รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาการควบคุมการแสดงออกของยีนและบทบาทการทำงานของโปรตีนแชเปอโรนิน 60 ภายใต้เครียดจากสภาพแวดล้อมในโมเดลสาหร่ายสีเขียวเซลล์เดียว *Chlamydomonas reinhardtii*

Regulation of gene expression and functional analyses of chloroplast-localized chaperonin 60 under environmental stresses in the model unicellular green alga *Chlamydomonas reinhardtii*

โดย รองศาสตราจารย์ กิตติศักดิ์ หยกทองวัฒนา

เดือน ปี ที่เสร็จโครงการ

พฤศจิกายน พ.ศ. 2560

สัญญาเลขที่ RSA5680032

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยมหิดล

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ชื่อโครงการ: โครงการการศึกษาการควบคุมการแสดงออกของยีนและบทบาทการทำงานของโปรตีนแชเปอโรนิน 60 ภายใต้เครียดจากสภาพแวดล้อมในโมเดลสาหร่ายสีเขียวเซลล์เดียว

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ระยะเวลาโครงการ: 3 ปี

โมเลกุลาร์แชเปอโรน หรือ ฮีทช็อคโปรตีน เป็นกลุ่มโปรตีนที่มีบทบาทที่สำคัญต่อการดำรงชีวิตของสิ่งมีชีวิตทุกชนิด ในบรรดาโปรตีนในกลุ่มนี้นั้น ข้อมูลทางวรรณกรรมเกี่ยวกับ HSP90B และ CPN60 นั้นยังมีอยู่น้อยมาก ในโครงการวิจัยนี้ ได้ศึกษาถึงการแสดงออกรวมทั้งการศึกษาโปรโมเตอร์ของยีน *HSP90B* ในสาหร่ายโมเดล *Chlamydomonas reinhardtii* ผลการทดลองพบว่ายีน HSP90B นั้นถูกกระตุ้นได้ด้วยอุณหภูมิที่สูงขึ้นและการกระตุ้นให้เกิด ER stress การศึกษาโปโมเตอร์ด้วย promoter truncation และ chromatin immunoprecipitation พบว่าโปรโมเตอร์ทำงานอยู่ในช่วง –1 to –253 bp จากตำแหน่งเริ่มการถอดรหัส และยังพบด้วยว่าส่วนของโปรโมเตอร์ที่เลย –253 bp ขึ้นไปเล็กน้อยน่าจะเป็นตำแหน่งที่ repressor มาจับเพื่อควบคุมการแสดงออกของยีน นอกจากนี้ โครงการวิจัยนี้ยังได้แสดงให้เห็นว่าการถ่ายยีนที่สร้างหน่วยย่อยของโปรตีน CPN60 จาก *Chlamydomonas* ไปยังแบคทีเรีย *E. coli* ยังสามารถสร้างโปรตีนลูกผสมระหว่างหน่วยย่อยของ CPN60 กับ GroEL อีกด้วย

คำหลัก : แชเปอโรนิน; สาหร่าย Chlamydomonas; CPN60; ฮีทช็อคโปรตีน; HSP90; การศึกษาโปรโมเตอร์; หน่วยย่อยของโปรตีน

Abstract

Project Code: RSA5680032

Project Title: Regulation of gene expression and functional analyses of chloroplast-localized chaperonin 60 under environmental stresses in the model unicellular green alga *Chlamydomonas reinhardtii*

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Project Period: 3 years

Molecular chaperones or heat shock proteins are a large protein family with important functions in every cellular organism. Among all types of the heat shock proteins, information on the ER-localized HSP90 protein (HSP90B) and CPN60 along with their encoding genes is relatively scarce in the literature. In this study, expression profiles as well as promoter sequence of the *HSP90B* gene were investigated in the model green alga *Chlamydomonas reinhardtii*. We have found that *HSP90B* is strongly induced by heat and ER stresses, while other short-term exposure to abiotic stresses, such as salinity, dark-to-light transition or light stress does not appear to affect the expression. Promoter truncation analysis as well as chromatin immunoprecipitation using the antibodies recognizing histone H3 and acetylated histone H3, revealed a putative core constitutive promoter sequence between –1 to –253 bp from the transcription start site. Our results also suggested that the nucleotides upstream of the core promoter may contain repressive elements such as putative repressor binding site(s). In addition, we also demonstrated that the chloroplast chaperonin 60 subunits, when heterologous expressed in *E. coli*, could interact with and assemble into high-molecular-weight tetradecameric complex.

Keywords : Chaperonin; Chlamydomonas; CPN60; heat-shock protein; HSP90; promoter analysis; subunits

เนื้อหางานวิจัย

1. Introduction and Literature Review

Rationale and significance of this research

Almost every country in the world today is facing with economic difficulty due to the rising price of fossil fuels. Alternative energy sources are being actively sought to help stabilize the retail petrol price. One of Thailand's top policies that the government has emphasized is the mixture of biodiesel with that refined from petroleum oil (to the ratio of 3-5%, termed B3 and B5, respectively). At present, the major source of biodiesel production in Thailand is palm oil, the yield of which significantly varies each year. When the harvest of palm seeds is low, the shunt of palm oil toward biodiesel production greatly affects the supply for human consumption. Thus, finding a new alternative source of oil for biodiesel production is of great interest. Microalgae have been touted as one of the most promising organisms for lipid accumulation capable of producing biodiesel (Halim et al. 2012). It was estimated that microalgae, in theory, can produce biomass that generates 100 times more lipids than soybean on equal land area of farming (Hu et al. 2008). The actual yield, however, is far from such calculated number.

Environmental stresses during cultivation, i.e. excessive irradiances, high salinity, heat stress, etc., are pinpointed as the key factors hampering real-world microalgal biomass/lipid productivity. In order to overcome such limitations, attempts have been made to screen for the most productive algal strains that can tolerate extreme environments while can still deliver the highest amount of lipids. This approach, however, is difficult and no one can guarantee that such algae do exist. Another approach is to genetically engineer the existing promising algal strains to have specific phenotypes that enhance lipid productivity (Larkum et al 2012). In order to do that, basic molecular understanding of how microalgae can successfully acclimate to various stress factors and maintain photosynthetic activity is needed. Scientists around the world have long been studying algal physiology in response to all kinds of abiotic stresses. Information on the desired physiological traits have been formulated (for review, see Wilhelm and Jakob 2011; Work et al. 2012). Relatively fewer interests, on the other hand, are focused on investigating structure and functions of specific proteins that play important roles under suboptimal growth conditions. Examples of such proteins include antioxidant enzymes, heat shock proteins or molecular chaperones, specific ribosomal proteins, etc.

Heat shock proteins are generally known for their classical function in assisting protein folding (reviewed by Bukau et al. 2006). Under stresses, a number of proteins within the cells undergo denaturation and aggregation. The chaperones, hence, function to refold the aggregated proteins and restore their functional properties (Liberek et al. 2008). In addition to the function in assisting protein folding, other unique functions, such as protein trafficking, protein translation, etc., have also been reported on several groups of chaperone proteins (Nielsen et al. 1997; Jackson-Constan et al. 2001; Yokthongwattana et al. 2001; Schroda 2004).

Part of the research data obtained from the existing TRF grant that the PI is holding (RMU5380037) also revealed possible active role of certain heat shock protein during algal adaptation to high salinity. We found that transcript level of *cpn60B1* and *cpn60B2*, which encoded for B1 and B2 subunits of chloroplast-localized chaperonin 60, were greatly enhanced in the unicellular green model alga *Chlamydomonas reinhardtii* that successfully acclimated and could actively grow in liquid media containing 300 mM NaCl (Salt Adapted, Fig. 1). (It must be noted that NaCl concentration of 150 mM is already lethal to higher plants.) Such enhanced transcript accumulation was not observed in *C. reinhardtii* control cells growing in normal recipe of the growth medium or in the cultures subjected to short-term salinity stress of the same 300 mM NaCl (Control and Salt Shocked in Fig. 1, respectively). This result suggested that the chloroplast-localized chaperonin 60 might play important roles in cellular adaptation to salinity stress of microalgae.

To date, there is very limited knowledge in the literature regarding the control of gene expression, structural arrangement of the subunits, and the natural substrates, of the chloroplastic chaperonin 60 (CPN60) under abiotic stress conditions. This project aimed to elucidate the structure and function as well as regulation of gene expression of this protein during abiotic stress response in microalgae. In addition, the research in this project was extended toward investigation of gene expression profile and promoter characterization of another stress-responsive molecular chaperone, HSP90B. Basic knowledge gained from this research can supplement the existing ideas on specific target for genetic manipulation to enhance real-world biomass and lipid production of microalgae.

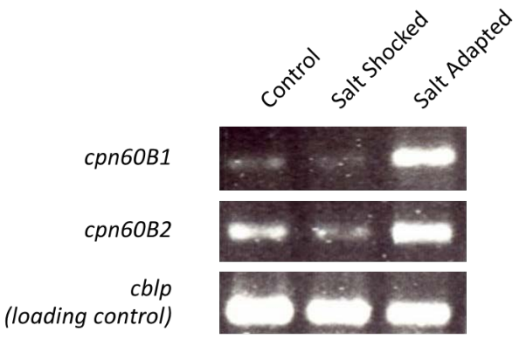

Figure 1. Semi-quantitative RT-PCR showing transcript accumulation of chloroplast-localized chaperonin 60 subunits B1 and B2 in the unicellular green alga *Chlamydomonas reinhardtii*. Cells were either grown under control growth condition or subjected to salt shocked with 300 mM NaCl for 2 h or salt-acclimated cells that were able to grow in media containing 300 mM NaCl. Expression of *cblp* gene is serving as loading control.

Different classes of molecular chaperones

Molecular chaperones are a group of proteins found in all living organisms from archaea to human. Classically, they are known for their function in assisting folding folding of other proteins as well as renaturing the unfolded or aggregated ones (Liberek et al. 2008). Based on amino acid homology and conserved motifs, molecular chaperones can be classified into 5-6 major groups as: HSP100/Clp family, HSP90 family, HSP70 family, chaperonins, small heat shock proteins, and calnexin/calreticulin (Miernyk 1999; Schroda 2004). Homologs for each of the classes have been identified in almost all major cell compartments of every organism. Most of the heat shock proteins have been extensively studied in great detail in terms of structure and functions. Below is the summary of what is known about each of the chaperone family in plants and algae.

<u>HSP100/Clp family</u>

HSP100 is also large protein family found in both prokaryotes and eukaryotic organisms (Schirmer et al. 1996). The chaperones belonging to this class vary in their molecular mass from 84 to 104 kDa (Miernyk 1999). According to numbers and structural arrangement of conserved motifs within the polypeptide chain, HSP100 family proteins can be divided into 2 subclasses as class 1 and class 2. Class 1 possesses an N-terminal domain, two nucleotide-binding domains (NBD), one variable middle domain and a C-terminal domain (Schroda 2004). Class 2, on the other hand, is smaller in size and only contains one NBD and the C-terminal domain. In its native

form, HSP100 proteins exist as a hexamer complex, the assembly of which requires ATP (Gallie et al. 2002). Some of the complexes also have proteolytic activity leading to the other name of the protein as Clp (standing for caseinolytic protease). In terms of the molecular mechanism, it was reported in prokaryotes that ClpB functions together with other HSP100 proteins to disintegrate the aggregated proteins in the cytosol (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 by working together with the translocon complex (Nielsen et al. 1997; Jackson-Constan et al. 2001).

HSP90 family

The actual sizes of proteins in this group are also variable between 82-96 kDa (Miernyk 1999). The much conserved primary structure, from prokaryotes to eukaryotes, can be separated into 3 distinct domains: an N-terminal domain, a middle domain and a C-terminal domain (Young et al. 2001). In addition to its independent function in maturation of proteins in the signaling cascades inside the cell, HSP90 proteins have also been found to work in association with other chaperones and cochaperones (Buchner 1999; Brychzy et al. 2003).

