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Master of Science in
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**UV-B-INDUCED EXPRESSION OF HEAT SHOCK
PROTEINS ON TOMATO (*Solanum lycopersicum*)
PLANTS**

by

ÖMER FARUK KARABULUT

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APPROVAL PAGE

This is to certify that I have read this thesis written by Ömer Faruk KARABULUT and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biology.

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ABSTRACT

The use of some chemical substances may have unfavourable effects on living and non-livings. Some of these substances can damage the ozone layer, which has critical role in filtering harmful rays such as ultraviolet. Depletion of ozone layer causes loss of filtering feature of stratosphere, therewithal harmful rays can pass ozone layer without absorption. Ultraviolet light can have a damaging effect on skin and lead to skin cancer. Ultraviolet radiations pose a threat to not only humans, but also all living organisms and inanimate. Plants use solar energy for growth and food production. However, ultraviolet rays come along with photosynthetic rays to the Earth's surface and this situation can cause some adverse effects on plants. Naturally, all living organisms have some defence mechanisms to protect themselves against abiotic and biotic stresses. HSPs (Heat Shock Proteins), also named as Stress Proteins play a critical role in protecting living organisms against biotic and abiotic stress types. In this study, we have tried to analyse expression rates of *Hps* genes (*Hsp17.4*, *Hsp17.7*, *Hsp17.8*, *Hsp20*, *Hsp21.5*, *Hsp23.8*, *Hsp70*, *Hsp90* and *Hsp100*) under high dose UV-B light by using quantitative real-time PCR method. Our results demonstrated that most of the *Hsp* genes were down-regulated except *Hsp23.8* and *Hsp90*. *Hsp23.8* expression was not significantly changed. *Hsp90* gene expression was found to be up-regulated under UV-B. These results showed that *Hsp90* can play critical role in protecting tomato plant against UV-B.

Keywords: Tomato, Ultraviolet-B Effects, Heat Shock Protein, Stress

DOMATES (*Solanum lycopersicum*) BİTKİSİNDE ISI ŞOK PROTEİNLERİNİN UV-B SEBEPLİ EKSPRESYONU

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ÖZ

Bazı kimyasal maddelerin kullanımı canlı ve cansız varlıklar üzerinde negatif etkiler oluşturmaktadır. Bu maddeler aynı zamanda ozon tabakasına zarar vermektedir. Ozon tabakasının incilmesi, stratosfer tabakasının filtreleme özelliğinin kaybolmasına sebep olmaktadır ve böylelikle zararlı ışınlar ozon tabakasında filtre edilemeden dünyaya ulaşabilmektedir. Ultraviyole gibi zararlı ışınlar insan cildine zarar vermekte ve cilt kanserine sebep olabilmektedir. Bu ışınlar sadece insanlara değil, aynı zamanda yaşayan tüm canlı ve cansız varlıklara karşı da bir tehdit oluşturmaktadır. Örneğin, tarım sektörü gıda üretiminde dünyada birinci sırada yer almaktadır. Tarım alanında kullanılan bitkiler gibi tüm fotosentetik bitkiler de gelişmek ve besin üretmek için güneş ışınlarını kullanmaktadır. Fakat ultraviyole ışınlar diğer fotosentetik ışınlarla birlikte dünyaya ulaştıklarından bu durum bitkiler üzerinde olumsuz etkiler oluşturabilmektedir. Doğal olarak tüm canlı varlıklar kendilerini biyotik ve abiyotik stress türlerine karşı korumak için savunma sistemlerine sahiptirler. Stres proteini olarak da adlandırılan HSP'ler (Heat Shock Protein – Isı Şok Proteini) sadece isminde yer alan ısı stresine karşı değil aynı zamanda tüm çevresel ve yaşamsal stres koşullarına karşı yaşayan canlıyı korumakta kritik bir rol oynamaktadır. Bu çalışmada kantitatif Real-time PCR metodu kullanılarak, *Hsp17.4*, *Hsp17.7*, *Hsp17.8*, *Hsp20*, *Hsp21.5*, *Hsp23.8*, *Hsp70*, *Hsp90* ve *Hsp100* genlerinin güçlendirilmiş UV-B ışığı altındaki ekspresyon seviyelerinin analizi yapılmaya çalışılmıştır. Sonuç olarak, *Hsp23.8* ve *Hsp90* genleri dışındaki tüm genlerin ekspresyon seviyesinde azalma gözlemlenmiştir. Ancak *Hsp23.8* geninin ekspresyon seviyesindeki artış bir anlam ifade etmemektedir. *Hsp90* genin ekspresyon seviyesindeki artışın UV-B sebepli olduğu görülmektedir. Bu sonuçlar gösteriyor ki, *Hsp90* genleri UV-B stresine karşı domates bitkisini korumada kritik rol oynayabilir.

