	89.1 / 5.8	92.7 / 5.3	517	2941	1773
4732	Heat shock protein 70A	*C. reinhardtii*	gi|159486599	80.1 / 5.4	71.5 / 5.2	1466	2370	1720
Down-regulated proteins Molecular chaperone								
4923	Heat shock protein 70E	*C. reinhardtii*	gi|159475503	93.5 / 5.4	88.1 / 5.2	663	1264	641
3921	Heat shock protein 70E	*C. reinhardtii*	gi|159475503	93.3 / 5.3	88.1 / 5.2	1133	949	603

Table3. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	6 h (HL)
Down-regulated proteins Molecular chaperone								
3624	T-Complex protein theta subunit	*C. reinhardtii*	gi|159490756	66.4 / 5.2	58.0 / 5.6	265	907	607
Carbohydrate metabolism								
6639	ADP-glucose pyrophosphorylase small subunit	*C. reinhardtii*	gi|159467349	59.9 / 5.9	55.9 / 8.4	736	1148	701
7621	ADP-glucose pyrophosphorylase small subunit	*C. reinhardtii*	gi|159467349	61.0 / 6.1	55.9 / 8.4	205	866	330
3532	Fructose-1,6-bisphosphatase	*C. reinhardtii*	gi|159465323	53.9 / 5.1	44.9 / 5.6	86	2562	1587
3628	Galactose kinase	*C. reinhardtii*	gi|159487006	67.7 / 5.3	60.0 / 6.2	141	774	471
6743	Phosphoglucomutase	*C. reinhardtii*	gi|159479834	76.9 / 6.0	64.8 / 7.1	238	442	185
7537	Phosphoglycerate kinase	*C. reinhardtii*	gi|159482940	49.8 / 6.2	49.2 / 8.9	304	970	629
Down-regulated proteins Amino acid metabolism								
7625	Acetohydroxy acid isomeroreductase	*C. reinhardtii*	gi|159489328	67.2 / 6.3	60.6 / 6.0	329	1382	807
9524	Agmatine iminohydrolase	*C. reinhardtii*	gi|159484436	57.3 / 6.7	46.1 / 6.5	173	719	459
6735	Isopropylmalate synthase	*C. reinhardtii*	gi|159477008	77.8 / 5.8	48.0 / 8.4	190	499	310

Table3. Continued

Spot	Matched Protein	Organism	NCBI Accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	6 h (HL)
Down-regulated proteins								
Amino acid metabolism								
8546	LL-Diaminopimelate aminotransferase	*C. reinhardtii*	gi|159469820	55.4 / 6.6	48.3 / 8.3	763	980	586
9520	LL-Diaminopimelate aminotransferase	*C. reinhardtii*	gi|159469820	56.2 / 6.8	48.3 / 8.3	943	951	639
Cytoskeleton and cell movement								
3530	Alpha tubulin 1	*C. reinhardtii*	gi|159467393	59.9 / 5.3	50.2 / 5.0	687	1715	1028
8734	Dynamin-related GTPase	*C. reinhardtii*	gi|159485798	78.5 / 6.6	67.8 / 6.5	73	959	515
4830	Flagellar associated protein	*C. reinhardtii*	gi|159476808	92.2 / 5.4	90.9 / 5.3	61	871	284
Proteins with other function								
144	Hypothetical protein	*C. reinhardtii*	gi|159465102	17.9 / 4.2	14.2 / 8.6	61	1344	612
1418	Protein phosphatase 2C	*C. reinhardtii*	gi|159477373	49.0 / 4.6	39.1 / 4.7	201	1309	765
2324	14-3-3-like protein-related protein	*C. reinhardtii*	gi|74272601	32.9 / 4.9	29.7 / 4.9	114	2742	1759
5525	Predicted protein	*C. reinhardtii*	gi|159487851	56.7 / 5.7	53.9 / 6.3	232	410	188
6535	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase	*C. reinhardtii*	gi|159486551	58.1 / 6.0	51.9 / 6.2	392	501	314