HSP70 family

HSP70 chaperones are highly conserved and can be found in almost all living organisms on this planet and are present in every cellular compartment. Despite of the conserved amino acid sequences, the observed molecular weight of the HSP70s can range from 68-110 kDa (Miernyk 1999). In plants and algae, the cytosolic HSP70A resembles that of other eukaryotic proteins. The chloroplast and mitochondrial version (HSP70B and HSP70C, respectively), however, is more closely related to the prokaryotic type (DnaK). Mechanism of action for chaperones in the HSP70 family has been elucidated in great detail (for extensive review, please see Mayer and Bakau 2005; Bakau et al. 2006). Other than their classical function in protein renaturation, certain members of the HSP70 chaperones have been found to perform other specialized functions. The first evidence supporting the active role of molecular chaperone 70 during plant adaption to irradiance stress came from the work from 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. Thus, it has been proposed that HSP70B plays important roles in the PSII repair process (Schroda et al. 2001; Yokthongwattana et al. 2009).

Chaperonins

Bacterial chaperonin GroEL is perhaps one of the heat shock proteins that have been studied almost thoroughly (Sigler et al. 1998). Based on sequence homology and evolutionary relationship, the chaperonins can be classified into two subgroups: Group I and Group II. Group I chaperonins are found in eubacteria, mitochondria and chloroplast while Group II covers that of archaea and eukaryotes (Boston et al. 1996). The large subunit of 60 kDa (GroEL) functions together with the smaller chaperonin GroES (~10 kDa).

Small heat shock proteins

Sometimes referred to as smHSPs or sHSPs, this class of molecular chaperones is small in size (15-30 kDa). sHSPs contain a conserved C-terminal domain similar to that of vertebrate's eye lens (Narberhaus 2002). Although the subunits are small in size, the functional complex can be as large as 400 kDa (Miernyk 1999).

Calnexins and calreticulins

These two groups of chaperones function inside the ER. Both calnexins and calreticulins bind calcium in high capacity and cooperate together to facilitate folding of protein de novo biosynthesized into the RER (Croft and Denecke 1998).

Structure and function of the chaperonin 60

Eukaryotic chaperonin 60s (CPN60s) belong to Group I and are homologs of the bacterial GroEL. Structural configuration of the function chaperonin complex is the same as that of the GroEL which is available in any biochemistry textbook. Briefly, individual subunits (~60 kDa each) assemble into two asymmetrically-stacked heptameric donut rings forming a barrel-like structure (Boston et al. 1996). The overall tetradecameric double-ring structure binds to the target denatured protein. One of two rings of GroELs is in a conformation that the inner surface becomes hydrophobic to facilitate the substrate binding. Subsequent ATP hydrolysis promotes a conformational change that expands the volume inside the barrel as well as a switch of the inner surface from hydrophobic to hydrophilic, allowing the denatured substrate to refold. Concomitantly, the co-chaperone GroES binds and closes the barrel. Another round of ATP hydrolysis releases the now-correctly-folded substrate as well as the GroES and the cycle repeats again (Bukau and Horwich 1998).

In bacteria and mitochondria, there exists only one gene copy for GroEL or chaperonin 60 and one copy for GroES or chaperonin 10. However, it is very interesting to note that in plant chloroplast, the GroEL homologs exist in more than one form: A and B (sometimes referred to as α and β, see Schroda 2004). Each form can also exist in more than one copy. The two isoforms share ~50% amino acid identity. Higher plant model, *Arabidopsis thaliana*, contains 2 genes coding for CPN60A, and 4 genes for CPN60B (Schroda 2004). In the unicellular green alga *Chlamydomonas reinhardtii*, the model organism to be used in this research, there are 3 genes coding for plastid chaperonin 60s: one gene coding for isoform A and two genes coding for isoform B. The three *Chlamydomonas* proteins are hereafter referred to as CPN60A, CPN60B1 and CPN60B2. It has been reported that isoform Bs of the chloroplast CPN60 can be assembled into the active tetradecameric structure by themselves whereas isoform A can only be incorporated into the complex together with the B isoform (Dickson et al. 2000; Peng et al. 2011).

In terms of function, the chloroplast CPN60 has been proven to be essential for folding and assembly of the large subunits of the CO_2 fixation enzyme, RuBisCo (Boston et al. 1996; Feiz et al. 2012). Genetic studies also revealed other the important functions of the chloroplast chaperonin 60. In higher plant, *Arabidopsis* mutant defective in both cpn60A and cpn60B genes

manifested defective chloroplast development (Suzuki et al. 2009). In addition to its roles in protein folding, the chloroplast-localized CPN60 was reported for a novel function in RNA splicing (Balczun et al. 2006).

Despite of several reports on different combinations for oligomerization of isoform A and isoform B of the chloroplast chaperonin 60, very little is known about how each of the subunits assemble together. Even worse is the fact that there is only one research paper (from PubMed search) reporting on chloroplast CPN60 in the model alga *C. reinhardtii*, describing its function in RNA maturation (Balczun et al. 2006). What are the possible combinations? Under what condition does each of the combination is preferred? Which region within the protein plays important roles in association of A-B or B-B subunits? How are the gene coding for this chaperonin proteins regulated, especially under stress treatments? The research in this project tried to complement the lacking knowledge for the mechanism of action of this plastid CPN60 in microalgae, particularly when the organism is subjected to abiotic stresses.

HSP90B **gene expression and regulation**

In the same way as other molecular chaperone families, HSP90 can be found in diverse groups of organisms from bacteria to human. Their amino acid sequences, constituting to a mature protein size of ~80–94 kDa, are highly conserved (Young et al. 2001). HSP90 protein is active as a homodimer, of which the monomer contains 3 distinct domains: an ATP-binding N-terminal domain, a substrate-binding conserved flexible middle region and a C-terminal domain (Chong et al. 2015; Schroda 2004). HSP90 proteins can be constitutively detected in most organisms, although, their gene expression has been shown to be influenced by environmental stresses such as heat, cold and salinity (Krishna et al. 1995; Schmollinger et al. 2013; Wang et al. 2012). Based on substrate specificities, HSP90s have been postulated to facilitate maturation of signal transduction proteins such as hormone receptors, cytosolic signaling protein kinases, casein kinase II and oncogene products, nitric oxide synthase and calcineurin (García-Cardeña et al. 1998; Imai and Yahara 2000; Wang et al. 2004). Functionally, HSP90 proteins play important roles in cellular processes, developments and stress responses. In plant, expression of HSP90s could be detected throughout multiple developmental stages (Koning, et al. 1992; Reddy et al. 1998). Lower levels of functional Hsp83, a homolog of HSP90, led to developmental

abnormalities and morphological changes in *Drosophila* (Rutherford and Lindquist 1998). Nevertheless, yeast cells with overexpression of Hsc82 are hypersensitive to high salt (Imai and Yahara 2000).

In *Chlamydomonas reinhardtii*, three genes encoding for HSP90s have been annotated in the Genbank database. *HSP90A* encodes for a cytosolic form of the protein. *HSP90B* has been predicted to produce an ER-localized polypeptide while *HSP90C* generates the chloroplast-targeted versions, respectively (Schroda 2004). In higher eukaryotes and land plants, there are a number of studies on characterization of the HSP90 proteins, but very few in *C. reinhardtii* exists in the literature. These include the study by Schmollinger et al. (2013) who investigated heat stress response in *C. reinhardtii* and found that expressions of HSP90A and HSP90C proteins followed the same dynamic patterns as that of Heat Shock Factor 1 (HSF1), a transcription factor regulating responses of plants to heat and other abiotic stresses. Inhibition of HSP90 activity by radicicol and geldanamycin also prolonged the effect of heat stress (Schmollinger et al. 2013). In addition, HSP90C was shown to interact with HSP70B (Willmund and Schroda 2005) to form a multichaperone complex (Willmund et al. 2008). Putative post-translational modification of HSP90A and HSP90C under NaCl stress was discerned in a previous proteomic study (Yokthongwattana et al. 2012). For the putative ER-localized version of the HSP90B, there is only one report on its seasonal expression in *Iris pumila* (Manitasević et al. 2007). Recently, HSP90B has been reported to be up-regulated in *C. reinhardtii* cells adapted to grow under high concentration of NaCl (Sithtisarn et al. 2017), suggesting its possible role in long-term salinity tolerance. In this research, promoter element and gene regulation of the *HSP90B* in *C. reinhardtii* were thus further investigated. Using promoter deletion analysis, treatments with inhibitors of DNA methylation or histone modifying enzymes and chromatin immunoprecipitation, we have demonstrated an active promoter region as well as the putative repressive element(s).

2. Experimental Plans and Results

Gene Expression Analysis and Promoter Characterization of Chlamydomonas HSP90B

HSP90B gene structure and expression profile

To comprehensively characterize the promoter of the *Chlamydomonas HSP90B* gene (Fig. 2), full-length cDNA (Fig. 3) of the gene was cloned by RT-PCR together with 5' and 3' RACEs. The sequence was analyzed and deposited to the Genbank database (accession# KY555127). The transcript is 3188 bp in length, consisting of 134 bp of 5' UTR, 2460 bp of coding sequence, and 594 bp of 3' UTR (Fig. 4). Upon alignment of the transcript sequence with the *Chlamydomonas* genome database (Merchant et al. 2007), the *HSP90B* gene appears to contain 13 exons and 12 introns.

Figure 2. Nucleotide sequence of ~1.2 kb upstream promoter of *Chlamydomonas HSP90B* gene in this study. Primer biding sites of each 5' truncated promoter are underlined and indicated. Yellow highlight indicates transcription start site (TSS).