Anahtar Kelimeler: Domates, Ultraviyole-B Etkisi, Isı Şok Proteinleri, Stres

This thesis is dedicated to my mother, father, sister and my wife; they are my most valuable friends in this life

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TABLE OF CONTENTS

| | |
|--|-----|
| ABSTRACT | iii |
| ÖZ | iv |
| DEDICATION | v |
| ACKNOWLEDGMENT | vi |
| TABLE OF CONTENTS | vii |
| LIST OF TABLES | ix |
| LIST OF FIGURES | x |
| LIST OF SYMBOLS AND ABBREVIATIONS | xii |
| CHAPTER 1 INTRODUCTION | 1 |
| 1.1 UV-B | 1 |
| 1.1.1 Ozone Depletion Stimulates UV-B Radiation | 1 |
| 1.1.2 General Effects of UV-B Radiation | 2 |
| 1.1.3 Effects of UV-B Radiation on Plants | 3 |
| 1.2 Heat-Shock-Proteins (HSPs) | 7 |
| 1.2.1 HSP and Abiotic Stress | 8 |
| 1.2.2 Types of HSP and Roles of Them | 10 |
| 1.2.2.1 HSP60 | 10 |
| 1.2.2.2 HSP70 | 11 |
| 1.2.2.3 HSP90 | 12 |
| 1.2.2.4 HSP100 | 13 |
| 1.2.2.5 sHPS (Small Heat-Shock-Protein) | 13 |
| CHAPTER 2 MATERIALS AND METHODS | 17 |
| 2.1 Methods | 17 |
| 2.1.1 Preparation Plant Growth & Sampling | 17 |
| 2.1.1.1 Sterilization of Tomato Seeds | 17 |
| 2.1.1.2 Germination of Tomato Seeds | 17 |
| 2.1.1.3 Preparation of Hydroponic System and Hoagland Solution | 17 |

| | |
|---|----|
| 2.1.1.4 Transferring of Seedlings to Hydroponic System..... | 18 |
| 2.1.1.5 UV-B Treatment..... | 19 |
| 2.1.1.6 Sampling from Plants..... | 19 |
| 2.1.2 Analyzes..... | 19 |
| 2.1.2.1 RNA Isolation from Plant Tissues..... | 19 |
| 2.1.2.2 Monitoring of RNA..... | 20 |
| 2.1.2.3 Quantitation of RNA..... | 20 |
| 2.1.2.4 Primer Design..... | 21 |
| 2.1.2.5 1 st Strand cDNA Synthesis..... | 21 |
| 2.1.2.6 Reverse-Transcriptase PCR (RT-PCR)..... | 24 |
| 2.1.2.7 Real-Time PCR..... | 25 |
| 2.1.2.8 Calculation of Relative Quantitation Data..... | 26 |
| CHAPTER 3 RESULTS..... | 27 |
| 3.1 Morphological Effects of UV-B..... | 27 |
| 3.2 Screening of Total RNA..... | 28 |
| 3.3 Real-Time PCR and Relative Quantitative Data..... | 28 |
| 3.3.1 Hsp17.4..... | 32 |
| 3.3.2 Hsp17.7..... | 33 |
| 3.3.3 Hsp17.8..... | 34 |
| 3.3.4 Hsp20..... | 35 |
| 3.3.5 Hsp21.5..... | 36 |
| 3.3.6 Hsp23.8..... | 37 |
| 3.3.7 Hsp70..... | 38 |
| 3.3.8 Hsp90..... | 39 |
| 3.3.9 Hsp100..... | 40 |
| 3.3.9 Actin..... | 41 |
| CHAPTER 4 DISCUSSION & CONCLUSION..... | 42 |
| CHAPTER 5 REFERENCES..... | 46 |