Table3. Continued

Spot[a]	Matched Protein	Organism	NCBI[b] Accession	Observed MW (kDa) / pI	Theoretical[d] MW (kDa) / pI	MOWSE[e] Search Score	Spot Density at Time (arbitrary unit)	
							0 h (LL)	6 h in HL
Proteins with other function[f]								
6738	N-Ethylmaleimide sensitive fusion protein	*C. reinhardtii*	gi|159480686	79.9 / 5.9	78.7 / 5.7	54	424	235
6829	Hypothetical protein CHLREDRAFT_82920	*C. reinhardtii*	gi|159488381	84.0 / 6.0	77.2 / 5.7	188	721	474
7218	Predicted protein	*C. reinhardtii*	gi|159470065	30.6 / 6.1	31.3 / 8.2	177	796	527
7825	Putative chloroplast 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase precursor	*C. reinhardtii*	gi|61742128	83.8 / 6.1	75.2 / 5.8	239	1016	158
8221	4 Ran-like small GTPase	*C. reinhardtii*	gi|159467397	30.2 / 6.5	25.7 / 6.3	184	1415	715
8629	S-Adenosylmethionine synthetase	*C. reinhardtii*	gi|159477124	59.3 / 6.5	43.1 / 6.0	1039	1764	837
Up-regulated proteins Proteins with other function								
3625	ATP synthase CF1 beta subunit	*C. reinhardtii*	gi|41179057	60.3 / 5.3	53.2 / 5.2	298	2298	3780
7530	Phosphoglycerate kinase	*C. reinhardtii*	gi|1172455	49.8 / 6.3	49.3 / 8.8	250	661	1281

a. Spot number in gel b. NCBI accession number c. Mass and pI protein from 2-DE gel

d. Mass and pI protein from data base e. Score hits f. Function of protein

It is surprising that down regulation of several molecular chaperones was observed in our proteomic screen in this study. The averaged spots intensities of these proteins were then compared at each time point to see the trend of down-regulation processes (Fig. 3). Expression of chaperonin 60, β subunit, was significantly decreased just after 3 h of transition from LL to HL and remained at this level at 6 h. Levels of the other proteins, HSP70A, HSP70E, and ClpC (HSP100) did not significantly change during the first 3 h of HL exposure but become drastically reduced in the 6th h.

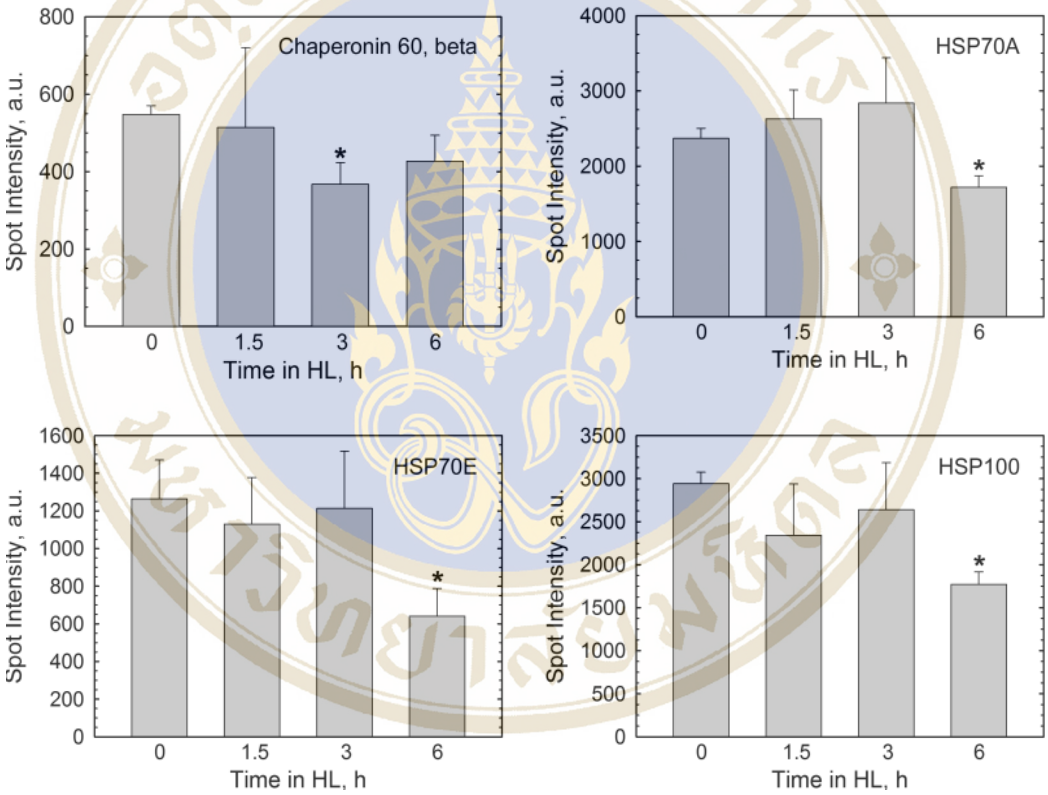


Figure 3. Comparative protein spot intensity of various molecular chaperone proteins that are found to be down-regulated during the LL→HL shift. Average spot intensities from each time point are illustrated with error bars represent SE. The asterisks (*) indicate significant difference statistically based on student *t*-test with *P* value < 0.05 when compare to 0h.