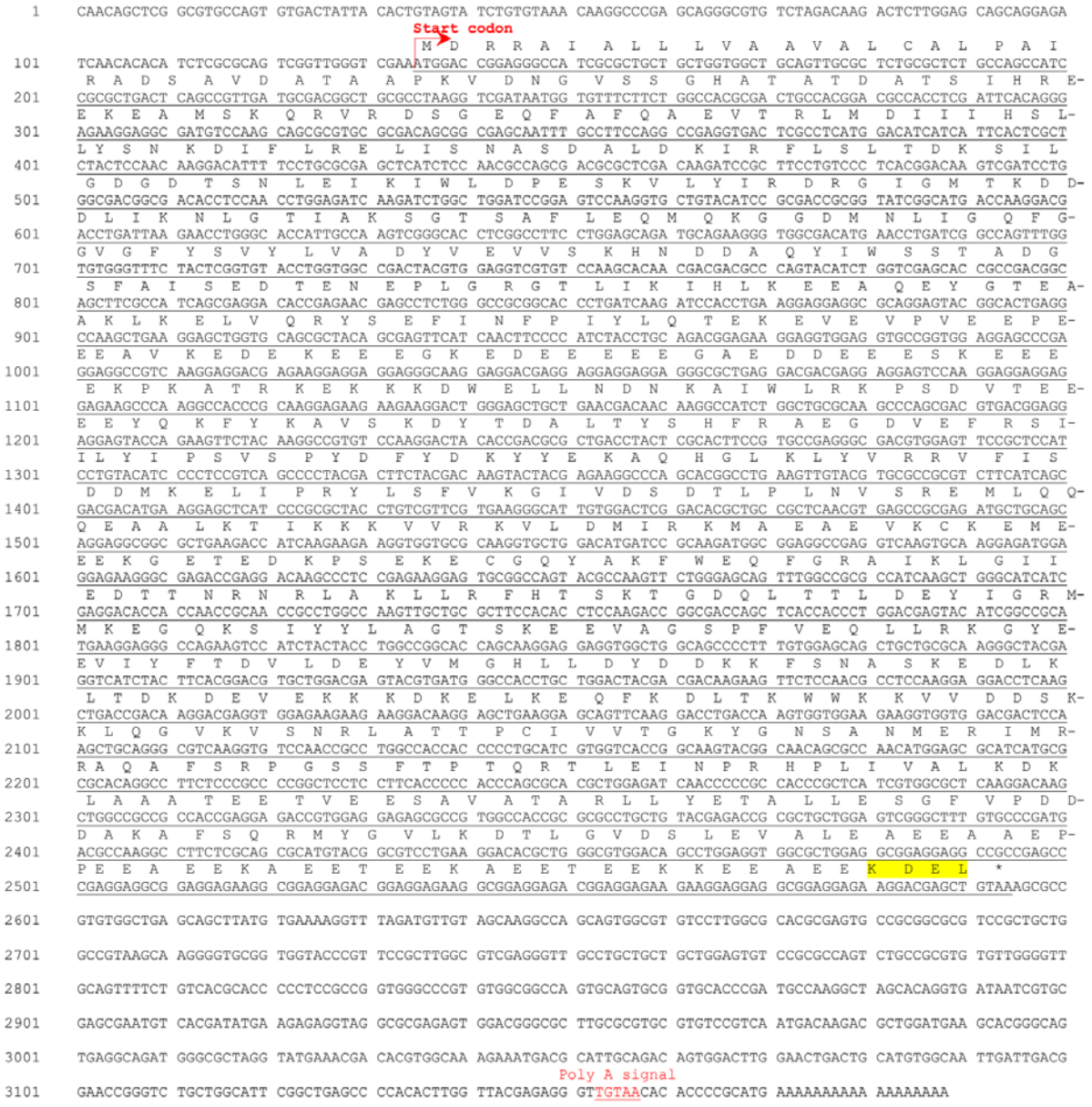

Figure 3. Nucleotide sequence of full-length cDNA and deduced amino acid sequence of *Chlamydomonas HSP90B* gene. Coding sequence is illustrated as underlined text. ER-localization signal is highlighted in yellow. Start codon and poly A signal is marked by red texts.

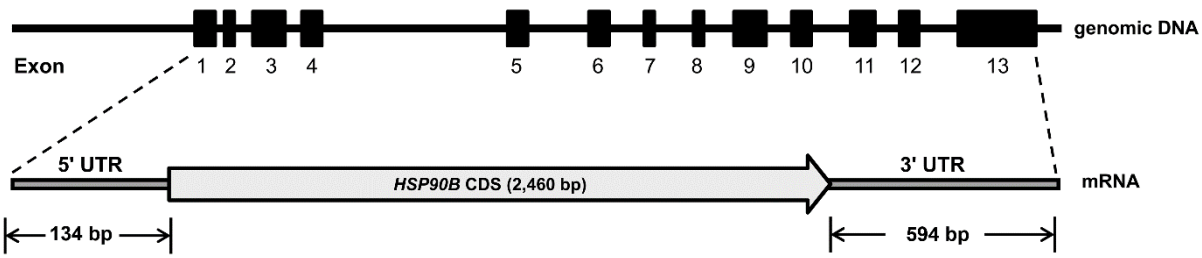

Figure 4. Diagram showing gene structure and mRNA of the *Chlamydomonas HSP90B*. Top panel illustrates the gene organization containing 13 exons and 12 introns. The lower panel displays the transcript configuration of the gene consisting of 134 bp of 5' UTR, 2,460 bp of coding sequence and a 594 bp of 3' UTR.

Specific primers were then designed to assess gene expression profile under various stress conditions by RT-qPCR. As expression of *RCK1* (*Cblp*) gene has been demonstrated to be constant under various stresses (Mus et al. 2007; Shi et al. 2017), it has been commonly used as a control gene for RT-qPCR normalization (Im and Grossman 2001; Iwai et al. 2014; Jokel et al. 2015; Kwak et al. 2017). We, thus, also used the *RCK1* as our control gene for normalization as its expression did not significantly change during the stress treatments we employed (Fig. 5). When growth temperature was raised from 25 to 37 ºC for 1 h, the *HSP90B* gene transcript level was approximately 15-fold increased (Fig. 6A). Transition from normal growth irradiance of 50 µmol photons m^{-2} s^{-1} to high light of ~1,000 µmol photons m^{-2} s^{-1} for the same period of time did not lead to significantly change in *HSP90B* mRNA level. Abrupt elevation of NaCl concentration in the growth medium from 0 to 50 mM, as well as transition from darkness to normal light intensity for 1 h led to slight decrease in the *HSP90B* gene expression (Fig. 6A).

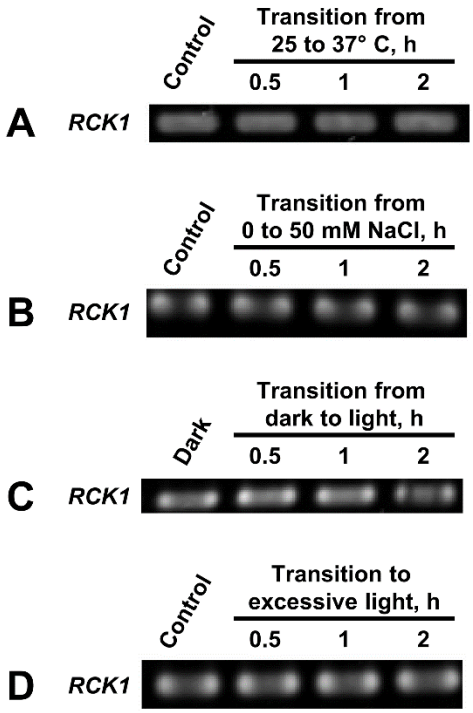

Figure 5. Semi-quantitative RT-PCR of *RCK1* gene under abiotic stress treatments of *C. reinhardtii* cells. Cells were grown under normal conditions until mid-logarithmic phase before subjected to the following stress treatments: (A) high temperature, (B) NaCl, (C) transition from dark to light and (D) transition from low light (50 µmol photons m^{-2} s^{-1}) to high light (1,000 µmol photons m^{-2} s^{-1}). Cells were collected after 0 (control), 0.5, 1 and 2 h and subjected to RNA isolation and RT-PCR analysis. As 30 min was the time that showed consistently expression of the *RCK1* gene, this time point was employed to study *HSP90B* gene expression as shown in the main paper.

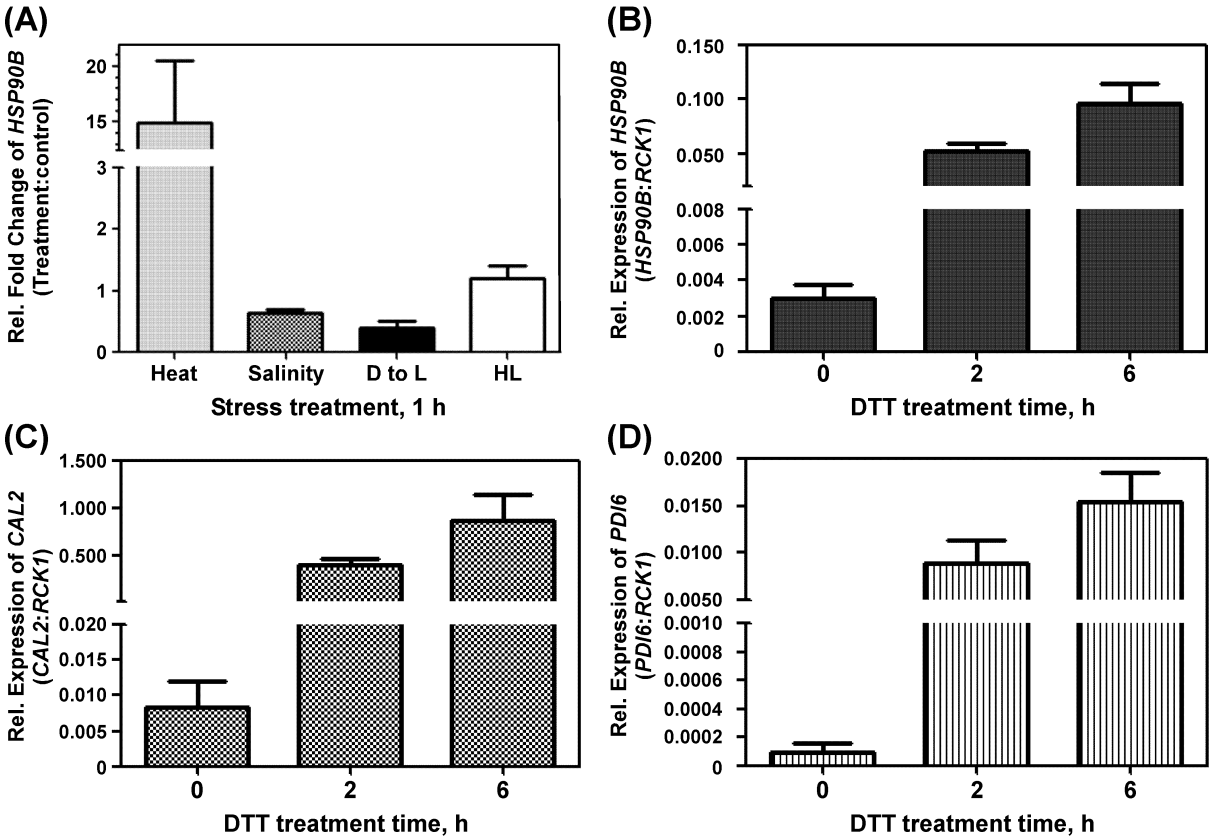

Figure 6. Relative gene expression profiles determined by RT-qPCR. (A) Relative fold change of *Chlamydomonas HSP90B* gene after 30 min of: heat treatment (transition from 25 to 37 ºC), exposure to mild salinity (from 0 to 50 mM NaCl) and irradiance stress (shifting from 50 to 1,000 µmol photons m^{-2} s^{-1}). Relative expression of the gene after stress treatments were normalized to that of the untreated control cells. Expressions of the *HSP90B* gene (B) as well as known marker genes for ER-stress response such as *CAL2* (C) and *PDI6* (D) after 0, 2 and 6 h of DTT treatment was assessed by RT-qPCR. Expression of *RCK1* gene was used as a control for normalization in each of the experiments. All data points/bars are averages of 3 independent experiments ± SD.

In *Arabidopsis thaliana*, *HSP90B* protein has been shown to correlate with ER stress (Chong et al. 2015). Hence, we also addressed whether or not expression of the *Chlamydomonas HSP90B* gene could be modulated by the ER stress. Dithiothreitol (DTT), which has been demonstrated to stimulate ER stress in *Chlamydomonas* (Kim et al. 2013; Pérez-Martín et al. 2014), was added to the algal cultures at the final concentration of 3 mM. Cell aliquots were taken at 0, 2 and 6 h after treatment before subjecting to RNA isolation and RT-qPCR analysis. Expression of the *HSP90B* gene was almost 20 times enhanced after 2 h of DTT treatment and was elevated to ~30 folds by 6 h (Fig. 6B). Similarly, two marker genes for ER stress such as

CAL2 and *PDI6* also showed similar trends (Fig. 6C and 6D), suggesting that DTT-induced ER stress can also stimulate *HSP90B* gene expression.