LIST OF TABLES

TABLE

| | | |
|-----|---|----|
| 1.1 | Cell stressors that induce Heat Shock Proteins | 9 |
| 1.2 | Families of HSPs in human beings, their site and suggested functions | 10 |
| 1.3 | Expression of sHsps in different conditions other than heat stress | 14 |
| 2.1 | Hoagland solution for hydroponic growth system. | 18 |
| 2.2 | Measurement ratio of absorbance values and concentration of RNA samples. | 21 |
| 2.3 | Accession, Sequences and T _m information of used Hsp primers. | 21 |

LIST OF FIGURES

FIGURE

| | | |
|------|--|----|
| 1.1 | Ultraviolet and visible light location on spectrum chart | 1 |
| 1.2 | Analysis of Phenolic compounds and anthocyanins under UV-B | 3 |
| 1.3 | Analysis of photosynthesis, stomatal conductance, transpiration under UV-B | 4 |
| 1.4 | The negative effect of different doses of UV-B rays | 5 |
| 1.5 | Under UV-B growth condition, changes in leaf and plant morphology | 7 |
| 1.6 | Model of the ATPase cycle of Hsp90 | 12 |
| 2.1 | Growth conditions of tomato plants | 19 |
| 2.2 | DNase-1 Treatment / Step-1 | 22 |
| 2.3 | 1st Strand cDNA Synthesis / Step-2 | 23 |
| 2.4 | 1st Strand cDNA Synthesis / Step-3 | 23 |
| 2.5 | 1st Strand cDNA Synthesis / Step-4 | 24 |
| 2.6 | Real-time PCR experiment steps..... | 25 |
| 2.7 | Real-time PCR condition | 26 |
| 3.1 | Morphological condition of tomato plants after UV-B treatment..... | 27 |
| 3.2 | Checking of Isolated RNA by using Gel Electrophoresis | 28 |
| 3.3 | Melting curve analysis results | 29 |
| 3.4 | Amplification Plot results..... | 30 |
| 3.5 | Comparing of Gene Expressions..... | 31 |
| 3.6 | Real Time PCR results of Hsp17.4 expression analysis | 32 |
| 3.7 | Real Time PCR results of Hsp17.7 expression analysis | 33 |
| 3.8 | Real Time PCR results of Hsp17.8 expression analysis | 34 |
| 3.9 | Real Time PCR results of Hsp20 expression analysis | 35 |
| 3.10 | Real Time PCR results of Hsp21.5 expression analysis | 36 |
| 3.11 | Real Time PCR results of Hsp23.8 expression analysis | 37 |
| 3.12 | Real Time PCR results of Hsp70 expression analysis | 38 |
| 3.13 | Real Time PCR results of Hsp90 expression analysis | 39 |

| | | |
|------|---|----|
| 3.14 | Real Time PCR results of Hsp100 expression analysis | 40 |
| 3.15 | Real Time PCR results of Actin expression analysis | 41 |
| 4.1 | Ozone hole area and minimum ozone for each year | 42 |

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

| | |
|------|-------------------------------------|
| A | Ampere |
| ATP | Adenosine triphosphate |
| ATP | Adenosine triphosphate |
| cDNA | Complementary Deoxyribonucleic Acid |
| DOC | Dissolved Organic Carbons |
| E | Element |
| ER | Endoplasmic Reticulum |
| FEC | Final Element Concentration |
| H | Hour |
| HSP | Heat Shock Protein |
| MC | Molecular Weight |
| mRNA | Messenger Ribonucleic Acid |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PCR | Polymerase Chain Reaction |
| ROS | Reactive Oxygen Species |
| sHSP | Small Heat Shock Protein |
| SSC | Stock Solution Concentration |
| SV | Stock Volume |
| TBE | Tris-borate-EDTA |
| UV | Ultraviolet |
| UV-B | Ultraviolet-B |
| V | Volt |
| W | Watt |

CHAPTER 1

INTRODUCTION

1.1 UV-B

Ultraviolet lights are located between 100 nm and 400 nm. These lights are divided into three groups, which are UV-C (Ultraviolet-C: 100 nm-280 nm), UV-B (Ultraviolet-B: 280 nm - 315 nm) and UV-A (Ultraviolet-A: 315 nm - 400 nm). UV-A radiations can pass ozone layer (stratosphere) easily without losing any energy, but all incoming UV-C and %90 of UV-B radiations are absorbed by stratospheric ozone layer (Horneck, 1995).