CHAPTER V
DISCUSSION

Although the overall responses of plants to irradiance stress are somewhat conserved among different photosynthetic organisms, the molecular mechanisms underlying such process are complex. Proteomic analysis of the model green alga, *C. reinhardtii*, in this study revealed several well known HL responses as well as a key finding that could possibly explain its light-sensitive nature. State transition is the well-known response of plants to excessive light. The phosphorylated LHC complexes detach from the reaction center core of the PSII resulting in slower rate of light absorption, hence, lowering the chance of the D1 protein damage. In *Chlamydomonas*, such state-transition could be discerned after 1.5 h of HL exposure. Increase expressions of the proteins involved in carbohydrate metabolisms could also help accelerate the utilization of electron generated by the photosynthetic light reactions, also minimizing the risk of superoxide formation. Another typical response of plants to irradiance stress is the increasing rate of chlorophyll and carotenoids biosynthesis. In *C. reinhardtii*, such response could be observed as an increase in spot intensities of the PDS and Mg chelatase enzymes (Table 1).

In the literature, primary functions of the molecular chaperones are thought to be for assisting protein folding/refolding. However, as molecular chaperones are the housekeeping proteins, they could have many other specialized functions, including translation, protein trafficking, proteolytic cleavage, etc. The first evidence supporting the active role of molecular chaperones during plant adaption to irradiance stress came from the work from Michael Schroda and his colleagues in Germany. Using *C. reinhardtii* as a model, this group of scientists discovered that down-regulation of a chloroplast-localized heat-shock protein 70 (HSP70B) by antisense technique makes the transformants more susceptible to photo-oxidative damage than wild type (Schroda et al. 1999). On the contrary, the transformants overexpressing such protein are more resistant to high light compared

to the wild type counterpart (Schroda et al. 1999). Yokthongwattana et al. (2001) further demonstrated that HSP70B could be part of the PSII repair intermediate complex. In this study, the increasing level of HSP70B after 3 h of transition to HL is consistent with the previous evidence in the literature regarding its functions.

However, to our surprise is the finding that several important molecular chaperones are down-regulated in *Chlamydomonas* exposed to excessive growth irradiance for up to 6 h. In particular, CPN60, ClpC, HSP70A and 70E were found to be underexpressed. HSP70 is a large protein family found in all living organisms. Although HSP70 chaperones have been reported to carry out a wide range of specialized cellular functions, including the PSII repair process, their predominant role is thought to be for helping renature the unfolded or misfolded proteins during stresses. HSP70A is a well-known cytosolic protein (Müller et al. 1992) believed to function as a typical chaperonin. HSP70E, on the other hand, was identified during the *C. reinhardtii* genome sequencing (Merchant et al. 2007) as an ORF that shares some degree of homology to the HSP70 and HSP110 protein family (Schroda 2004). It is predicted to encode for a cytosolic protein of about 87 kDa, the function of which has not been characterized. It is possible that both HSP70A and 70E may function in facilitating the transport/trafficking of nuclear-encoded proteins important for HL acclimation into the chloroplast. Down regulation of these two proteins, therefore, might results in the alga unable to cope with the excessive irradiance.

CPN60 or HSP60, which is a plastid homologue of bacterial GroEL (Viitanen et al. 1995), was suggested to help refold the denatured proteins by the same mechanism as that of the famous bacterial GroES/GroEL system. The primary substrate for the CPN60 could be Rubisco large subunit (RbcL) proteins (Schroda 2004). Decreasing level of the CPN60 may perhaps lead to accumulation of the Rubisco enzyme in the inactive form. Lacking of the active Rubisco leads to an imbalance between the rate of CO_2 assimilation and the rate of photon absorption and electron transport events. Therefore, lowering in the amount of the CPN60 could well explain the lower threshold of irradiance in *C. reinhardtii*.

