ANALYSIS OF HEAT-SHOCK PROTEIN GENE EXPRESSION PROFILES IN *CHLAMYDOMONAS REINHARDTII* SUBJECTED TO DIFFERENT ABIOTIC STRESSES

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ABSTRACT

Heat shock proteins (HSPs) are important proteins in living cells that play key roles in cellular adaptation to various stress conditions. The strict correlations between plant *HSP* gene expressions and abiotic stresses, albeit in bits and pieces, have been well-documented and reported. In this thesis, 25 *HSP* gene expression profiles in a model unicellular green alga *Chlamydomonas reinhardtii* were simultaneously observed under 4 abiotic stresses compared to the normal growth condition by semi-quantitative RT-PCR and, for selected genes, by qPCR. Such stresses included temperature rises, increasing salinity, exposure to excessive irradiance, and the transition from darkness to normal light intensity. The results showed that expression of 17 out of 25 *HSP* genes could be detected under normal growth conditions. Under abiotic stress treatments, 12 genes were up-regulated by heat stress, while *CLPB1* and *HSP33* were down regulated. Transcripts of 5 genes were enhanced by increasing irradiance. When the alga was subjected to increasing salinity for 2 hours, 7 genes were found to be up regulated. After overnight dark incubation followed by exposure to normal light conditions, mRNA of 7 genes were elevated. Four *HSP* genes showing significant up regulation profile by the semi-quantitative RT-PCR were selected for validation by qPCR. Analyses by qPCR correlated with the RT-PCR results. Notably, *HSP22A* gene significantly increased its expression profile under heat stress, as determined by qPCR, with a calculated fold change of about 3,500 times, making this gene a good candidate for further studies on heat-induced regulation of gene expression. Putative promoter sequences of the 4 selected genes (about 500 bp upstream of the start codon) were obtained from the genome database and the selected abiotic stress responsive elements were located using a computer program. The promoter analysed data suggested that the space between heat shock elements (HSEs) on their putative promoter sequences might play a key role in heat-induced gene expression.

KEY WORDS: *CHLAMYDOMONAS REINHARDTII* / HEAT SHOCK PROTEIN GENE / ABIOTIC STRESS / GENE EXPRESSION PROFILES / STRESS RESPONSIVE ELEMENTS

102 page

การศึกษาการแสดงออกของยีนฮีทช็อคโปรตีนในสาหร่าย *CHLAMYDOMONAS REINHARDTII* ที่เลี้ยงภายใต้สภาพความเครียดต่าง ๆ

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บทคัดย่อ

ฮีทช็อคโปรตีนเป็นหนึ่งในบรรดาโปรตีนที่มีความสำคัญในการดำรงชีพของเซลล์สิ่งมีชีวิตเพื่อรับมือกับสภาพความเครียดต่าง ๆ มีการศึกษามากมายที่แสดงให้เห็นว่าสภาพความเครียดมีความสัมพันธ์กับการเหนี่ยวนำให้ยีนฮีทช็อคโปรตีนมีการแสดงออก ในการศึกษานี้ยีนฮีทช็อคโปรตีนของสาหร่าย *Chlamydomonas reinhardtii* จำนวน 25 ยีนถูกเลือกมาเพื่อศึกษาการแสดงออกในสภาวะเครียดที่มีอุณหภูมิเพิ่มสูงขึ้น, การเพิ่มความเข้มข้นของเกลือแกง, การเพิ่มความเข้มแสง และเปลี่ยนจากที่มืดสู่สภาวะเลี้ยงในที่มีแสงด้วยเทคนิค semi-quantsitative RT-PCR และสำหรับยีนฮีทช็อคโปรตีนที่ถูกเลือกจะนำไปทำ qPCR เพื่อยืนยันผล RT-PCR ผลการศึกษาพบว่ามียีนฮีทช็อคโปรตีน 17 ยีนแสดงออกในสภาวะปกติ 12 ยีนมีการแสดงออกเพิ่มขึ้นเมื่อเพิ่มอุณหภูมิ ในขณะที่ยีน *CLPB1* และ *HSP33* ถูกลดการแสดงออก เมื่อเพิ่มความเข้มแสงมี 5 ยีนที่ถูกเพิ่มการแสดงออก เมื่อเพิ่มความเข้มข้นของเกลือในระยะเวลา 2 ชั่วโมงพบว่ามี 7 ยีนที่มีการแสดงออกเพิ่มขึ้น เมื่อให้เซลล์อยู่ในความมืดข้ามคืนก่อนรับแสงที่ความเข้มปกติในวันรุ่งขึ้น พบว่ามี 7 ยีนที่มีการเพิ่มการแสดงออก ยีนฮีทช็อคโปรตีนสี่ยีนที่พบว่าเพิ่มการแสดงออกอย่างมีนัยสำคัญในสภาพเครียดได้ถูกเลือกเพื่อยืนยันผลการแสดงออกด้วยเทคนิค qPCR ซึ่งผลการทดลองที่ได้สอดคล้องกับ semi-quantitative RT-PCR เป็นที่น่าสังเกตว่ายีน *HSP22A* เพิ่มการแสดงออกสูงขึ้นในสภาวะเพิ่มอุณหภูมิถึงราว 3,500 เท่า ซึ่งน่าสนใจและควรนำไปเป็นต้นแบบสำหรับศึกษาการตอบสนองต่อความร้อนในระดับการสร้างอาร์เอ็นเอ ลำดับดีเอ็นเอที่เชื่อว่าเป็นโปรโมเตอร์ของทั้งสี่ยีน (500 คู่เบสก่อนถึงโคดอนเริ่มต้น) จากฐานข้อมูลจีโนมถูกนำมาค้นหาและแสดงลำดับดีเอ็นเอที่สนองตอบความเครียดด้วยโปรแกรมคอมพิวเตอร์เป็นแผนผัง ซึ่งแผนผังที่ได้บ่งชี้ว่าระยะห่างระหว่างลำดับเหล่านั้นอาจมีบทบาทสำคัญต่อการเหนี่ยวนำให้เพิ่มการแสดงออกด้วยความร้อน

102 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLE

LIST OF TABLE (cont.)

LIST OF FIGURE

LIST OF FIGURE (cont.)

LIST OF FIGURE (cont.)

LIST OF ABBREVIATIONS

%E	Percent of PCR amplification efficiency
bp	Basepairs
cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
E	PCR amplification efficiency
HSP (protein) or *HSP* (gene)	Heat shock protein
HSE	Heat shock element (heat responsive element)
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
TAP	Tris-acetate-phosphate medium

CHAPTER I
INTRODUCTION

Plants must perform many biological processes to survive and reproduce themselves. Such processes involve several systems in the whole plant body that have responsibility to maintain their integrities and functionalities. Achievement of the processes rely on the cooperation of many macromolecules. Of particular interest is how plants respond to environmental changes such as light intensity or temperature changes during day and night. The environmental changes generally affect many enzymatic pathways and somehow disrupt them. Animal can avoid such hazard conditions by moving away to find more suitable environments. Plants, however, cannot do such thing. They must stay (sessile or immobile) where they germinate and have to sustain any environmental changes. The most impressive thing is that many plants can survive, grow and even propagate themselves.

1.1 Plants' life relies on photosynthesis

1.1.1 Photosynthesis takes place inside the chloroplast

As autotrophs, sunlight, water and CO_2 are essential components for plants to live. The sunlight is an energy source while water is as electron donor and CO_2 serves as carbon source for producing many other forms of reduced carbon compounds. The process, so called photosynthesis, takes place in a type of plastids called chloroplast. Note that there are several types of plastids in plant cells depending on sites and tissue functions. For chloroplast, the photosynthetic membrane structure inside was developed as shown in Figure 1.1.

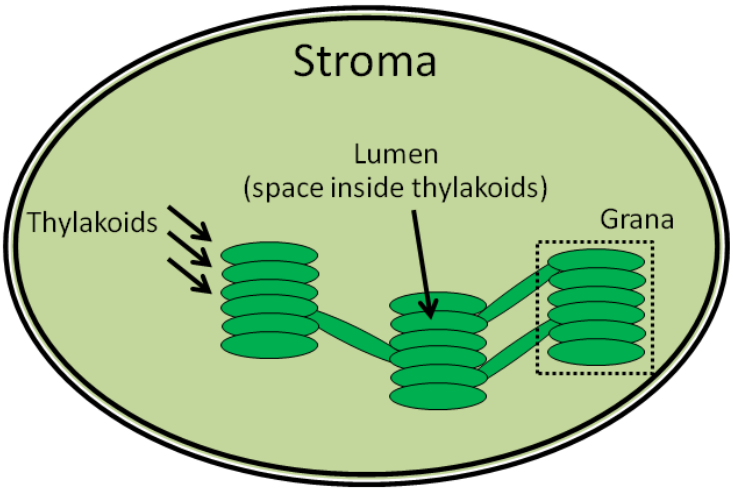

Figure 1.1 Double-membrane organelle chloroplast. It composes of aqueous part called stroma and photosynthetic membrane structure called thylakoids. A stack of thylakoid called grana and the space inside thylakoid called lumen.

There are two major reaction series involved in photosynthesis; light dependent reactions and carbon assimilation reactions. The first one occurred in the thylakoids. The membrane of thylakoids contain photosynthetic machineries involved in light energy conversion, electron transfer and finally convert into ATP and NADPH at the stroma-exposed surface. The carbon assimilation reactions take place in the stroma phase of the chloroplast where the enzymes involved in CO_2 fixation and regeneration of its substrate exist.

1.1.2 Energy conversion in light-dependent reaction

There are three components involved in the light-dependent reactions of photosynthesis: photosystem II reaction center (PSII), photosystem I reaction center (PSI) and Cytochrome b_6f complex. The first and the second components are multisubunit pigments-binding proteins on the thylakoid membrane. They both contain light harvesting or antenna proteins that bind chlorophyll and carotenoids in a specific arrangement for light energy absorption and transfer. PSII core subunits absorb light at wavelength 680 nm while the PSI core absorbs light at wavelength 700 nm.

Photosynthetic reactions start by light energy absorption using light harvesting pigments. Upon photon absorption, chlorophylls are activated to undergo transition from ground state to excited state. The excitation energy is then transferred

to nearby pigments and eventually to the reaction center core. When the energy is passed along to the reaction center, it promotes an electron transfer to a specific acceptors. This is called photochemical reaction. High energy electrons from this step are passed through a series of electron carriers and ultimately transfer to $NADP^+$, so called linear electron flow, as shown in Figure 1.2.

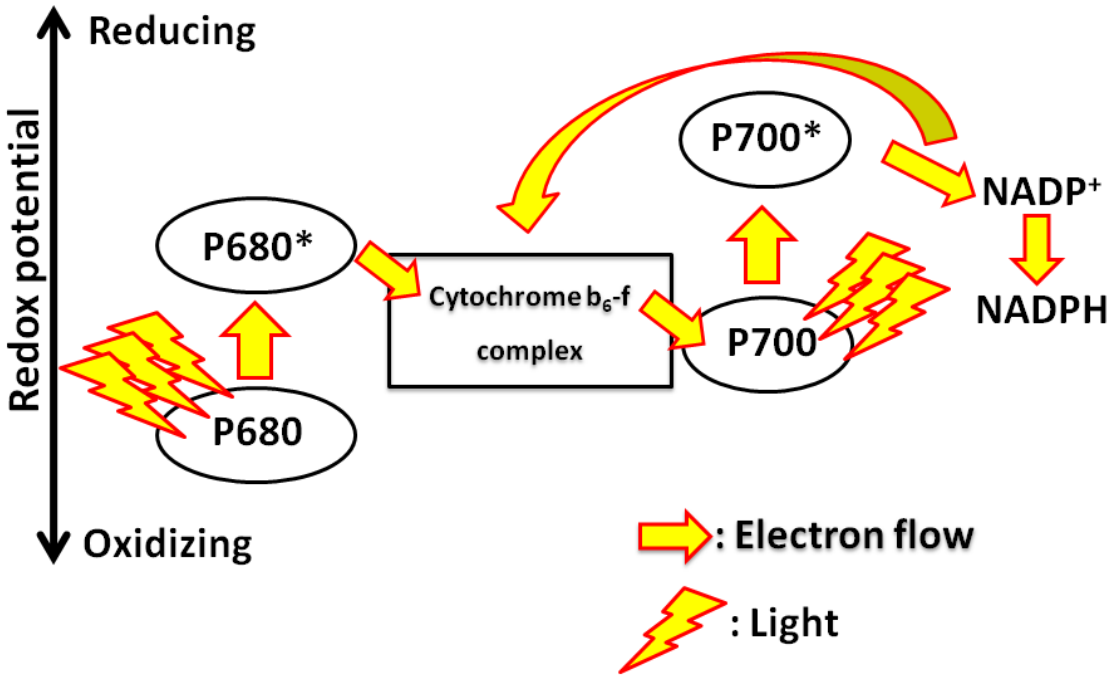

Figure 1.2 A brief Z scheme (zigzag scheme) of photosynthesis. Electrons are transferred from excited P680 (PSII) to $NADP^+$ to become NADPH. Cyclic electron flow around P700 (PSI) happens when electrons from P700* are transferred to cytochrome b_6f complex via ferredoxin. Asterisk (*) defines excited state of the photosystems.

After electron transfer, PSII was deficient of electron and cannot return to ground state. Here, oxygen-evolving complex, plays a key role. This complex uses light energy to oxidize water molecules then capture and transfer electrons to PSII. Finally, PSII receives electrons, return to ground state and becomes capable to perform photochemical reaction again.

The oxidation reactions performed by photosynthesis not only yield electrons but also protons (H^+) and molecular oxygen. The protons are deposited into

the lumen. Protons inside the thylakoid lumen come from two sources, one from oxidation of water as describe previously and another is from electron transfer from plastoquinol to Cytochrome *c* at the Cytochrome b_6f complex. This makes proton concentration in the lumen higher than stroma creating electrochemical potential. This is a form of energy that can be converted to ATP by using ATP synthase. Protons move across thylakoid via this membrane bounded enzyme drives ATP synthesis. This process is similar to ATP synthesis in mitochondria and some prokaryotic bacteria and so called chemiosmotic coupling.

All processes happen at the thylakoid membrane because all of enzymes and electron carrier proteins are membrane bound. But ATP synthesis and the reduction of $NADP^+$ to NADPH take place in the stroma. Those ATP and NADPH molecules were used to drive the carbon-assimilation reaction.

1.1.3 Carboxylation and Oxygenation reaction catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) enzyme

While light-dependent reactions take place in the thylakoids, carbon-assimilation reactions take place in stroma. There are three steps involved CO_2 fixation, reduction and regeneration of acceptor. In the first step, CO_2 form covalent bond with five carbon atoms CO_2 acceptor, ribulose 1,5-bisphosphate (RuBP), to become an unstable intermediate. Then the intermediate is cleaved into two molecules of 3-phosphoglycerate. Those two reactions are catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase or so called rubisco. Its optimal condition is at high pH (>7.5) and with high concentration of Mg^{2+} (>5 mM). This condition is achieve when light-dependent reactions are operating. ATP and NADPH molecules are used in the second step. Regeneration of RuBP back to perform CO_2 fixation again is achieve in the third step.

As its name implied, rubisco not only catalyzes carboxylation reaction but also oxygenation reaction. Its substrate binding pocket cannot discriminate between CO_2 and O_2. So, the O_2 can compete with CO_2 in the reaction. If the oxygenation happened, O_2 form covalent bond with RuBP, the product will be one each of 3-phosphoglycerate and 2-phosphoglycolate. This reaction is called photorespiration. The 2-phosphoglycolate is then regenerate to 3-phosphoglycerate via glycolate pathway which migrated the substrate to peroxisome and mitochondria. For glycolate pathway in mitochondria, CO_2 is released. This means photorespiration draws some of 3-phosphoglycerate out of the carbon-assimilation reaction leading to loss of CO_2, which is opposite to the carbon-assimilation pathway. This eventually disrupts overall plant growth.

1.2 Abiotic stress obstructs plant growth by disrupting photosynthesis

As sessile organisms, plants are certainly affected by environmental changes. For example in one day the sun light intensity swings and directly affected both temperature and air humidity. Wind blow can harm physical structure of plants. Sometimes they were eaten by herbivores which in turn can cause infection. Please keep in mind that those things can happen in nature simultaneously. In this study, there is a term that should be introduced: abiotic stress. To be clarify, “stress” for plants means external factor(s) which serve disadvantageous influence on them (Taiz and Zeiger 1991) and “abiotic” means not alive which is proper for our experimental design and results analysis. Three not alive factors were included herein: the irradiance by the exposure to excessive irradiance (excess light) and the transition from darkness to normal irradiance (shifting from the darkness), the rising of temperature (heat stress) and the increasing of salt concentration (salinity stress).

For photosynthetic organisms, light is an essential energy source. It drives photochemical reactions and series of electron transfer from water to $NADP^+$ via both photosystems as shown in Figure 1.2. If irradiance is in excess of the photosynthetic capacity, the amount of high energy electrons in the system are increase because the amount of $NADP^+$ is limited. Increasing of electron flux density then promotes production of various reactive oxygen species (ROS), increase reduction state of some

electron transport components such as plastoquinone and thiols which eventually inhibits photosynthesis (Li et al. 2009). At the same time, the protons in stroma will be saturated as a result of more electrons transported via plastoquinone. The protons were moved to thylakoid and causes the decrease of lumenal pH. This triggers the qE (energy-dependent quenching), and activates violazanthin de-epoxidase (VDE), an enzyme in a part of xanthophylls cycle. Both will be described more in the next topic below.

The effect of heat is tricky and complicated because it affects many cellular processes. At the cellular level from outside to inside, heat can cause the cell membrane becoming more fluid. For metabolic pathways, it can cause imbalance of metabolism by altering the rate of various enzymatic reactions. For proteins, it can disrupt their folding and complex assembly. And for DNA, it can disrupt DNA replication and repair (Schroda et al. 2015). However, all those happen on one basis: heat affects weak interactions that stabilized macromolecules' structure. Proteins, such as metabolic enzymes, are macromolecules that are responsible for many processes of life. If they cannot maintain their native structure, they are malfunction, dysfunction or even aggregated by hydrophobic interaction and eventually leading to cell death.

Furthermore, heat disrupts photosynthesis by impairing the carboxylation reaction (Taiz and Zeiger 1991 and Schroda et al. 2015). The mechanism is based on the amount and solubility of two gases, CO_2 and O_2. The atmosphere contains CO_2 and O_2 of about 0.03 % and 21 %, respectively. When the temperature rises, the solubility of these gases is decreased, but the CO_2 amount is very low compared to O_2. This causes significant decreasing in the amount of CO_2 concentration but not for O_2. As such, the photosynthesis is affected by decreasing the chance of carboxylation reaction and increasing the chance of oxygenation reaction at the same time. This means the increasing of temperature forced the plants to perform more photorespiration and photosynthesis is simultaneously diminished.

The increasing of salt concentration (salinity), NaCl, for instance, causes osmotic and ionic stresses. When Na^+ amount outside the cells is high, the cytoplasmic water diffuses out. At the same time, Na^+ can diffuse into the cells. The toxicity is based on its physiochemical properties (for example, ionic radius), which is similar to

K^+. So, it can compete at potassium binding site(s), resulting in disruption of various enzymatic reactions (Shabala and Cuin 2007).

1.3 Plants response against abiotic stresses

To survive, plants have various strategies to cope with many types of abiotic stresses depending on the stress exposure time period. If it is a short period, their strategies may only be to restrict and avoid more damage from the stressors. If stressor(s) exist for a long time, the strategies will change to acclimate, gain resistance (harden) or even gain tolerance.

The excess light promotes production of ROS and decreasing of lumenal pH. Plants response to that situation by up-regulating gene expression of enzymes involved in ROS scavenging such as superoxide dismutases (SODs), catalases and peroxidases. Upon lowering of lumenal pH, violazanthin de-epoxidase (VDE) is activated (Li et al. 2009). Then its substrate violaxanthin will be converted to antheraxanthin and zeaxanthin. Both of them contain carotenoid ring structure. They can promote qE (energy-dependent quenching), the dissipation of excess absorbed light energy as heat.

The heat disrupts weak interactions that stabilize macromolecules' structure. For proteins, plants respond by increasing gene expression of the components that stabilize denatured proteins and recover them back to native state. Those components are called heat shock proteins (HSPs). Their details will be described more in the next topic below.

The increasing of salt concentration (salinity) caused osmotic and ionic stresses. Plants use two strategies to respond. The first is exclusion of excess Na^+ out of the cells via the plasma membrane Na^+/H^+ antiporter. The second is adjustment of the osmotic pressure inside the cells by accumulation of more organic, compatible solute such as glycine betaine (*N*,*N*,*N*-trimethylglycine-betaine), certain amino acids and oligosaccharides (Mahajan and Tuteja 2005).

1.4 Heat shock proteins (HSPs) and their aspects in our model organism

1.4.1 From heat shock proteins (HSPs) to molecular chaperones

When the cells are subjected to abiotic stresses, their cellular milieus is changed. This affected most of functional units such as proteins at the molecular level. Heat, for example, disturbs weak interactions that stabilize protein structure. When proteins cannot maintain their native structure, their functions were demolished. Moreover, non-native proteins sometimes expose their hydrophobic regions. By hydrophobic interaction, the aggregation happens. Losing functional units and accumulation of aggregates can lead to cells becoming dysfunction and eventually die. To avoid this, living organisms need to respond against abiotic stresses.

In 1962, there was a notice in one publication performed in salivary glands of fruit fly, *Drosophila busckii* about a new set of chromosomal puffs which was found when the temperature was shifted (Ritossa 1962). Later work described about *D. melanogaster* chromosomal puffs that they were produced within a few minutes after induced by heat stress, associated with newly synthesized RNAs and proteins (Tissières et al. 1974). This similar responses were also found in other organisms such as bacteria, yeasts and plants. After that, the name *h*eat *s*hock *p*roteins (HSPs) were designated to those proteins. Long time passing with more extensive studies, the HSPs were realized as molecular chaperones (Gething and Sambrook 1992; Georgopoulos and Welch. 1993) with these following characteristics; (i.) They can bind specifically to non-native proteins. (ii.) They do not form covalent bond with the interacting proteins. (iii.) They do not add, cleave or modify any covalent bond of the interacting proteins. This feature distinguishes molecular chaperones form foldase enzymes like *p*rotein *d*isulfide and *p*eptidyl-*p*rolyl *i*somerases (PDI and PPI, respectively). And (iv.) the ATP-dependent molecular chaperones have their specific conformational change functions such as facilitate folding of newly synthesized (nascent) proteins or promote renaturation of aggregated proteins.

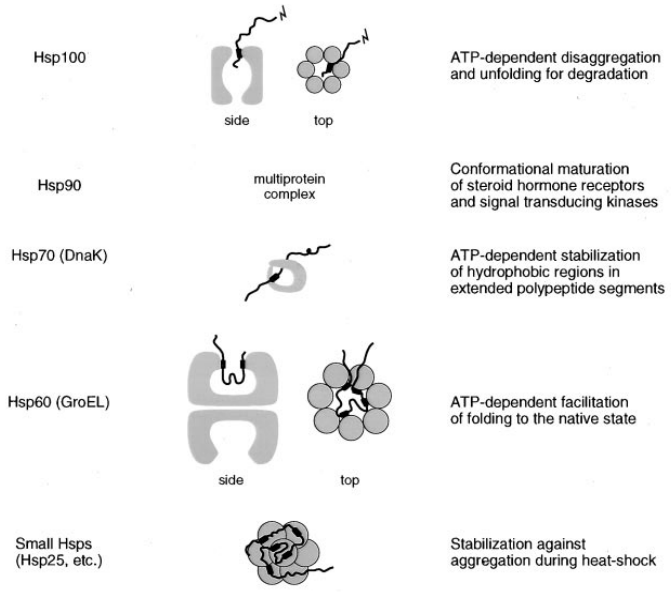

Figure 1.3 Five groups of well-known molecular chaperones. The number indicates each individual approximate subunit size in kDa. HSPs arrangement and topology was roughly depicted and their functions were briefly described. Note that small HSPs are not ATP-dependent (modified from Bukau and Horwich 1998).

Their binding with non-native proteins is usually mediated by recognition of hydrophobic residues and/or unstructured backbone regions in their proteins which is typically exposed under non-native state (Hartl and Hayler-Hartl 2002). Molecular chaperones also have other unusual roles, for example, interaction with proteolytic subunits (Schroda 2004), association with vesicle secretion, recycling, and regulating assembly and/or disassembly of protein complexes (Young et al. 2003). Combined all of those useful functions listed above, it is not surprise why all cellular compartments have molecular chaperones inside. They are essential for cells (or more specific, for other proteins) in both normal and stress conditions.

1.4.2 Model organism *Chlamydomonas reinhardtii*

The unicellular green alga *C. reinhardtii* is an excellent model organism for studying various aspects in cell biology. The important advantages of this alga include: it has standard medium for mixotropic growth, is haploid vegetative cells with 8-12 hr doubling time and can be synchronized by exposing to periodic light. Moreover, its nuclear, chloroplast and mitochondrial genomes have been completely

sequenced and available on many well-known online databases and its genetic toolbox grows up continuously (Blaby et al. 2014).

Another thing that makes *C. reinhardtii* attractive is it contains both animals and plants characteristics. With two flagella, it can swim and glide in water. Its flagella are also similar to flagella and cilia in mammalian cells. So many studies associated with cilia and flagella select it as a model organism. And with chloroplast, it can perform photosynthesis and lives as autotrophs by using carbon dioxide if there is no acetate in the medium as carbon source. Because of their photosynthetic machineries and auxiliary compartments are similar to higher plants, it is very useful for photosynthesis studies about its mechanisms and/or photosynthetic gene function.

The first wild type *C. reinhardtii* was isolated from potato field's soil sample taken near Amherst, Massachusetts in 1945 (Harris 2009). Nowadays many strains were submitted and collected at Chlamydomonas Resource Center (Chlamydomonas Genetic center, c/o Dr. Elizabeth H Harris, DCMB Box 9100, Duke University, Durham NC 27708-1000, USA; www.chlamy.org). Researchers can enter the website (www.chlamycollection.org) and find out which wild type or mutant strains are suitable for their study requirements before decide to order and obtain.

1.4.3 The controversial discussion of heat stress response

In *C. reinhardtii*, increasing of *HSP* transcripts and HSP proteins happen when it was subjected to heat stress. The key regulator of heat stress response is heat shock factor 1 (HSF1) (Schulz-Raffelt 2007 and Schmollinger et al. 2010). HSF1 at mRNA and protein level are also heat shock inducible. Under heat stress, HSF1 protein becomes hyperphosphorylated and can induce the expression of many *HSP* genes which their products are essential for maintaining cellular functions.

Until now, there is no definite description about how it senses heat. Many hypotheses have been discussed controversially. The first hypothesis is based on proteins interaction between HSF1 and molecular chaperone complex(es) which are thought to act as its repressor (Voellmy and Boellmann 2007). The hypothesis is that increasing amount of non-native proteins under stress conditions pull those molecular chaperone complex(es) away from HSF1, in turn, allowing it to become phosphorylated. Another work on higher plants proposed that the sensing of heat stress

may come from changing of membrane fluidity that triggers CNGC type calcium channels (Finka et al. 2012). According to this model, it is assumed that heat and/or non-native proteins affected plasma membrane integrity which may cause calcium ions influx through stress-sensing Ca^{2+} channels. Then via unknown kinase(s) cascade finally results in hyperphosphorylation of HSF1. Recently, a work that combined pharmaceuitical and RNAi approaches suggested the regulation of heat stress response via HSF1 may be achieved by stress-activated kinases and constitutively active phosphatases (Schmollinger et al. 2013). All are summarized in Figure 1.4.

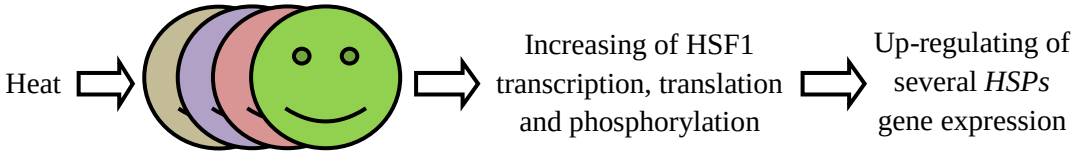

Figure 1.4 Heat shock response of *C. reinhardtii*. The smile faces represented unknown processes/cascade(s) which are triggered when the algae senses heat stress.

1.4.4 Abiotic stresses induce the expression of *HSP* genes

From the previous study (Schroda and Vallon 2009), not only that heat can induce *HSP* gene expression but also light, oxidative stress and others as shown below in table 1.1. This is an example that information about the induction of *HSP* gene transcription was limited and fragmented. Most works were focused on specific gene(s) at one stress condition. So we did not know how it senses abiotic stresses as shown in Figure 1.5.