Figure 1.1 Ultraviolet and visible light location on spectrum chart.

Ultraviolet-B radiations are between 280 nm and 315 nm in spectrum charts, but only 290 nm and over radiations can reach the earth's surface. These conditions can be changed according to weather, meteorology, latitude and ozone layer thickness (Madronich, 1995).

1.1.1 Ozone Depletion Stimulates UV-B Radiation

Ozone depletion is the most important reason for reaching of ultraviolet rays on the earth. All living organisms on the world expose the ultraviolet radiation. Density of the ultraviolet radiation can be changed with some important reasons that are incidence

angle of solar radiation and thickness of the ozone layer in stratosphere (Madronich et al., 1998; Kakani et al., 2003).

Reaching level of UV radiation can be changed according to cloud cover, solar elevation and ozone depletion are some of several factors (Udelhofen et al., 1999). The largest ozone depletion was observed at high latitudes in spring season. This condition stimulated chlorine and bromine catalyzed ozone destruction (SORG, 1996).

Montreal Protocol was accepted with some states in the Vienna in 1985 to protect ozone layer. According to Montreal Protocol, harmful substances to the ozone layer were limited for using and production. Released halogenated chlorofluorocarbons into the air cause slimming of the ozone layer (Kerr and McElroy, 1993).

1.1.2 General Effects of UV-B Radiation

Known as, UV radiations have harmful effects on human, plant, terrestrial and aquatic ecosystem. At the same time, UV radiation has beneficial effects on some livings such as production of D vitamin (Norval et al., 2011).

If we give some examples for the general harmful effects of Ultraviolet radiations, we can short these: Solar ultraviolet radiation causes degradation of Dissolved Organic Carbons (DOC) (Naganuma et al., 1996). UV-induced decomposition of the Dissolved Organic Matters (DOM) stimulates formation of the photosensitizers. Nevertheless, the ultraviolet rays stimulate Reactive Oxygen Species (ROS) and free radical formation (Herndl, 1996).

Humid substances absorb ultraviolet radiations highly. Bacteria can protect themselves in these humid substances. But, these humid substances are degraded by ultraviolet radiation and new substances can be occurred such as formaldehyde, acetaldehyde, glyoxylate and pyruvate because of ultraviolet rays. And bacteria can absorb these new produced toxic substances easily (Wetzel et al., 1995).

The interaction of the ultraviolet lights with heavy metals in many plankton species can inhibit nutrient intake, enzyme activities, carbon fixation and ATP production (Rai et al., 1996; Rai and Rai, 1997).

1.1.3 Effects of UV-B Radiation on Plants

Direct UV-B protection mechanism mediated by photoreceptors is the production of some different UV-B absorbers that are flavonoids, hydroxycinnamate and phenolic compounds especially in the epidermis of the leaves (Fohnmeyer et al., 1997; Caldwell et al., 2003; Fedina et al., 2007).

These responses types don't mean a damage response or stimulation of expression of some particular genes. In fact, these type responses lead to regulation of transcription of UV-B signal transduction processes and light detection systems (Jenkins et al., 1997).

Subepidermal and epidermal layers contain these pigments that protect, absorb and reduce penetration of UV-B deeper in the mesophyll cells (Bornman et al., 1997; Fedina et al., 2007; Fedina et al., 2010). These systems prevent the transmittance of 95 to 99% of incoming UV-B radiation and effects on the plants (Robberecht and Caldwell, 1986).