HSP100 or Clp is also a large protein family found in both prokaryotes and eukaryotic organisms (Schirmer et al. 1996). The renowned function of Clp chaperones, especially ClpB, is their ability to dissociate protein aggregates and help

them refold (Goloubinoff et al. 1999). In the chloroplast stroma of plants and green algae, ClpC and ClpD are the two homologues of the HSP100 protein family (Zheng et al. 2002). So far, the only reported function of ClpC is believed to facilitate protein import into the chloroplast (Nielsen et al. 1997). Decrease amount of ClpC, together with the cytosolic HSP70s, may lead to less availability of the nuclear-encoded proteins, which could have important function in photoprotection/acclimation to irradiance stress. The steady-state amount of such imported proteins factors may not abundance enough for detection by the Comassie staining; therefore, they might not be detected in our work.

Altogether, our proteomic study showed that *C. reinhardtii* manifested a down-regulation of several of the key chaperones in response to irradiance stress. Only the chloroplast-localized HSP70B was increase in expression after 3 h of HL exposure, consistent with its proposed function in the PSII repair process. However, Förster et al. (2006) also reported that when *Chlamydomonas* was exposed to very high light intensities for longer period of time (24 h), the level of HSP70B also declined. The results presented in this work, thus, provide an important evidence for explanation of the light sensitive nature of this model alga. When these molecular chaperones are down-regulated, the chloroplast may have lower threshold of saturating irradiance due to (1) lacking of important key nuclear-encoded protein factors required for photoprotection/successful acclimation to HL, (2) several of the key enzymes/proteins that become inactive as the results of the excessive irradiance could not be adequately refolded/repaired.

CHAPTER VI
CONCLUSION

The work in this thesis demonstrates that in *Chlamydomonas reinhardtii*, a variety of molecular changes occur in response to irradiance stress. Many of the responses are similar to that of the existing reports in the literature regarding the same stress condition. The particular highlight in this thesis is the finding that several of the molecular chaperones, including HSP70A, HSP70E, CPN60 and ClpC, are down-regulated during the HL exposure of this alga. These heat-shock proteins have been reported to play important functions both for renaturing proteins and for translocation of proteins between cytosolic and organellar compartments. It is concluded that, therefore, the decrease in expression of these chaperones may render *Chlamydomonas reinhardtii* more sensitive to HL than many other organisms.