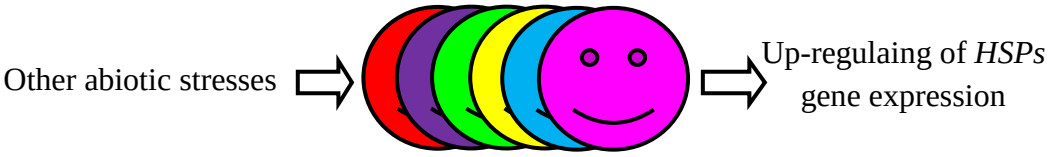

Figure 1.5 Other abiotic stresses response of *C. reinhardtii*. The smile faces represented unknown processes/cascade(s) which were trigged when it sense other abiotic stresses.

Table 1.1 Some *C. reinhardtii HSP* genes and their inducers

Gene	Inducers	Reference
HSP70A	Heat	von Gromoff et al. 1989
	Light	von Gromoff et al. 1989
	Singlet oxygen	Leisinger et al. 2001
	Hydrogen peroxide	Shao et al. 2007
HSP70B	Heat	von Gromoff et al. 1989
	Light	von Gromoff et al. 1989
CPN60A	Heat	Thompson et al. 1995
CPN60B1	Heat	Thompson et al. 1995
HSP22A	Heat	Grimm et al. 1989
	Light	Ish-Shalom et al. 1990
HSP22E	Sulfur starvation	Zhang et al. 2004
	Oxidative stress	Fisher et al. 2005
	Phosphorus starvation	Moseley et al. 2006
HSP22F	Sulfur starvation	Zhang et al. 2004
	Oxidative stress	Fisher et al. 2005
	Phosphorus starvation	Moseley et al. 2006

1.4.5 Known responsive elements of *HSP* genes and others

Gene expression depends on its own promoter and other regulation components such as specific factor responsive elements. For *HSP* genes in *Chlamydomonas*, *HSP70A* promoter is one of the best studied (Kropat et al. 1995; von Gromoff et al. 2006; Lodha et al. 2008). The *HSP70A* gene is composed of two independent promoters called P_{A1} and P_{A2}. Both have their own transcriptional start site. The upstream P_{A1} contains four heat responsive elements, so called *h*eat *s*hock *e*lements (HSEs) which serve for HSF1 binding. And the downstream P_{A2} contains two *p*lastid *r*esponse *e*lements (PREs) which responsible for light induction via Mg-protoporphyrin IX (MgProto), one intermediate of chlorophyll biosynthesis, regardless of its orientation and distance. So, it is qualified as enhancer. Other responsive elements related to abiotic stress are shown below in table 1.2.

Table 1.2 Responsive elements related to abiotic stresses

Responsive element	Inducer	Sequence*	Reference
HSE	Heat	repeated of NGAAN and NTTCN	Lodha et al. 2008
HLR1-like	Light	TTACAA	Park et al. 2013
SORLIP1AT	Light	GCCAC	Hudson and Quail 2003
SORLIP2AT	Light	GGGCC	Hudson and Quail 2003
PRE	MgProto	$SCGAYNR(N)_{15}HD$	von Gromoff et al. 2006
SP1-like	Singlet oxygen	CCRCCC	Fisher et al. 2009
CRE/AP-1	Singlet oxygen	TGACGCCA	Fisher et al. 2009
MYBR	Salinity	TGGTTAG	Yamagushi-Shinozaki and Shinozaki 2005
ABRE	Salinity	ACGTGKC	Yamagushi-Shinozaki and Shinozaki 2005

*Those letters followed the nomenclature for incompletely specified bases in nucleic acid sequences (Cornish-Bowden 1985), see Appendix A for more detailed.

From table 1.2, only HSE, PRE, SP1-like and CRE/AP-1 were discovered in *C. reinhardtii*. Note that original SP1 was found in maize (*Zea mays*), and rice (*Oryza sativa*), (Data from PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). For three light responsive elements, SORLIPAT1 and SORLIPAT2 were discovered in *Arabidopsis* as reference herein and the original *HLR1* was discovered in *Synechocystis*, which is a cyanobacterium (Eriksson et al. 2000). Those three elements were found at LIP (*l*ight *i*nducible *p*rotein gene) promoter from salt tolerant green alga *Dunaliella* sp. (Park et al. 2013). Two salinity responsive elements were selected from the list in that reference. The *C. reinhardtii* has MYB transcription factor. We assumed it can serve for MYBR, MYB recognition site.

Abscisic acid (ABA) is known as a signal of abiotic stress in higher plants. Exogenous treated ABA partly released growth suppression of *C. reinhardtii* caused by NaCl (Yoshida et al. 2004). So, ABRE (ABA-responsive element) was selected.

Because *HSP* gene expression is strictly correlated with abiotic stress, the *HSP* gene expression profiles can match a group of induced genes with specific stress. Then the selected responsive elements will be used to find whether they were located on putative promoter sequence of those *HSP* genes or not. These will help predict some unknown abiotic stress responsive elements and/or provide a guidance to track down the signaling cascade of *HSP* gene induction which in turn, required for elucidation of the *HSP* genes transcription regulation.

CHARPTER II
OBJECTIVES

This thesis has three following objectives:

2.1 Comparison of selected *C. reinhardtii HSP* gene expression between normal and abiotic stress conditions.

Although the *HSP* gene sequences are available on many online databases nowadays, some of their existent at both mRNA and protein level in algal cells have not been confirmed. Here we checked the existent of their mRNA indirectly and quantified the difference of gene expression by using semi-quantitative PCR.

2.2 Confirmation of interested *HSP* gene expression

From the semi-quantitative PCR results, *HSP* genes that manifest increased or decreased gene expression will be selected to perform real-time PCR validation. The fold change difference can strongly relate the *HSP* gene expression to the specific abiotic stress.

2.3 Investigating the relation between interested *HSP* gene expression and abiotic stress responsive elements.

From knowing that the responsive elements can regulate gene expression, each of the putative promoter sequence of the selected *HSP* genes will be obtained. Many of abiotic stress responsive elements will be reviewed, selected and mapped on those putative promoter sequences. This may help understand the relationship between *HSP* gene expression and abiotic stress.

CHAPTER III
MATERIALS AND METHODS

3.1 Algal strain, normal growth condition and abiotic stress conditions

The unicellular green alga *C. reinhardtii* strain CC-503 (cw-92 mt^+) were obtained from the Chlamydomonas Resource Center (http://chlamycollection.org/). The cells about 20 µl from the previous stock culture were started growing in standard TAP medium (Gorman and Levine 1965) of about 20 ml at temperature ~25°C under continuous 50 µmol photons m^{-2} s^{-1} illumination. The culture cells, which reached mid-logarithmic phase after three days of cultivation, were collected by taking 5 ml of them to centrifugation at 3,000 rpm for 1 min. Heat stress treatment was performed by shifting the cultures into a water bath at 37°C or 42°C (heat-shocked). Salinity stress was performed by collecting about 15 ml of the algal cells and subjected to brief centrifugation. Supernatant was discarded while the cells pellet was resuspended into a new TAP medium containing 50mM NaCl (TAP-NaCl, salt-shocked). After cultivation in the salt-containing medium for a specific time interval, the cells were collected by centrifugation at 5,000 rpm for 2 min. High light stress was implemented by shifting the cultures grown under normal light intensity to a continuous 1,000 µmol photons m^{-2} s^{-1} illumination. The transition from darkness to normal light intensity was performed by incubating the 4 days old culture in the darkness for about 18 hr. before moving them back to continuous light of about 50 µmol photons m^{-2} s^{-1} illumination. Each stress treatment condition was performed and cells were collected at three different time periods (half an hour, 1 hr. and 2 hr.).

3.2 Bacterial strain using for cloning techniques

Escherichia coli strain DH5α [*fhuA2, lacΔU169, phoA, glnV44 Φ80', lacZΔM15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*] was used as a general host strain for plasmid transformation and propagation.

3.3 Semi-quantitative PCR Primer design and verification

Twenty five *HSP* mRNA sequences of *C. reinhardtii* cc-503 (cw-92 mt^+) and other information about them were obtained from http://www.ncbi.nlm.nih.gov/gene. Those data were categorized into many groups then sequence alignment was performed to select each individual specific regions for primer design. Each specific primer was designed individually then verified for their specific product, annealing temperature and other parameters by using Oligo Analyzer 3.1 (http://sg.idtdna.com/analyzer/applications/oligoanalyzer/), NCBI Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Vector NTI Suite 8. Those verified primer sequences were ordered and synthesized by Bio Basic Inc.

3.4 RNA extraction and cDNA conversion

The cells (~5 ml) were collected into 1.5 ml microtube by centrifugation at 3,000 rpm, 1 min (for salinity stress, the cells were harder to collect and needed to centrifuged at 5,000 rpm, 2 min). The supernatant was discarded and the pelleted cells were resuspended in TRIzol reagent (Life Technologies). The principle is based on phenol-chloroform extraction method. The protocol was modified from the instruction manual. Briefly, cell suspension was mixed with 1 ml of TRIzol and incubated at room temperature for 5 min. Then 200 µl of cold (-21°C) chloroform was added and mixed by inverting the tube until the solution was homogeneous. The lysate was incubated at room temperature for 1 min. After that the solution was centrifuged for 10 min at 12,000 g. The clear supernatant which contains RNA was transferred into to a new microtube before adding 500 µl of cold (-21°C) isopropanol to precipitate RNA. The tube was inverted briefly before incubation at room temperature for 5 min. The RNA

can be collected as white pellet after centrifugation at 12,000 g for 5 min. The RNA pellet was washed twice with 70% ethanol in sterile DEPC treated distilled water. The RNA pellet were kept in 70% ethanol at -21°C until use.

The conversion of RNA to cDNA was performed as described below. First, the 70% ethanol was discarded then the remaining liquid was pipetted out and evaporated by heat at 65°C using heat blocks. Note that the RNA pellet was kept a bit moist before dissolving in 30-40 µl of sterile distilled water then heat at 65°C for about 10 s. The process was repeated until the solution is homogeneous. RNA concentration was measured using NanoDrop 2000 spectrophotometer (Thermo scientific) The purity of RNA was determine by OD_{260}/OD_{280} ratio. Conversion of RNA to cDNA was performed by reverse transcription using M-MuLV Reverse Trannscriptase. The reaction mixture was composed of the following: (Note that RNA was diluted to become 0.2 µg/µl before use).

Solutions	Volume (µl)
0.2 µg/µl RNA	10.0
sterile distilled water	4.5
100 µM oligo dTs	0.5
10 mM dNTPs	1.0

The solution was gently mixed by pipetting then incubated at 65 °C for 5 min using T100 thermal cycler (BIO-RAD) followed by chilled on ice for 1 min. After that another reaction mixture composed by these following was added.

Solutions	Volume (µl)
10x MuLV-RT buffer	2.0
200 U/µl MuLV-RT	0.5
RNase inhibitor	0.5
sterile distilled water	1.0

The solution was gently mixed by pipetting and incubated at 42 °C for 90 min followed by 70 °C for 20 min to inactivate the reverse transcriptase. Finally, the cDNA

was synthesized at the total volume of 20 µl. Its concentration was expected to be 100 ng/µl and must be diluted 10 times to become 10 ng/ µl before use. It can be kept at -21 °C.

3.5 Polymerase chain reaction

After the primers were obtained, they were dissolved as 100 mM stock by adding sterile distilled water. The working primers were prepared by diluting the stock solution to 10 mM using sterile water. Each individual gene-specific primer set was tested for their expected product size, optimal annealing temperature and cycle numbers to avoid product saturation. cDNA synthesized from total mRNA isolated from *C. reinhardtii* CC-503 strain grown under normal condition was subjected to PCR at a its calculated annealing temperature and varied cycle numbers (20, 24, 28 and 35). PCR amplification was performed using 2X GeNei Red Dye PCR Master Mix (Merck) with 100 ng of cDNA template. The reaction mixture was composed of the following:

Solutions	Volume (µl)
2X GeNei Red Dye PCR Master Mix	12.5
10 mM Forward primer	1.0
10 mM Reverse primer	1.0
10 ng/µl cDNA template	10.0
sterile distilled water	0.5

Then the thermocycler was programmed as these following.

Cycles	Temperature (°C)	Time (min:sec)
Cycle 1 (1x) Step 1	94	3:00
Cycle 2 (20, 24, 28 or 35) Step 1	94	0:30
Step 2	(see the primer table)	0:30
Step 3	72	0:45
Cycle 3 (1x) Step 1	72	5:00

3.6 Analysis of PCR products by agarose gel electrophoresis

The PCR products were separated by agarose gel electrophoresis using Mupid-exU submarine electrophoresis system (ADVANCE) under 0.5x TAE (Tris-Acetate-EDTA). The gel percentage was varied (1% or 2% agarose in 0.5x TAE) depending on the expected product size. The gel was set by heat for completely dissolved in 0.5x TAE then stayed at room temperature about 10 min. The cooled gel solution (~ 50 °C) was casted by pouring onto a gel tray equipped with comb. It was allowed to solidify for about 20 min. To perform gel electrophoresis, standard DNA ladder and PCR products were loaded into the wells. If loading samples didn't come from Red Dye PCR Master Mix, 10x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) was added at final concentration of 1x. The electrophoresis was carried out at 100V constant voltage for about 30 min. After finishing the run, the gel was stained by ethidium bromide for about 1 min followed by destaining in distilled water for about 2 min. Basic gel documentation (gel doc) system from Syngene was used to visualize the PCR products. The stained gel was illuminated under UV light using SYNGENE G:BOX EF2 (Syngene) connected with computer. Gel pictures were captured using software GENESys Version 1.0.9.0 with database version 1.47.

3.7 PCR product validation

The PCR products which had expected size longer than 250 basepairs were purified, concentrated and sent for DNA sequencing (Macrogen). The gel was stained using ethidium bromide followed by illuminated using TFX-20.MC UV Table 254/312 nm (VILBER LOURMAT). The band at expected size was excised using a razor blade. GenepHlow Gel/PCR Kit (Geneaid) was used to perform gel extraction purification as described by instruction manual. Finally, the purified PCR products were rechecked by agarose gel electrophoresis before sending to sequencing (Macrogen). The DNA sequencing results were used to perform sequence alignment compare to their reference mRNA sequence of *HSP* genes individually using Vector NTI Suit 8.

For the PCR products which had expected size less than 250 basepairs, additional DNA cloning techniques were required to introduce the fragment into plasmid before sending them to DNA sequencing. Firstly, the PCR products were purified by agarose gel electrophoresis and concentrated by gel extraction purification using the kit as previously described. Second, the ligation was performed using pGEM-T Easy Vector Systems (Promega) as described by instruction manual. Then, the competent *E.coli* DH5α was prepared by cold $MgCl/CaCl_2$ method (see below). Transformation was performed by heat shock method (see below) then plated on 100 μg/ml Ampicilin LB agar. After that, transformant clones were selected to subculture on another 100 μg/ml Ampicilin LB agar plate as reference plate and perform colony PCR with their specific primers at appropriate annealing temperature and cycle numbers. The reference plate was incubated at 37°C about 4 hr. For the positive clones, they were selected from reference plate to grow overnight (about 12-14 hr.) in 80 μg/ml Ampicilin LB liquid medium then plasmid purification using HiYield Plasmid Kit Mini (Real Genomics) was performed as described by instruction manual. Finally, those plasmids were checked, purified before sending to perform DNA sequencing. The results were used to perform sequence alignment as describe above.

3.8 Preparation and transformation of competent *E.coli*

These following protocols were modified from The Condensed Protocols from Molecular Cloning: A Laboratory Manual (Sambrook and Russell 2006).

3.8.1 Preparation of bacterial competent cells

The stock *E.coli* was inoculated into 5 ml LB liquid media at 37°C overnight. New LB liquid media was prepared by adding 1% by volume of overnight culture (1% inoculum). This new culture was swirled at 220 rpm, 37°C until the absorbance at 600 nm reached 0.4-0.5. Then collected them into the pre-chilled sterile 50 ml centrifuge tubes and placed on ice for 10 min followed by centrifugation at 5,000 rpm, 10 min at 4°C. The supernatant was discarded and the pellet cells were resuspended in 1/5 original culture volume of pre-cooled 80 mM $MgCl_2$, 20 mM

$CaCl_2$ solution. Keep the cells on ice for 30 min and followed by centrifugation at 5,000 rpm, 10 min at 4°C. The supernatant was discarded and the pellet cells were resuspended into cold 0.1 M $CaCl_2$ at 1/50 original culture volume and left on ice for 1 hr to establish the competency. Finally sterile glycerol were added to the competent cells to final concentration of 15% (v/v) and dispensed into pre-chilled sterile 1.5 ml microtube. They were stored at -80°C until use.

3.8.2 Bacterial transformation

The frozen competent cells were thawed on ice. The ligation reaction was added to the cells and briefly vortexed. The reactions were incubated at 4°C on ice for 30 min. Then the reactions were heat shock by shifted from ice to 42°C for exactly 2 min followed by 4°C for exactly 2 min. After that 800 µl of LB liquid medium was added. The reactions were incubated at 37°C for 45 min before collecting the cells by centrifugation at 9,000 rpm, 1 min. The supernatant was discarded and the pellet cells were resuspended. The cells were spread on 100 µg/ml Ampicilin LB agar and incubated at 37°C overnight. The transformants were selected to perform colony PCR.

3.9 Semi-quantitative PCR

PCR reactions and products detection were prepared similar to the previous step except the cycle numbers and annealing temperatures were optimized for each primer individually as shown in this following table.

Table 3.1 Specific primer sequences and their optimal conditions.

Genes	Primer sequences (FW-Forward, RV-Reverse)	Annealing temperature (°C)	Optimized cycle numbers
CLPB1	FW: TATTGCGTAGACCGGCCCCG RV: GGCCATGCGGCTCCTCGAAG	62	28
CLPB3	FW: TGCCAGCAGCAGCCGAACTC RV: GCCGCCGTATGTAGGCGTCC	65	28
CLPB4	FW: TGCCGGCGCACCAAGAACAA RV: AAGCGCCGGAACTTGTCGGG	65	28
CLPD1	FW: CTGTGGCCCGTATGATGCGC RV: CCGTTGCAGTCCGGCATCGA	65	24
HSP90A	FW: GAGGCCCCCGTGGAGACCTT RV: CCCACGCCGAACTGGCCAAT	55	24
HSP90B	FW: ATCGTGGTCACCGGCAAGT RV: TTACAGCTCGTCCTTCTCCTCCG	61	24
HSP90C	FW: GTGTGCGATTTCAGAGCCCAG RV: ATGCCAGAGGACAGACCGC	61	35
HSP70A	FW: AGGCCCCCGCTATCGGTATT RV: CCTGAGCGGTCTCCTTCATC	61	24
HSP70B	FW: GCAAGTGAACAATACTCCTCAAC RV: TGTCGTTCAGGAACTTGGC	61	24
HSP70D	FW:TGCAAGTTACCTAGATGCAGTAGA RV: CACCTCCACGGTGCCTG	61	28
HSP70E	FW: CCATGGAGGACTGGCTCTACGA RV: TTAGTTGGTCTCCATGGGCG	61	28
HSP70F	FW: CGGGACGGCAGGCCACG RV: GAACGTCACTGCCACTGCCG	65	28
HSP70G	FW: CAGTCTACACGGTCAAGGAG RV: CTCGATGATGCGCTTCAG	57	28

Table 3.1 Specific primer sequences and their optimal conditions (cont.).

Genes	Primer sequences (FW-Forward, RV-Reverse)	Annealing temperature (°C)	Optimized cycle numbers
HSP70G	FW: CAGTCTACACGGTCAAGGAG RV: CTCGATGATGCGCTTCAG	57	28
CPN60A	FW: CAATGGCGCAGTCTCAGCTTGCC RV:AGGCCGTAGTGGATCATCTCGCGC	65	24
CPN60B1	FW: ATCCTTGGCCGGGTGACCG RV: GGTGCCTGCCGACACGATCT	55	24
CPN60B2	FW: ATGCGGTCGTTCCAGGCGAA RV: CGGTCTTCTCCATGCCGCGG	55	24
HSP22A	FW: CGCTCCAGGGAAAGCTGG RV: CGGGCCACAATTTGATAGTACAC	61	24
HSP22B	FW: CGCACGAGCGGGACAATATC RV: GTCTCTTATTGTTGGCGTACCG	61	28
HSP22C	FW: GAGTTTGCTTTGCCGAATAG RV: GCCAGCTCAGCGGTGAG	61	28
HSP22D	FW: GAAACAACCCACTCTCACAA RV: AGCCCAGGAACAGGCG	61	35
HSP22E	FW: GTAGCGTGGGGATTGTTG RV: ACGTTGTGTTACAGGTATGG	61	24
HSP22F	FW: GCAAATCATGTTGAGGCACG RV: AACCAGCATGCGCTCAGG	61	24
HSP22G	FW: ACCGCAGCGGCAGTG RV: CATGGCTGTCTGCTGCTG	61	28
HSP22H	FW: AGATCGACCGCGCCATG RV: GGTGCCAGTGCCGGTG	61	28
HSP33	FW: AGGTCAGCGTGCTGGTG RV: CCAGACCCAGTGCCGAG	61	35
RACK1	FW: ACGGCTACGTCAACACCG RV: TGGTGATGTTGAACTCGG	55	24

3.10 Real-time PCR

Four *HSP* genes and one housekeeping gene were selected to design new primer set. Stock and working primer solutions were prepared similar to the PCR topic. Each individual gene-specific primer set was tested for their dissociation curve and amplification efficiency. All real-time PCR experiments were performed on Mx3000P QPCR Systems (Agilent Technologies) or ABI 7500 (Applied Biosystems) connected with computer. To setup reaction types, protocols and results analysis, software MxPro - Mx3000P v4.10 Build 389, Schema for Mx3000P and Sequence Detection Software Version 1.4.0.25, 7500 System SDS Software for ABI 7500 were used. KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal (KAPA BIOSYSTEMS) was selected. The test reaction composed of these following.

Solutions	Volume (µl)
2X KAPA SYBR FAST qPCR Kit Master Mix	10.0
10 mM Forward primer	0.4
10 mM Reverse primer	0.4
ROX reference dye Low (50X)	0.4
2 ng/µl cDNA template	8.0
sterile H_2O	0.8

For no template control (NTC), sterile water was used instead of template. Thermal profile was programmed as these following.

Segments	Temperature (°C)	Time (min:sec)
Segment 1 (1X) Step 1	95	3:00
Segment 2 (40X) Step 1	95	0:03
Step 2	(calculated value)	0:20
Segment 3 (1x) Step 1	95	1:00
Step 2	55	0:30
Step 3	95	0:30

For amplification efficiency, standard curve of each primer sets were prepared by using 10-fold dilution of cDNA. Then 8 µl of cDNA template was added followed by adjusted primers and sterile water amount. The standard curve will be plotted using C_Ts as Y axis against the cDNA amount in ng as X axis. The thermal profile was programmed as the previous with step 3 added at the segment 2: 72°C with 32 sec. The segment 3 (dissociation curve segment) was deleted. The sterile water must be added to fulfill the reaction volume if the primer amount is less than 0.4 µl. Asterisks (*) indicated the primer sequences that similar to the semi-quantitative PCR primers.

Table 3.2 Real-time PCR specific primer sequences and their optimal conditions.

Genes	Primer sequences (FW-Forward, RV-Reverse)	Annealing temperature (°C)	Primer amount (µl)
HSP90B	FW: TATCGGCATGACCAAGGACG RV: ACACCAAACTGGCCGATCAG	61	0.35
HSP70A	FW*: AGGCCCCCGCTATCGGTATT RV : TGGCGGCATCACCAATCAG	64	0.30
CPN60B1	FW: AGCCCTGGAGTGTCTTATGGTG RV: ACCCGCCGCAGCATAGC	58	0.35
HSP22A	FW : TGTGTTCGTTGACACGAGCAG RV*:CGGGCCACAATTTGATAGTACAC	61	0.30
RACK1	FW : AGTTCTGCCTGACTGGCTC RV : TCCACAGCTTGATGGTCTTG	60	0.40

3.11 Abiotic stress responsive elements mapping

The sequence (500 nucleotides) upstream of the start codon from the selected *HSP* genes was obtained from NCBI database. Nine abiotic stress responsive elements from the table 1.2 were mapped those sequences using Vector NTI suit 8. Only HSEs were truncated based on its consensus sequence. Underline alphabets in Table 3.3 indicate the cut nucleotides.

Table 3.3 HSEs and their truncation.

Label	Putative HSE (Lodha et al. 2008)	Truncated HSE
HSE1	GCGTCCAGAAGGCGCCATACGG	GTCCAGAAGGCGC
HSE2	GGGGAAGCTCTGGAAGGGCCGCGATGG	GGAAGCTCTGGAAGGGCC
HSE3	ATGAAGCTACAGGACTG	GAAGCTACAGGAC
HSE con_1	repeated of NGAAN and NTTCN	NGAANNTTCN
HSE con_2	repeated of NGAAN and NTTCN	NTTCNNGAAN

CHAPTER IV
RESULTS

4.1 Observation of *C. reinhardtii HSP* genes information and their expression at normal condition

There are 34 potential (co-)chaperone genes in *C. reinhardtii* (Schorda 2004). As several of those genes function together (as co-chaperone) only 25 candidate genes were selected. Their mRNA sequences and other information were obtained from http://www.ncbi.nlm.nih.gov/gene. Those genes were categorized and shown in Table 4.1. Then gene-specific primers were designed, verified and ordered.

From 25 individual specific primers subjected with 100 ng cDNA template to performed PCR, only 17 of them shown PCR products at the expected size.

Table 4.1 Predicted localization and their expression under normal condition of 25 selected *C. reinhardtii HSP* genes.

Genes	Predicted localization	Expression under normal growth condition
CLPB1	cyt	Yes
CLPB3	cp or mt	No
CLPB4	Organelle-target	No
CLPD	cp	Yes
HSP90A	cyt	Yes
HSP90B	ER	Yes
HSP90C	cp	Yes
HSP70A	cyt	Yes
HSP70B	cp	Yes
HSP70D	cp	No
HSP70E	Unknown	No
HSP70F	Organelle-target	No
HSP70G	ER	No

Table 4.1 Predicted localization and their expression under normal condition of 25 selected *C. reinhardtii HSP* genes (cont).

Genes	Predicted localization	Expression under normal growth condition
HSP70F	Organelle-target	No
HSP70G	ER	No
CPN60A	cp	Yes
CPN60B1	cp	Yes
CPN60B2	cp	Yes
HSP22A	cyt	Yes
HSP22B	cyt	Yes
HSP22C	cp	Yes
HSP22D	cp	Yes
HSP22E	cp	Yes
HSP22F	cp	Yes
HSP22G	cp	No
HSP22H	cyt	No
HSP33	cp	Yes

Abbreviations: cyt – cytoplasm; ER – endoplasmic reticulum; cp – chloroplast; mt- mitochondria

To perform semi-quantitative PCR, the condition must be optimized. The optimal cycle numbers were tested by performing PCR at 20, 24, 28 and 35 cycles with 100 ng cDNA template. Then the optimal cycle numbers were selected to avoid product saturation as shown in Figure 4.1.

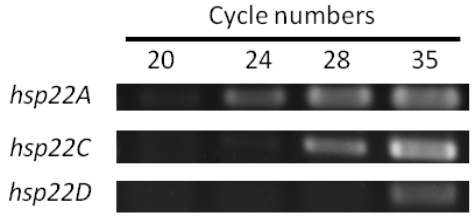

Figure 4.1 Selecting optimal cycle numbers compared between three *HSP* genes. The optimal cycle numbers at 24 28 and 35 cycles were selected for *HSP22A*, *HSP22C* and *HSP22D*, respectively.

All PCR products were purified, concentrated and sent for DNA sequencing. AlignX of Vector NTI Suite 8.0 and NCBI BLAST (nucleotide blast) were used for sequence alignment. The alignment shown that all PCR products at the expected size had similar sequence to their specific genes at the selected region shown in Table 4.2. See the Appendix B for the DNA sequencing results.

Table 4.2 NCBI reference sequences of 17 *HSP* genes and *RACK1* as a housekeeping gene, the selected regions and product sizes.

Genes	NCBI reference	Selected region	Product size (bp)
CLPB1	XM_001698754.1	83-632	550
CLPD	XM_001698581.1	127-634	508
HSP90A	XM_001695212.1	118-488	371
HSP90B	XM_001701833.1	1861-2307	447
HSP90C	XM_001702932.1	1-422	422
HSP70A	XM_001701274.1	81-473	393
HSP70B	XM_001696380.1	27-616	590
CPN60A	XM_001703640.1	57-468	412
CPN60B1	XM_001701061.1	86-496	411
CPN60B2	XM_001692452.1	53-500	448
HSP22A	XM_001700963.1	656-975	320
HSP22B	XM_001700778.1	190-697	508
HSP22C	XM_001695665.1	3-368	366
HSP22D	XM_001689903.1	1-321	321
HSP22E	XM_001689769.1	871-1227	357
HSP22F	XM_001690229.1	921-1362	442
HSP33	XM_001700394.1	201-641	441
RACK1	XM_001698013.1	581-838	258

4.2 Analysis of *C. reinhardtii HSP* gene expression under abiotic stress conditions

Normally, *HSP* genes were up-regulated after heat stress (von Gromoff et al, 1989). The studies about *HSP* gene transcription accumulation of *C. reinhardtii* under heat stress were performed either at 40°C using cc125 mt^+ (von Gromoff et al, 1989) or at 42 °C using cc124 mt^+ (Voss et al, 2011). Both strains had normal cell

wall. However, the model strain used in this study, cc-503 cw 92 mt^+, is a cell wall deficient mutant. The lack of cell wall may render the alga more sensitive for heat. So, the proper temperature should be investigated. Three temperatures (37, 40 and 42°C) and three durations (30, 60 and 120 min) were selected to test. Little cell debris was found when the cells were shifted from room temperature to 37°C at all time periods. But when they were shifted to 40 and 42°C, the cell debris was increased in time dependent manner. Few living cells were observed after shifted to 42°C for 2 hr. So we selected 37°C with all durations and 42°C at 30 min to observe the expression profiles.