As other isoforms of the HSP90 proteins have previously been shown to be crucial for plant growth and development (Koning, et al. 1992; Reddy et al. 1998), we followed the *HSP90B* gene expression in *C. reinhardtii* during growth from fresh inoculation in standard TAP medium (initial cell density was approximately $6x10^5$ cells ml^{-1}). Cell density increased rapidly during the first 2 days and reached the stationary phase by day 3 (Fig. 7). Interestingly, the steady-state *HSP90B* transcript, as determined by RT-qPCR, showed an inverse trend with growth of the culture. The gene expression was relatively high on day 1 after inoculation when cell density was relatively low. Then the transcript gradually decreased to a low level by day 3, concomitantly with the cell reaching stationary growth phase (Fig. 7). These results altogether suggested that *HSP90B* could be in under tight regulation in conjunction with cell growth stages.

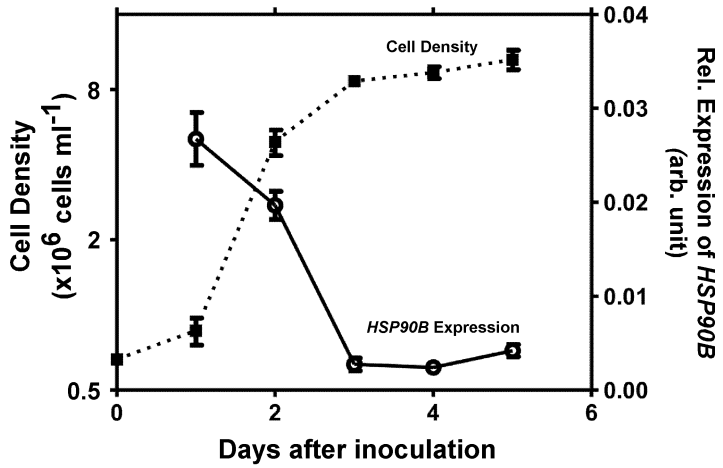

Figure 7. Reverse relationship of the *HSP90B* gene expression and cell densities during growth of the culture. Cell densities were obtained via counting under microscope while the relative expression of the *HSP90B* gene (over that of the *RCK1* control) were obtained by RT-qPCR.

253 bp of *HSP90B* promoter conferred constitutive expression

The promoter sequence of the *HSP90B* gene was investigated using promoter truncation analysis, one of the conventional and effective methods for identification of regulatory regions in a gene promoter. As the exact location of the promoter is unknown, we amplified a ~1.2 kb region upstream of the transcription start site (TSS) and placed in front of a codon-optimized *LUC* reporter gene (referred to as 1177P herein). Series of the promoter 5' truncations were subsequently generated as –795 bp (795P), –614 bp (614P), –392 bp (392P), –311 bp (311P),

–253 bp (253P) of the TSS. The constructs with the *LUC* gene without any promoter sequence (promoterless) and one driven by *PSAD* promoter were used as negative and positive control, respectively. Each promoter truncation construct was transformed into *C. reinhardtii* CC-503 cells. Positive transformants were isolated and verified for existence of the *LUC* transgene (Fig. 8).

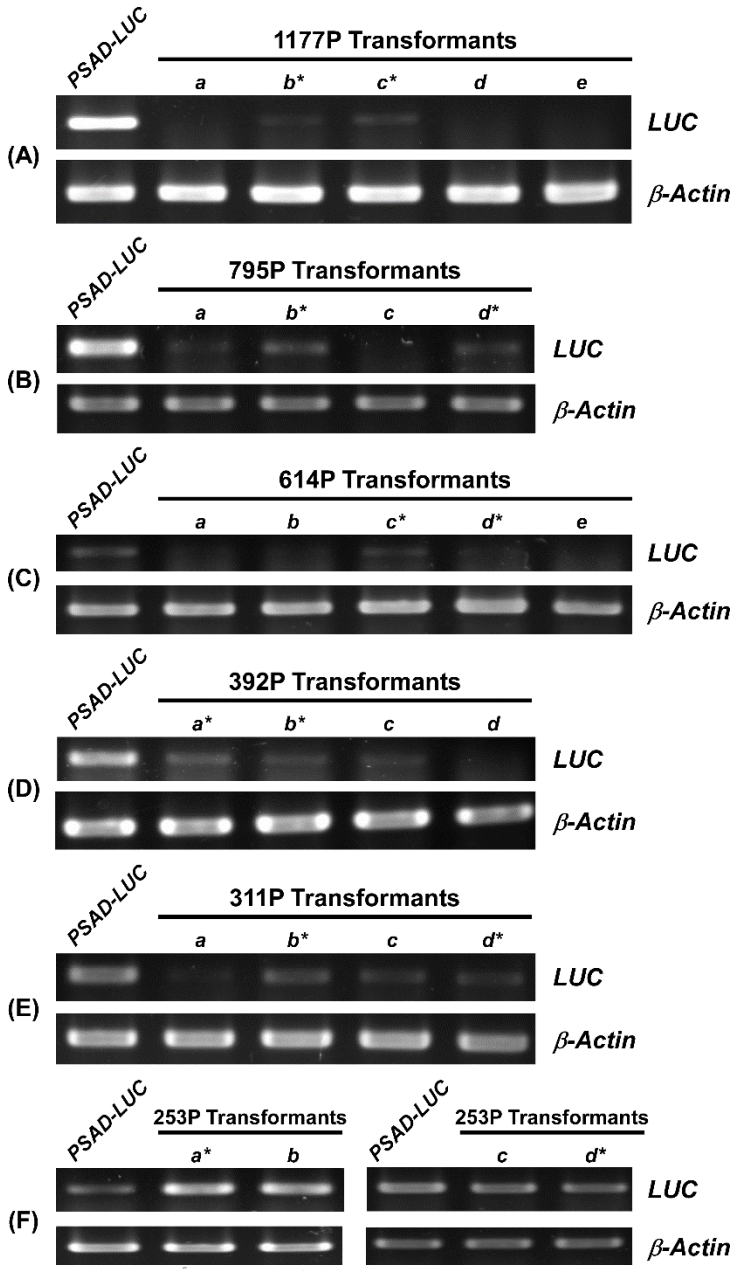

Figure 8. Semi-quantitative RT-PCR of *LUC* transgene from *Chlamydomonas* transformants harboring *HSP90B* promoter truncation cassettes as following: (A) 1177P, (B) 795P, (C) 614P, (D) 392P, (E) 311P and (F) 253P. Expression of *β-actin* gene was used as a control for equal cDNA template. Individual transformant lines are signified as small italic alphabets while * denotes selected transformants for the studies in the project.

Two independent transformant lines from individual truncated-promoter constructs were selected for further studies. The selected transgenic lines were culture for several generations to ensure that the transgene was stably integrated into the nuclear genome. As the subsequent comparative expression (mRNA level) of the *LUC* transgene were used as the reporter for promoter activity, it was important to verify that each of the transformant lines contained only a single transgenic insert. To determine the copy number of the *LUC* insert, genomic qPCR was performed using primers specific to the transgene. For comparison, the endogenous *HSP90B* gene existing as a single copy in the nuclear genome was used as a normalizer for the genomic qPCR. If the *LUC* transgene was present as a single copy insert, after genomic qPCR amplification it should give approximately 1:1 ratio of the amplicon to that of the *HSP90B*. None of the selected transformants showed relative amplification signal of *LUC*:*HSP90B* higher than 1, indicating that each of them contained single insert of the transgene (Fig. 9A).

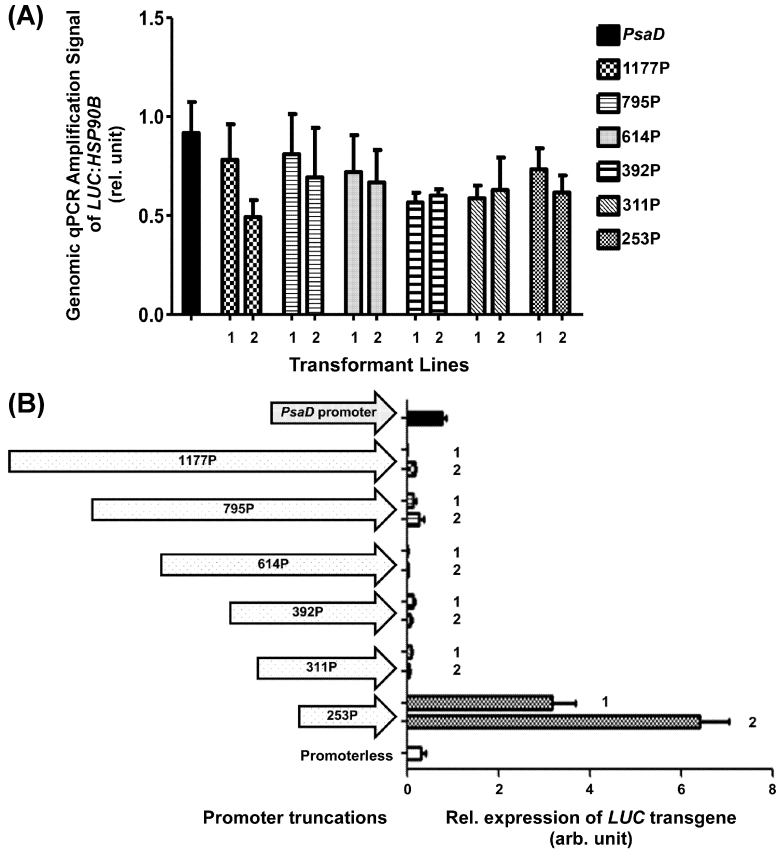

Figure 9. (A) Determination of copy number of the vector insert the transformant lines carrying each of the promoter truncation constructs. Relative genomic qPCR amplification signal of the *LUC* reporter transgene was normalized to that of the endogenous *HSP90B* gene. (B) Relative expression of *LUC* transgene driven by individual promoter truncation construct was assessed by RT-qPCR. *RACK1* was used as the control normalizer for the qPCR technique. All data are averages of triplicate experiments ± SD.

Next, the transformant lines for each promoter truncation construct were grown in TAP medium for 4 days, prior to RNA isolation and RT-qPCR analysis of the *LUC* transgene expression. Transformants harboring the construct 1177P, 795P, 614P, 392P, and 311P expressed miniscule amount of the *LUC* transgene, even lower than that of the promoterless transformant (Fig. 9B). Note that these representative tranformants for each of the promoter truncation constructs were the best performers in terms of *LUC* transgene expression during the screening process (Fig. 8). Remarkably, expression of the transgene in the 253P transformants was highly and significantly greater than that of other promoter truncation constructs. When compared to the *PSAD* promoter commonly used by several transformation vectors, *LUC* expression driven by the 253P promoter was about 3-6 times higher (Fig. 9B), indicating that the core constitutively-active promoter might reside around the –253 bp upstream of the TSS. The results in Fig. 9B also suggested that sequences beyond –253 bp may still contain other *cis* elements that regulate transcription of the reporter gene, including the ones that repressed the *HSP90B* gene expression during stationary phase of growth (Fig. 7).

Epigenetic regulation of *HSP90B* gene

The next questions we asked were whether DNA methylation and histone modifications, two major epigenetic marks, have a role in epigenetic control on the *HSP90B* gene expression. To address these questions, we observed the effects of treating the *C. reinhardtii* cells with inhibitors of either DNA methyltransferase enzyme (5-aza-2'-deoxycytidine or 5aza-dC) or histone deacetylase or HDAC enzyme (trichostatin A or TSA). For the contribution of the DNA methylation, upon fresh inoculation *C. reinhardtii* CC-503 cultures were subjected to daily supplements of 5aza-dC (to the final concentration of 50 μM). The control samples were added with 50% acetic acid at the same volume. After 4 days, both control and the treated cells were harvested and subjected to RNA isolation followed by RT-qPCR analysis of the *HSP90B* gene expression. In the absence of 5aza-dC, relative expression of the endogenous *HSP90B* gene (normalized to that of the constitutively expressed *RCK1* gene) was about five times lower than that of the cells treated with the inhibitor (Fig. 10A). This result indicates that inhibition of DNA methylation could de-repress the *HSP90B* gene in *C. reinhardtii* on day 4 after inoculation. To further examine the contribution of histone acetylation in regulating the expression of *HSP90B*,

4-day-old *C. reinhardtii* cultures were also treated with TSA at the final concentration of 100 ng/ml. Cells aliquots were taken at different time intervals and subjected to RT-qPCR analysis of the *HSP90B* gene expression as before. During the first 60 min after supplementation of TSA, relative expression of the *HSP90B* gene did not significantly change. Interestingly, the expression gradually increased thereafter, and reached ~20 times higher level at 6 h as compared to the zero time point (Fig. 10B), suggesting that repression of the gene on day 4 of growth (Fig. 7) could be under regulation of histone acetylation.