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APPENDIX

Process mascot searching

1. Fill in the setting in the blank following below panel

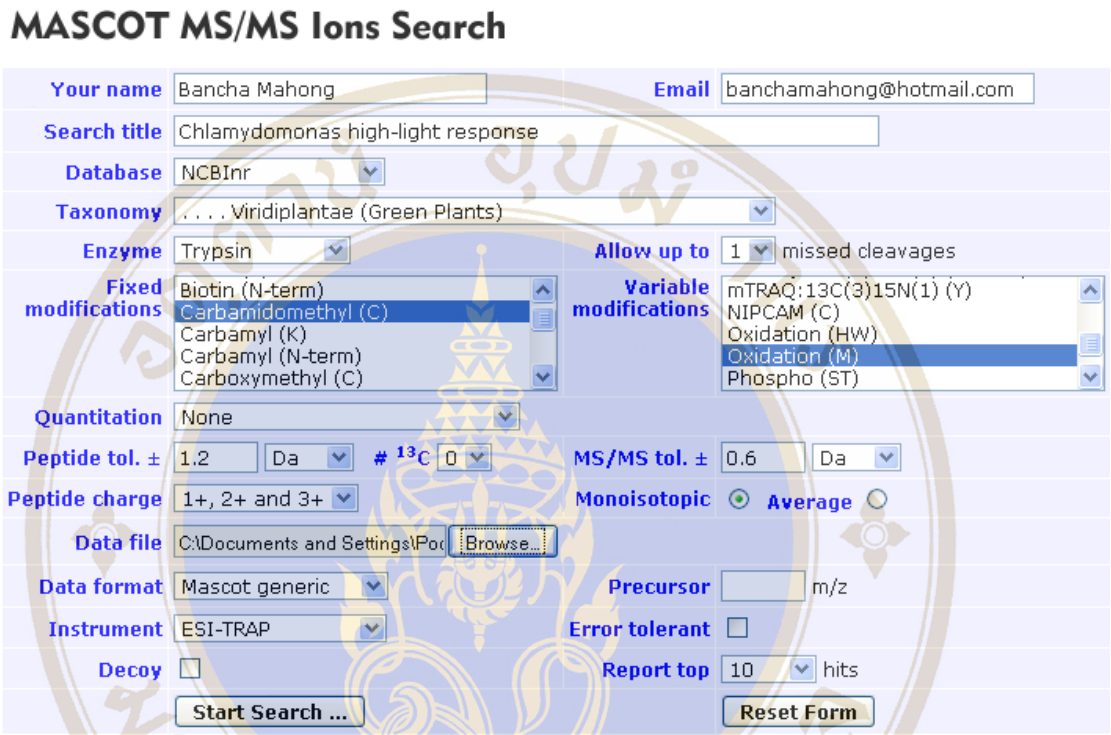

Fig3. The feature of MASCOT online program

2. Press browse button to choose any one mass spectrum file
3. Press start search button to initiate protein searching

```
Timestamp       : 27 Sep 2009 at 04:21:34 GMT
Protein hits    : gi|159482940 phosphoglycerate kinase [Chlamydomonas reinhardtii]
                  gi|159483707 glutamine synthetase [Chlamydomonas reinhardtii]
                  gi|129915    RecName: Full=Phosphoglycerate kinase, chloroplastic; Flags: Precursor
                  gi|145347850 predicted protein [Ostreococcus lucimarinus CCE9901]
                  gi|102140037 phosphoglycerate kinase, chloroplast, putative [Musa acuminata]
                  gi|223975935 unknown [Zea mays]
                  gi|147792805 hypothetical protein [Vitis vinifera]
                  gi|159472138 predicted protein [Chlamydomonas reinhardtii]
                  gi|116058864 unnamed protein product [Ostreococcus tauri]
                  gi|226459197 predicted protein [Micromonas pusilla CCMP1545]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 48 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

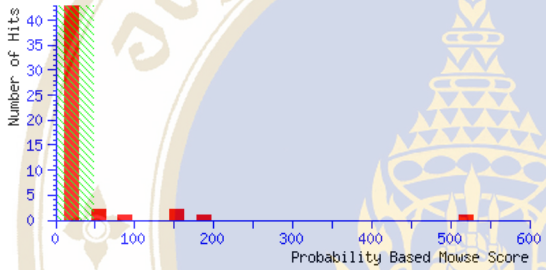

Fig4. The displayed feature of MASCOT program result after peptide searching

4. After pressing starch button, the window result is shown in above panel, 10 hits candidate proteins exhibiting in NCBI accession number. The top protein is the highest probably proteins which correlate the spot protein in gel.
5. According to this graph, the bars representing hitting proteins, raising outside the shade area is possible relievable candidate proteins and the far from shade area directly related the higher probability based Mowse score which the highest score is most possible correlation protein in the gel
6. Whenever click the highest score proteins, its detail is exhibited as figure.5.

1. gi|159482940 **Mass:** 49172 **Score:** 519 **Queries matched:** 10