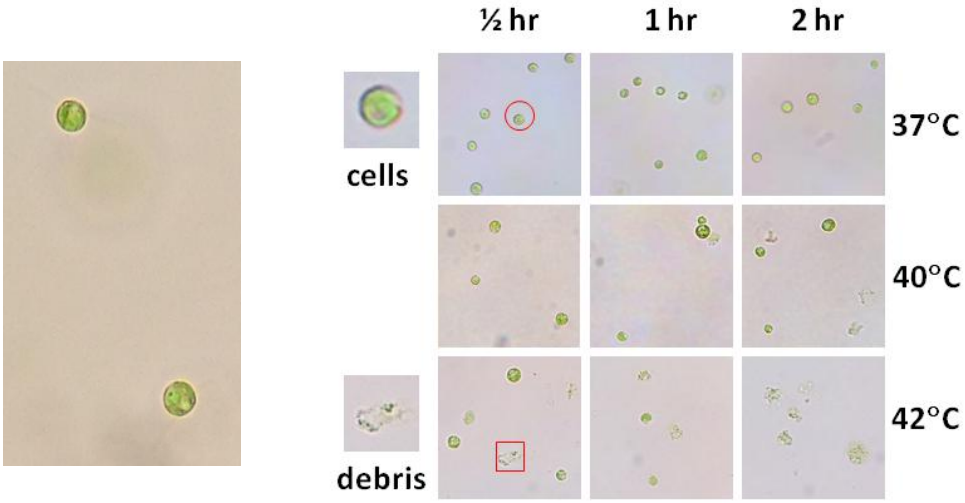

Figure 4.2 Normal condition (left) and heat shocked *C. reinhardtii* cc-503 at difference time period (right) under light microscope at magnification of 400 times. The durations were defined as column and temperatures as row. Normal condition cells were heat shocked at given temperatures and durations. The morphology of cells in circle and the debris in rectangular were magnified as shown.

For heat shocked at 37°C, most of *HSP* genes were increased in their expression compared to normal condition as shown in Figure 4.3. Three genes, *CLPD*, *HSP90C* and *HSP70B* were similar to normal condition and the expression of *CLPB1* and *HSP33* were decreased compared to normal condition. Note that *RACK1* (receptor of activated protein kinase C 1, sometimes called *CBLP* - *Chlamydomonas* beta

subunit-like polypeptide) is constitutively expressed and was used as control (Schloss 1990).

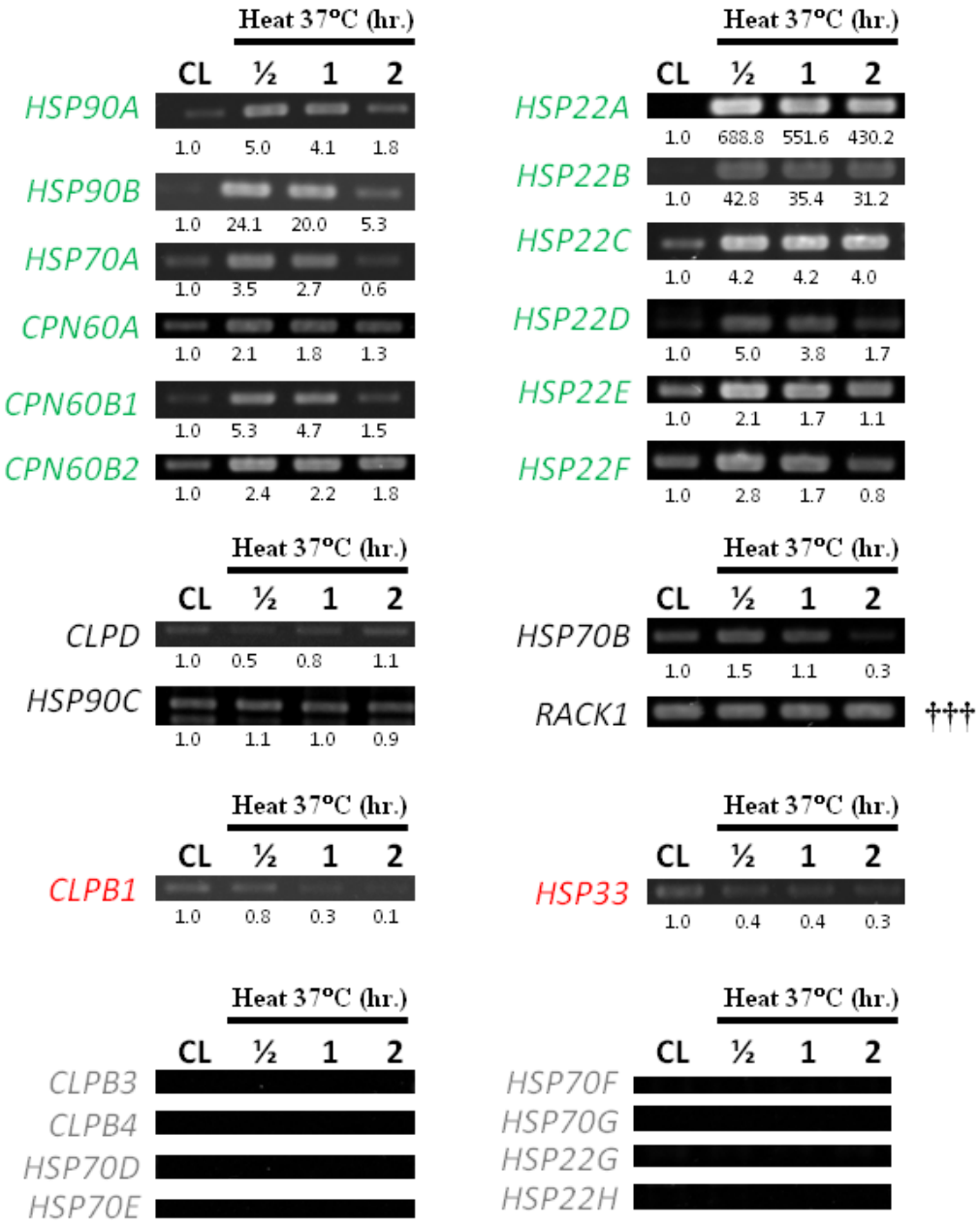

Figure 4.3 Semi-quantitative RT-PCR *HSP* gene expression profiles under heat shock at 37°C for different durations. Numbers under the bands indicated their comparative intensity. Each of them was normalized to *RACK1* at their duration then compare with its expression under normal condition (CL). Band intensity quantification was performed by ImageJ 1.48v (http://imagej.nih.gov/).

But for heat shocked at 42°C, only 9 *HSP* genes were increased their expression. Two genes were similar to normal and 4 genes were decreased their expression. Some of them had different expression profiles compare to heat shocked at 37°C.

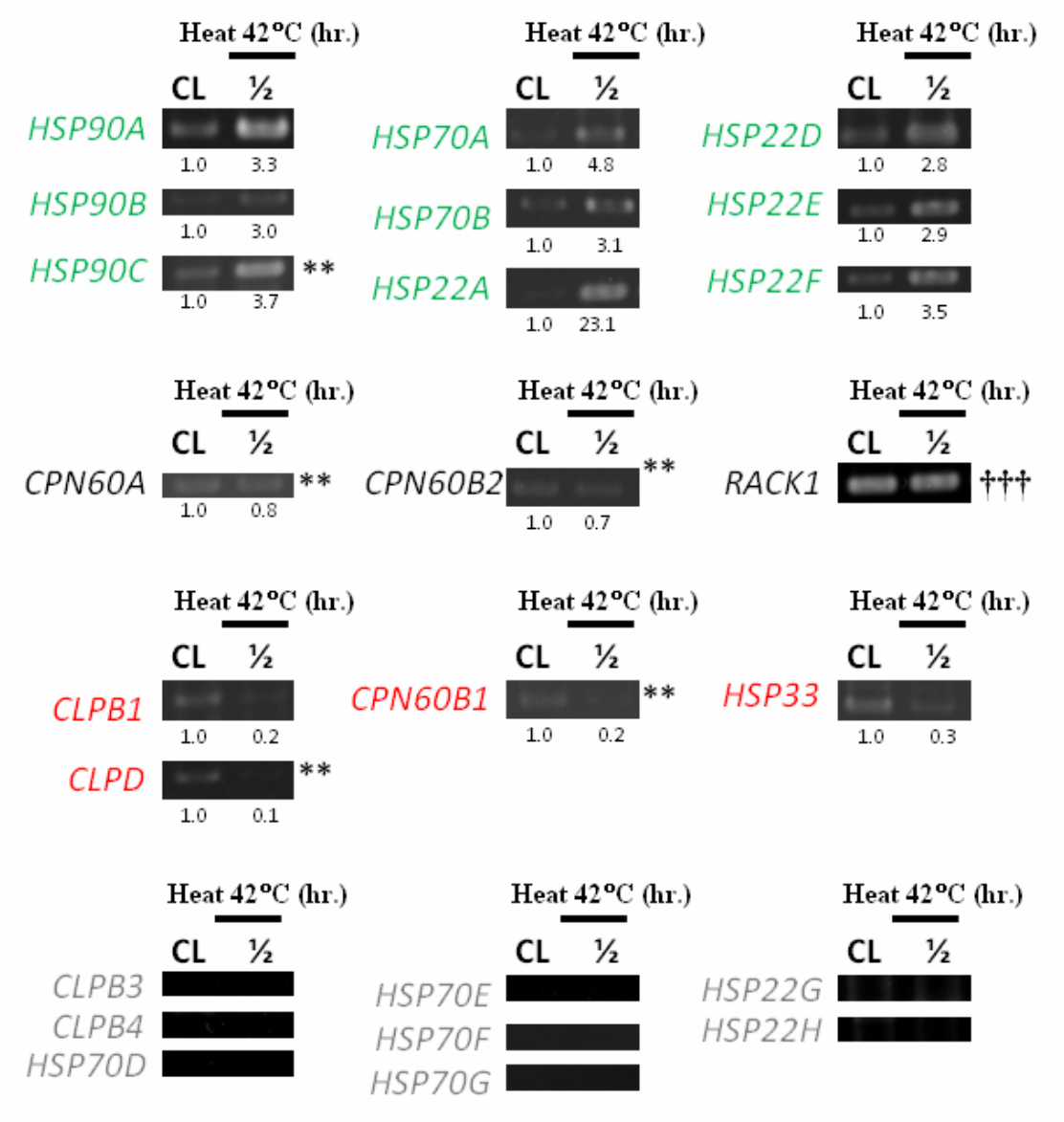

Figure 4.4 Semi-quantitative RT-PCR *HSP* gene expression profiles under heat shock at 42°C for different durations. Each of them was normalized to *RACK1* (†††) then compare with its expression under normal condition (CL). Asterisks (**) indicated the gene expression which were different from heat shock at 37°C.

For salt-shocked condition, seven *HSP* genes included *HSP90A*, *HSP90B*, *HSP70A*, *HSP70B*, *HSP22A*, *HSP22E* and *HSP22F* were increased. Other *HSP* genes were expressed similar to normal.

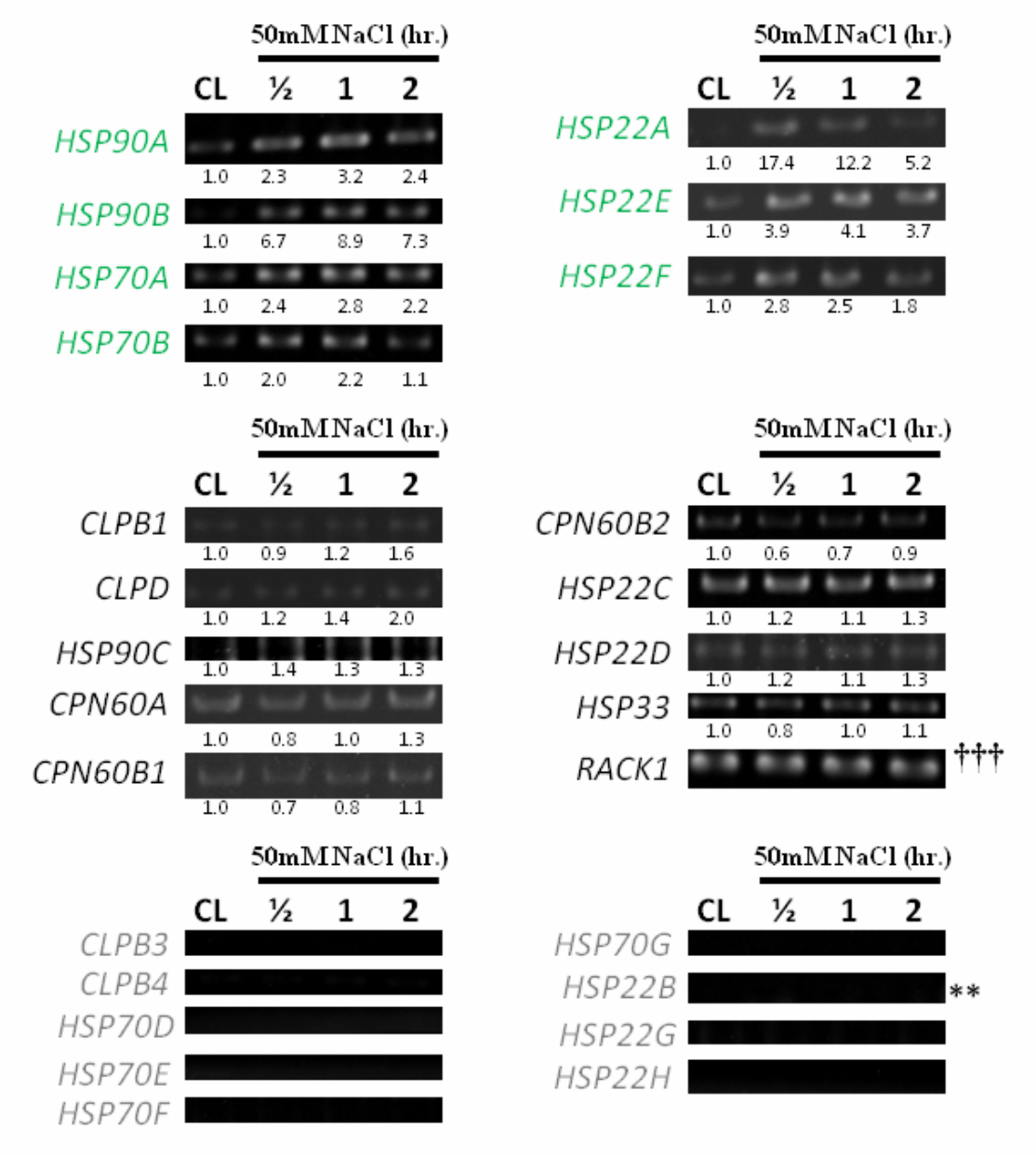

Figure 4.5 Semi-quantitative RT-PCR *HSP* gene expression profiles under salt-shocked condition for different durations. The intensity of each band was normalized to *RACK1* (†††) then compared with its expression under normal condition (CL).

For the transition from darkness to normal light intensity condition, 7 *HSP* genes included *HSP90A*, *HSP90B*, *HSP70A*, *HSP70B*, *CPN60A*, *CPN60B1* and *HSP22F* were increased compared to the cells that continuing dark incubation.

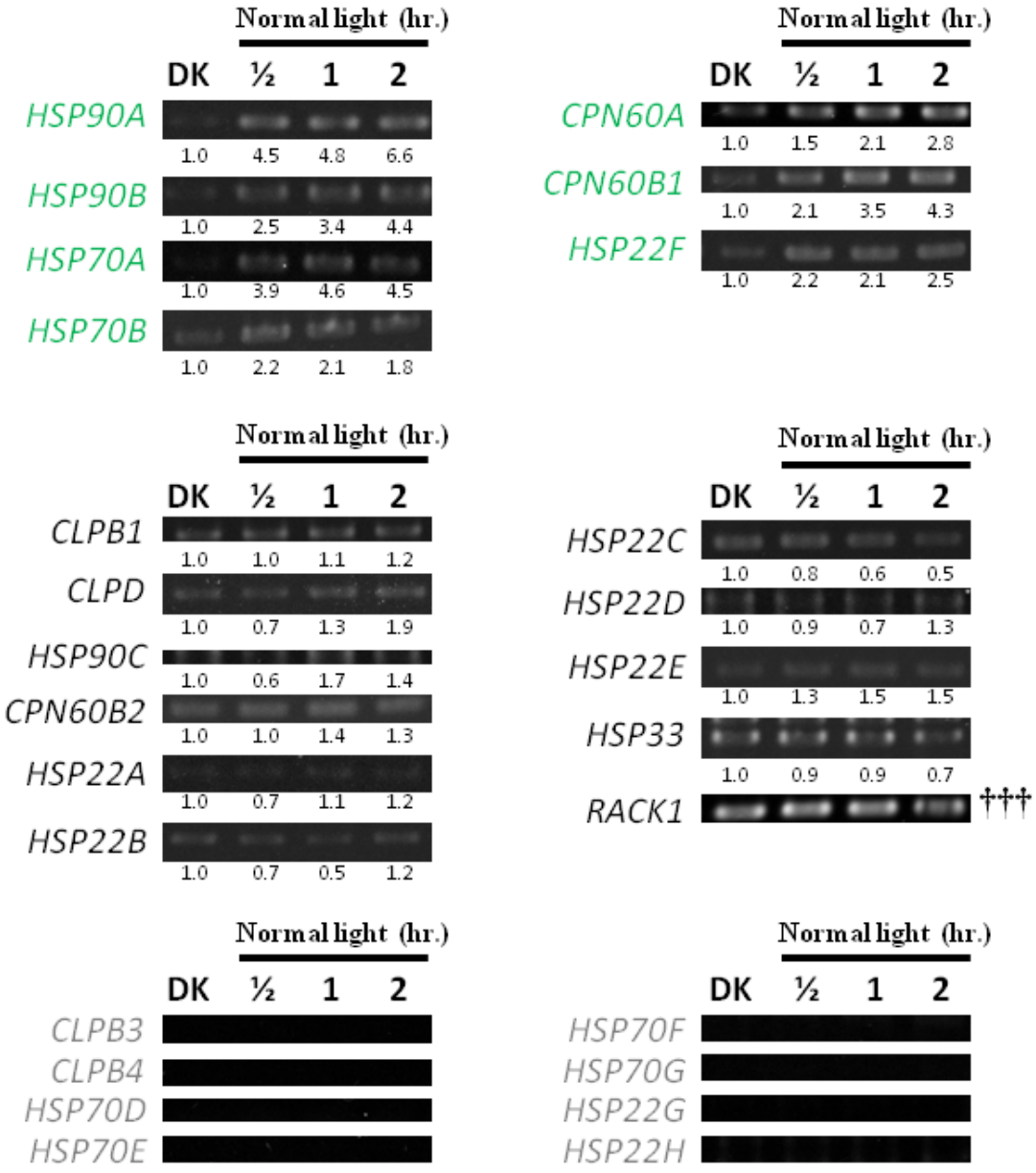

Figure 4.6 Semi-quantitative RT-PCR *HSP* gene expression profiles under transition from darkness to normal light intensity for different durations. The intensity of each band was normalized to *RACK1* (†††) then compared with its expression under dark incubation (DK).

Gene expression in the algal culture grown at normal condition was also performed along with those dark incubation cells. By comparing its profiles to those dark incubation and the transition cells, the expression of *HSP22B* was shown an interested property. Under normal condition, its expression was hardly detected but after dark incubation it was appeared and still remains as shown below.

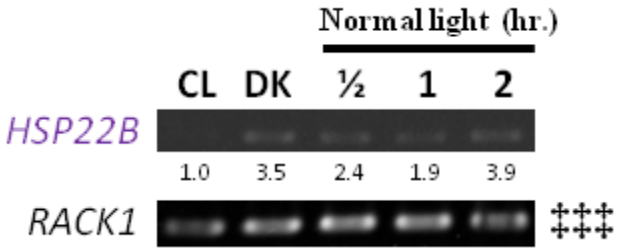

Figure 4.7 Semi-quantitative RT-PCR *HSP22B* gene expression profiles under normal condition, dark incubation and the transition from darkness to normal light intensity for different durations. The intensity of each band was normalized to *RACK1* (‡‡‡) then compared with its expression under normal condition (CL).

For exposure to excessive irradiance condition, 5 *HSP* genes include *HSP90A*, *HSP90B*, *HSP22A*, *HSP22B*, and *HSP22F* were increased compared to normal condition as shown in Figure 4.8.

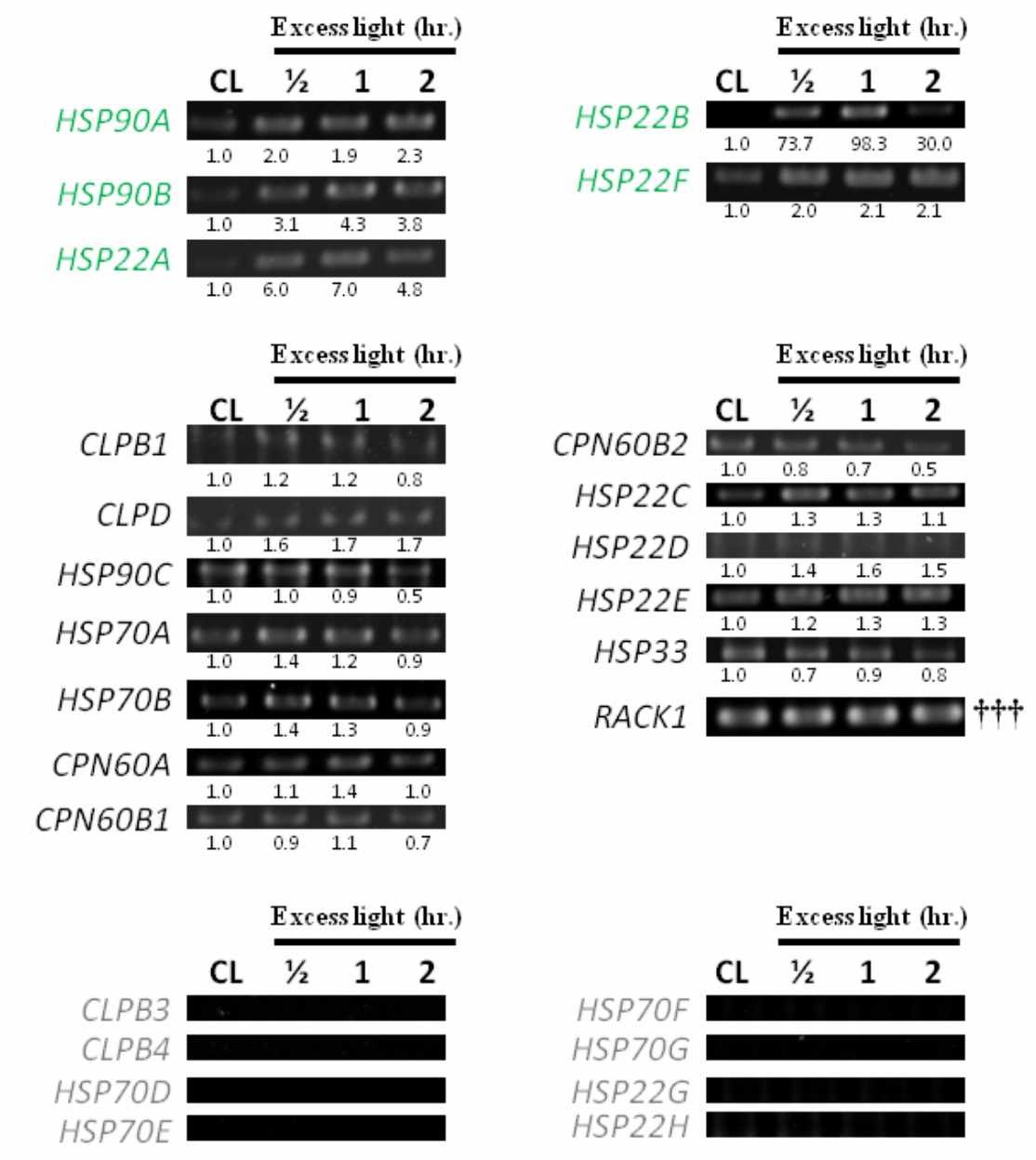

Figure 4.8 Semi-quantitative RT-PCR *HSP* gene expression profiles under increasing irradiance for different durations. The intensity of each band was normalized to *RACK1* (†††) then compared with its expression under normal condition (CL).

4.3 Analysis of the *HSP* gene expression using real-time PCR

From those semi-quantitative PCR results, four *HSP* genes were selected to observe their expression level under four stress conditions included heat shocked 37°C, salt-shocked 50 mM NaCl, transition from darkness to normal light intensity and exposure to excessive irradiance. In our lab, *HSP90B* and *CPN60*s were on focus. The *HSP70A* was well-documented and might serve as positive control. For small HSPs, many genes were interesting. The *HSP22A* was selected because its expression profile was highly increase compared to the others small *HSP* genes and the protein existent was confirmed by a previous study (Grimm et al. 1989). Those gene expression profiles at 30 min compared to CL (or dark incubation) were shown in table 4.3.

Table 4.3 Semi-quantitative results of four *HSP* genes expression at 30 min compared to CL or dark incubation.

Genes	heat shocked 37°C	salt-shocked 50 mM NaCl	transition	excessive irradiance
HSP90B	Increase	Increase	Increase	Increase
HSP70A	Increase	Increase	Increase	Not Change
CPN60B1	Increase	Not Change	Increase	Not Change
HSP22A	Increase	Increase	Not Change	Increase

The efficiency-correlated comparative quantitation method (Pfaffl 2001) was selected instead of $2^{-(\Delta\Delta C_T)}$ method (Livak and Schmittgen 2001) because its mathematical model includes the difference of PCR amplification efficiencies (in converted form, %E, and will be described below) of both control and unknown. So, the amplification efficiency of each specific primer was required. The KAPA SYBR FAST qPCR kit achieved consistently high amplification efficiency at amplicon lengths 86-249 bp. Hence, alternative primer sets were designed. Each of them was verified their dissociation curve, %E, their product size and sequence.

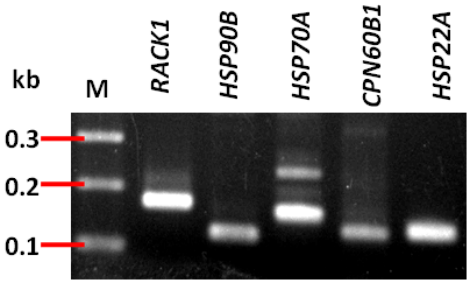

Figure 4.9 Real-time PCR products of interested *HSP* genes and *RACK1* housekeeping gene. The dominant bands were in range of the expected size.

All PCR products were purified, concentrated and ligated to plasmid pGEM-T. Then those ligated plasmids for each individual PCR products were checked by colony PCR, purified, and sent to perform DNA sequencing. AlignX of Vector NTI Suite 8.0 was used for sequence alignment. The alignment was shown all PCR products have similar sequence to their specific genes at the selected region shown in table 4.4. See the Appendix C for the DNA sequencing results.

Table 4.4 Selected regions and expected product sizes of four interested *HSP* genes and *RACK1* as housekeeping gene.

Genes	Selected region	Expected size (bp)
HSP90B	375-497	123
HSP70A	81-239	159
CPN60B1	2268-2400	133
HSP22A	841-975	135
RACK1	230-400	171

The dissociation curve was shown after analysing the results by MxPro program as shown below. The peak that was equal to or higher than 80°C is corresponded to the specific DNA product. See the Appendix D for all dissociation curves.

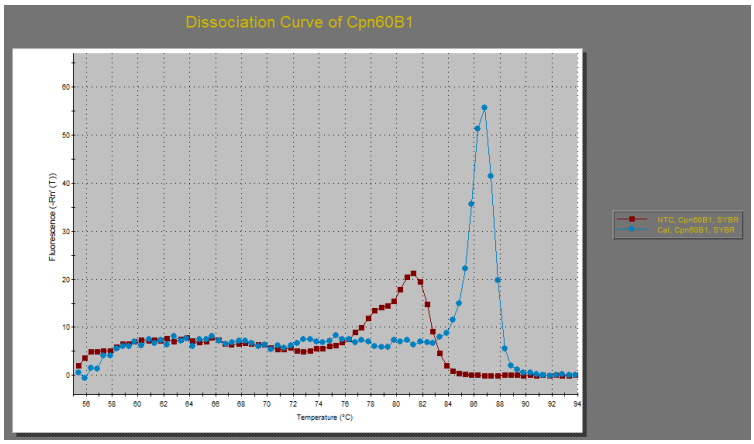

Figure 4.10 Dissociation curve of *CPN60B1* alternative primer. The Y axis is based on the first derivative of the normalized fluorescence reading multiplied by -1. It composed of no template control. (NTC, squares) and calibrator (Cal, circles). The small peak of NTC indicates non-specific product such as primer dimers.