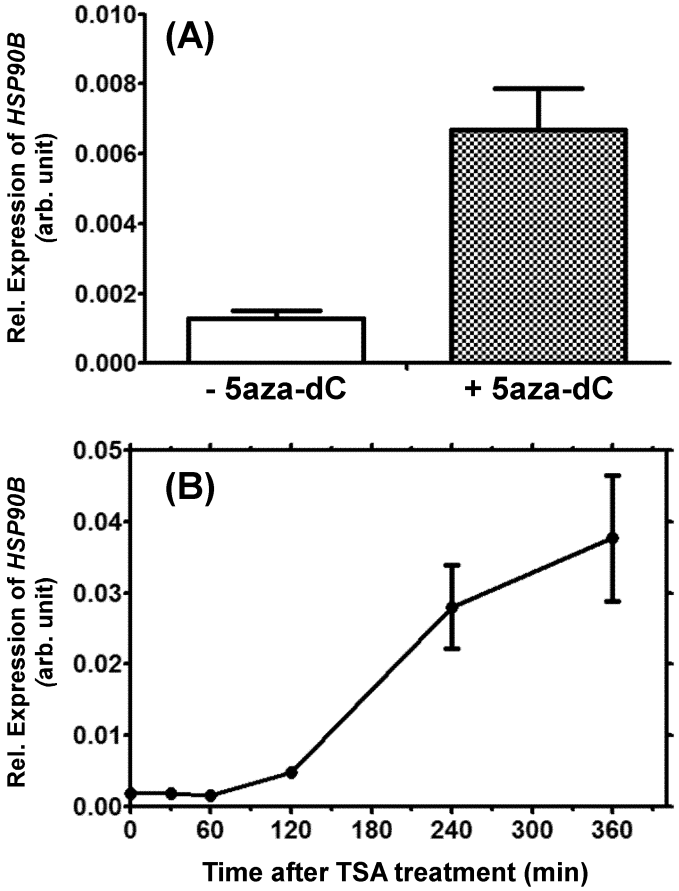

Figure 10. Effects of epigenetic inhibitor treatments on expression profile of *Chlamydomonas* endogenous *HSP90B* gene. Relative expression profiles of the gene over that of the *RCK1* control were determined by RT-qPCR. (A) Transcript abundance in the absence and presence of DNA methyltransferase inhibitor, 5-aza-dC. (B) Effects of treating or not treating the cells with TSA, an HDAC inhibitor. Each data set is an average of 3 independent experiments ± SD.

Effects of 5aza-dC and TSA on reactivation of the truncated promoters

As shown in Fig. 10, re-activation of the endogenous *HSP90B* gene could be observed upon 5aza-dC or TSA treatments. We further examined whether the diminutive expressions of

our *LUC* transgene driven by the truncated promoter constructs could be explained by epigenetic repression as well. Each of the transformant lines harboring the individual truncated promoter:*LUC* construct was subjected to 5aza-dC and TSA treatments the similar to what described in Fig. 10. Unlike that of Fig. 10A, results in Fig. 11A clearly showed that 5aza-dC treatment did not lead to significantly change in the expression profile of the *LUC* transgene compared to that of the control untreated cells. TSA, on the other hand, seemed to have little more effects on significant re-activation of the *LUC* transgene in several promoter truncation lines (Fig. 11B). It is noteworthy that independent lines of the transformants harboring the same construct exhibited different degree of de-repression of the transgene. As the plasmid construct was randomly inserted into the genome of *C. reinhardtii* during the transformation process, it is possible that the difference among tranformants harboring the same construct was partly interfered by positional effect of the local genomic sequences and chromatin states. These results suggested that parts of the promoter sequence, at least in part, was regulated by histone modification and DNA methylation.

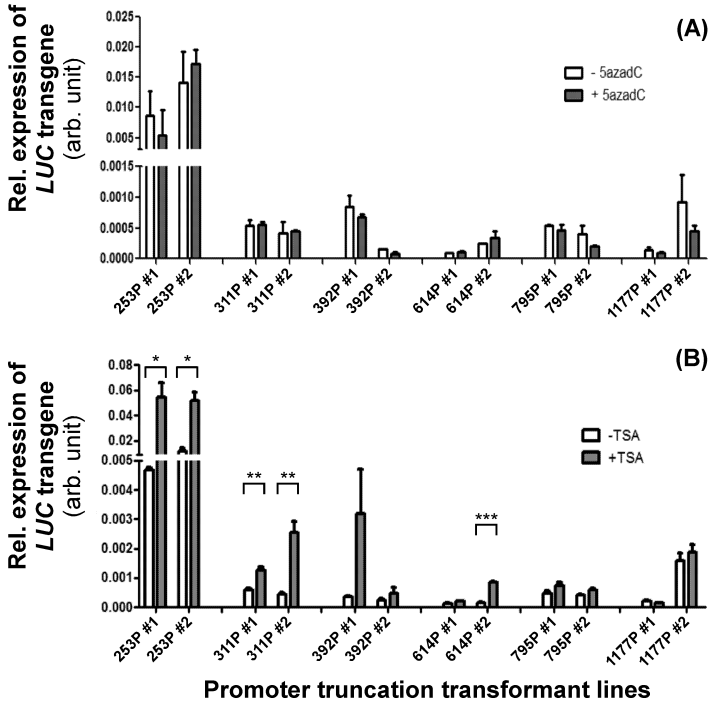

Figure 11. Effects of epigenetic inhibitor treatments on expression of *LUC* reporter transgene in individual transformant lines. Relative expressions of *LUC* over that of the *RCK1* were evaluated by RT-qPCR. (A) In the absence or presence of 5-aza-dC. (B) In the absence or presence of TSA. Data are averages of 3 experiments ± SD. * represent P value < 0.05, ** < 0.01 and *** < 0.001 as determined by pair-wise *t* test.

Cis regulatory elements in the *HSP90B* promoter

We have shown that suppressive effect conferred by the promoter sequences beyond –253 bp could be overcome by the TSA treatment. However, the degree of de-repression was still significantly lower than that of the untreated 253P transformants (Fig. 11B). In addition, the results in Fig. 9 clearly showed that deletion of only a small region of 59 bp between –253 and –311 bp could drastically reactivate the *HSP90B* gene. It is possible that regulatory *cis* elements could be occupied by another putative repressor protein, and might also play a role in repressing the gene during stationary growth. To investigate this hypothesis, the 1.2-kb promoter sequence was subjected to *in silico* prediction of the *cis*-acting elements via the online tools PlantCARE and PlantPAN (Chow et al. 2016; Lescot et al. 2002) as well as manually searched for known motifs. Table 1 illustrates the *cis* elements found within the 1.2 kb promoter of the *HSP90B* gene. Common core *cis* elements such as TATA box (2 positions), CAAT box (6 positions) and GC box (17 positions) were found. Typical for molecular chaperones, 3 heat shock elements (HSEs) were found scattering between –27 to –789. Although *HSP90B* gene expression did not seem to respond to light, we could identify 20 motifs that have been reported as light-responsive elements. Two hormonal (ABA and MeJA) regulatory elements as well as those responsive to abiotic stresses were also detected. Interestingly, none of these regulatory elements except the CAAT box was found in the –253 to –311 bp promoter, which was critical for controlling *HSP90B* as well as the *LUC* transgene expression. On the other hand, if we consider only the core active promoter between –253 and +1, several key regulatory elements were confidently predicted (Table 1). Example of such *cis*-elements located within the core active promoter include ABRE (–67) for ABA response, SP1 (–15), AE box (–25) and GATA motif (–25, –114, –116, –133, –141, –150, and –155) for light response, ARE (–227) for anaerobic induction, CuRE (–144) for copper response, G box (–67), GC-box (–15, –110, and –213) HSE (–27 and –102), MBS (–159) for drought response, O2 site (–157), PRE (–131) for plastid responsiveness, and zinc finger element (–75).

Table 1. *In silico* analysis of *cis*-acting elements within the 1.2 kb promoter sequence of the *Chlamydomonas reinhardtii HSP90B* gene.

Element	Organism	Consensus seq.	Predicted function	No.	Position	DB
A-box	*P. crispum*	CCGTCC	Regulatory element	2	–400, –609	PC
AAGAA-motif	*A. sativa*	GAAAGAA	Unknown	1	–928	PC
ABRE	*A. thaliana*	CACGTG	ABA response	3	–67, –625, –636	PC
ACE	*P. hortense*	ACGTGGA	Light response	1	–638	PC
AE-box	*A. thaliana*	AGAAACTT	Light response	1	–25	PC
AP2	*C. reinhardtii*	ggCCGGCt	Abiotic stress responses	1	–495	PP
ARE	*Z. mays*	TGGTTT	Anaerobic induction	1	–227	PC
CAAT-box	*A. thaliana*	CCAAT	Common cis-acting	4	–282, –429, –517, –913	PC
	B. rapa	CAAAT	element	1	–858	
	P. hybrida	TGCCAAC		1	–754	
CAT-box	*A. thaliana*	GCCACT	Meristem expression	3	–672, –716, –1070	PC
CGTCA-motif	*H. vulgare*	CGTCA	MeJA-response	3	–954, –1042, –1079	PC
CuRE	*C. reinhardtii*	GTAC	Copper-response	3	–144, –631, –694	M
G-box	*A. thaliana*	CACGTG	G-box	3	–67, –625, –636	PC
	Z. mays	CACGAC		1	–741	
GATA-motif	*A. thaliana*	GATAGGA	Light response	1	–116	PC
	A. thaliana & C. reinhardtii	nGATAn		5	–114, –150, –271, –917, –1048	PP
		(G/T)GATG		6	–133, –141, –155, –266, –279, –643	PP
		(G/A)GATT		6	–428, –772, –796, –809, –860, –869	PP
GC-box	*Mammalians & plants*	GGGCGG consensus	Core element	17	–15, –110, –213, –301, –316, –353, –435, –448, –459, –504, –514, –728, –844, –896, –1057, –1076, –1137	M
HSE	*D. melanogaster*	nGAAnnGAAnnCT TnnGAAn	Binding site for HSF	1	–789	M
		nGAAnnCTCn		1	–102	
		nGAAnnTTCn		1	–27	

MBS	*A. thaliana*	(C/T)AACTG	Drought response	4	–159, –603, –619, –1025	PC
O2-site	*Z. mays*	GTTGACGTGA	Zein metabolism	1	–157	PC
PRE	*C. reinhardtii*	aggtaacgtagcagttg cgGTCGC	Plastid responsiveness	1	–1036	PP
		atgtgtttccgccagtcct ATCGG		1	–131	
Sp1	*O. sativa*	GGGCGG	Light response	3	–15, –504, –842	PC
	Z. mays	CC(G/A)CCC		1	–504	
TATA-box	*A. thaliana*	TATA	Core promoter element	1	–1101	PC
	C. reinhardtii	TATTA		1	+25	
TF_motif_seq0364	*C. reinhardtii*	atGTTCG	*Nia1* gene repression	1	–950	PP
		aaGTGCG		1	–372	
TF_motif_seq0400	*C. reinhardtii*	ccACTCA	mRNA stability	1	–671	PP
TGACG-motif	*H. vulgare*	TGACG	MeJA-response	3	–954, –1042, –1079	PC
Unnamed	*Z. mays*	CGTGG	Unknown	5	–68, –637, –892, –899, –1160	PC
Unnamed	*Z. mays*	CCCCGG	Unknown	1	–301	PC
WRKY	*C. reinhardtii*	cgaGTCAAca	Stress responses	1	–578	PP
Zinc-finger	*C. reinhardtii*	CACCT	Zinc finger	1	–75	M

Cis elements are reported for both + and – strand of the promoter sequence.