phosphoglycerate kinase [Chlamydomonas reinhardtii]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
44	538.6420	1075.2694	1075.5509	-0.2815	0	19	72	2	K.ATLAITDDTR.I
48	581.3650	1160.7154	1161.6315	-0.9160	0	48	0.1	1	K.ITVIEALMEK.C + Oxidation (M)
59	710.7670	1419.5194	1419.6882	-0.1687	0	86	7.7e-06	1	K.ELDYLDGAVSNPK.R
60	724.3330	1446.6514	1446.8599	-0.2084	0	53	0.021	1	K.FLKPSVAGFLLQK.E
61	738.2620	1474.5094	1474.7894	-0.2800	0	53	0.013	1	K.IIIGGGMIFTFYK.A + Oxidation (M)
63	534.8800	1601.6182	1601.8512	-0.2330	1	(21)	22	3	K.VGSSLVEDDKIELAK.K
64	801.8430	1601.6714	1601.8512	-0.1797	1	59	0.0044	1	K.VGSSLVEDDKIELAK.K
67	887.8790	1773.7434	1773.9625	-0.2190	0	56	0.0071	1	K.FANGTVSIANTLAGLTPK.G
69	901.8310	1801.6474	1801.8880	-0.2406	0	86	5.7e-06	1	K.MSHISTGGGASLELLEGK.V + Oxidation (M)
70	640.2430	1917.7072	1917.9333	-0.2261	0	60	0.0017	1	K.LAANADLYVNDAFGTAHR.A

2. gi|159483707 **Mass:** 42689 **Score:** 172 **Queries matched:** 3
glutamine synthetase [Chlamydomonas reinhardtii]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
37	505.2290	1008.4434	1008.5393	-0.0958	1	41	0.28	1	R.SIFKDPFR.G
45	550.6930	1099.3714	1099.4678	-0.0964	0	67	0.00063	1	R.FACAEVMEK.A + Oxidation (M)
52	601.7790	1201.5434	1201.6666	-0.1232	0	64	0.0018	1	K.IGSLLDQSITR.H

3. gi|129915 **Mass:** 49980 **Score:** 151 **Queries matched:** 3
RecName: Full=Phosphoglycerate kinase, chloroplastic; Flags: Precursor
Check to include this hit in error tolerant search

Fig5. Array of hit peptides with different parameters and also exhibiting their sequences. The possible peptide that their mass from mass spectrum correlate to the mass from data base, including particularly some protein modification probability.

Search Parameters

Type of search	: MS/MS Ion Search
Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 1.2 Da
Fragment Mass Tolerance	: ± 0.6 Da
Max Missed Cleavages	: 1
Instrument type	: ESI-TRAP
Number of queries	: 83