Before the standard curve was generated, each primer was tested for optimal annealing temperature and amount. Those optimal conditions were shown in section real-time PCR of materials and methods. The % Efficiency (%E) of each specific primer was calculated and shown in table 4.5. See the Appendix E for all standard curves.

Table 4.5 Real-time PCR primer genes and its %E.

Genes	%E
HSP90B	104.0
HSP70A	131.9
CPN60B1	147.8
HSP22A	107.7
RACK1	113.6

The concept of %E comes from two fundamentals; (i) the increasing of PCR product during each cycle of exponential amplification and (ii) the equal separating of C_T values when the template is diluted. The PCR amplification efficiency, E, is calculated from the slope of standard curve using the following formula:

$$E = 10^{(-1/slope)}$$

Ideally, the amount of PCR product will be double during each cycle of exponential amplification which means 2-fold increasing (E=2). The (ii) combined with $R^2 > 0.95$ is needed as verification. This means the standard curve is linear which in turn means the slope is reliable. When any slope value was substituted into the formula, the E value can be calculated. It can be converted to %E by minus one then multiply by one hundred. So, an ideal reaction has %E = 100 %.

Normally, the mathematical model $2^{-(\Delta\Delta C_T)}$ (Livak and Schmittgen 2001) was used to calculate the approximate fold change as described below

$$\Delta\Delta C_T = (C_T \text{ GOI}-C_T \text{ NORM})_{UNK} - (C_T \text{ GOI}-C_T \text{ NORM})_{CAL}$$

The term GOI and NORM designated the gene; GOI as *g*ene *o*f *i*nterested such as *HSP* genes and NORM as *norm*alize which is *RACK1*. The term CAL and UNK designated the sample; CAL as *cal*ibrator which is normally untreated (normal condition; CL) and UNK as *unk*nown which comes from the cells subjected to stress condition. The selected mathematical model, efficiency-correlated comparative quantitation method (Pfaffl 2001), has the equation and description as described below. The designations are similar to the previous.

$$\text{Fold change} = \frac{[1 + (\%E_{GOI}/100)]^{(C_T CAL - C_T \text{ UNK})_{GOI}}}{[1 + (\%E_{NORM}/100)]^{(C_T CAL - C_T \text{ UNK})_{NORM}}}$$

The calculation includes the PCR amplification efficiency which is different from $2^{-(\Delta\Delta C_T)}$ that assumes both GOI and NORM have an ideal (100) percent amplification efficiency (See appendix F). Compared with $2^{-(\Delta\Delta C_T)}$, this method is more flexible and reliable. Furthermore, the standard curve helps verifying. The reliable C_Ts of NORM should stay in range that fit to the standard curve.

For the heat shocked 30 min at 37°C, all *HSP* genes were increased their expression to more than 10 fold. The response of *HSP22A* was interesting because the calculated fold change was remarkable (about 3,500 fold). All their C_Ts and calculated fold changes were shown in the table 4.6.

Table 4.6 The C_Ts of selected *HSP* genes and *RACK1* with their fold changes compare between normal (CAL) and under 30 min heat shocked 37°C (UNK).

Genes	C_T CAL	C_T UNK	Fold change
HSP90B	23.0±1.0	19.2±0.2	11.6
HSP70A	20.4±0.4	16.1±0.3	29.9
CPN60B1	21.9±0.4	18.7±0.4	15.7
HSP22A	26.5±1.3	15.3±0.1	3,502.8
RACK1	17.5±0.2	17.3±0.1	Not compare

For the salt-shocked 50 mM NaCl, The *HSP90B* and *CPN60B1* were decreased their expression a bit to 0.7 and 0.5 fold, respectively. The *HSP70A* and *HSP22A* expression were increased a bit to 2.2 and 3.4 fold, respectively. All the C_Ts and fold changes were shown in the Table 4.6.

Table 4.7 The C_Ts of selected *HSP* genes and *RACK1* with their fold changes compare between normal (CAL) and under 30 min salt-shocked 50 mM NaCl (UNK).

Genes	C_T CAL	C_T UNK	Fold change
HSP90B	21.1±0.2	21.2±0.2	0.7
HSP70A	18.8±0.2	17.4±0.3	2.2
CPN60B1	21.5±0.1	21.6±0.3	0.5
HSP22A	23.1±0.3	20.8±0.3	3.4
RACK1	17.4±0.3	16.8±0.5	Not compare

For the transition from darkness to normal light intensity, The *CPN60B1* and *HSP22A* were increased their expression a bit to 3.3 and 3.0 fold, respectively. The *HSP70A* was increased its expression to 10.1 fold. The *HSP90B* expression was decreased a bit to 0.4 fold. All the C_Ts and fold changes were shown in the Table 4.8.

Table 4.8 The C_Ts of Selected *HSP* genes and *RACK1* with their fold changes compare between dark incubation (CAL) and the transition from darkness to normal light intensity for 30 min (UNK).

Genes	C_T CAL	C_T UNK	Fold change
HSP90B	23.8±1.2	25.0±0.1	0.4
HSP70A	21.9±1.9	18.9±0.3	10.1
CPN60B1	23.0±0.8	21.5±0.1	3.3
HSP22A	26.4±0.2	24.6±0.6	3.0
RACK1	17.7±0.2	17.4±0.1	Not compare

For the excessive irradiance, The *CPN60B1* was decreased their expression a bit to 0.5 fold. Other *HSP* genes were not change their expression (had the fold change about 1). All the C_Ts and fold changes were shown in the Table 4.9.

Table 4.9 The C_Ts of Selected *HSP* genes and *RACK1* with their fold changes compare between normal (CAL) and 30 min excessive irradiance (UNK).

Genes	C_T CAL	C_T UNK	Fold change
HSP90B	21.6±0.2	21.2±0.5	1.1
HSP70A	21.6±0.2	21.2±0.5	1.2
CPN60B1	21.4±0.1	21.9±0.4	0.5
HSP22A	25.0±0.2	24.7±0.4	1.1
RACK1	17.8±0.1	17.6±0.3	Not compare

4.4 Localization of abiotic stress responsive elements on the putative promoter sequence of interested *HSP* genes

After the putative promoter sequences were obtained, the different sets of abiotic stress responsive elements composing of heat, salinity and light intensity were manually input to the Vector NTI suit 8 then the search was performed and the localizations were displayed. The percentage on the picture showed the similarity of

detected responsive elements compared with the input sequences. The "site" means the actual number of responsive elements. The "group" means the number of location that may has only one responsive element alone or many responsive elements intercept/intact together. All putative promoter sequences were shown in Appendix G.

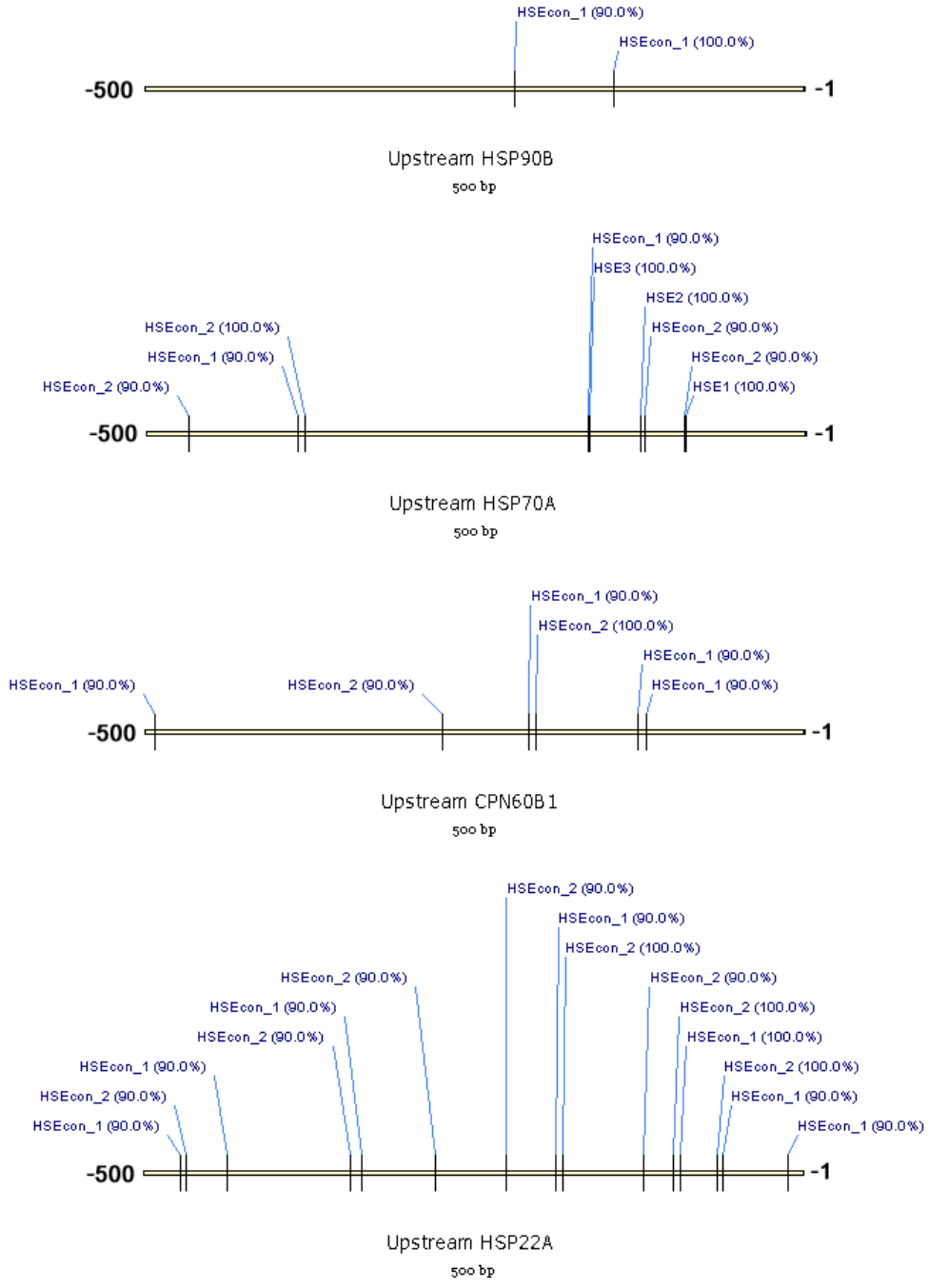

Figure 4.11 Localization of HSEs on the putative promoter of selected *HSP* genes. Minus numbers indicate the upstream of bases from transcription start site (+1).

Table 4.10 The counting of HSEs sites and groups.

Genes	Sites	Groups
HSP90B	2	2
HSP70A	9	5
CPN60B1	6	4
HSP22A	15	10

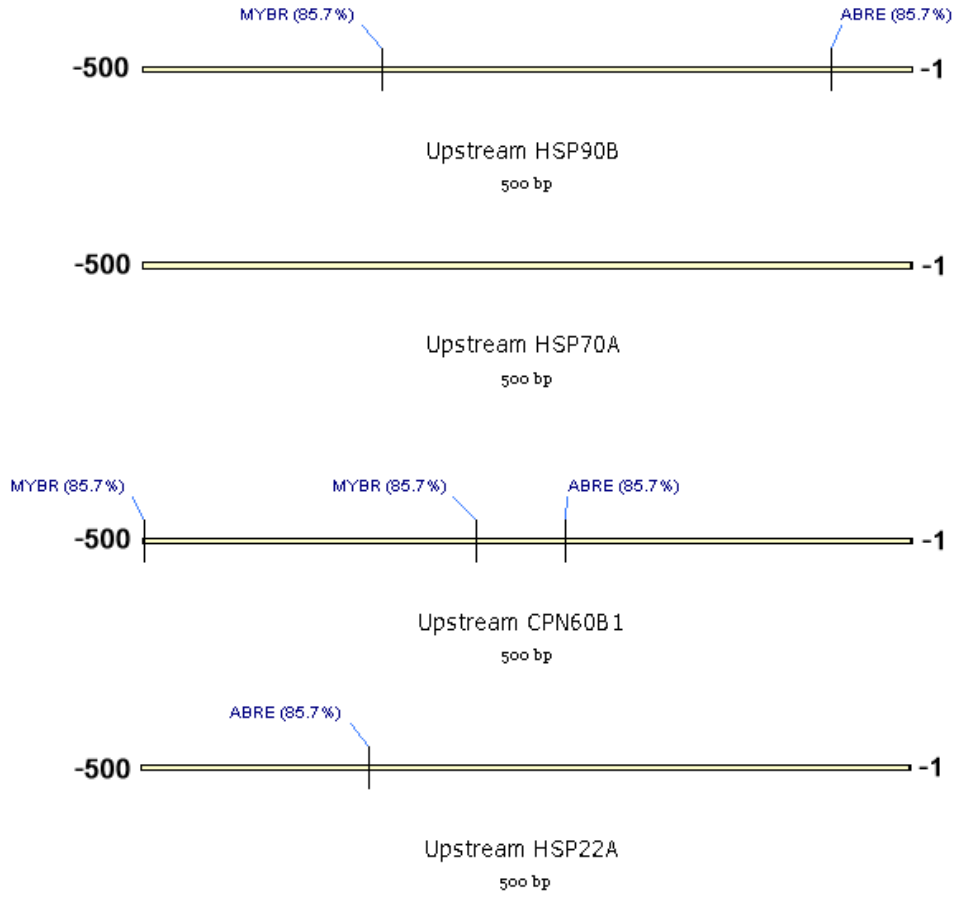

Figure 4.12 Localization of salinity responsive elements on the putative promoter of selected *HSP* genes.

Table 4.11 The counting of salinity responsive elements.

Genes	ABRE	MYBR
HSP90B	1	1
HSP70A	0	0
CPN60B1	1	2
HSP22A	1	0

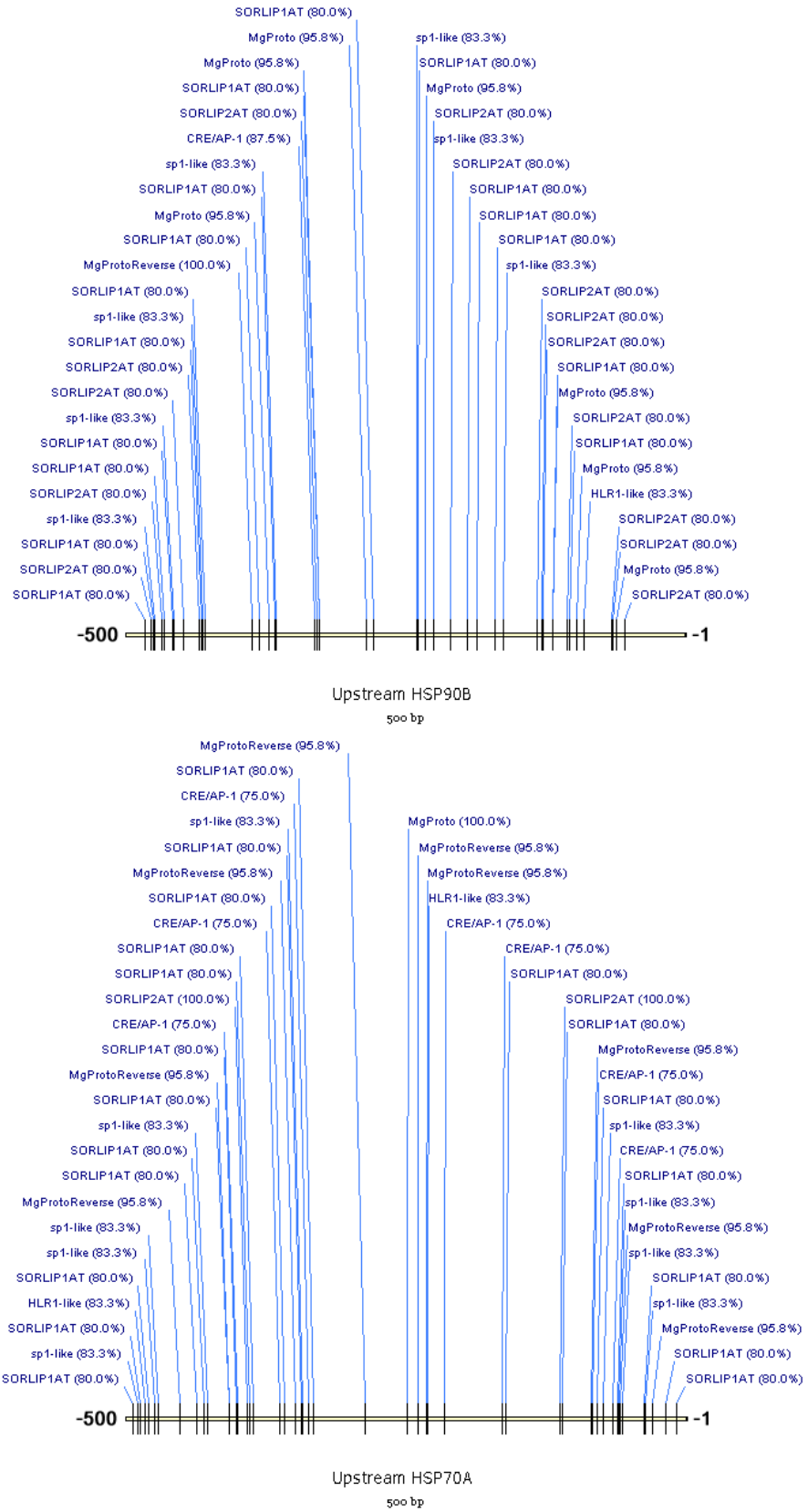

Figure 4.13 Localization of light responsive elements on the putative promoter of *HSP90B* and *HSP70A*

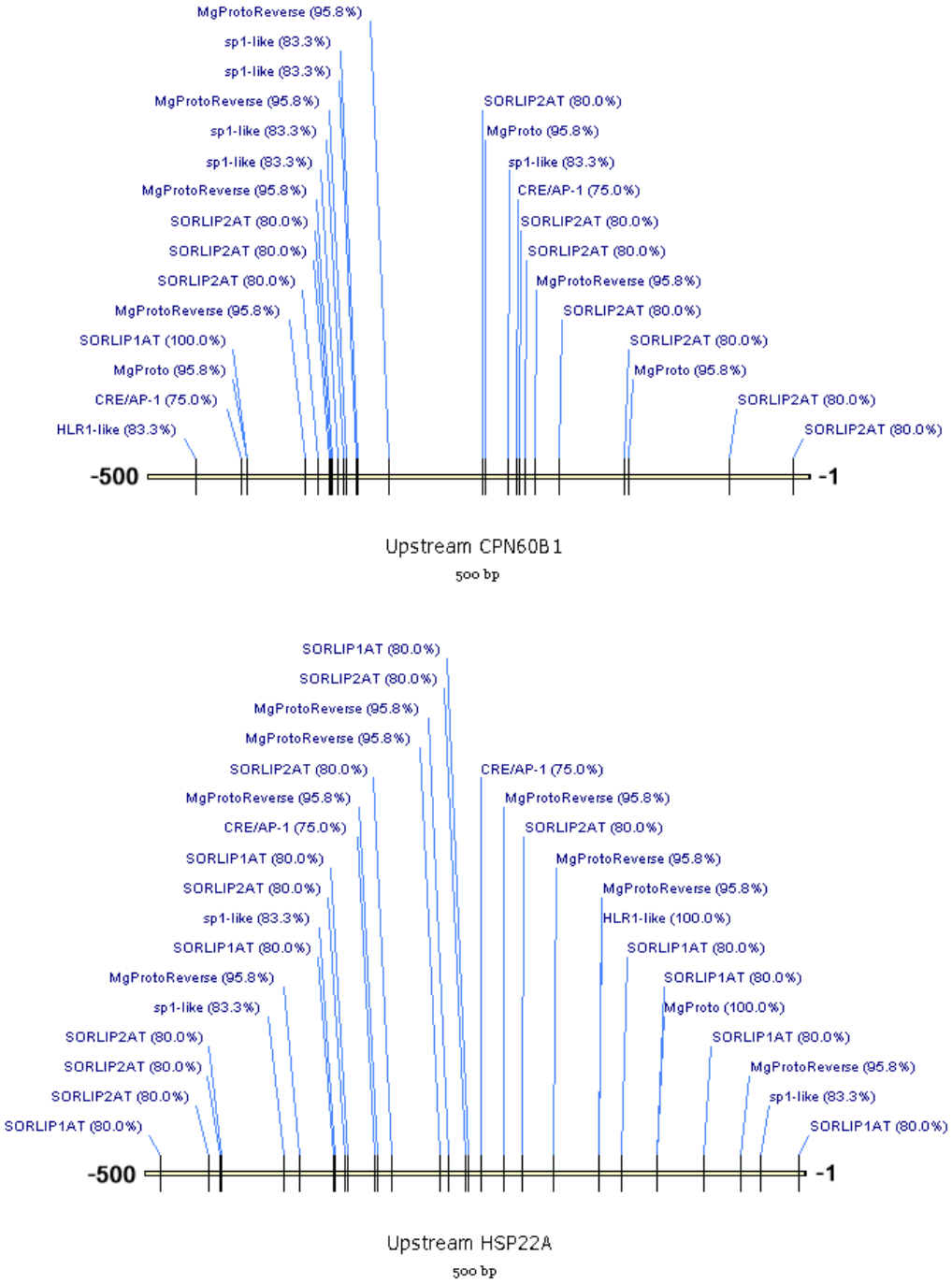

Figure 4.14 Localization of light responsive elements on the putative promoter of *CPN60B1* and *HSP22A*

Table 4.12 The counting of light responsive element sites and groups.

Genes	Sites	Groups
HSP90B	47	18
HSP70A	49	11
CPN60B1	27	9
HSP22A	30	16

Table 4.13 The counting of light responsive elements.

Genes	SORLIP 1/2AT	CRE/AP-1	HLR1-like	MgProto	Sp1-like
HSP90B	16 / 14	1	1	8	7
HSP70A	19 / 2	7	2	10	9
CPN60B1	1 / 10	2	1	8	5
HSP22A	8 / 7	2	1	9	3

In addition, the putative promoter sequences were submitted into PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) an online tool for analysis of the promoter sequence in higher plants. The results related to our abiotic stresses were collected and shown in many tables and figures below. Those responsive elements were different from the previous and located at other sites compared to the previous localization.

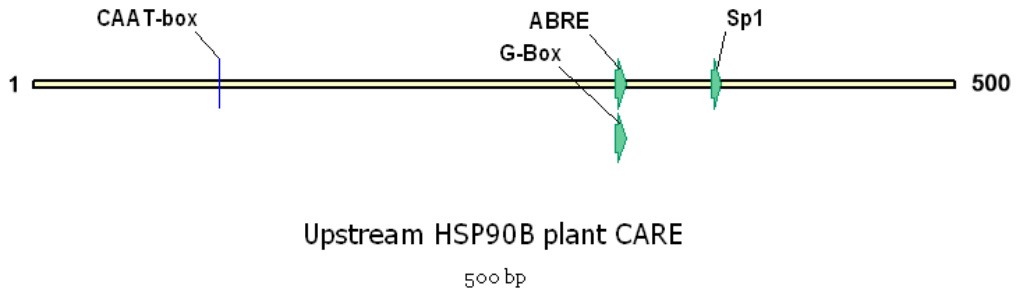

Figure 4.15 Localization of responsive elements on the putative promoter of *HSP90B* from PlantCARE. Numbers indicate the bases respect to the Appendix G.

Table 4.14 The details of responsive elements on the putative promoter of *HSP90B* from PlantCARE.

Position	Sequence	Name	Organism	Description
101	CAAT	CAAT-box	*Hordeum vulgare*	CAAT-box
316	CACGTG	G-box	*Pisum sativum*	*cis*-element involved in light responsiveness
316	CACGTG	ABRE	*Arabidopsis thaliana*	abscisic acid responsive element
368	GGGCGG	Sp1	*Oryza sativa*	Light responsive element

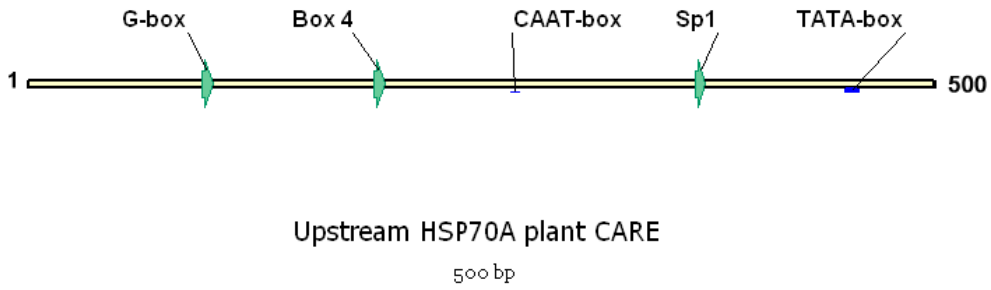

Figure 4.16 Localization of responsive elements on the putative promoter of *HSP70A* from PlantCARE. Numbers indicate the bases respect to the Appendix G.

Table 4.15 The details of responsive elements on the putative promoter of *HSP70A* from PlantCARE.

Position	Sequence	Name	Organism	Description
96	CACGTC	G-box	*Zea mays*	*cis*-element involved in light responsiveness
191	ATTAAT	Box 4	*Petroselinum crispum*	A part of conserved DNA module involved in light responsiveness
266	CAAAT	CAAT-box	*Brassica rapa*	CAAT-box
368	GGGCGG	Sp1	*O. sativa*	Light responsive element
452	TATAAA	TATA-box	*A. thaliana*	TATA-box

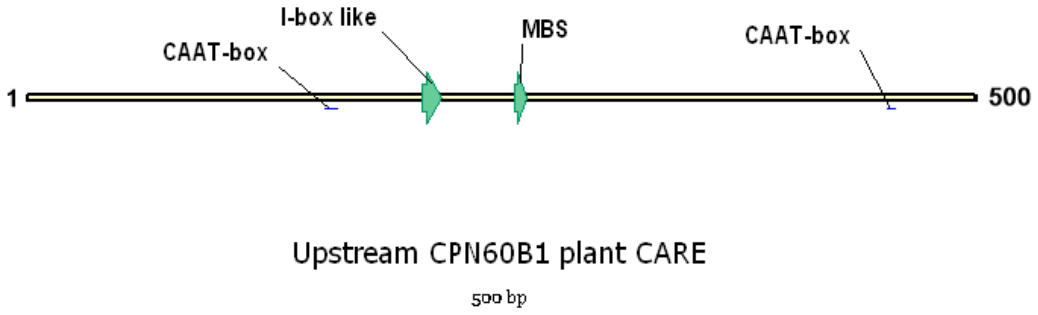

Figure 4.17 Localization of responsive elements on the putative promoter of *CPN60B1* from PlantCARE. Numbers indicate the bases respect to the Appendix G.

Table 4.16 The details of responsive elements on the putative promoter of *CPN60B1* from PlantCARE.

Position	Sequence	Name	Organism	Description
156	TGCCAAC	CAAT-box	*Petunia hybrida*	CAAT-box
208	cCATATCCAAT (original is cCATATCCATT)	I-box like	*Flaveria trinervia*	A part of light responsive element
257	CAACTG	MBS	*A. thaliana*	MYB binding site involved in drought inducibility
453	CAATT	CAAT-box	*Glycine max*	CAAT-box

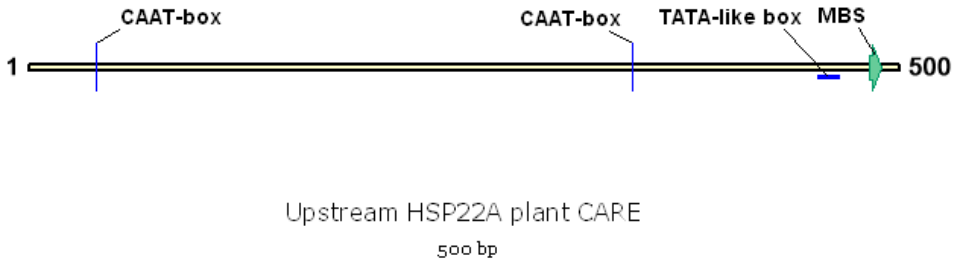

Figure 4.18 Localization of responsive elements on the putative promoter of *HSP22A* from PlantCARE. Numbers indicate the bases respect to the Appendix G.

Table 4.17 The details of responsive elements on the putative promoter of *HSP22A* from PlantCARE.