No – number of the element found within the promoter sequence.

DB – database prediction: PC = PlantCARE; PP = PlantPAN 2.0; M = manually search

Nucleosome occupancy within the *HSP90B* promoter

Previous studies in eukaryotes including plants have described overlapping sites intrinsically preferred by nucleosomes and certain transcription factors, and in essence, DNA binding of one protein may restrict DNA accessibility of the other (Charoensawan et al. 2012; Cortijo et al. 2017; Dai et al. 2017; Sura et al. 2017; Teichmann et al. 2012). Importantly, their antagonistic binding to cognate sites are modulated by environmental cues and stresses, allowing dynamic expression regulation of stress responsive genes. Here, we investigated whether or not the interplay between nucleosomes and cis-factors also plays a role in controlling the transcription

of the *HSP90B* gene. The same 1.2 kb sequence as well as its truncated versions were subjected to nucleosome occupancy prediction using "Online Nucleosomes Position Prediction" at https://genie.weizmann.ac.il/software/nucleo_prediction.html (Field et al. 2008; Kaplan et al. 2009; Segal et al. 2006). The sequences between –1177 and –392 bp were predicted to have relatively high nucleosome occupancy of 40-50% (Fig. 12A), whereas from –311 bp down to the TSS was predicted to be relatively nucleosome-depleted region of approximately 20%.

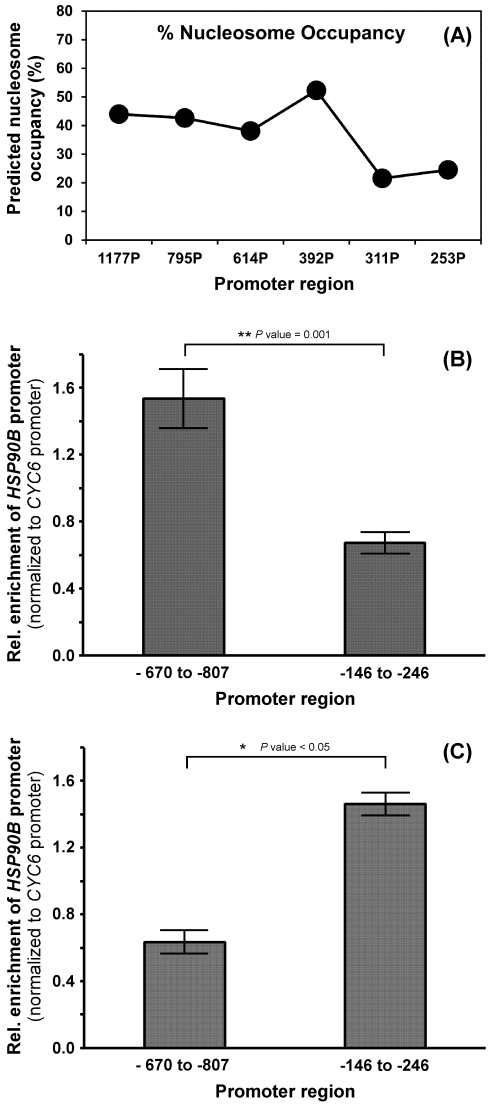

Figure 12. Nucleosome/histone occupancy on the promoter of *Chlamydomonas HSP90B* gene. (A) Predicted percentage of nucleosome occupancy on various versions of the promoter truncations as predicted by online tool (see text). Chromatin immunoprecipitation analyses were performed. Chromatins were pulldown by anti-H3 histone (B) and anti-acetylated histone (K9/K14) (C) antibodies before subjected to qPCR. The qPCR signals were normalized to that of the *CYC6* promoter. Specific primer sets were designed for the distal promoter (–670 to –807 bp of the TSS) and proximal promoter (–146 to –246 bp of the TSS). Data are averages of 3 independent experiments ± SD. *P* values were determined by pair-wise *t* test.

To verify this *in silico* prediction, chromatin immunoprecipitation (ChIP) was performed on the endogenous *HSP90B* promoter. Fragmented chromatins were pulled down by anti-histone H3 (monoclonal). The precipitated chromatins were amplified by qPCR using two specific primer sets, one residing within the proximal promoter from –1 to –253 bp and another one located at the distal promoter beyond the –253 bp. It is important to note that most of the primer sets we designed to amplify the distal promoter sequences beyond the –253 bp did not yield a single PCR product, thus were not suitable for qPCR (results not shown). The only distal promoter region that could be amplified as a single PCR product was located around –670 to –807 bp (130 bp amplicon) and was thus used in the ChIP-qPCR assay. Enrichment of the cytochrome c_6 (*CYC6*) promoter was used as a normalizer (Strenkert et al. 2011). In consistent to the nucleosome occupancy prediction, relative enrichment of the *HSP90B* distal promoter region beyond –253 bp (–670 to –807 bp) was about two folds greater than that of the proximal region (–146 to –246 bp, Fig. 12B). This demonstrates that less abundance of histone H3 is associated with the proximal promoter, whereas the DNA sequence beyond –253 bp have relatively higher histone H3 occupancy. Nevertheless, as several reports have shown that histone acetylation is associated with the active promoter of genes (Fernández-Sánchez et al. 2013; Srivastava et al. 2014), we further checked whether the low histone occupancy of the –253 bp active promoter sequence of the *HSP90B* gene was enriched in acetylated histone. Similar ChIP-qPCR was performed but this time using the antibodies recognizing acetylated histone H3 (K9/K14). The opposite results was obtained as the proximal promoter was indeed enriched with the acetylated histone while such association at distal site was significantly lower (Fig. 12C), strengthening the notion that the core active promoter of *Chlamydomonas HSP90B* is residing around –253 bp upstream of the TSS.

Subunit Assembly of Chloroplast-Localized Chaperonin 60 in *Chlamydomonas*

The original scopes on characterization of chaperonin 60 proteins in this research project recently received a lot of attention from other researchers, including the group from China. As a result, there have been several papers coming out in recent years coinciding with the research we had been carrying out. We, therefore, had to modify the aims and scopes of the project to suit the current situation. From the original aim of investigating the subunit assembly within the

model alga *Chlamydomonas reinhardtii*, we shifted the scope to subunit assembly of the chaperonin 60 in bacteria model *Escherichia coli*.

Vector constructs for heterologous expression of *Chlamydomonas* CPN60 subunits in *E. coli*

For heterologous expression of the *Chlamydomonas* chaperonin 60 subunits in *E. coli*, pET duet expression cassette (Novagen) was purchased and employed. We have designed and created 7 constructs for different combinations of the expression (Fig. 13). The first construct (Fig. 13A) was designed to express two copies of CPN60A, each tagged with different epitopes (His and HA tags, respectively). The two different epitope tags were employed to verify the self-interaction between the CPN60A subunits. Similar approaches were performed for the expression of CPN60B1 (Fig. 13B) and CPN60B2 (Fig. 13C) but instead using the combination of His and S tags. To co-express two different subunits of the *Chlamydomonas* CPN60 in *E. coli*, genes encoding for the corresponding subunits were ligated into the same plasmid behind the T7 promoter and, again, tagged with two different antigenic epitopes. Such combinations included B1~A (Fig. 13D), A~B2 (Fig. 13E), B1~B2 (Fig. 13F) and B1~B2~A (Fig. 13G).

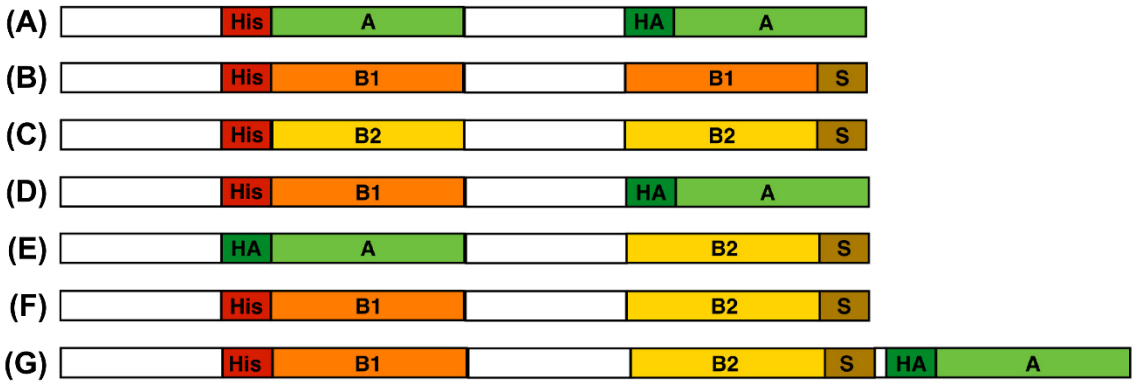

Figure 13. Diagrams showing vector constructs for heterologous expression of *Chlamydomonas* CPN60 subunits in *E. coli* using pET duet system (Novagen). (A) A construct expressing CPN60A linked with two different tags while similar constructs for CPN60B1 (B) and CPN60B2 (C) were also generated. Constructs (D), (E), (F) contain different combination between CPN60A, B1 and B2. The final construct (G) could express all three subunits in *E. coli*.

Interaction between *Chlmydomonas* CPN60 subunits in *E. coli*

To assess the interaction between the *Chlamydomonas* CPN60 subunits in the bacterial environment, individual constructs illustrated in Fig. 13 were separately transformed into *E. coli*

BL-21 (DE3) cells. Positive transformants were subjected to verification of the ability to heterologous express the foreign proteins. The transformants expressing each of the vector constructs were subjected to protein isolation and immunoprecipitation using specific antibodies against each of the epitope tag (His, HA and S tags).