List of mascot parameters that were used in the protein searching. These parameters were chosen for mascot MS/MS ion searching and also their effects can be used to calculate the Mowse score. Variable modifications parameter, peptide mass tolerance, and fragment tolerance are able to readjust to suit each.

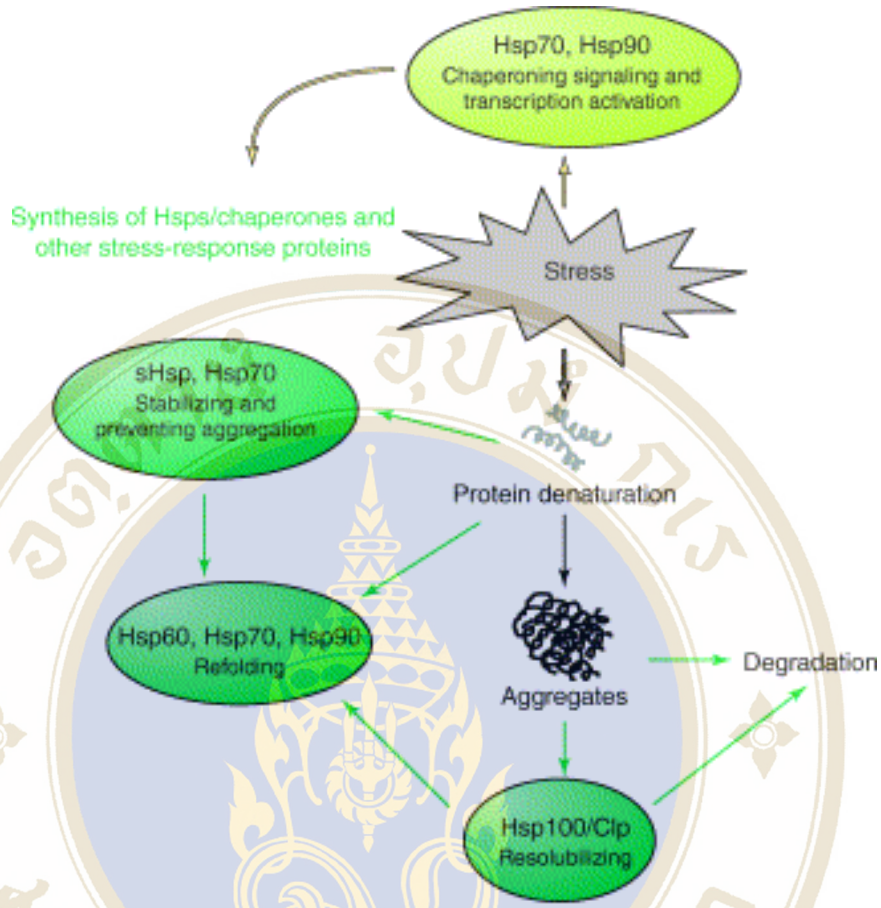

Fig.6 Important functions of several heat shock proteins for control homeostasis inside the cells. HSPs show a lot of involving stress response both abiotic and biotic stress by such as transcriptional signaling regulated other stress response proteins, prevent denatured protein aggregation, folding/refolding general proteins, and contribute resoluble proteins. Whenever, lack or reducing level of HSPs may be cause of tremendous proteins degradation.

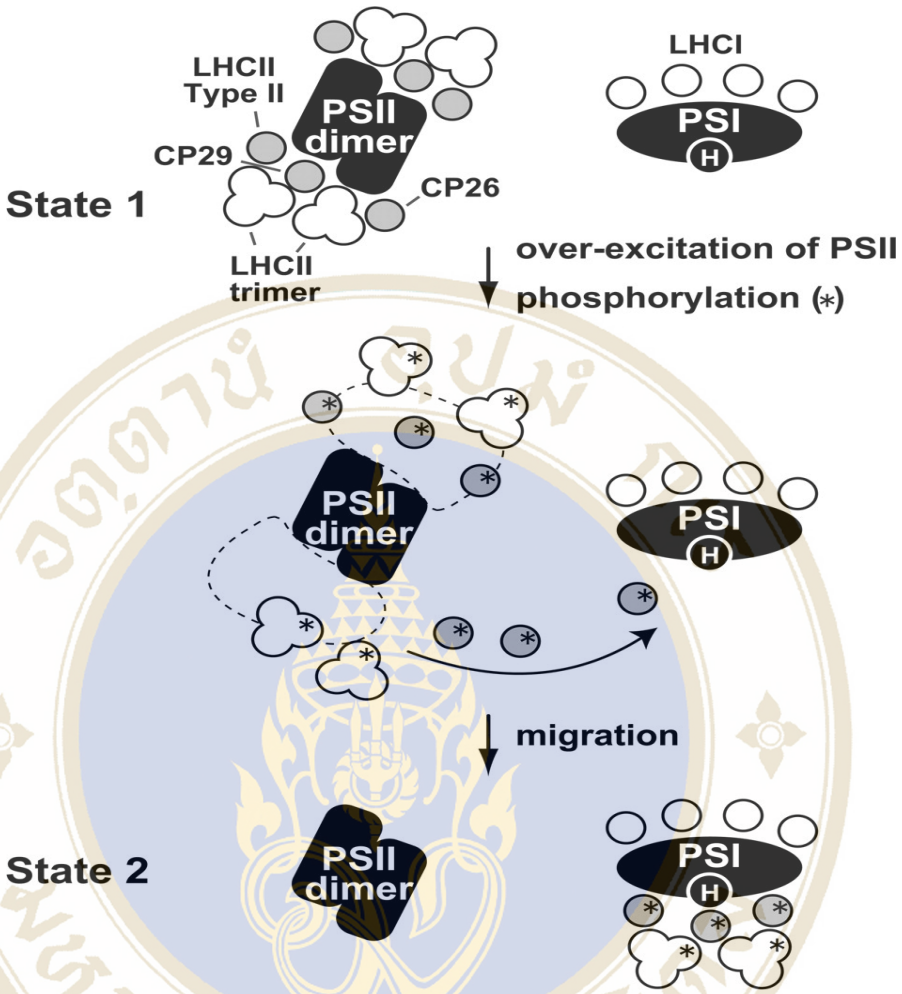

Fig 7. The diagram of state transition in relieving harmfulness of light intensity. In low light intensity a number of LHCII proteins that are responsible to bind amount of chlorophylls associate PSII as shown in white three monomers and gray circles. The both types contribute chlorophyll to absorb energy from light and further transfer toward PSII reaction center as so-called state 1. Upon high light environment raised level of kinase protein and consequence LHCII proteins are phosphorylated (asterisks) and leaded to LHCII proteins to be phosphorylated and detaching from PSII to association PSI. The results reduce number of PSII-absorbed energy.

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POSTER PRESENTATIONS

1.Bancha Mahong, Suttiruk Roytrakul, Narumon Phaonaklop, Kittisak Yokthongwattana, "Proteomic analysis of *Chlamydomonas reinhardtii* in response to high-light stress" in 2nd Biochemistry and Molecular Biology Conference (BMB) 2009, The Science Society of Thailand(May 7-8, 2009)

2. Bancha Mahong and Kittisak Yokthongwattana, "Proteome analysis of the respondses of the green alga *Dunaliella salina* to high light stress"in 2nd Annual Symposium of Protein Society of Thailand Odysseys in Protein Research. Chulabhorn Research Institute Conference Center, Bangkok, Thailand. (20-21 September, 2007)

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