Position	Sequence	Name	Organism	Description
39	CAAT	CAAT-box	*H. vulgare*	CAAT-box
347	CAAT	CAAT-box	*H. vulgare*	CAAT-box
455	ccTATTAAaa (original : ccTATAAAaa)	TATA-like box	*A. thaliana*	TATA-box
484	CGGTCA	MBS	*Z. mays*	MYB binding site

CHARPTER V
DISCUSSION

Generally, the *HSP* genes were well known for their increase expression after the organisms were subjected to stress such as heat. Although knowing that, the mechanism(s) behind this is unknown and still controversial. One important cause is the lacking of strong evidence about their signaling cascade(s). Lacking of other *HSP* genes study is secondary. The obvious example is the *HSP* gene study in *Chlamydomonas* which only *HSP70A* promoter was focused on but the others were drowning out. The *Chlamydomonas HSP70A* promoter was used to study in many aspects such as finding of the hydrogen peroxide and singlet oxygen responsive element (Shao et al. 2007 and Leisinger et al. 2001) and the counteract of epigenetic silencing (Strenkert et al. 2013). Also PRE and the most important HSE of *Chlamydomonas* were discovered from this promoter study as described earlier. However, the gathered knowledge is not enough to provide the defined understanding about heat shock response and regulation at transcriptional level. Hence, the observation of *HSP* gene expression profiles served the need. This study revealed other potential *HSP* gene that can be a model to fulfill the missing piece of information.

5.1 Screening of *C. reinhardtii HSP* gene expression.

PCR is the conventional technique used to amplify the target DNA sequence. Combined with the reverse transcription using oligo(dT) as a primer hybridized to the poly (A) tail of mRNA and the rough adjusting of cycle numbers, the expression of interested genes can be observed as semi-quantitative.

Most of the *HSP* genes were induced by heat. The following table symbolized the increasing of HSP gene expression as (✓). Decreasing of *HSP* gene expression was found at *CLPB1* and *HSP33* under heat 37 °C stress and symbolized

(X). The others were similar to normal and shown as (-). Note that the *HSP70E* had no expected PCR product.

Table 5.1 Five groups of expressed *HSP* genes and their expression under abiotic stresses

Genes	Heat 37°C	Heat 42°C	Salt 50 mM	Shift from dark to light	High light
CLPB1	X	X	-	-	-
CLPD	-	X *	-	-	-
HSP90A	✓	✓	✓	✓	✓
HSP90B	✓	✓	✓	✓	✓
HSP90C	-	✓ *	-	-	-
HSP70A	✓	✓	✓	✓	-
HSP70B	-	✓ *	✓	✓	-
CPN60A	✓	- *	-	✓	-
CPN60B1	✓	X*	-	✓	-
CPN60B2	✓	- *	-	-	-
HSP22A	✓	✓	✓	-	✓
HSP22B	✓	✓	-	-	✓
HSP22C	✓	✓	-	-	-
HSP22D	✓	✓	-	-	-
HSP22E	✓	✓	✓	-	-
HSP22F	✓	✓	✓	✓	✓
HSP33	X	X	-	-	-

From the Table above, 17 *HSP* genes were expressed at normal condition. The *CLPD* gene that did not change its expression may not contain any stress responsive elements involved in this study. Alternatively, it is also possible that the abiotic stresses tested in this thesis were below its threshold for gene activation or those stresses are not its particular trigger such as cold shock (Schorda 2004). But the expression of 8 genes (*CLPB3*, *CLPB4*, *HSP70D*, *HSP70E*, *HSP70F*, *HSP70G*, *HSP22G* and *HSP22H*) was not detected even under abiotic stresses. Those except *HSP70E* may have no function under normal and the selected stresses in this work. They probably serve as backup genes to compensate the function of the other members in the family upon null mutation. Note that *CLPB3* and *HSP70G* were founded in microarray (Voss et al. 2011) and *HSP70E* was found in northern blot (Vasileuskaya

et al. 2004). The absent of *HSP70E* gene expression in this study should be investigated further. By these limited results, the mutation or deletion of this gene at the selected site for primer design may be a possible cause. But the degradation of primer should not be excluded.

When the heat shock 37˚C was applied, 13 *HSP* genes were up regulated. Most of them were correlated with the previous study from microarray (Voss et al. 2011). Although the *C. reinhardtii* CC-124 (has normal cell wall) was applied to heat stress by shifting from 23˚C to 42˚C, 45 min, this study used CC-503 (cw-92 mt^{+}) which cw-92 indicate a very clean wall-deficient mutant. The down regulation of *CLPB1* was also counted on. The similar results from different degree of heat shock might indicate a relation between cell wall and heat sensing. Otherwise, it might support the hypothesis that the cell membrane is involved with algal cells heat sensing (Finka et al. 2012 and Schroda et al. 2015).

The *HSP22D* and *HSP22E* were not found to be up-regulated in microarray (Voss et al. 2011). This observation needs more experiments to confirm especially for *HSP22D* which had very low amount but detectable with the cycle number at 35. For *HSP22E* it was probably not found because of some limitations of the technique included; first, the comparison between *HSP22E* and *HSP22F* shown 98% identity nucleotide at coding sequence, second, its probe was 45-60 nucleotides and third, the probe melting temperature must reach 80˚C. All of this might explained the microarray results that found only up regulated *HSP22F* but not *HSP*22E.

Comparison between the results of heat shock 37˚C and 42˚C for 30 min indicated the algal cells might sense the degree of heat stress. So, this affected *HSP* gene expression profiles as shown by asterisks. Some of the *HSP* genes were expressed similar to normal (*CPN60A*, *CPN60B2*), up regulated (*HSP90C*) or even down regulated (*CPN60B1, CLPD*). Yet there is no other study showing that the *CLP* genes and other *HSP* genes were down regulated at heat stress.

The *CPN60B2* may sense and up regulated only 37˚C heat shock but not others included in this study. This interesting property may be suitable for heat shock response study at transcriptional level because at least 3 abiotic stress conditions may not affect those genes' transcript.

For salinity stress, the promoter of *HSP90A*, *HSP90B*, *HSP70A*, *HSP70B*, *HSP22A*, *HSP22E* and *HSP22F* may have potential for further investigation for salt responsive elements. The salinity stress response should dissect to investigate whether the response happened from osmotic and/or ionic stress.

Light can affect the photosynthetic organisms by several ways. For overnight dark incubation followed by normal light, the promoter of *HSP90A*, *HSP90B*, *HSP70A*, *HSP70B*, *CPN60A*, *CPN60B1* and *HSP22FB* may have potential to investigate further for the light responsive elements. The light response should be dissected to find out whether it was induced by MgProto (like PRE from the previous study) or other factors. For excess irradiance, the promoter of *HSP90A*, *HSP90B*, *HSP22A*, *HSP22B* and *HSP22F* may have potential to investigate further for the responsive elements that responsible for singlet oxygen or other ROS. Note that *HSP22B* was not up regulated only by heat and excess irradiance but also by the transition to darkness as shown in Figure 4.7. The algal cells in the darkness should be less stress compared to the normal because they obtained less energy. The regulation of this gene and also the protein function should be investigated in more detail.

From the expression profiles, the *HSP90A* and *HSP90B* shown that they were up regulated in all abiotic stress included in this study. The function of HSP90s protein is involved in the maturation of signal transduction proteins and receptors (Schorda 2004) which is a large number of proteins. Hence, if their mature client proteins begin to be denatured, they must need HSP90s too. It was possible that abiotic stress affected protein structure. Then it might be sensed and eventually trigged the up regulation of both *HSP90* genes. The expression level of two genes may be used as abiotic stress indicator because both *HSP90* genes up regulated by all abiotic stresses included above.

From the results there were many interested *HSP* genes which increased or decreased their expression compared to normal as shown earlier. Although most of them may be heat inducible and may also light, high light and salinity inducible, only these alone are weak. Making the conclusion from semi-quantitative RT-PCR results were risky because this technique has two pitfalls. One, the PCR reaction is end-point. The difference of band intensity on agarose gel may come from excess amplification at the extension step (cycle 3) of the protocol. And two, if error(s) occur at later steps

after the PCR was performed such as loading volume error or the agarose gel thickness was not proper; the appearance of band intensity after UV illumination would be imprecise. By those reasons, we need another method to help us confirm those results.

5.2 Confirmation of the interested *C. reinhardtii HSP* gene expression.

Because of those semi-quantitative RT-PCR pitfalls, the real-time PCR must takes responsibility. This technique measures signal of PCR products in the close system at the DNA polymerization step of every cycle. So, it is more accurate and precise. But it needs the very specific primer at the product length of 100-250 bp., the optimization of the reaction parameters and generating of the reliable standard curves. Those are the reason why it was not selected at the first time even it is better than the semi-quantitative RT-PCR. Putting all candidate genes into real-time PCR is a wasteful and labor-intensive work. When both techniques pros and cons were considered, it was found that both of them can compensate each other. Semi-quantitative RT-PCR has less parameters so it is easier to optimize and suitable for screening. Real-time PCR has more parameters to concern but its result is accurate and precise. Hence, it was used to confirm a few selected genes after the screening was performed.

The evaluation of real-time PCR results is essential. To say a gene was increased or decreased in its expression compared to the normal condition was based on the criteria described here. The calculated fold change is ≤ 0.1 or ≥ 10 (decrease or increase 10 fold compare to the control) with the difference of C_T more than 3 and both C_T value have their SD less than 0.5. The SD may be less considered if the difference of C_T was high. The explanation comes from the relationship between E and slope of the standard curve generated by 10-fold serial dilution. When the E was subtracted by 2, the reliable standard curve in this case gave the slope value at -3.22. The absolute value of slope is similar to the difference of C_T from two adjacent points on that standard curve. Hence, their concentration was different by 10 fold.

The %E of *HSP70A* and *CPN60B1* alternative primers were higher than 100 may be due to accumulation of non-specific PCR product. The small peak which

appeared only at dissociation curves of both primers support this notion (see Appendix D). Note that although there were many steps to verify the primer as shown before in the topic materials and methods, sometimes the limitation is itself (gene sequence) which has less suitable regions for primer design. Then only few primers have excellent specificity to overcome both computational and experimental verifications.

Table 5.2 Comparison of calculated fold change between two techniques under 30 min heat shocked 37°C

Genes	Semi-quantitative RT-PCR	Real-time PCR
HSP90B	24.1	11.6
HSP70A	3.5	29.9
CPN60B1	5.3	15.7
HSP22A	688.8	3,502.8

Increasing gene expression after subjected to heat stress or heat inducible is the well known property of most *HSP* genes. At 37°C heat shock for 30 min, the *HSP90B*, *HSP70A*, *CPN60B1* and *HSP22A* were shown that property. The fold change of *HSP22A* was correlated between two techniques. The fold change values of each gene from real-time PCR were more than 10. Although the C_T of *HSP90B* in CL had 1.0 SD, the C_T of heat shock had 0.2 SD which meant it is consistent. The *CPN60B1* had the fold change more than *HSP90B*. For the expectation as positive control, the famous *HSP70A* was increased very high at 29.9 fold change. And interestingly, the *HSP22A* was increased significantly with the difference of C_T 10.2 that gave the calculated fold change equal to 3,502.8. It is correlated to a recent study (Kobayashi et al. 2014) which found that *HSP22A* was about 1,000-fold up regulated after subjected to heat shock at 36°C for 20 min.

Table 5.3 Comparison of fold change between two techniques under 30 min salt-shocked 50 mM NaCl

Genes	Semi-quantitative RT-PCR	Real-time PCR
HSP90B	6.7	0.7
HSP70A	2.4	2.2
CPN60B1	0.9	0.5
HSP22A	17.4	3.4

However, in salinity stress the fold change values of semi-quantitative RT-PCR were not related to real-time PCR. All of them did not change their expression level.

Table 5.4 Comparison of fold change between two techniques under excessive irradiance

Genes	Semi-quantitative RT-PCR	Real-time PCR
HSP90B	3.1	1.1
HSP70A	1.4	1.2
CPN60B1	0.9	0.5
HSP22A	6.0	1.1

In excessive irradiance, the fold change values of semi-quantitative RT-PCR were not related to real-time PCR. All of them did not change their expression level.

Table 5.5 Comparison of fold change between two techniques under the transition from darkness to normal light intensity

Genes	Semi-quantitative RT-PCR	Real-time PCR
HSP90B	2.5	0.4
HSP70A	3.9	10.1
CPN60B1	2.1	3.3
HSP22A	0.7	3.0

In the darkness shift to normal light condition, the fold change values of semi-quantitative RT-PCR were not related to real-time PCR. The expression of *HSP70A* may be increased. Although the dark incubation samples gave 1.9 SD, the shifting to normal light samples gave only 0.3 SD. And again, this gene can serve as positive control because the previous study shown its light inducible property (von Gromoff et al. 1989). The difference is time period of the darkness. In that study, the algal cells were grown in the darkness for 4-5 days plus 17 hr. but in this study the algal cells were grown 3-4 days in the normal light before shifted to the darkness about 18 hr. then brought them back to the normal light.

As shown in the table 5.2 to 5.5, only the fold change of *HSP22A* was high and correlated between two techniques but others were not. This dissonant might came

from the pitfalls as discuss earlier plus the unknown %E of semi-quantitative RT-PCR primers.

5.3 Relationship between the *HSP* gene expression in abiotic stress and the localization of responsive elements.

There are many macromolecules playing their roles and co-operation in every steps of transcription. To be specific, many of stress responsive elements were selected to analyze this study. Most of them were transcription factors binding site or assumed to be in the case of SORLIP1AT and SORLIP2AT. The PRE is an enhancer. Only the original HLR1 is a repressor binding site which its study suggested the affinity of repressor complex was increased when the light intensity decreased.

The putative promoter sequences were selected from the NCBI database at the length of 500 bp. before transcription start site. Although most of the core promoters were ranged about 300 bp. for example, the *HSP70A* core promoter was about 300 bp., this makes sure that some elements were not excluded. The putative promoter sequence of *HSP90B* was modified from NCBI database by shifting above the reported site because of the study from Ph.D candidate. He found that the mRNA of this gene should be longer than the previously report (data not shown).

Heat inducible is a well known property of *HSP* genes. The real-time PCR results and the observation under light microscope (Figure 4.2) indicated both of the temperature and the time period was proper. It stimulated heat shock response at the transcription level without killing them. The comparison between HSE localized on *HSP70A* putative promoter and the others revealed some interested information. First, the number of HSEs may relate to this increasing as shown in Table 4.1. Second, the distribution of HSE along the putative promoter may relate to this increasing as shown by their localization. Third, the space between HSEs may play a role in this increasing. There's an interesting point about the number of HSE on putative promoter and the calculated fold change. *HSP70A* was very effective. They had not much HSE groups compare to *HSP90B* and *CPN60B2* but gave the higher calculated fold change. The size of well-documented *HSP70A* core promoter was about 300 bp. This lead to the

last point, the core promoter of *HSP90B*, *CPN60B1* and *HSP22A* may be less than 500 bp. similar to the *HSP70A*.

Salinity stress affected cells' milieu by making the water diffused out and the excess amount of Na^+ ion that diffused into the cells can disrupted many enzymes. The results indicated two possibilities either the selected responsive elements didn't involve in salinity stress or the stress condition (salt concentration and/or duration) is too less to stimulate the response.

Light is an essential energy source of photosynthetic organisms. Naturally, the light intensity changes smoothly from day to night as always. The shifting from the darkness to light affected the algal cells by instantaneous starting the photosynthesis while its machineries such as electron transport proteins were not prompt. This can be accounted to be a stress condition because the ROS were expected to generate. At this condition, only the *HSP70A* was increased. But at high light stress which also caused more ROS generation, none of them was changed their expression. Here the light intensity was comparable to a previous study (Mahong et al. 2012) which shifted from 50 to 1,200 µmol photons m^{-1} s^{-1}. So, may be the duration was too less to observed the response. The analysis of light responsive elements localized on their putative promoters shown no relationship between them in both conditions. It's surprised that the PRE was shown in all putative promoters but only *HSP70A* was light inducible at 30 min. This suggested there're more factors than PRE that accounted on to make a gene light inducible.

For abiotic stresses included in this study, no relationship was found between the expression profiles and the localization of abiotic stress responsive elements from PlantCARE. This might came from the different of responsive elements between higher plants and single cell green algae. But at least the results shown those putative promoter sequence were contain some common sequences which similar to CAAT-box and TATA-box. This indicated the core promoter of selected genes might be there.

CHARPTER VI
SUMMARY

In this study, expression profiles of 25 *HSP* genes were observed the under 4 abiotic stress conditions using semi-quantitative RT-PCR. Four of them were selected to confirm and validate by qPCR along with analysis of stress responsive elements on their putative promoter sequences. From all experimental results and the analysis, the information can be summarized as described below.

6.1 Not all *HSP* genes were expressed and some of them were not induced by abiotic stresses.

The 17 out of 25 selected *HSP* genes were expressed at normal condition. The *HSP* gene expression profiles from semi-quantitative RT-PCR technique can be summarized as the previous table 5.1. Most of the *HSP* genes were up regulated by heat. Several genes may up regulated by salt stress, transition from darkness to normal light intensity and excessive irradiance. The expression of 8 genes (*CLPB3*, *CLPB4*, *HSP70D*, *HSP70E*, *HSP70F*, *HSP70G*, *HSP22G* and *HSP22H*) was not detected even under abiotic stresses. The *HSP22B* was up regulated not only at heat and excess irradiance but also at the transition to darkness.

6.2 Real-time PCR confirmed heat and light induction of *HSP* genes.

Four *HSP* genes included *HSP90B*, *HSP70A*, *CPN60B1* and *HSP22A* were selected for validation by real-time PCR. From four abiotic stress conditions with duration 30 min, all of them were shown heat inducible property but only *HSP70A* was shown light inducible property. None of them was shown that they could induced by salt or high light intensity. The *HSP22A* was significantly increased its expression

which correlated to the recent study (Kobayashi et al. 2014). It could be use as a model to study the heat stress response and regulation at the transcription level.

6.3 The expression profiles from salinity stress, the transition to normal light and excessive irradiance stress were not related to the selected responsive elements.

There was no relationship between salt and light responsive elements localized on the putative promoter sequences and the real-time PCR results of salinity stress, the transition to normal light and excessive irradiance stress.

6.4 Only heat induced expression was related to the HSE localization.

The selected abiotic stress responsive elements were grouped and mapped on the putative promoter sequences of four *HSP* genes based on this experimental abiotic stresses. For heat stress, both number of HSEs and their distribution the putative promoter may relate to this increasing. So, the space between HSEs may also play a role too. And base on the *HSP70A* core promoter size, the core promoter of *HSP90B*, *CPN60B1* and *HSP22A* may be less than 500 bp.

6.5 Abiotic stress responsive elements localized by PlantCARE were not related to the expression profiles.

In addition, the online tool PlantCARE was used to find and locate the abiotic stress responsive elements. No relationship between the expression profiles and the localized responsive elements. The sequences similar to CAAT-box and TATA-box were localized on 4 putative promoter sequences.

CHARPTER VII
CONCLUSION

All experimental and analytical information from this study can be concluded as these following.

The 17 out of 25 *HSP* gene candidates were successfully observed for their expression profile by using semi-quantitative RT-PCR. Most of them were up regulated under heat stress either 37ºC or 42ºC while a few genes did not change their expression or even down regulated compared to normal condition. Salinity stress, the transition to normal light and excessive irradiance stress can trigger expression of some *HSP* genes. The expression of other 8 genes was not detected under normal and abiotic stresses included in this study.

Four selected genes were successfully validated for their expression by using real-time PCR. The results related their up regulated with heat shock 37ºC for 30 min. The up regulated of *HSP70A* also related to the transition to normal light for 30 min. The expression of those genes under salinity stress and excessive irradiance stress for 30 min was similar to normal.

The relation between 4 selected *HSP* gene expression and abiotic stress responsive elements on their putative promoter sequences was investigated. The localization of abiotic stress responsive elements on selected putative promoter sequences shown that they are related with the expression profiles under heat shock 37ºC for 30 min. The up regulation of *HSP* genes under that heat shock might depended on number of HSE and the distribution along putative promoter. No relationship was found between salinity and light responsive elements and the expression of selected *HSP* genes under those related abiotic stresses. PlantCARE, an online promoter analysis tool, was used. The analysis also showed no relationship between and light responsive elements and the expression of selected *HSP* genes under those related abiotic stresses. The sequence similar to CAAT-box and TATA-box were found. This indicated the core promoter sequences might be there.

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APPENDICES

APPENDIX A

Nomenclature for incompletely specified bases in nucleic acid sequences

Nucleic Acids Research

Table 1. Summary of single-letter code recommendations

Symbol	Meaning	Origin of designation
G	G	Guanine
A	A	Adenine
T	T	Thymine
C	C	Cytosine
R	G or A	puRine
Y	T or C	pYrimidine
M	A or C	aMino
K	G or T	Ketone
S	G or C	Strong interaction (3 H bonds)
W	A or T	Weak interaction (2 H bonds)
H	A or C or T	not-G, H follows G in the alphabet
B	G or T or C	not-A, B follows A
V	G or C or A	not-T (not-U), V follows U
D	G or A or T	not-C, D follows C
N	G or A or T or C	aNy

Figure 9.1 Summary of single-letter code recommendations.

APPENDIX B

DNA sequencing results of semi-quantitative PCR products

CLPB1 PCR product with forward primer

```
  1 CAGGGCCCCC TTTGGTCTTG CGGGTCTAAT GGAGACCTCG GGACGGCTCT ACAGAAACCG
 61 GGAGAGCTCG GGAGCACTCT GGAGAATGCC GGAATGCTCT CGAACGCGCT CAGCCAAGTC
121 GCGATGCAGC GCCCCGCCAG GGCCGCCAGT ATATAATAGG TATTGGGACC ACACGTGCAT
181 TCACAATCAG CCATTGCTAT ACACACAATT TAGAGCTGCT ACCCAACTCG ACACTGCCAG
241 TTGTACCGAC ACACGGAACA AGCTTCAATT CTAGCAGTAT TCAATTGCAG CGGAATACAA
301 GCTTCTAATC TACTGATTTA TTACACGTAA ATATAGCAGA ACCGCCTTTG GCGACCACTT
361 GCTTTAGCAC CGTCACCGGC GCCTGGAAAG ATGTCGTTTG ACACCAAGAA AGCGACAGAA
421 AAGGTCAATA ATGTGCTTGG AGAGGCGATT AACCTAGCCA AGGAGGACAA GCACGCGGCG
481 CTGACGCCGA CTCATCTAGC GGTTGTTCTC TTCGAGGAGC CGCATGGCCA AAAAGGAACC
541 TACCCGGATT TTAGTAGTTT TATGGTTAGT GTGTGTGTCC AATTATAAAG AGCAATATCG
601 TTATGTCTCA
```

CLPB1 PCR product with reverse primer

```
  1 CCCCCAAAAA TTTTATTGCG TAGACCGGCC CCGGCCCTCC AGCATTTGGT CTTGCGGGCC
 61 TAATGGAGAC CTCGGGACGG CTCTACAGAA ACCGGGAGAG CTCGGGAGCA CTCTGGAGAA
121 TGCCGGAATG CTCTCGAACG CGCTCAGCCA AGTCGCGATG CAGCGCCCCG CCAGGGCCGC
181 CAGTATATAA TAGGTATTGG GACCACACGT GCATTCACAA TCAGCCATTG CTATACACAC
241 AATTTAGAGC TGCTACCCAA CTCGACACTG CCAGTTGTAC CGACACACGG AACAAGCTTC
301 AATTCTAGCA GTATTCAATT GCAGCGGAAT ACAAGCTTCT AATCTACTGA TTTATTACAC
361 GTAAATATAG CAGAACCGCC TTTGGCGACC ACTTGCTTTA GCACCGTCAC CGGCGCCTGG
421 AAAGATGTCG TTTGACACCA AGAAAGCGAC AGAAAAGGTC AATAATGTGC TTGGAGAGGC
481 GATTAACCTA GCCAAGGAGG ACAAGCACGC GGCGCTGACG CCGACTGATT TTGTGTTT
```

CLPD PCR product with forward primer

```
  1 GGGGGGAAAC AGACCTACGT CAGGGGGCGG CCCTCGGGCG GCAGCCCCGC AGCAGTTGGC
 61 GATGGCCTCC AAGCTCCCCC GGCCCCAGCG CGCTACCGTG AACCGGCGAC AGGCGCTGAA
121 GGTCCAGGCG GTGTTTGAGC GCTTCACCGA GCGTTCCATC AAAACTGTGA TGATTGCACA
181 GGCGGAAGCG AAGGCGTTCG GGCACACGGA GGTCAACACT GAGCACATCC TGCTGGGCCT
241 GGTCGCGGAG GAGAGCCTCA GCAAGAACGG CTACTTGAAC AGCGGGGTCT CATCGGAGCG
301 CGCCAAGGCA GCTGTGGAGG CGTTGTTTGG CCGCAAGCGC CCCGTGAGCC ATGGCGAGAG
361 CATTCCCTTC AGCCGTGAAG TTCGCAAGAT GTTCGAGAAC GCCACACACG AGTGCAAGCG
421 CTCCAACGTC AACTGGATCT CGCCCGAGCA CATCCTGCTG GCGATGCTGT CGATGCCGGA
481 CTGCAACAG
```

CLPD1 PCR product with reverse primer

```
  1 TTTCCGTGGC CCCCCTGATG CGCAGTATCG CGCTGACAGC GTCAAGGGGC GGCCCTCGGG
 61 CGGCAGCCCC GCAGCAGTTG GCGATGGCCT CCAAGCTCCC CCGGCCCCAG CGCGCTACCG
121 TGAACCGGCG ACAGGCGCTG AAGGTCCAGG CGGTGTTTGA GCGCTTCACC GAGCGTTCCA
181 TCAAAACTGT GATGATTGCA CAGGCGGAAG CGAAGGCGTT CGGGCACACG GAGGTCAACA
241 CTGAGCACAT CCTGCTGGGC CTGGTCGCGG AGGAGAGCCT CAGCAAGAAC GGCTACTTGA
301 ACAGCGGGGT CTCATCGGAG CGCGCCAAGG CAGCTGTGGA GGCGTTGTTT GGCCGCAAGC
361 GCCCCGTGAG CCATGGCGAG AGCATTCCCT TCAGCCGTGA AGTTCGCAAG ATGTTCGAGA
421 ACGCCACACA CGAGTGCAAG CGCTCCAACG TCAACTGATC TCGCCCGAGC ACTACTGGGA
481 GTG
```

HSP90A PCR product with forward primer

```
  1 CACCCTGGGG AATCACAGGC TGCTGTCGCT GATTATCAAC ACCTTCTACT CGAACAAGGA
 61 AATCTTCCTG CGCGAGCTTA TCTCCAACGC CTCCGATGCT CTGGACAAGA TCCGTTACAT
121 GTCGCTCACT GACAAGTCGG TGCTGGACAA CAACCCGGAG CTGTACATCC ACCTGCAGCC
181 CAACAAGGCG GATGGCACCC TGGCCATCAC CGACTCGGGT ATCGGTATGA CCAAGGCGGA
241 CCTGATCAAC AACCTGGGTA CCATCGCGCG CTCGGGCACC AAGGCGTTCA TGGAGGCCCT
301 CTCCGCCGGC GCCGACGTGT CGATGATTGG CCAGTTCGGC GTGGGAAAA
```

HSP90A PCR product with reverse primer

```
  1 TTGAGGCCCC CGNGTGAGGC CCCCGTGGAG ACCTTCGCCT TCCAGGCGGA GATCAACCAG
 61 CTGCTGTCGA TGATTATCAA CACCTTCTAC TCGAACAAGG AAATCTTCCT GCGCGAGCTT
121 ATCTCCAACG CCTCCGATGC TCTGGACAAG ATCCGTTACA TGTCGCTCAC TGACAAGTCG
181 GTGCTGGACA ACAACCCGGA GCTGTACATC CACCTGCAGC CCAACAAGGC GGATGGCACC
241 CTGGCCATCA CCGACTCGGG TATCGGTATG ACCAAGGCGG ACCTGATCAA CAACCTGGGT
301 ACCATCGCGC GCTCGGGCAC CAAGGCGTTC ATGGAGGCCA TGTCCGCACG CGCCCGATTG
361 CCCT
```

HSP90B PCR product with forward primer

```
  1 AGCGCCACTG GAGCGCATCA TGCGCGCACA GGCCTTCTCC CGCCCCGGCT CCTCCTTCAC
 61 CCCCACCCAG CGCACGCTGG AGATCAACCC CCGCCACCCG CTCATCGTGG CGCTCAAGGA
121 CAAGCTGGCC GCCGCCACCG AGGAGACCGT GGAGGAGAGC GCCGTGGCCA CCGCGCGCCT
181 GCTGTACGAG ACCGCGCTGC TGGAGTCGGG CTTTGTGCCC GATGACGCCA AGGCCTTCTC
241 GCAGCGCATG TACGGCGTCC TGAAGGACAC GCTGGGCGTG GACAGCCTGG AGGTGGCGCT
301 GGAGGCGGAG GAGGCCGCCG AGCCCGAGGA GGCGGAGGAA AAGGCGGAGG AGACGGAGGA
361 GAAGGCGGAG GAAACGCACG AGGTTGGCGC TGTTGCCGAA CATGGCGGCG AACCACAGAT
421 GATC
```