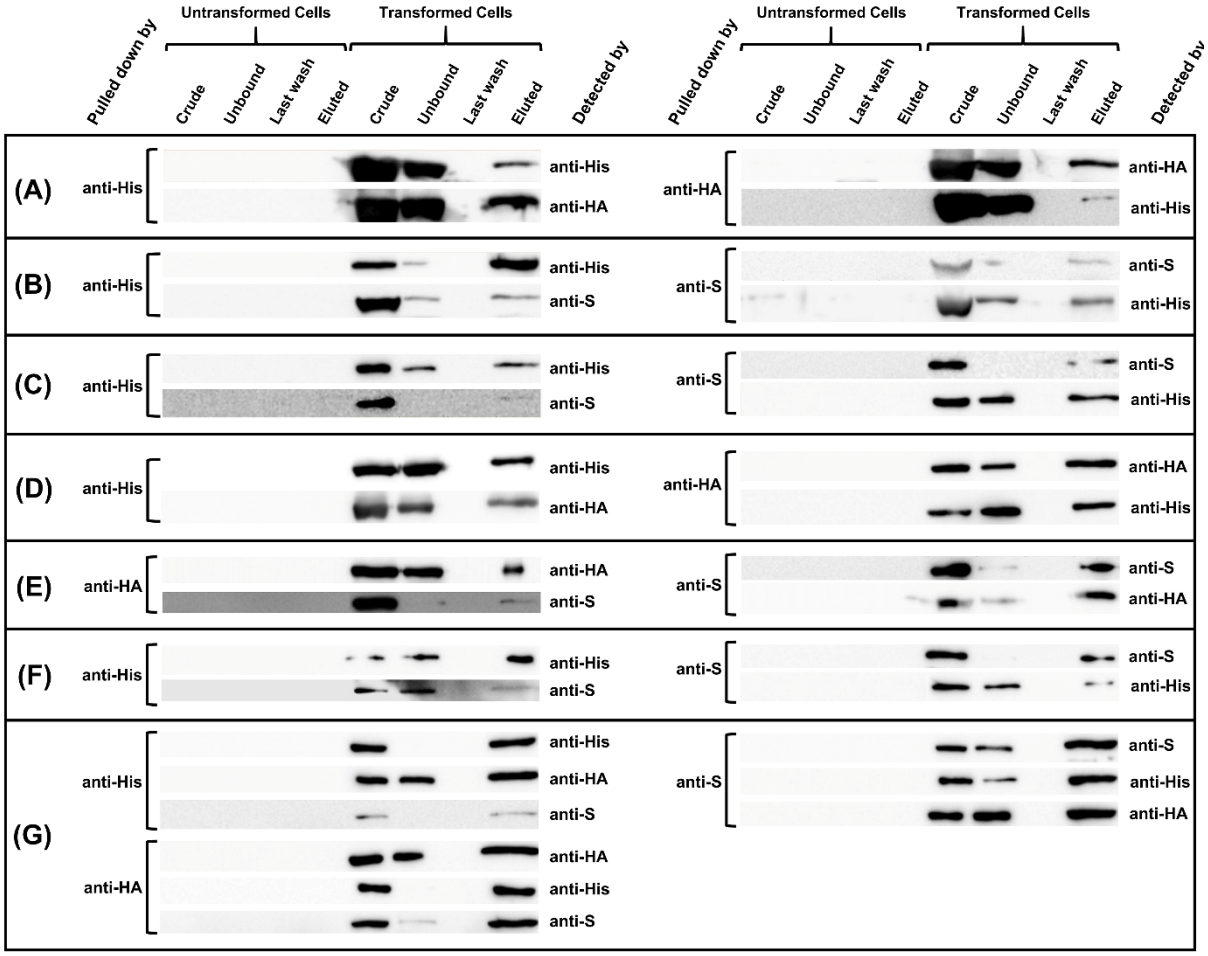

Figure 14. Immunoprecipitation of heterologous-expressed CPN60 subunits in *E. coli*. Each of the subunits was linked to different epitope tags for verification of their interaction in the bacterial cells. Total proteins were isolated and subjected to pull-down assay by antibodies recognizing one of the epitope tags. The eluates were subjected to SDS-PAGE followed by Western blot analysis using antibodies recognizing the employed tags. (A) to (G) represent experiments performed using cells expressing vector constructs corresponding to those shown in Fig. 13. Untransformed cells were used as negative control. Left and right panels indicate pull-down and reverse pull-down experiments.

Total proteins isolated from the first transformants expressing two copies of CPN60A were first pulled down by anti-His antibodies. The eluate fraction was then subjected to SDS-PAGE

and Western blot analysis using anti-His and anti-HA antibodies. Proteins from the untransformed cells were not cross-reacted by both anti-His and anti-HA antibodies, indicating the specificity of the antisera used in this study. If the CPN60A can interact with one another, the eluate fraction should be detected by both antibodies. Indeed that was the result we observed (Fig. 14A, left panel). We further reversed the pull-down process using anti-HA antibodies. The same results were obtained, suggesting that CPN60A subunits could interact with one another in *E. coli*. This finding was surprising as it has been accepted in the literature and was recently proved that the CPN60A cannot interact with one another (Bai et al. 2015). Similar results from the immunoprecipitation followed by Western blot analysis were observed when two copies of the CPN60B1 and CPN60B2 were expressed in *E. coli* (Fig. 14B and 14C). Self-interaction between B1 and B2 subunits observed by this assay is consistent with the existing data that the beta subunits could self-assemble into functional chaperonin protein (Zhang et al. 2016). Moreover, interaction between all heterologous expressed subunit could be verified by the immune-pull-down assay as well (Fig. 14D-G).

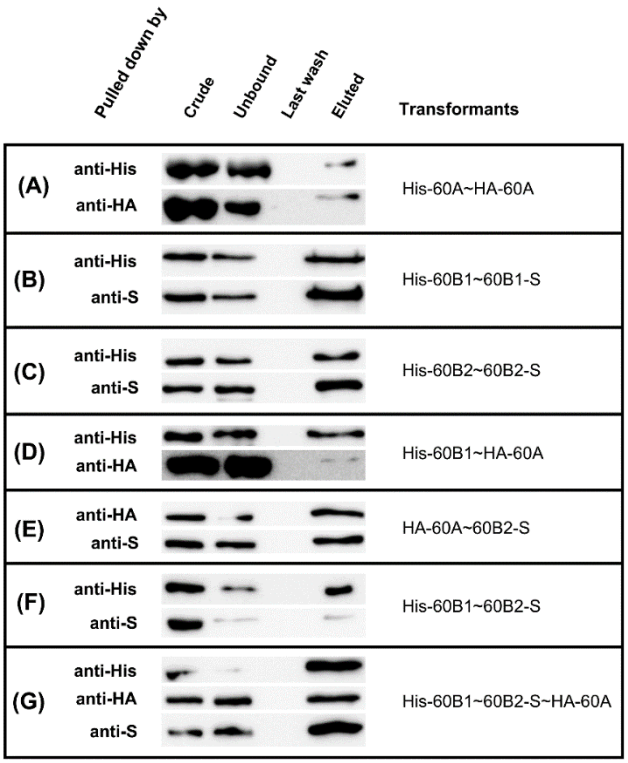

Figure 15. Western blot analysis of the immunoprecipitation assay shown in Fig. 14 using anti-GroEL antibodies. Experiments were performed as before with the exception that the antibodies used for Western blot analysis was anti-GroEL.

As mentioned above, our finding that CPN60A could self-interact or self-assemble was intriguing. However, we must consider the fact that the *E. coli* strain used in this study also contain the homolog of these chaperonin subunits, GroEL. It is possible that self-assembly of CPN60A, as well as those of the rest of the combinations, could be assisted by GroEL. To verify such notion, the same pull-down assays shown in Fig. 14 were subjected to Western blot analysis using specific antibodies for GroEL. The results in Fig. 15 clearly showed that GroEL could be detected in every pull-down combination, suggesting that GroEL could interact with all the heterologous expressed *Chlamydomonas* CPN60 subunits. We then further investigated this cross-interaction between GroEL and CPN60 subunits as this notion has never been reported before in the literature.

The next question were asked was whether the interactions observed in Fig. 15 exist as a dimer, oligomer or as a full-functional chaperonin complex. To verify this question, the representative transformant cells expressing the His-60A~HA-60A, His-60B1~60B1-S and His-60B2~60B2-S were employed. Total proteins were isolated and subjected to gel filtration chromatography (Superdex-200). Proteins from all of the transformants gave the same gel filtration pattern as shown in Fig. 16, having a high-molecular-weight elution peak at ~750 kDa (calculated). The >750 kDa size corresponds well with the full tetradecamer (14 subunits) of the chaperonin/GroEL complex.

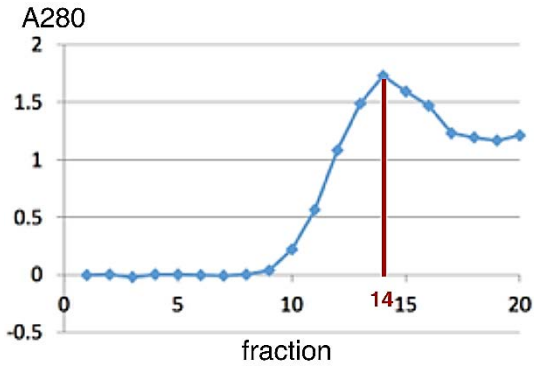

Figure 16. Representative of gel filtration chromatograms. Total proteins isolated from the transformants harboring *Chlamydomonas* CPN60 subunits were subjected to gel filtration (Superdex-200). The eluted fractions were detected by UV-visible spectrophotometer at A280nm.

The eluate fractions from 12 to 16 were further subjected to native gel electrophoresis (Fig. 17). We could consistently observe a high molecular weight band around the 669 kDa marker size. Note that the native marker could have a certain range of error for calculating the actual size.

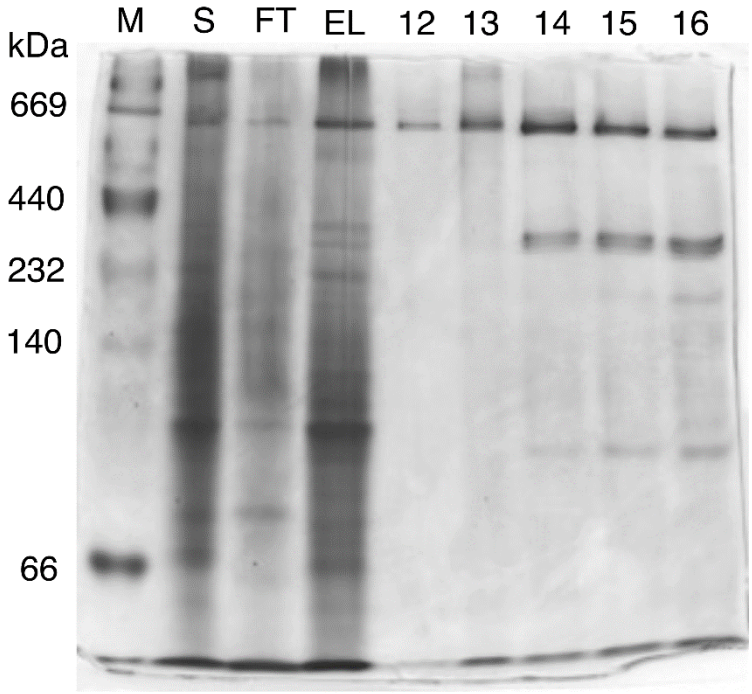

Figure 17. Native PAGE of proteins isolated from E. coli cells expressing *Chlamydomonas* CPN60 subunits. Total proteins were subjected to DEAE column followed by gel filtration and separation on the native PAGE. M = native PAGE markers; S = supernatant or crude total proteins; FT = flow through, the unbound fraction from DEAE column; EL = eluate from DEAE column; 12-16 = fraction numbers from gel filtrations. Protein bands were visualized by Coomassie staining.

As there could be many other type of proteins having such high molecular weight size in their native configuration, further analysis was performed. First we tried using specific antibodies recognizing the epitope tags linked to individual proteins for immunoblot analysis. That approach did not work as not of the antibodies could recognize that high molecular weight complex discerned in Fig. 17. Nevertheless, when we consider the fact that the epitope tag was fused to the protein either at the N or C terminus, the negative cross-reaction by the native-immunoblot made more sense. When the CPN60 subunits are assemble into native tetradecamer complex, both N and C termini of the subunits are located in the middle of the barrel structure. Thus, they are hidden inside and are not readily accessible by the antibodies. To prove this hypothesis, the

high molecular weight bands were excised from the native gel and subjected to the 2nd dimension SDS-PAGE followed by Western blot using specific antibodies against the epitope tags. Results in Fig. 17 clearly demonstrate that our hypothesis was correct. When the high molecular weight band was excised from native gel of proteins isolated from untransformed cells, the only antibodies that could detect the 2nd-dimension SDS-PAGE band was anti-GroEL (Fig. 17, Negative lane). Positive control used in this experiment was the crude total proteins isolated from the transformant cells, thus, containing all the expressed proteins (Fig. 17, Positive lane). All the excised bands gave rise to the monomeric subunits after the 2nd-dimension SDS-PAGE detectable by all the antibodies used (Fig. 17A, 17B and 17C). These results altogether suggested that the *Chlamydomonas* CPN60 subunits could cross-assemble with GroEL in *E. coli* into the full-functional tetradecameric complex.