HSP90B PCR product with reverse primer

```
  1 AAGGTTGACT GTGATTGTGA TTATGCCCCT CTCGCTAATT TCCCTGCATC GAGGCAGGCC
 61 TGGCCCCACC AAATGTCTTC GTGGTCACCG GCAAGTACGG CAACAGCGCC AACATGGAGC
121 GCATCATGCG CGCACAGGCC TTCTCCCGCC CCGGCTCCTC CTTCACCCCC ACCCAGCGCA
181 CGCTGGAGAT CAACCCCCGC CACCCGCTCA TCGTGGCGCT CAAGGACAAG CTGGCCGCCG
241 CCACCGAGGA GACCGTGGAG GAGAGCGCCG TGGCCACCGC GCGCCTGCTG TACGAGACCG
301 CGCTGCTGGA GTCGGGCTTT GTGCCCGATG ACGCCAAGGC CTTCTCGCAG CGCATGTACG
361 GCGTCCTGAA GGACACGCTG GGCGTGGACA GCCTGGAGGT GGCGCTGGAG GCGGAGGAGG
421 CCGCCGAGCC CGAGGAGGCG GAGGAGAAGG CGGAGGAGAC GGAGGAGAAG GCGGAGAGAC
481 GAGAGATATG AGTTTAAGT
```

HSP90C PCR product with forward primer

```
  1 GGCGGAGTCT AGCATGCAGT AGCCCGGCGA TGGGTGTTGC ACTGTGCAAC TCACGTCTCA
 61 AGCAGGCGTG AGGCTTCGCT ACGGATGACA GTGTTAACGG CGTGTGAAAT TTTGATGAAG
121 AAGGAGACTT CTGAAGAGGG TTCTCGAAAT CCAGGTGCAC TTTGCATGAG CGGGGTTCTG
181 GAAGTCGCAG CGGCACCACA TGCAACCAGC ACCTTGCATT TGCCCCAAAC TTTACTTGCG
241 CTGCATTCAC AAGCTAACTT GACGATCTAA ACCTAGCAGA GGCTTGAGTG GGTTCCACTC
301 GGCCCAGAGT TTTCGATCAT TTCGGCGGCT TCCGTGTTTG CTGACGCGCG CAAGCGCTTG
361 ACCACCATGA TGCTACGCGG TCTGCCCATC TGGCATAAA
```

HSP90C PCR product with reverse primer

```
  1 TTTTGTGGGA ATTTTGAGCC CAGAAAGAAC CGTGCTAAAG CCATGCAGTA GCCCGGCGAT
 61 GGGTGTTGCA CTGTGCAACT CACGTCTCAA GCAGGCGTGA GGCTTCGCTA CGGATGACAG
121 TGTTAACGGC GTGTGAAATT TTGATGAAGA AGGAGACTTC TGAAGAGGGT TCTCGAAATC
181 CAGGTGCACT TTGCATGAGC GGGGTTCTGG AAGTCGCAGC GGCACCACAT GCAACCAGCA
241 CCTTGCATTT GCCCCAAACT TTACTTGCGC TGCATTCACA AGCTAACTTG ACGATCTAAA
301 CCTAGCAGAG GCTTGAGTGG GTTCCACTCG GCCCAGAGTT TTCGATCATT TCGGCGGCTT
361 CCGTGTTTTG CTGACGGCGC GGGCA
```

HSP70A PCR product with forward primer

```
  1 TAGGGTTTCG TTAAGCTGCG TGGGTGTCTG GCAGAATGAC CGCGTGGAGA TTATTGCCAA
 61 CGATCAGGGC AACCGCACCA CTCCCTCGTA CGTGGCCTTC ACGGACACTG AGCGTCTGAT
121 TGGTGATGCC GCCAAGAACC AGGTCGCTAT GAACCCGCGC CACACGGTGT TCGACGCCAA
181 GCGCCTGATT GGCCGCAAGT TCTCGGACCC CATTGTCCAG GCGGACATTA AGCTGTGGCC
241 TTTCCAGGTT CGCGCCGGCG CGCACGATGT GCCCGAGATC GTTGTCTCCT ACAAGAACGA
301 GGAGAAGGTC TTCAAGGCTG AGGAGATCTC CTCGATGGTG CTTATCAAGA TGAAGGAACC
361 GCTCAGGAGA TTTTGCTATT GACTTCCAGG GTGATGATAT CTCCGCGGCG GTGCTTAACA
421 AAATTAAGGA AACCGCTAAG GATTT
```

HSP70A PCR product with reverse primer

```
  1 TTAGGTCACC GCCATCGGTA TTGACCTGGC CACCACGTAC AGTTGAGTGG GGGTCTGGCA
 61 GAATGATCAA CTTGTTAGGC CCCCCGGCAT CGGTATTGAC CTGGGCACCA CGTACAGCTG
121 CGTGGGTGTC TGGCAGAATG ACCGCGTGGA GATTATTGCC AACGATCAGG GCAACCGCAC
181 CACTCCCTCG TACGTGGCCT TCACGGACAC TGAGCGTCTG ATTGGTGATG CCGCCAAGAA
241 CCAGGTCGCT ATGAACCCGC GCCACACGGT GTTCGACGCC AAGCGCCTGA TTGGCCGCAA
301 GTTCTCGGAC CCCATTGTCC AGGCGGACAT TAAGCTGTGG CCTTTCCAGG TTCGCGCCGG
361 CGCGCACGAT GTGCCCGAGA TCGTTGTCTC CTACAAGAAC GAGGAGAAGG TCTCAAGGCT
421 GAGAGTTCCT TTTGTTC
```

HSP70B PCR product with forward primer

```
  1 CGATTAGCGA TAACGAGCGC AGCTAGCACA CGACCCAACA ATGCCGGTTC AGCAGATGAC
 61 TTCTATGCGC AGCCAGTCCC TGGCTGGCGC TCCAGTGGCA CCCGTCAAGG CGGGCCGCGC
121 TGGCGTTTCG CGCCGTGGCC TGGCCGTCAG CGTCCGTGCT GAGAAGGTCG TGGGTATCGA
181 TCTGGGCACG ACCAACTCGG CTGTGGCTGC CATGGAGGGT GGCAAGCCGA CCATCATCAC
241 CAACGCTGAG GGCGGCCGCA CATCTTTCGG TTGGGGGGAT CCCCCAAAAC CGGCGACCCT
301 CTGGGTTGGG CCAAAATTCC CAACCCCCGG GGTGGGGGGA ACCCCCAAAA AAATTTTTTT
361 TTTCCCCAAA AAGGGTTTTT CCCCCCCCCC CCAGGGAAAA AAGGCCCCCC AAAAACCCCC
421 CCAAGGGGTT TCCGGGGGAT GAAAGGGGGG GGCCCCCAAA AAAAAAAAAA GCCCCCCCCC
481 CCCCGCAAAA ATTTTTCCCC CCCCAGAAAA AAAACCCCCC GGGTTTCTCC CGGGGAAAGG
541 GAGCCCCCCC CTTTTTTTTT ATAAAAAAAA AAAAAAG
```

HSP70B PCR product with reverse primer

```
  1 TGAAGTGGGA AAACTACTCC TCAACAATAC CTGCTGCACT TTAGCGATAA ACGAGCGCAG
 61 CTAGCACACG ACCCAACAAT GCCGGTTCAG CAGATGACTT CTATGCGCAG CCAGTCCCTG
121 GCTGGCGCTC CAGTGGCACC CGTCAAGGCG GGCCGCGCTG GCGTTTCGCG CCGTGGCCTG
181 GCCGTCAGCG TCCGTGCTGA GAAGGTCGTG GGTATCGATC TGGGCACGAC CAACTCGGCT
241 GTGGCTGCCA TGGAGGGTGG CAAGCCGACC ATCATCACCA ACGCTGAGGG CGGCCGCACC
301 ACCCCCTCGG TGGTGGCATT CACCAAGACC GGCGACCGTC TGGTTGGCCA GATTGCCAAG
361 CGCCAGGCTG TGGTGAACCC CGAGAACACT TTCTTCTCCG TAAAGCGCTT CATCGGCCGC
421 CGCATGTCGG AGGTCGGCTC GGAGTCCACC CAAGTGCCTT ACCGGGTGAT TGAGGACGGC
481 GGCAACGTGA AGATCAAGTG CCCCAACGCC GGTAAGGACT TCGCTCCCGA GGAGATCAGC
541 GCGCAGTGCT GCGCAAGAAG
```

CPN60A PCR product with forward primer

```
  1 NNGNNCTCGC AGACACTGGT CGTCCTTCCA GAACAAGCCG GCCCGCGCTG CGCGCCGCCT
 61 GGTGATCCGC GCTGCTGACG CTAAGGAGAT TGTGTTCGAC CAGGAGTCGC GCCGGAGGCT
121 GCAGGCGGGC ATCAACAAGG TGGCCGATGC CGTCGGTGTG ACCCTGGGCC CCCGCGGCCG
181 CAACGTGGTG CTGGAGCAGA AGTTCGGTGT GCCCCAGGTT ATCAACGATG GCGTGTCCAT
241 TGCTCGCGCT ATCGAGCTGA AGGACCCCGT GGAGAACGCC GGTGCCCAGC TCATCAAGGA
301 GGTGGCCGGC CGCACCAACA ACGCCGCGGG TGACTCCACC ACAACCACAT CCCTGCTGTT
361 GCTTGAGATA ATCCACTGAG ANATAGCT
```

CPN60A PCR product with reverse primer

```
  1 TCTATGGCGC TTGTTTCAGC TTGCCAAGGG CCTTCGCCAG ACCACTGGTC GTCCCTTCCA
 61 GAACAAGCCG GCCCGCGCTG CGCGCCGCCT GGTGATCCGC GCTGCTGACG CTAAGGAGAT
121 TGTGTTCGAC CAGGAGTCGC GCCGGAGGCT GCAGGCGGGC ATCAACAAGG TGGCCGATGC
181 CGTCGGTGTG ACCCTGGGCC CCCGCGGCCG CAACGTGGTG CTGGAGCAGA AGTTCGGTGT
241 GCCCCAGGTT ATCAACGATG GCGTGTCCAT TGCTCGCGCT ATCGAGCTGA AGGACCCCGT
301 GGAGAACGCC GGTGCCCAGC TCATCAAGGA GGTGGCCGGC CGCACCAACG ACGCCGCGGG
361 TGACGGCACC ACCACCGCTC GTTGC
```

CPN60B1 PCR product with forward primer

```
  1 TNNGNNNGGG CGTGANCCGG AGCAGGCGCA GCGTGGTTAG TTCGCGCTGC CAAGGAGCTG
 61 CACTTCAACA AGGACATGCA AGCGCTGAAG CGTATGCAGG CGGGTGTGGA CAAGTTGGCG
121 ACTGTGGTTG GCGTCACCAT CGGCCCCAAG GGTCGCAACG TGGTGCTTGA GTCCAAGTTT
181 GGTGCGCCCA AGATCGTGAA CGACGGCGTG ACCATCGCCC GCGAGGTGGA GCTGTCTGAC
241 CCTGTGGAGA ACATTGGTGC CACCCTGGTC CGCCAGGCCG CCGCCCGCAC CAACGACACG
301 GCGGGCGACG GCACCACCAC CGCCACCGGC CTGTCCGCCG CCTTCATCGG TGAGGGCATG
361 AAGATCGTGT CGGCAGGCAC ATA
```

CPN60B1 PCR product with reverse primer

```
  1 GTGAGTTGCT TATCCTTGGG GCCGGGTGAC CGGCAAGACC GCTGCCAAGG GCGGCGTGAG
 61 CCGGAGCAGG CGCAGCGTGG TTGTTCGCGC TGCCAAGGAG CTGCACTTCA ACAAGGACAT
121 GCAAGCGCTG AAGCGTATGC AGGCGGGTGT GGACAAGTTG GCGACTGTGG TTGGCGTCAC
181 CATCGGCCCC AAGGGTCGCA ACGTGGTGCT TGAGTCCAAG TTTGGTGCGC CCAAGATCGT
241 GAACGACGGC GTGACCATCG CCCGCGAGGT GGAGCTGTCT GACCCTGTGG AGAACATTGG
301 TGCCACCCTG GTCCGCCAGG CCGCCGCCCG CACCAACGAC ACGGCGGGCG ACGGCACCAC
361 CACCGCCACC GTCCTTCCGC TGCCTCATCG AAAGCC
```

CPN60B2 PCR product with forward primer

```
  1 AGGGGGGGCT GGGCTGCGGC GCGTCCGTGG CAGGCGCAGC GTGGTCGTTC AGGCCGCCAA
 61 GGAGCTGCAC TTCAACCGCA ACATGGAGGC GCTGAAGAAG ATGCAGGCTG GTGTGGACAA
121 GCTGGCCACC GTGGTCGGTG TGACCATCGG CCCCAAGGGT CGCAACGTGG TGTTGGAGTC
181 CAAGTTCGGC TCGCCCAAGA TTGTGAACGA CGGTGTGACC ATTGCTCGCG AGGTGGAGCT
241 GGAGGACCCC GGGGAGAACA TCGGCGCCAA GCTGGTCCGC CAGGCCGCTG CCCGCACCAG
301 CGACTCCGCT GGTGATGGCA CCACCAGGGC GACCGGCCGG TCCGCCGCCT TCATCGCTAA
361 GGGCATGAAG ATGGTGGCGA CCGGCACCAA CCACGTGCAG CTGAACCGCG GCATGGAAAG
421 ACAACGTTAA AACCGGTGTG GCGGTGCCGG CCCACCATGG AGGGGAACAC AGCGCGAAGG
481 TCTTCATCAC AATCAAAGTC AGCCTT
```

CPN60B2 PCR product with reverse primer

```
  1 GCCTCGGGTC GGGGCCCCGT CCCTGCCAGG CGCAGCCTGG TATTTCAGGC CGCCAAGGAG
 61 CTGCACTTCA ACCGCAACAT GGAGGCGCTG AAGAAGATGC AGGCTGGTGT GGACAAGCTG
121 GCCACCGTGG TCGGTGTGAC CATCGGCCCC AAGGGTCGCA ACGTGGTGTT GGAGTCCAAG
181 TTCGGCTCGC CCAAGATTGT GAACGACGGT GTGACCATTG CTCGCGAGGT GGAGCTGGAG
241 GACCCCGTGG AGAACATCGG CGCCAAGCTG GTCCGCCAGG CCGCTGCCCG CACCAACGAC
301 ACCGCTGGTG ATGGCACCAC CACCGCCACC GTCCTGTCCG CCGCCTTCAT CGCTGAGGGC
361 ATGAAGATCG TGCCGCCGCA CCACCCCCCT CCTTT
```

HSP22A PCR product with forward primer

```
  1 AGGGGGAAGA CCGGTGCTGG TATGCGGGTG GTGCGGTGCG ATTGAGGCGT GAGGATGACG
 61 GGCAGTTGGT GGTGCCCGTA CGAGTTGAGT GGGTAGTTGT TGTGGCTAGT GGCAGGCTGG
121 GGAACGGGGT GCCACGGGCG TGTGTATGTG CCGGCTGTGT TCGTTGACAC GAGCAGTGAC
181 GCTCACTTGC GTGGGCCGGC AAGCCCCACA ACATTGACTT TGTGGCTCCG TAGAGACTTC
241 ATGCCGGGCT GAATCTGCTA TATGTTTGTG TACTATCAAA TTGTGGCACG AAGAATGAAG
301 TCTCTACGGA GCCACAAAGT CAAAGTTGTG GGGCTTGCCG GCCCCCCGTT AGCGTCCTGC
361 TCTGTCACGA AAAAGCCCGA AATACCAGGC CGTTGCCCCC CTTCCCCGCC GCCCTAGCCA
421 CAACAACTAC CCCCTCCAC
```

HSP22A PCR product with reverse primer

```
  1 TTTTCAGCAA ATTTTTGTGA GGCACGGCAA GCGCTTGCAG AGTGGGGTCT TGCAAGCAAG
 61 CTAAGGGGCC GTTGGCATCG CTTCCGGGTA AGTTGGGTCA ATGGATTGCC AGGATGGATT
121 GGGTTGTTTA TTTCGCTCCA GGGGAGCTGG GCGCTGCATG TGTGGGTGAT GACCGGTGCT
181 GGTATGCGGG TGGTGCGGTG CGATTGAGGC GTGAGGATGA CGGGCAGTTG GTGGTGCCCG
241 TACGAGTTGA GTGGGTAGTT GTTGTGGCTA GTGGCAGGCT GGGGAACGGG GTGCCACGGG
301 CGTGTGTATG TGCCGGCTGT GTTCGTTGAC ACGAGCAGTG ACGCTCACTT GCGTGGGCCG
361 GCAAGCCCCA CAACATTGAC TTTGTGGCTC CGTAGAGACT TCATGCCGGG CTAATCTG
```

HSP22B PCR product with foward primer

```
  1 CCCGCGCACG AAGCTGGGAA CTTTCCGGGC CACAGCGCCC ATGGACATTG TAGAAACTCC
 61 AAGCGGGTAT GAGTTGCATG CCGACGCCCC GGGCTTGGGC CCACGCGACG TGAAGGTCGA
121 GCTGCATAAC GGCGTTCTCC AGATCAGTGG CTCCCGCAAG TTGCACCACG AGTCCAAGGA
181 CTTGCGCGGC CGCCTGCTCC GTCGCGAGCG CACCGCCTAC AGCTTCAGCC GCGCCTTCAG
241 CCTGCCGGAG AACGCCAACC CGGACGGCAT CACCGCCGCC ATGGACAAGG GAGTGCTGGT
301 GGTCACCGTG CCCAAGCGGA TTGCCGCGGC GGCGGCGCCT GCCGCGAAGC TGCAGAATGG
361 GCGAACCTGT ACCATCATGA GCTGCCCCGA GCCGGCCAGG GTTCAGGGAT GCAATTGGCT
421 GCCGGTACGC CAACATT
```

HSP22B PCR product with reverse primer

```
  1 CCGTGGGTGG TCGCACGATC GGGACAATAT GATGCTTAAC CTTCGCACGA GGCGGACAAT
 61 ATCGCAACGC CGCGCGACTC GCACGAGGCG TACAATATCG CAACGCCGCG CGACCAGAGC
121 TGGGATCTTT CCGGGCCAAC AGCGCCCATG GACAGTGTAG AAACTTCAAG CGGGTATGAG
181 TTGCAAGCCG ACGTCCCGGG CGTGGGTCCA CGCGACGTGA AGGTCGAGAT GCATCACGGC
241 GTTTTCCAGA TCAGGGGCTC CCGCAAGTTG CATCACGAGT CCACGGACTT GAGCGGCAGC
301 CTGATCTGTA GCGAGCGCAC CGCCGACAGC TTCAGCCGCG CCTTCAGCGC GCCGGAGAAC
361 GCCACCCCGG ACGGCATCAC CGCCGCCATG GACAAGGGAG TGCTGGTGGT CCCGGTGCCC
421 AAGCGGATTG CGGTGGCGGC GGCGCCTGCC GCGAAGCTGT AGTAGCGGCG AAGCTGTAGC
481 ATCGTGAGCT GCCCCGAGCC GGCCCAGGTT TCAGGGGATT
```

HSP22C PCR product with forward primer

```
  1 CCGTTTTAGN TGGTTGCCCA CCACCAACCC GAGCACCGAG CTTCAATTCT ACAGCGATAC
 61 CGCGATGTCG TTCAACCACG CACTTCGGAG GTCTGCCTCC CAGCTCCTAG CTCGCGCGGC
121 CGCTGCGAAC ACTGCAGCCG AGCTGCCGAC CATGGCGATG GGCACCGTGC GCAACCTATC
181 CTCGCGCTCC GCTGGCGACC TGGTCCTCGG CCCGTTCCGT GGCTGCTCGC CAAGATACAG
241 GTCCCTGGCC CCCTTCCTCC AGCCGTCCAG CCTGGGCAGC CTTTCACGAG CCATGGATGA
301 GCTGCTTCAG ATGGACCGCC ACCTCACCGT GNAAGCTGGC AAAA
```

HSP22C PCR product with reverse primer

```
  1 TTTTGAGTTT GCTTTGCCGA ATAGCTGCCA CTTTTGCAAG TGGTTGCCAA CCAACCAACC
 61 CGAGCACCGA GCTTCAATTC TACAGCGATA CCGCGATGTC GTTCAACCAC GCACTTCGGA
121 GGTCTGCCTC CCAGCTCCTA GCTCGCGCGG CCGCTGCGAA CACTGCAGCC GAGCTGCCGA
181 CCATGGCGAT GGGCACCGTG CGCAACCTAT CCTCGCGCTC CGCTGGCGAC CTGGTCCTCG
241 GCCCGTTCCG TGGCTGCTCG CCAAGATACA GGTCCCTGGC CCCCTTCCTC CAGCCGTCCA
301 GCCTGGGCAG CCTTCACGAG CCATGAGAGT GTCAGCAGGT CCCCCCC
```

HSP22D PCR product with forward primer

```
  1 AAAAATCGNC ATACGNGCAG CAGTAGTGCT ACTGTCGCAA GCACGGTACA CGAGCATCGC
 61 TCCCGCTTTC AGTTTGCCTC AAGCTTACCC CACCACAAAC ACCATGGCCC TTAGCGTGAC
121 TGTGCTTCAC GTTGTCAACA ACGTCGCGGC CCGCGCCGGG CGCTGCCAAA CGCTGCCAAA
181 CGCGTGCGTG CCACCCCAAC GGCCTCAGCC TGGCCACAGC TCGCCCCAGC CAGCCCTGGC
241 CAGCCCCAGC CATCTGGCCC GCCGGATGCT GGACCCGGCA CGCCTGTTCC TGGGCTAAAA
```

HSP22D PCR product with reverse primer

```
  1 TTTGAAACAA CCCACTCTCA CAAATAAGAA GCTCCGTCAA TACGGCAGCA GTAGTGCTAC
 61 TGTCGCAAGC ACGGTACACG AGCATCGCTC CCGCTTTCAG TTTGCCTCAA GCTTACCCCA
121 CCACAAACAC CATGGCCCTT AGCGTGACTG TGCTTCACGT TGTCAACAAC GTCGCGGCCC
181 GCGCCGGGCG CTGCCAAACG CTGCCAAACG CGTGCGTGCC ACCCCAACGG CCTCAGCCTG
241 GCCACAGCTC GCCCCAGCCA GCCCGGCCAG CCCCAGCCAT CTGCCCGCCG ATGGCCCC
```

HSP22E PCR product with forward primer

```
  1 GGGGGGGCGG GAAGGGGGGG GTGATGTACA GTATGTGCCG TGTCGGCGTG TACCATATTG
 61 GTTTCCTGGA TTGTCTCCGC AGTTGCGAGA TGCACATGCG CTGGGAGGCA GCGAGCGCGA
121 TGTTGTTGCA GTATATAAAG CACGTACGGT ACTTCGTGTG TTTGGGTTGT GGTGTTGCGA
181 AGGGGAATGC TTAACAACCT AACACAGCAC AACCGCATGC TGTGCAAGGA GTGGCTTTTG
241 TTTAATGTGG CAAAGGTGTC TGCGACATGA TGCCGCAGCG CCTACATGGG TACAGCTGGC
301 CCTTACTTGC ATCACGGCCA TACCTGTAAC ACAACGTATG GTC
```

HSP22E PCR product with reverse primer

```
  1 TCAGTAGCGT GGTGGATTGT TGCCCAGTGC TATGGCCTTA GGTGTCCATG TACAATATGT
 61 GCCGTGTCGG CGTGTACCAT ATTGGTTTCC TGGATTGTCT CCGCAGTTGC GAGATGCACA
121 TGCGCTGGGA GGCAGCGAGC GCGATGTTGT TGCAGTATAT AAAGCACGTA CGGTACTTCG
181 TGTGTTTGGG TTGTGGTGTT GCGAAGGGGA ATGCTTAACA ACCTAACACA GCACAACCGC
241 ATGCTGTGCA AGGAGTGGCT TTTGTTTAAT GTGGCAAAGG TGTCTGCGAC ATGATGCCGC
301 AGCGCCTACA TGGGTACAGC TGTCCT
```

HSP22F PCR product with forward primer

```
  1 CCCGGAAGTG GGGTCCTGCA AGCAGCTAAG GGGCCGCTGG CATCCTGTGG CATTGACTTC
 61 GTTAGCCCTC GTGTCAAATC CATAGGCATG ATTCCAGACA GTACTGGCAA GTGCGGGGAT
121 GTGCGGTAGC AATCTGACGT GGCGCAGCAT GTGTTTTGAG TGTTGTACCA TATCAAACGT
181 GATGTGGTGG CTCGGGATAA TTTCATGCAT GGCGTTGCAG AGTACAATTG AAACAGGCCT
241 GCATTGGGCA GTCGGACGAT TTATTGAGCG AGTGTGCTGC TAGCAGATGC TGCCTGATTA
301 CTCCCGTCCT GGGCCCTGCC GGAAAGTTGG AAACAGTGCG TTGCACCAGT GCGCACGCGA
361 CATTATCAAA GTGACCGTGG GTGAACTAAT GAGTCCCTGA GCGCATGCTG GTTAG
```

HSP22F PCR product with reverse primer

```
  1 TATTGCAAAT CCATTTGAGG CACGGCAAGC GCTTGCAGAG TGGGGTCCTG CAAGCAAGCT
 61 AAGGGGCCGC TGGCATCCTG TGGCATTGAC TTCGTTAGCC CTCGTGTCAA ATCCATAGGC
121 ATGATTCCAG ACAGTACTGG CAAGTGCGGG GATGTGCGGT AGCAATCTGA CGTGGCGCAG
181 CATGTGTTTT GAGTGTTGTA CCATATCAAA CGTGATGTGG TGGCTCGGGA TAATTTCATG
241 CATGGCGTTG CAGAGTACAA TTGAAACAGG CCTGCATTGG GCAGTCGGAC GATTTATTGA
301 GCGAGTGTGC TGCTAGCAGA TGCTGCCTGA TTACTCCCGT CCTGGGCCCT GCCGGAAAGT
361 TGGAAACAGT GCGTTGCACC AGTGCGCACG CGACATTATC AAAGTACCGT TGACATGT
```

HSP33 PCR product with forward primer

```
  1 AAATAAGGGG TAGCGTACGT TCATGCAGCG ACGCCCATGC TGGCGCCCAC CGTCACCTCC
 61 GCCATGGGCC GCACGCTGCT GGGCGCGCTG CTGATGGGGT CGTTCAGGAA GGAGGATGAG
121 CTGGTGCAGA TCACGTTCCA AGGCAACGGG CCAGCGGGCT CCATTCTGGC CAGCGCTGAC
181 ACGCGCGGCA ACGTCAAGGG GAAAATCAAC AACCCCGCAG CGGACCCTCC TCTGCGCCCT
241 GACGGCAAGC TGAATGTGGG CGGCGCTGTG GGGCCAGGTG TGTTGGCGGT GGTTCGAAGC
301 CACCCGCTGG AGCCACACCC GTACACAGGC ATGGGGCCCA TCGTAAGCGG CGAGGTGGCG
361 GAGGACCTCG CCAACTACCT GGTGGACAGG GAGCAGACTA ACTCGGAACT GGGTCTGGAA
421 CTTCTGTGAC AATAGGAGGC TTGGTAAAAA ATTTCCTAAC GCCATACAGC ACATTACGTA
481 TCTATAATTA TTATTGGGAT TATGAATCCT CATTCATAGT TTATTGTTTG CTTTGGGTCC
```

```
541 TGATTTATAT ATTTGTTTTT ATAATTGAAA TCGTCAAATA CAATAAGAAA AATATATCTT
601 TCCAGTAGGT GCTCAGTTCT ATTATTGGCA TATTTTATGA TGTTATTTAT ATTAATAAAA
661 GAAAATACGA ATATATGAAA ATAATAAAAA ATTTTAATCT GAGAAATTTT TTGATTATTT
721 AATAACATCC GAAATTTTAA TAGGTCAAAG AAGCTAAGTG AAGTAATAGC TATATTTATT
781 ATTTATGCTA TTATTATATT TGATATTATT AAGAAAATAT AAAAAAATAA ATGAAATTAT
841 TAAAATATAA TGTTATGATT ATATATGATA TTTATGATAC ATGCTCGTTA CTTAATTTAT
901 ATTTAAATAG ATAATATATA TAATAGAGAA CAATACTTTC GTTATAGAAA GCAATTTAAT
961 TCATGCACAT TAATCAAATT TTATGAGTAG TACATAGATC
```

HSP33 PCR product with reverse primer

```
  1 GAGGGGAGGC ATTCCAAGAT AGTTGGCGAG ATCGTGCGCA CGTCTCCGCT CGCGATGGGG
 61 ACCATGCCTG TGTACGGCTG CGGCTCCTGC GGATGGCTAC GAACCACCGC CAACACACCT
121 TGCCCCACAG CGCCCCCCAC ATTCAACTTG CCGTCAAGGC GCAAAGGAGG GTCCGCTGCG
181 GGGTTGTTGA TTTTCCCCTT GAC
```

RACK1 PCR product with forward primer

```
  1 AGGCCGACGG CTCGCTGTGC GCCTCGGGCG GCAAGGACGG CATTGCCATG CTGTGGGACC
 61 TGGCTGAGGG CAAGCGCCTG TACAGCCTGG ACGCCGGCGA CGTCATCCAC TGCCTGTGCT
121 TCTCGCCCAA CCGCTACTGG CTGTGCGCCG CCACCCAGTC CTCCATCAAG ATCTGGGACC
181 TGGAGAGCAA GAGCATCGTG GACGACCTGC GCCCCGAGTT CAACATCACC AACAGAAGGC
241 CCAGGTGCCC TACTGCGTGT CGCTGGCCTG GTCGGCCGAC GGCTCCACCC TGTACAGCGG
301 CTACACCGAC GGCCAGATCC GCGTCTGGGC CGTGGGCCAC TCCCTGTAAA TGCCCAGCCG
361 CGCCTAACTG CGGCCTGGCC CTGCAAAATA AGAACATCAA AAAACTAAAA GAATTAAAAA
421 AAAAAAAAAA AAATAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
481 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
541 AAAAAAAAAA AAAAAAAAAA AAAAAAAATA AGAAAAAAAT AATGAAAAAA ATAGAG
```

RACK1 PCR product with reverse primer

```
  1 TAGGCCCCCG CCATCGGTAT TGACCTGGGC ACCACGTACA GCTGCGTGGG TGTCTGGCAG
 61 AATGACCGCG TGGAGATTAT TGCCAACGAT CAGGGCAACC CACCACTCTT ACGCCCTCCA
121 CCCTTACTCT CCCGACGCTC GCTGGCGCTC GACGACAAGG ACGGCATGTC ATGCTGTGGA
181 CCTGGCTGAG GGCAAGCGCC TGTACAGCCT GGACGCCGGC GACGTCATCC ACTGCCTGTG
241 CTTCTCGCCC AACCGCTACT GGCTGTGCGC CGCCACCCAG TCCTCCATCA AGATCTGGGA
301 CCTGAGAGCA AGAGCTTTGG TT
```

The underline sequence indicate PCR product of *GPX5* primer.

```
   1 GGGTGGGCGA CGTCGCATGG CTTCCGGCCG CCATGGCGGC CGCGGGAATT CGATTAGAAG
  61 AGATGGAGTT GTCAGCCGTG TTCTGCAGGG CTAGCAAGCA ACGGCGTGTG TTGGCAGGAT
 121 GCAGCAGGGC GGTGAGGACT TGGAGGGAGA CTCGAACTAC TGCAAGCACC TGGGACAGCC
 181 CTGTTGAGGC ACCCGAGCTA TTTGCCTTTG GATAACTTGA AATGGAGAGG GAAGCATCAA
 241 TAACTTGTGA AATCACTAGT GAATTCGCGG CCGCCTGCAG GTCGACCATA TGGGAGAGCT
 301 CCCAACGCGT TGGATGCATA GCTTGAGTAT TCTATAGTGT CACCTAAATA GCTTGGCGTA
 361 ATCATGGTCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG CTCACAATTC CACACAACAT
 421 ACGAGCCGGA AGCATAAAGT GTAAAGCCTG GGGTGCCTAA TGAGTGAGCT AACTCACATT
 481 AATTGCGTTG CGCTCACTGC CCGCTTTCCA GTCGGGAAAC CTGTCGTGCC AGCTGCATTA
 541 ATGAATCGGC CAACGCGCGG GGAGAGGCGG TTTGCGTATT GGGCGCTCTT CCGCTTCCTC
 601 GCTCACTGAC TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA GCGGTATCAG CTCACTCAAA
 661 GGCGGTAATA CGGTTATCCA CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA
 721 AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG CTGGCGTTTT TCCATAGGCT
 781 CCGCCCCCCT GACGAGCATC ACAAAAATCG ACGCTCAAGT CAGAGGTGGC GAAACCCGAC
 841 AGGACTATAA AGATACCAGG CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC
 901 GACCCTGCCG CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC
 961 TCATAGCTCA CGCTGTAGGT ATCTCAATTC GGGGGTAGGT CGTTCGCTCC AAGCTGGGCT
1021 GTGTGGACCA AACCCCCCGT TTAACCCCAA CCGCTGGGGC TTATTCCGGG AACAATCGTC
1081 TTTGAGTCCA CCCCCGGGAG AACACAACTT ATTTGCCCCT GGGGGGCAGC ACCTGTGTAA
1141 AAGGATTATC CAAACCAAGG GTTGTTTTGC GGGCGTTACA AAATCTTTTA AAAGGGGGCC
1201 GGACCAATCG GCTCTCTTCA AAAAACAAAA ATTTTTTGTT TTTCGCCCCT CTCTTTAGAC
1261 CAATTTATTT CTCCCAAAAA AAGAATAGGT GGGTGCCTTG ATCCTGGGAA AACAACAACC
1321 CACCGGGGGG GGGGGGGGGT GTTTTTTTGT TTTTCTTCAC CCCCCAAATA ATCCCCCCCC
1381 CAAAAAAAAA AGATTTCTCT AAAAAAATCC TCTTGGATTT TTTTCTCTCC GGGGGTGCTG
1441 GCCCCCCCTT GGGGGACAGA AGAACCCCCC CTTAAAAGAG AATTTTTTGG GGTTGAGGGA
1501 ATTTTTTAAA AAAAGGAGTT TTTCCCCCCA AACCCCTCTT TTTTCTTTTT TA
```

APPENDIX C

DNA sequencing results of real-time PCR products

The underline sequences indicate real-time PCR products.

HSP90B PCR product with the alternative primer set

```
  1 TCTCGGGCCC TGGGGGCCCT CGGGGGGAAG GGAGAAAGGG GGACCAGGGA CCCGGAAAGC
 61 GGCAGGGGGG GAACAGGAGA GCGCACGAGG GAGCTTCCAG GGGGAAACGC CTGGTTTCTT
121 TTTTGTCCTG TCGGGTTTCG CCACCTTTGA CTTGAGCGTC GATTTTTGTG ATGCTCGTCA
181 GGGGGGCGGA GCCTATGGAA AAACGCCAGC AACGCGGCCT TTTTACGGTT CCTGGCCTTT
241 TGCTGGCCTT TTGCTCACAT GTTCTTTCCT GCGTTATCCC CTGATTCTGT GGATAACCGT
301 ATTACCGCCT TTGAGTGAGC TGATACCGCT CGCCGCAGCC GAACGACCGA GCGCAGCGAG
361 TCAGTGAGCG AGGAAGCGGA AGAGCGCCCA ATACGCAAAC CGCCTCTCCC CGCGCGTTGG
421 CCGATTCATT AATGCAGCTG GCACGACAGG TTTCCCGACT GGAAAGCGGG CAGTGAGCGC
481 AACGCAATTA ATGTGAGTTA GCTCACTCAT TAGGCACCCC AGGCTTTACA CTTTATGCTT
541 CCGGCTCGTA TGTTGTGTGG AATTGTGAGC GGATAACAAT TTCACACAGG AAACAGCTAT
601 GACCATGATT ACGCCAAGCT ATTTAGGTGA CACTATAGAA TACTCAAGCT ATGCATCCAA
661 CGCGTTGGGA GCTCTCCCAT ATGGTCGACC TGCAGGCGGC CGCGAATTCA CTAGTGATTT
721 ATCGGCATGA CCAAGGACGA CCTGATTAAG AACCTGGGCA CCATTGCCAA GTCGGGCACC
781 TCGGCCTTCC TGGAGCAGGT GCAGAAGGGT GGCGACATGA ACCTGATCGG CCAGTTTGGT
841 GTAATCGAAT TCCCGCGGCC GCCATGGCGG CCGGGAGCAT GCGAAGCTTG
```

HSP70A PCR product with the alternative primer set

```
   1 ACCCTCATTA TCAACCAACA GAAGCGGGGT GTGTATTTGT TTTGTTTTTT AGAAGAAATA
  61 ATTATAATAA GTGATTTTTT CACCTTTTTT TTTTTTATTT TCTGTGAGAG GTAAAAATTG
 121 GTGATTTTAA AGCAAAAGAG ACGACAGAAA AACACAAAAT TATTTTTTTT TTTTATGGTG
 181 TTTACCCGGT AGTTTAGGCC CCCCCACCTT CAAAAGAATT TTTTGTACCA CCGGCCTTAA
 241 AATCGCTCAA TTTTTTATAA ACCCGGTTAC CACGTGGGTG GTTGCCAGGG GGGGAAAAAT
 301 TTGTGGTTTT TCCCGGGTTG GGTTTCAAAG AGAGTTGGTT TCCGGATAAA GGGGCAAGGG
 361 GTCGGGGTTG AAACGGGGGG GTTTGTGCTC CCCAGCCCCA CTTGGGAGGG AATCGGACTT
 421 ACAACGGAAC TGGAAATCCT TTCCAGCGTG GAGTTCGGAG AAGGCGGCCA CGCTTTCCGG
 481 AAGGGGAGAA AGGGGGAACA GGTTTCCGGT AAGGCGGCAG GGTCGGAACA GGAGAGCGCA
 541 CGAGGGAGCT TCCAGGGGGG AACGGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC
 601 CTTTGACTTG AGCGTCGATT TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC
 661 GCCAGCAACG CGGCCTTTTT ACGGTTCCTG GCCTTTTGCT GGCCTTTTGC TCACATGTTC
 721 TTTCCTGCGT TATCCCCTGA TTCTGTGGAT AACCGTATTA CCGCCTTTGA GTGAGCTGAT
 781 ACCGCTCGCC GCAGCCGAAC GACCGAGCGC AGCGAGTCAG TGAGCGAGGA AGCGGAAGAG
 841 CGCCCAATAC GCAAACCGCC TCTCCCCGCG CGTTGGCCGA TTCATTAATG CAGCTGGCAC
 901 GACAGGTTTC CCGACTGGAA AGCGGGCAGT GAGCGCAACG CAATTAATGT GAGTTAGCTC
 961 ACTCATTAGG CACCCCAGGC TTTACACTTT ATGCTTCCGG CTCGTATGTT GTGTGGAATT
1021 GTGAGCGGAT AACAATTTCA CACAGGAAAC AGCTATGACC ATGATTACGC CAAGCTATTT
1081 AGGTGACACT ATAGAATACT CAAGCTATGC ATCCAACGCG TTGGGAGCTC TCCCATATGG
1141 TCGACCTGCA GGCGGCCGCG AATTCACTAG TGATTAGGCC CCCGCTATCG GTATTGACCT
1201 GGGCACCACG TACAGCTGCG TGGGTGTCTG GCAGAATGAC CGCGTGGAGA TTATTGCCAA
1261 CGATCAGGGC AACCGCACCA CTCCCTCGTA CGTGGCCTTC ACGGACACTG AGCGTCTGAT
1321 TGGTGATGCC GCCAAATCGA ATTCCCGCGG CCGCCATGGC GGCCGGGAGC ATGCGACTTC
1381 GGCTAAT
```

CPN60B1 PCR product with the alternative primer set

```
   1 CGTCGCTTTC TGTAAAAAAA AAACAACACA AAAAAAAAAA AAACCCAACC CGGCTTACAC
  61 CCCGGGCGGG GGGGGGGTTT GTTTTTGAGC CCGAAATCAA AAAAGGGTTA CCAAAATTTT
 121 TTTTTTTCAA AAAGGTAAAC CTGGGGTTTA GGCAGAGGCG GCAAAGAACA CAAAATTATT
 181 GTTTTTTTTA GGGTAACCGT TAGTTGGGGC CCCCACTTTC AAAAACTTTG TAAGCACCGC
 241 CTTACTTCCC CCGTTTTGTT AATCCTGTTA CCCAGGGCGG CTTGCCGGGG GGAATAATTG
 301 GTTTTTTACC GGGTGGAATT CAGGAGAATA GTTACCGGAT AAGCGCAGCC GTCGGGCTGA
 361 ACGGGGGGTT CGTGCACACA GCCCAGCTTG GAGGGAAGGA CTTCAACCGA ACTGAGATAC
 421 CTACAGCGTG AGTTATGAGA AAGCGCCACG CTTCCCGAAG GGAGAAAGGC GGACAGGTAT
 481 CCGGTAAGCG GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC
 541 TGGTATCTTT ATAGTCCTGT CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTTGTGA
 601 TGCTCGTCAG GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCTT TTTACGGTTC
 661 CTGGCCTTTT GCTGGCCTTT TGCTCACATG TTCTTTCCTG CGTTATCCCC TGATTCTGTG
 721 GATAACCGTA TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG AACGACCGAG
 781 CGCAGCGAGT CAGTGAGCGA GGAAGCGGAA GAGCGCCCAA TACGCAAACC GCCTCTCCCC
 841 GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG GAAAGCGGGC
 901 AGTGAGCGCA ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC
 961 TTTATGCTTC CGGCTCGTAT GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA
1021 AACAGCTATG ACCATGATTA CGCCAAGCTA TTTAGGTGAC ACTATAGAAT ACTCAAGCTA
1081 TGCATCCAAC GCGTTGGGAG CTCTCCCATA TGGTCGACCT GCAGGCGGCC GCGAATTCAC
1141 TAGTGATTAG CCCTGGAGTG TCTTATGGTG CAATGCACAT GTGGCACTTT CGCATGGCGG
1201 TGACAAGGCA CGGCGCGTTG ACCTGGCTTG AGTGATGCTC AGAAAGCATG AGAGCTGGGC
1261 CCCCGCTATG CTGCGGCGGG TAATCGAATT CCCGCGGCCG CCATGGCGGC CGGAGCCATG
1321 GANGTGGGCC AGCAC
```

HSP22A PCR product with the alternative primer set

```
   1 GGGTGGCCGA ATCCATGCTC CGGCCGCCTG GCGGCCGCGG GAATTCGATT TGTGTTCGTT
  61 GACACGAGCA GTGACGCTCA CTTGCGTGGG CCGGCAAGCC CCACAACATT GACTTTGTGG
 121 CTCCGTAGAG ACTTCATGCC GGGCTGAATC TGCTATATGT TTGTGTACTA TCAAATTGTG
 181 GCCCGAATCA CTAGTGAATT CGCGGCCGCC TGCAGGTCGA CCATATGGGA GAGCTCCCAA
 241 CGCGTTGGAT GCATAGCTTG AGTATTCTAT AGTGTCACCT AAATAGCTTG GCGTAATCAT
 301 GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC AATTCCACAC AACATACGAG
 361 CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT GAGCTAACTC ACATTAATTG
 421 CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA
 481 TCGGCCAACG CGCGGGGAGA GGCGGTTTGC GTATTGGGCG CTCTTCCGCT TCCTCGCTCA
 541 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG
 601 TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA GAACATGTGA GCAAAAGGCC
 661 AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC
 721 CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC
 781 TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
 841 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCATA
 901 GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC
 961 ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA
1021 ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAA
1081 CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAATGGGG GCCTAACTAC GGGTACACTA
1141 GAAGAACAGT TTTTGGGATC TGCGCTCTGC TGAAACCCAT TACCTCTCGA AAAAAAATTG
1201 GTAGCTCTTG TTCCGGAAAA AACCACCCCG GGGGGCGGGG GGTTTTTTGT TTGTAACAAC
1261 CAAATAACCC CCCAAAAAAA AGATTTCACA AAAACCTTTT ATTCTTTTTC TGGGGGTGAG
1321 GCTCCGGGAC GAAACACCCC CTCCGGGGTG TGTGTGGGGG TTTTTAAAAA
```

RACK1 PCR product with the alternative primer set

```
  1 GGTAGGCGTA GGTCGCATGG CTCCGGCCGC CATGGCGGCC GCGGGAATTC GATTAGTTCT
 61 GCCTGACTGG CTCGTGGGAC GGCACCCTGC GTCTGTGGGA CCTGAACACC GGCACCACCA
121 CCCGCCGCTT CGTGGGCCAC ACCAAGGATG TGCTGTCCGT GGCTTTCTCG GTGGACAACC
181 GCCAGATCGT GTCGGGCTCG CGCGACAAGA CCATCAAGCT GTGGAAATCA CTAGTGAATT
241 CGCGGCCGCC TGCAGGTCGA CCATATGGGA GAGCTCCCAA CGCGTTGGAT GCATAGCTTG
301 AGTATTCTAT AGTGTCACCT AAATAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG
361 TGAAATTGTT ATCCGCTCAC AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA
421 GCCTGGGGTG CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT
481 TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA
541 GGCGGTTTGC GTATTGGGCG CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC
601 GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA
661 TCAGGGGATA ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT
721 AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
781 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT
841 CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG
901 TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG TAGGTATCTC
961 AGTTCGGTGT AGGTCGTTCG CT
```

APPENDIX D

Dissociation curves

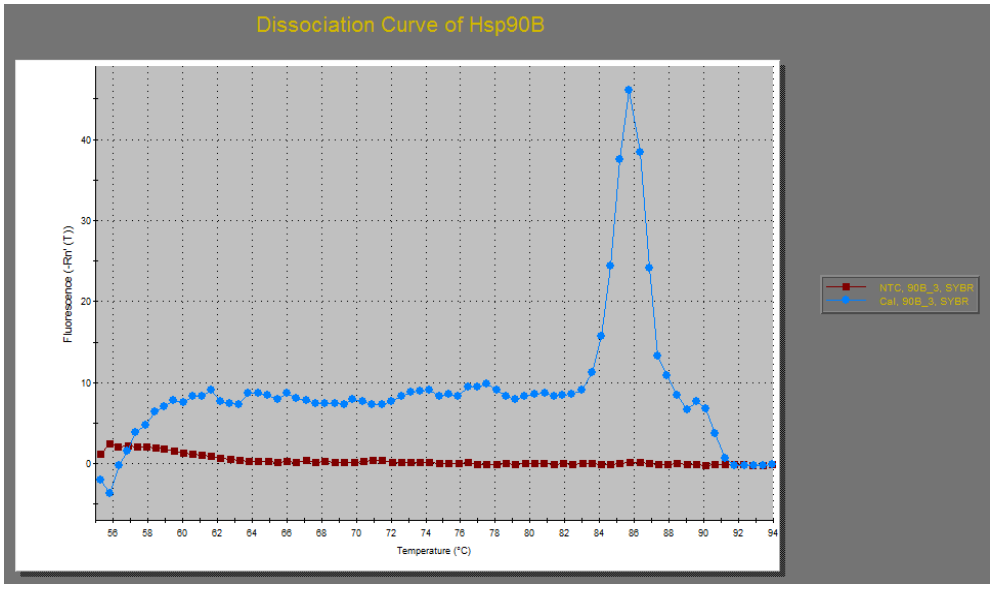

Figure 9.2 The dissociation curve of *HSP90B* PCR product of the alternative primer.

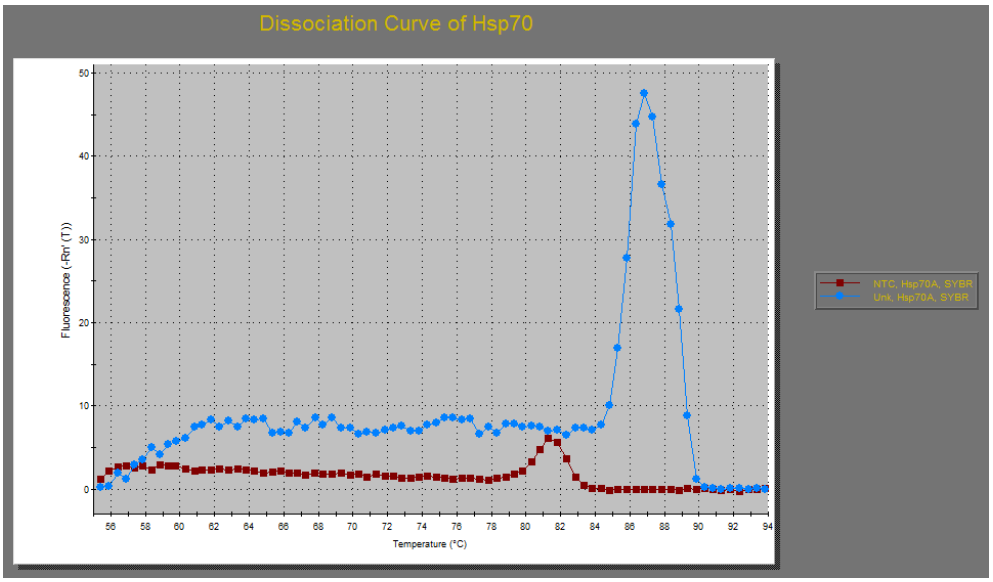

Figure 9.3 The dissociation curve of *HSP70A* PCR product of the alternative primer.

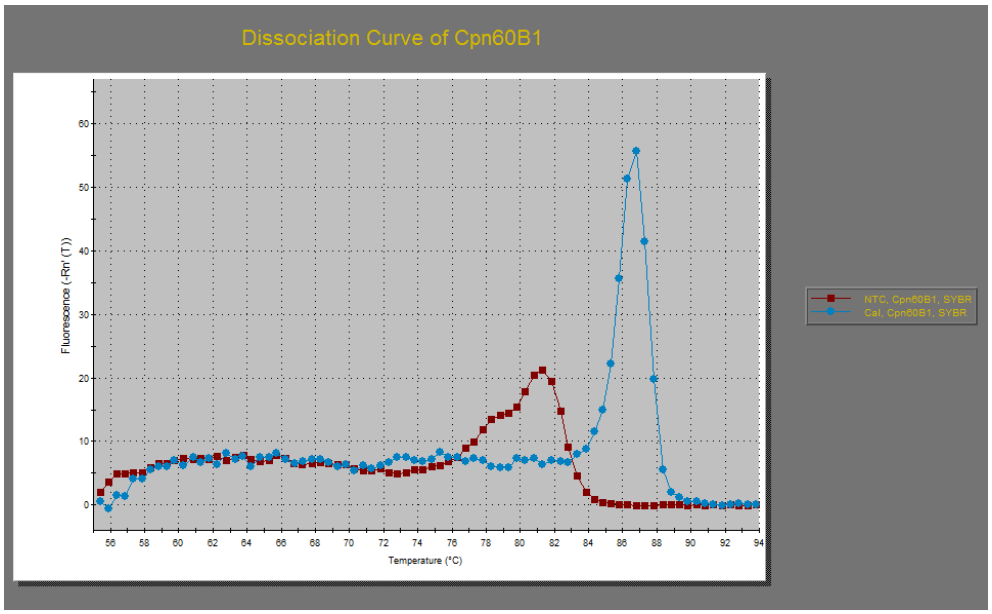

Figure 9.4 The dissociation curve of *CPN60B1* PCR product of the alternative primer.

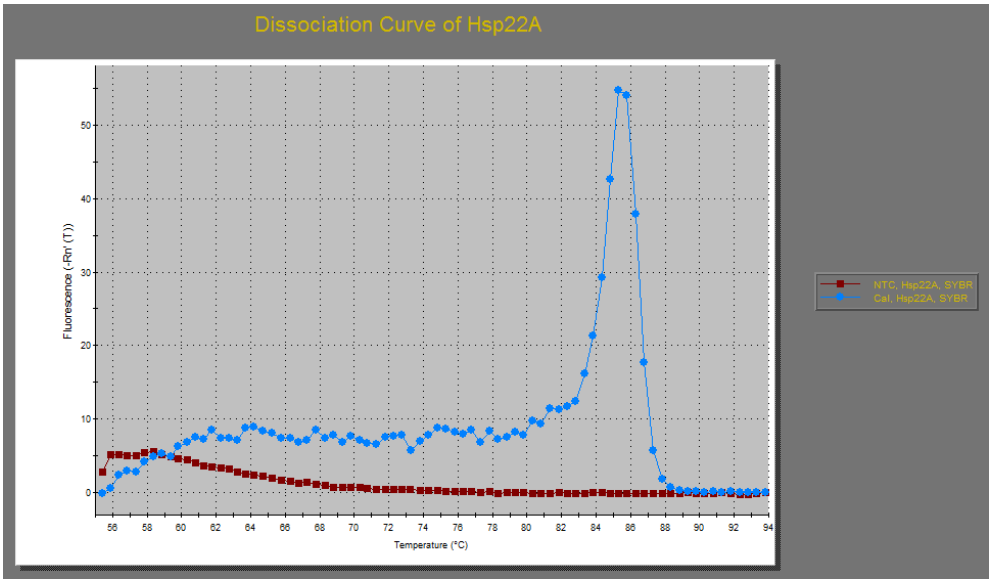

Figure 9.5 The dissociation curve of *HSP22A* PCR product of the alternative primer.

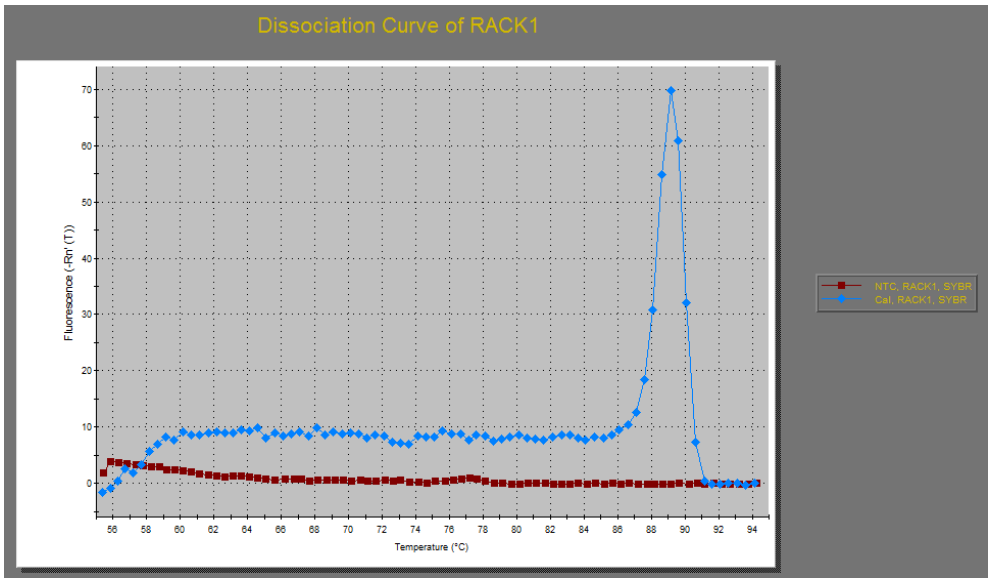

Figure 9.6 The dissociation curve of *RACK1* PCR product of the alternative primer.

APPENDIX E

Standard curves

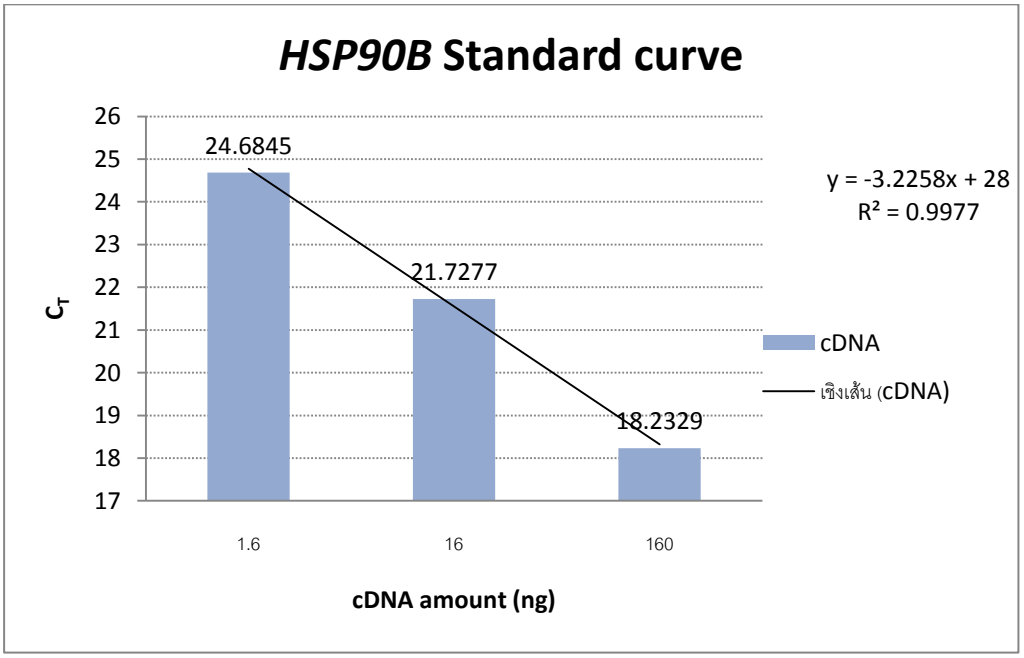

Figure 9.7 The standard curve of *HSP90B* product of the alternative primer.