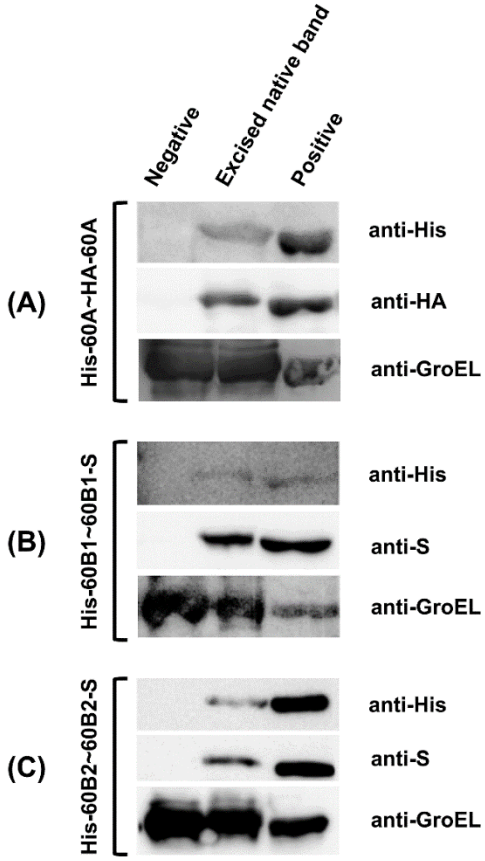

Figure 18. Second dimension SDS-PAGE followed by Western blot analyses. Total proteins from the transformants His-60A~HA-60A (A), His-60B1~60B1-S (B) and His-60B2~60B2-S (C) were subjected to DEAE follow by gel filtration before subjected to native page. The high molecular weight complex bands were excised from the native gel and rerun on SDS-PAGE before subjected to Western blots using antibodies recognizing the epitope tags as well as GroEL.

3. Discussion

HSP90B gene expression and promoter characterization

We have shown in this research the expression profiles of the *HSP90B* gene in the model unicellular green alga *Chlamydomonas reinhardtii*. Like that of other heat-shock proteins, its transcript was remarkably elevated upon significant rise in temperature when compared to other treatments (Fig. 6A). Furthermore, our study (Fig. 6B) showing that its gene expression was induced by DTT, an elicitor of the ER stress (Kim et al. 2013; Pérez-Martín et al. 2014), can support the concept that the HSP90B protein functions in the ER lumen. Of our particular notion was the fact that expression of the *HSP90B* gene could not be stimulated by other stress treatments employed in this study. It is possible that the other stress treatments employed in this study was not long enough to elicit the response at the level of *HSP90B* gene transcription.

Intriguingly, the *HSP90B* expression in *C. reinhardtii* exhibited inverse relationship to the stages of cell growth, higher expression in the fresh inoculum when cell density was low and gradually diminished to minimum level when the cells reached stationary phase (Fig. 7). This phenomenon was probably due to the requirement for biosynthesis of key ER proteins after cell division. It has been shown in mammalian cell that ER was subjected to reorganization during mitosis (Lu et al. 2009). Synthesis of the ER chaperone like HSP90B might play a role in restoration of the organelle's structure and function. When the rates of cell division slows down during the late-log to stationary phase, there is no need for new HSP90B protein synthesis. Under such condition, the gene would then be inactivated. Similarly, other isoforms of the HSP90 protein have also been shown to be in tight association with the key cellular processes required during cell division and development (Koning et al. 1992; Reddy et al. 1998; Rutherford and Lindquist 1998).

We have demonstrated a number of potential cis-acting elements responsive for different kinds of abiotic stresses predicted within the *HSP90B* promoter sequence. Nevertheless, one must be aware that most of the predicted cis-elements shown in Table 1 have been computationally derived from the known motifs reported in higher plants. Very few of those have been experimentally proven to exist or function in *Chlamydomonas*. Yet, our finding that both high temperature and ER-stress could enhance the *HSP90B* expression suggests that there must

be responsible cis-acting elements located within its promoter sequence. There are several lines of evidence suggesting that there could be an interplay between heat and ER stresses. High temperatures have been reported to stimulate ER stress in both animal and plants (Li et al. 2017; Liu et al. 2012). Ability of the organisms to activate heat-shock response (HSR) is crucial for alleviation of both high temperature and ER stresses (Deng et al. 2013; Liu and Chang 2008; Wang et al. 2017). Hence, it is possible that the requirement of HSP90B protein under heat and ER stresses could trigger its gene transcription.

Treating the cells with inhibitors of either the DNA methyltransferase or the HDAC enzymes, 5aza-dC or TSA, respectively, significantly reactivated the endogenous *HSP90B* gene that had been repressed on day 4 of growth (Fig. 7 and Fig. 10). The DNA methyltransferase enzyme adds a methyl group typically on cytosine residues within the DNA sequence. It is commonly known that the promoter region containing higher degree of the cytosine methylation is less active than the one with lower methylation level (Feng et al. 2010; Zemach et al. 2010). As such, inhibition of the DNA methylation process by the 5aza-dC could reactivate the suppressed gene. Additionally, when the histones, sitting at the core of the nucleosomes, occupy the DNA sequences as condensed chromatin, transcription of the affected gene cannot proceed (Guffanti et al. 2006; Polach and Widom 1995; Sebeson et al. 2015; Sekinger et al. 2005). In order to turn the gene on, cell needs to modify the histones in a way that loosens its interaction with the DNA, allowing transcription factors to bind and initiate the transcription process (Charoensawan et al. 2012; Cortijo et al. 2017; Owen-Hughes and Workman 1994; Teichmann et al. 2012; Whitehouse et al. 2007). Acetylation is one of the histone modification mechanisms that lead to decondensation of the chromatin through chromatin remodeling complex, leading to active transcription of the genes (Cairns 2009). Treating the cells with the HDAC inhibitor like TSA, thus, could prolong the active chromatin state, giving rise to sustained expression of the gene. It should be noted, however, that both 5aza-dC and TSA can bring about region-specific (Tabolacci et al. 2016) as well as broad-spectrum effects that involved protein homeostasis (Butler et al. 2015). Thus, it is possible that the enhanced expression of the *HSP90B* gene upon treatment with both inhibitors in this study might indirectly result from reactivation of the gene(s) encoding for its transcription activator(s) located elsewhere in the genome, or, from the inhibitor's effects on protein homeostasis leading to the unfolded protein response in the ER compartment.

Yet, it is noteworthy that 5aza-dC and TSA could not consistently de-repress the promoter truncation construct inserts (Fig. 11). It is possible that the epigenetic regulations of the endogenous *HSP90B* gene observed in Fig. 10 are locus-specific. When the promoter truncation constructs were randomly inserted in other positions, their expressions were under influence of the genome arrangement and epigenetic mark(s) nearby the insertion site (Cerutti et al. 1997; Rajeevkumar et al. 2015). Moreover, it is also possible that our number of transformants used in this study (two) were not sufficient to differentiate the positional effects from the intrinsic promoter property.

Results from promoter truncation analysis (Fig. 9B) as well as ChIP experiments (Fig. 12B and 12C) shown in this paper suggest that core constitutively-active promoter of the *HSP90B* gene could reside within the proximal region (–253 bp upstream from the TSS). Our finding that the acetylated histone H3 is associated more with the proximal promoter than the distal region (Fig. 12C) is consistent with previous reports in both animals and plants. Acetylated histones, including H3K9Ac, have been shown to predominantly localize around the TSS (Du et al. 2013; Ha et al. 2011; Wang et al. 2008). Interestingly, our results also showed that inclusion of a small nucleotide sequence of 59 bp between –253 and –311 was sufficient to suppress the constitutive core promoter (Fig. 9B, Fig. 11). Additionally, this small region was predicted to have low histone occupancy similar to that of the –253 core promoter sequence (Fig. 12A). Charoensawan et al. (2012) suggested that repressor proteins, rather than transcription coactivator, generally favor the binding sites within the regions on the DNA with low intrinsic histone binding preference. Thus, it is possible that this 59 bp sequence of the *Chlamydomonas HSP90B* promoter may contain repressor binding motif(s) unidentifiable by the online prediction tools we employed. Whether or not such putative repressor(s) and its/their binding site(s) really exists requires further studies.

Assembly of *Chlamydomonas* CPN60 subunits in *E. coli*

We have shown in this research project that subunits of chloroplast chaperonin 60 protein could assemble in combination with GroEL inside the bacterial cells into the tetradecameric complex. Such cross-interaction between CPN60 subunits and GroEL, although not unforeseeable, has never been experimentally demonstrated before in the literature. In term of evolution, this finding could pave a way toward understanding the diversification of this molecular

chaperone among organisms within the tree of life. Bacteria and archaea has only a single gene encoding for a single polypeptide that self-assemble into the fully-functional tetradecameric complex (Braig et al. 1994). During evolution, cyanobacteria have obtained two or three genes encoding for GroEL possibly through gene duplication event(s) (Lehel et al. 1993). Overtime, the duplicated genes diversified, via mutations, to become alpha and beta (A and B) subunits in eukaryotic chloroplasts. Studies have shown that one of the two GroEL from cyanobacteria could rescue heat-sensitive phenotype of the *groEL*-deletion mutant of *E. coli* while the other one could not (Furuki et al. 1996; Tanaka et al. 1997), supporting the notion of sequence diversification.

In our study here, the host bacteria still possess the wild type copy of the *groEL* gene. Thus, the additional isoforms of the homolog we introduced can mix and match with the existing GroEL copy to become multiple combinations. Whether any of these combinations would confer additional advantage for the bacteria similar to that of the cyanobacteria and higher plants requires further in-depth investigations.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ณ ขณะที่ส่งรายงานฉบับสมบูรณ์นี้ ยังไม่มีผลงานตีพิมพ์ได้รับการตอบรับ มีการส่งผลงานไปยังวารสารเพื่อขอตีพิมพ์ โดยได้รับความเห็นให้ minor revision กลับมา ณ ขณะนี้ได้ส่ง revision กลับไปยังวารสารแล้ว อยู่ระหว่างรอคำตัดสิน ส่วนผลงานอีกชิ้นหนึ่งอยู่ในระหว่างการรวบรวมเขียน manuscript และเติมเต็มการทดลองบางส่วนที่ยังไม่สมบูรณ์

1. Traewachiwiphak S, Yokthongwattana C, Ves-urai P, Charoensawan V, Yokthongwattana K* Gene Expression and promoter characterization of heat-shock protein 90B gene (*HSP90B*) in the model unicellular green alga *Chlamydomonas reinhardtii*. Plant Science, Revision submitted (awaiting for decision).#
2. Wongsamart R, Werapan B, Yokthongwattana K* *Chlamydomonas* CPN60 subunits interact with GroEL and confer additional stress tolerance in Escherichia coli. (Manuscript in preparation). #

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงพาณิชย์

N/A

2.2 เชิงนโยบาย

N/A

2.3 เชิงสาธารณะ

N/A

2.4 เชิงวิชาการ

2.4.1 มีการผลิตนักศึกษาระดับปริญญาเอก 2 คนคือนายสมโชค แตรวจีวิภาค และนางสาวรุ้งดาวรรณ วงค์สามารถ

2.4.2 มีการนำเนื้อหางานวิจัยบางส่วนไปใช้ในการเรียนการสอน

3. อื่น ๆ

N/A