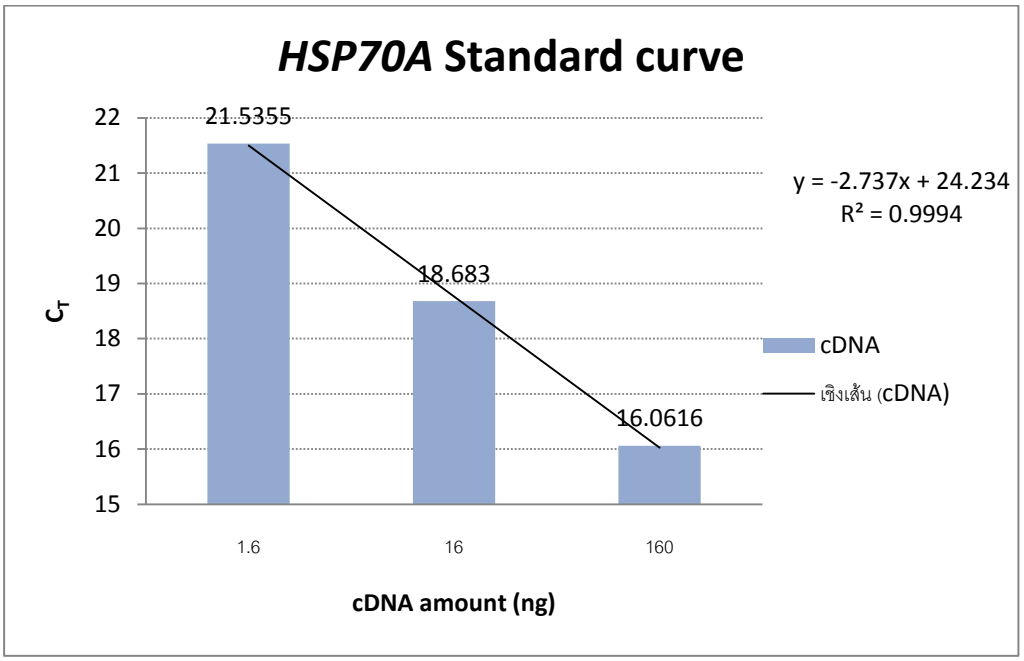

Figure 9.8 The standard curve of *HSP70A* product of the alternative primer.

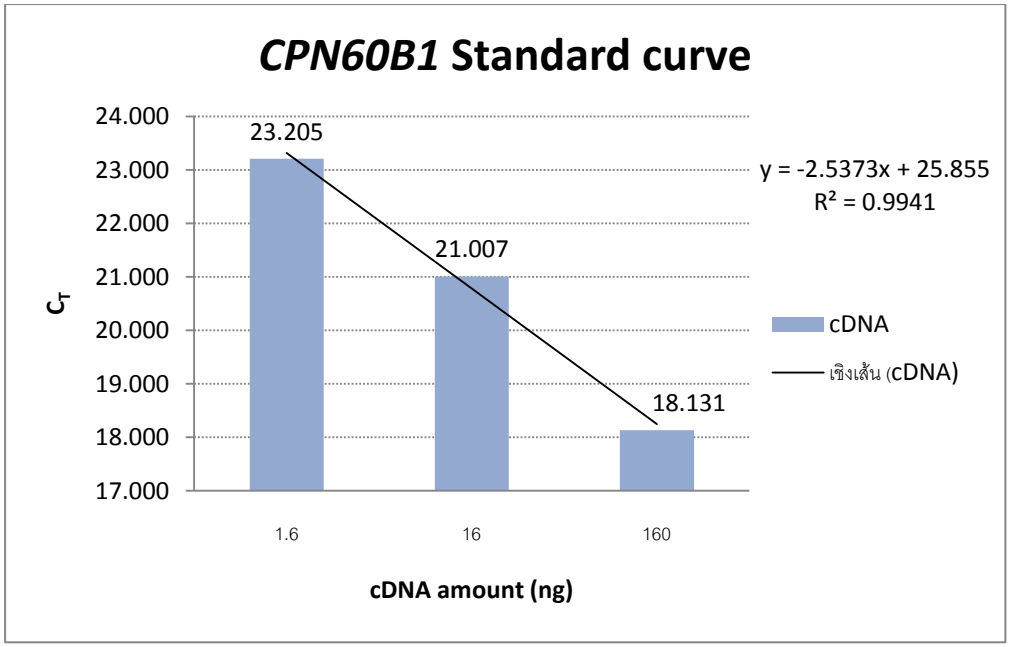

Figure 9.9 The standard curve of *CPN60B1* product of the alternative primer.

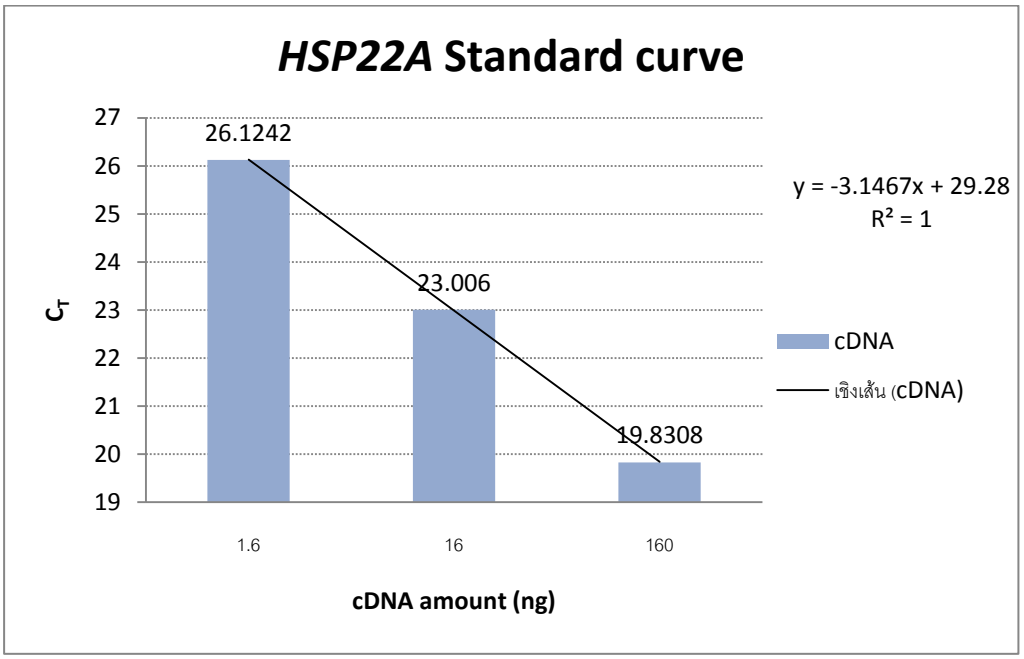

Figure 9.10 The standard curve of *HSP22A* product of the alternative primer.

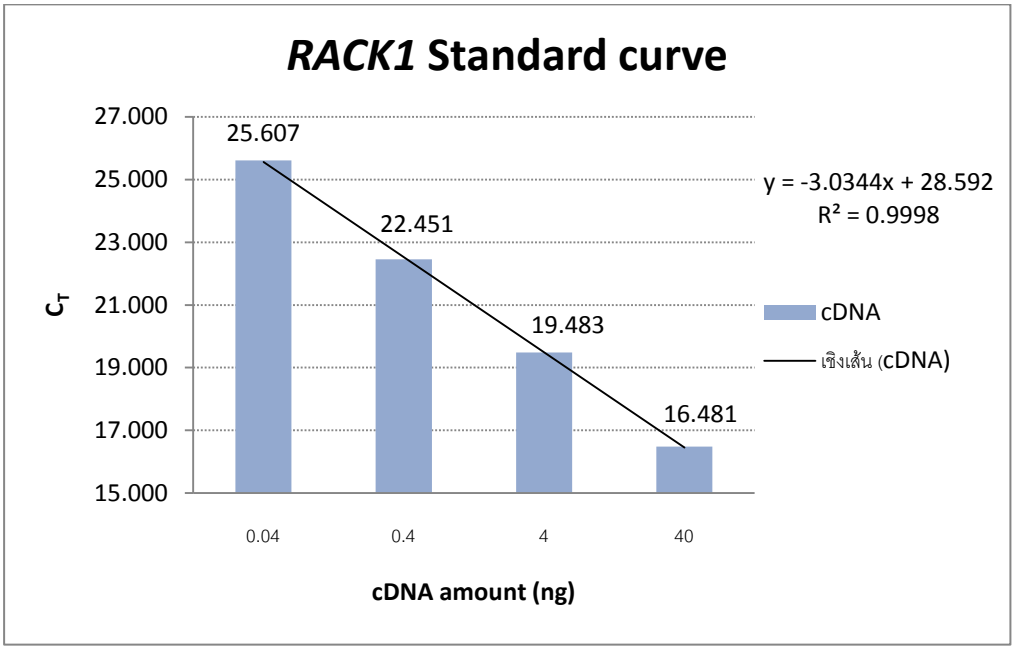

Figure 9.11 The standard curve of *RACK1* product of the alternative primer.

APPENDIX F

Two mathematic models can be equivalent

When both %E of housekeeping and interested gene are equal to 100, the efficiency-correlated model is equivalent to the $\Delta\Delta C_T$ model as these following. The designated of C_Ts were shown as sample/gene. For example; CAL/GOI means C_T from normal condition sample (CAL) using interested *HSP* gene primer (GOI).

$$\text{Fold change} = \frac{[1 + (\%E_{GOI}/100)]^{(C_T CAL/ - C_T\ UNK)_{GOI}}}{[1 + (\%E_{NORM}/100)]^{(C_T CAL - C_T\ UNK)_{NORM}}}$$

When both %E were subtracted by 100, they were 2 and their power can perform plus and minus. The following equations were derived by focusing on the C_Ts.

= (C_T CAL/GOI - C_T UNK/GOI) - (C_T CAL/NORM - C_T UNK/NORM)
= C_T CAL/GOI - C_T UNK/GOI - C_T CAL/NORM + C_T UNK/NORM
= C_T UNK/NORM - C_T UNK/GOI + C_T CAL/GOI - C_T CAL/NORM
= - (C_T UNK/GOI - C_T UNK/NORM) + (C_T CAL/GOI - C_T CAL/NORM)

In both bRACKets, the GOI minus NORM with the similar sample type belongs to the form of ΔC_T.

= - ΔC_TUNK + ΔC_TCAL
= - (ΔC_TUNK - ΔC_TCAL)
= $-(\Delta\Delta C_T)$

APPENDIX G

The putative promoter sequence of selected *HSP* genes

HSP90B putative promoter sequence

```
  1 GAAAGCTGCA AAGTGCGCAA CACGGCCAAC CGCGGCCATG TCCCACCGGA AGTGCCTAGA
 61 CCCGCTGGCC GCCATACTGC ACCGGGGTGA GCATATGGAG CAATGATGCA GTGATACATC
121 CACAACACCG GTAGCCGCAC AGCATGTTGT TTCCCTGGTT TGGGTGTTTG GCGCCATTAC
181 TGCGTCCAAG TCGCTGTGAA GGAGAGCTGC CTTTCCGAGC AGCCAGTTGA TGTGATATGT
241 ACATCAAGAG GATGTGTTTC CGCCAGTCCT ATCGGCGCCC AGAAAGCTCT GGGCAAGCAG
301 CTTGAGACAC CTCACCACGT GCACTTTTTG CCCCATTCCG CTCCATGCTC GGCATAGAAG
361 TTTCTATGGG CGGGTCCCTG CGCAACAGCT CGGCGTGCCA GTGTGACTAT TACACTGTAG
421 TATCTGTGTA AACAAGGCCC GAGCAGGGCG TGTCTAGACA AGACTCTTGG AGCAGCAGGA
481 GATCAACACA CATCTCGCGC
```

HSP70A putative promoter sequence

```
  1 GCTCCTGCTA CAGCCCCTTG CAACACCGCC GACCTCGGGA AGGTGGAGTT CTCAGCGCGG
 61 TGGCCGCTTG CCCCGGCCGG CAGCTCCGCA GGGCACACGT CACGCGAAGG GCCGCGACGG
121 TTCGAGAACC GACTTGAGGG CGCCAAACGA GCCCGAGCCG CCGTTGCGCC AGGCGAAACC
181 AGAACCGTAG ATTAATGCAC TTGAGCTATT CATTGGAGCG ATCTGCCGGG GACAGCGGGT
241 CTGGCGTGCG CGCGATTGGA GATCGCAAAT TACATATGTC TGCGTGACGG CGGGGAGCTC
301 GCTGAGGCTT GACATGATTG GTGCGTATGT TTGTATGAAG CTACAGGACT GATTTGGCGG
361 GCTATGAGGG CGGGGGAAGC TCTGGAAGGG CCGCGATGGG GCGCGCGGCG TCCAGAAGGC
421 GCCATACGGC CCGCTGGCGG CACCCATCCG GTATAAAAGC CCGCGACCCC GAACGGTGAC
481 CTCCACTTTC AGCGACAAAC
```

CPN60B1 putative promoter sequence

```
  1 GTGGCTAGGC AGTTTCTCGT TGTCACTGTT GTGTGTTTAC ATAAGCTGGA TGTCCCATTG
 61 CGTTTTGTGT AGAAGCCACA GATGCGTCCC CTTGCATGGC TGGTCGACAC AAGTCACGGC
121 TGTGTTCGGT GCCAGGATGG CCCCCACGCA CCCGCTGCCA ACCCAGACAA GCGTTCCGAG
181 GGAAAGCCAG TACCCTAGAA GCAGCTACCA TATCCATTGG TTGGAACTCT TGAAACACAA
241 AGGATGCAGT GAAGGGCAAC TGCAAAAGTA CACCAACCTG GCGCCGTGCC AGGAGCTTTC
301 TAGAAGAGGA GTGGCCTGTC CCACGGTTCC GCTTCGGCTC TTCAAGCTTG TATGGTGCAG
361 AGGCCGATAC ATCTGAAGAT TGCACCTTCG GAAGATAGCT CAAGTCCGCT GTGGTCACTG
421 TGCACGAGTT CGCTTGCATG GACCTCGGTC TGCAATTTTT CTCTAGACGC TCGTCTGCAC
481 TTGGGTGCGA GCCTTTCCCG
```

HSP22A putative promoter sequence

```
  1 GGCGTCGGCA TGCAACTCAT ACCCGCTTGG AGTTTCTACA ATGTCCATGG GCGCTGTTGG
 61 CCCGGAAAGA TCCCAGCTCT GGTCGCGCGG CGTTGCGATA TTGTCCCGCT CGTGCGTGTG
121 GCGCTCAGTG TGGCGCTTGC GCTGCCGCTC GTGGCCATGC TGGAAGGCAT CTTCCGACGC
181 AAAGCCGAGG CCTAGCGGCG TCAGCTCGGC ATCAAAAATG GGTTCAGAAA ACAGAGATAT
241 GAGAGCCATT ATCGTGATGC CGAATATCGT GGAAAATCTG GAAGTCGGGC TAGCTTTAAA
301 AGTTGTTGCT CGGGAGTATT CCCGAAAACT GCGAGCAAAG AGTTTACAAT GACAAGTAGG
361 AGCAACGCAG TTCTCGTTGG CTCTAGAAGC GACCGGAAGC CTTTCGGGAA CTTTCGGCGG
421 TTTGGCACAA ACCGCTTCTC GAAGCGTCGG TGCTCCTATT AAAACCCCGC CTGCGCTGCC
481 TTGCGGTCAG AAACTGCAAC
```

APPENDIX H

Other *HSP* gene expression profiles

There was an expression profiles which is not included in this study. At this time, the algal cells were treated with 4 mM H_2O_2 for ½, 1 and 2 hr. This concentration (4 mM) was selected from the screening shown in Appendix I. The expression of 8 genes (*CLPB3*, *CLPB4*, *HSP70D*, *HSP70E*, *HSP70F*, *HSP70G*, *HSP22G* and *HSP22H*) was excluded. Two new primer sets were added included *GPX5* (glutathione peroxidase) and *HSP33*AL (the alternative primer of *HSP33*). The *GPX5* PCR product was similar to the selected region. This confirmed by DNA sequencing. See the Appendix B for the DNA sequencing results. The *HSP33*AL PCR product was not confirmed.

Table 9.1 NCBI reference sequences, selected region and PCR product size of *GPX5* and *HSP33*AL primer

Genes	NCBI reference	Selected region	Product size (bp)
GPX5	XM_001698523.1	759-953	195
HSP33	XM_001700394.1	1-175	175

Table 9.2 Additional primer sequences and their optimal conditions.

Genes and label	Primer sequences (FW-Forward, RV-Reverse)	Annealing temperature (°C)	Optimized cycle numbers
*HSP33*AL	FW: AGCACATTTACACTGCACC RV: AAGGTAATCCTCGCGACC	57	35
GPX5	FW: AGAAGAGATGGAGTTGTCAG RV: TCACAAGTTATTGATGCTTC	56	22

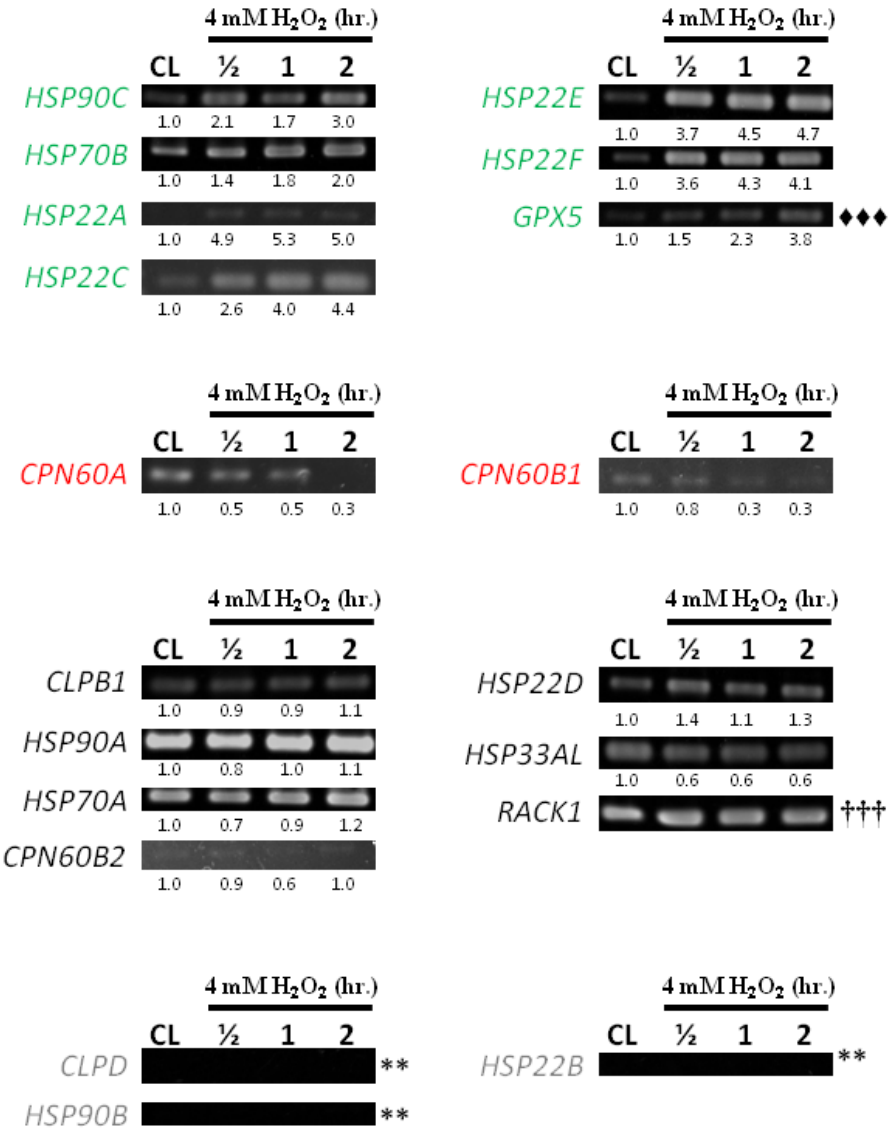

Figure 9.12 Semi-quantitative RT-PCR *HSP* gene expression profiles under 4 mM H_2O_2 for different durations. Each of them was normalized to *RACK1* (†††) then compare with its expression under normal condition (CL). The *GPX5* (♦♦♦) served as positive control. Asterisks (**) indicated the gene expression which may had problems and cannot be observed.

The increasing of *GPX5* expression compared to normal showed that it was proper to induce the cells. Six *HSP* genes included *HSP90C*, *HSP70B*, *HSP22A*, *HSP22C*, *HSP22E* and *HSP22F* were increased compared to normal condition. Two genes included *CPN60A* and *CPN60B1* were decreased compared to normal condition. Several *HSP* genes were expressed similar to normal condition.

APPENDIX I

The optimal concentration of ROS generating agents

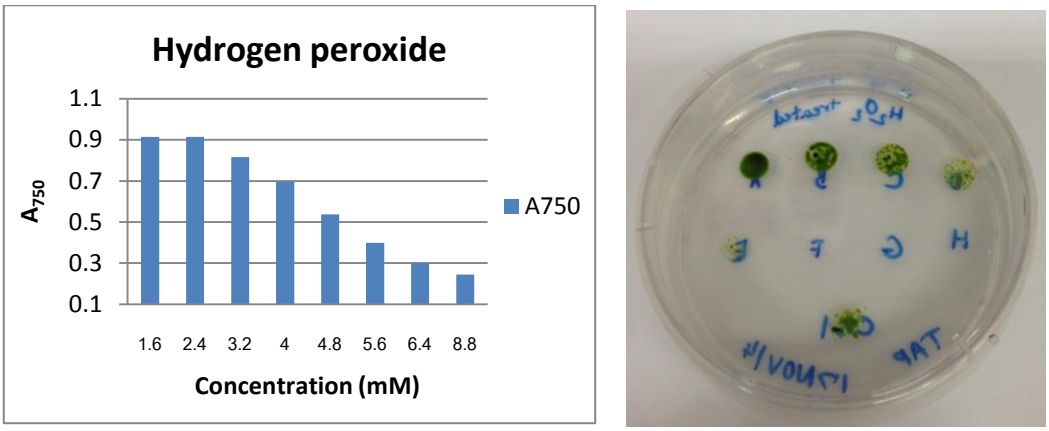

Figure 9.13 The Absorbance of H_2O_2 treated cells at various concentration (left) and their vitality test on TAP agar (right)**.** The alphabets (A-H) from left to right indicated H_2O_2 concentration from low to high compared to the left graph. The optimal concentration of H_2O_2 was 4 mM.

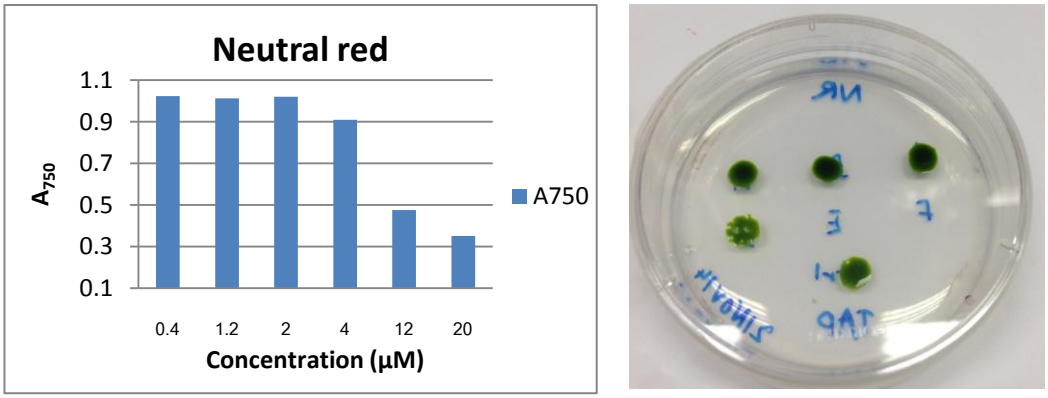

Figure 9.14 The Absorbance of neutral red treated cells at various concentration (left) and their vitality test on TAP agar (right)**.** The alphabets (A-F) from left to right indicated neutral red concentration from low to high compared to the left graph. The optimal concentration of neutral red was at least 4 mM but not equal to 12 mM.

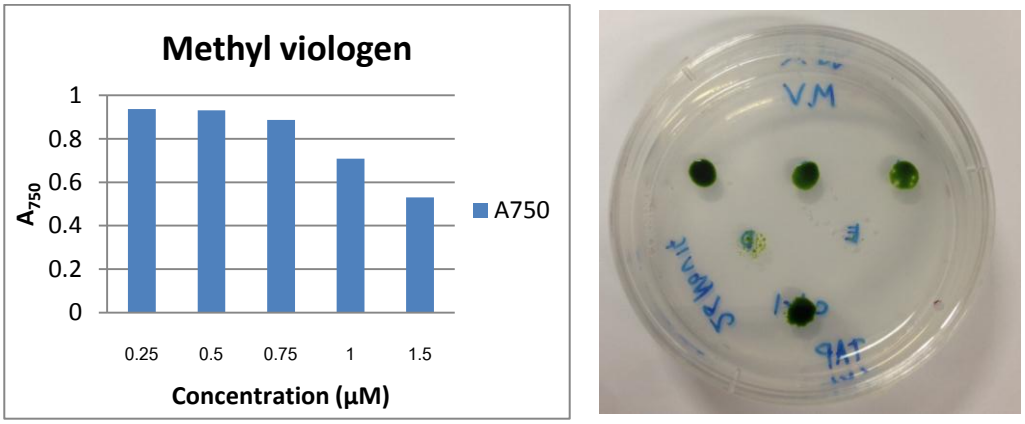

Figure 9.15 The Absorbance of methyl viologen treated cells at various concentration (left) and their vitality test on TAP agar (right)**.** The alphabets (A-E) from left to right indicated methyl viologen concentration from low to high compared to the left graph. The optimal concentration of neutral red was at least 1 µM but not higher than 1.5 µM.

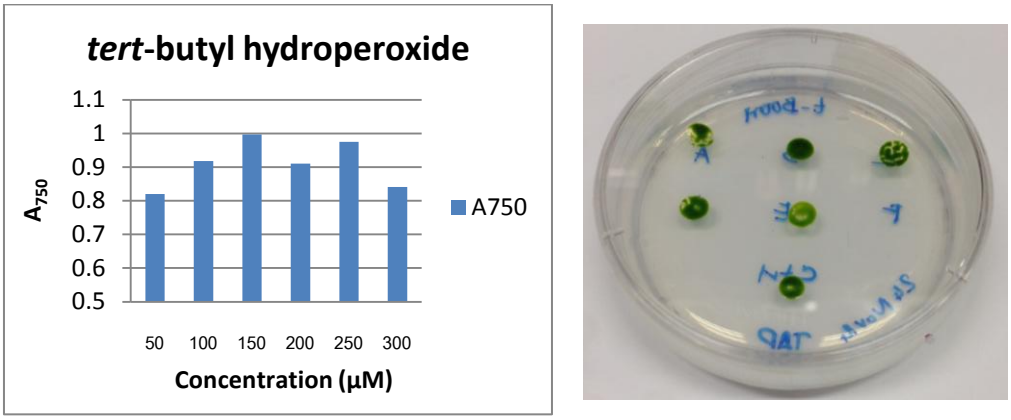

Figure 9.16 The Absorbance of *tert*-butyl hydroperoxide treated cells at various concentration (left) and their vitality test on TAP agar (right)**.** The alphabets (A-F) from left to right indicated *tert*-butyl hydroperoxide concentration from low to high compared to the left graph. The optimal concentration of *tert*-butyl hydroperoxide was 250 µM.

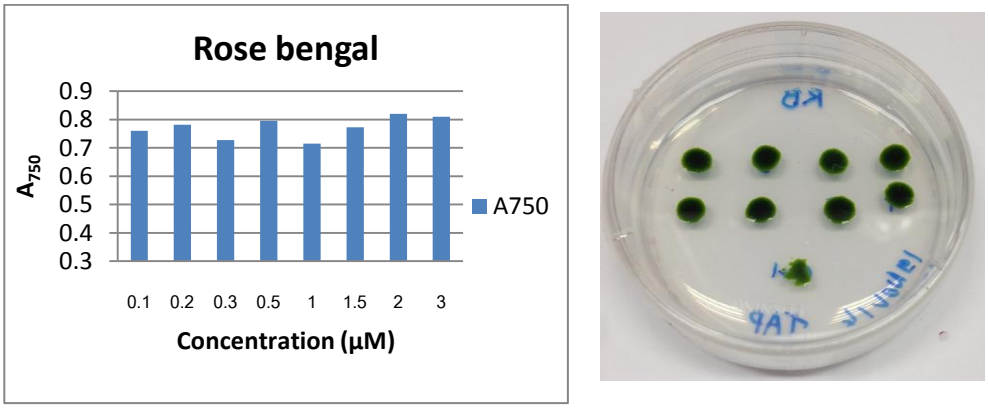

Figure 9.17 The Absorbance of rose bengal treated cells at various concentration (left) and their vitality test on TAP agar (right)**.** The alphabets (A-H) from left to right indicated rose bengal concentration from low to high compared to the left graph. The optimal concentration of rose bengal was at least 3 µM.

BIOGRAPHY

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