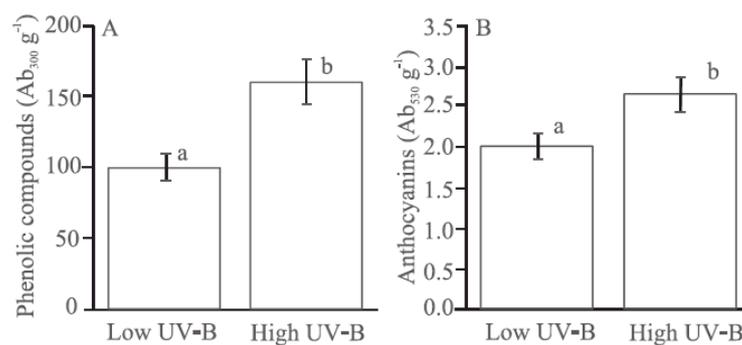


Figure 1.2 UV-B radiation for 16 days on yellow passion fruit; Accumulation of phenolic compounds (A) and anthocyanins (B) in plant. Bars represent the standard error (n = 4 to 5) (Cechin et al., 2012).

Cechin et al. (2012) showed that concentration of anthocyanin of UV-B induced Passion fruit plants had a 32% increase when compared to the control plants. (Figure 1.3-B) Flavonoids are increased %60 in UV-B exposed plants when compared to the control plants (Figure 1.3-A).

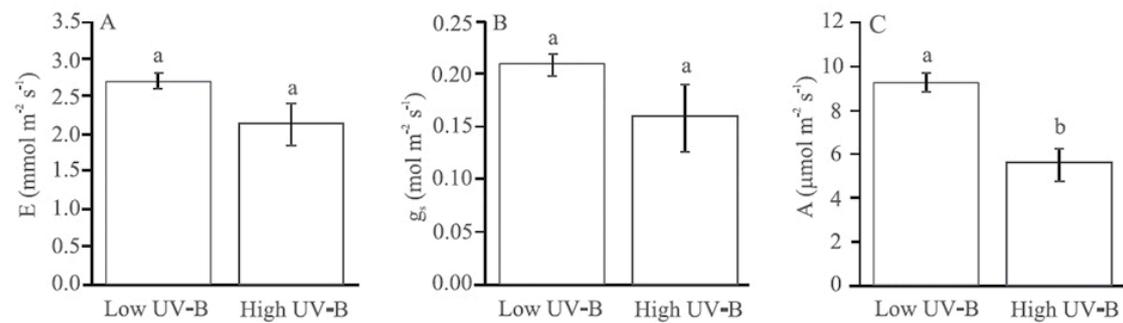


Figure 1.3 UV-B radiation for 16 days on yellow passion fruit; A, transpiration (E); B, stomatal conductance (gs) and C, photosynthesis (A). Bars represent the standard error (n = 7) (Cechin et al., 2012).

Cechin et al. (2012) reported that, after 16 days growth duration of yellow passion fruit plants, photosynthesis rates of the high and low UV-B-induced plants were induced by 41%. Stomatol conductivity and transpiration has also reduced when compared with control plant.

UV radiation can destroy photosynthetic pigments that cause reduction of photosynthetic capacity of plant, altered thylakoid integrity, increased stomatal diffusion and reduced Rubisco activity (Strid et al., 1990; Strid et al., 1994; Nogues and Baker, 1995).

UV-B-induced stress is the primary cause of reduction of RUBISCO activity that reduces photosynthesis. Harvesting complex protein and D1 polypeptide of PSII has also critical role on the efficiency of the photosynthetic system (Allen et al., 1997; Baker et al., 1997; A-H-Mackerness et al., 1997; Jordan et al., 1991).

Ultraviolet-B effects were studied extensively in previous researches. As results, UV-B caused reduction of RUBISCO activity, down-regulation of expression of photosynthetic genes, inactivation of photosystem II, reduction of thylakoid integrity and alteration of chloroplast structure (Strid et al., 1994; Teramura and Sullivan, 1994; Jansen et al., 1996; Friso et al., 1994; Vass et al., 1999; Greenberg et al., 1996).

UV-B effect capacity on plants can be changed with nutrient condition, visible radiation level and water regime (Balakumar et al., 1993).

Pigments in plants absorb photosynthetic lights. Pigments can be classified as three types in plants. One of them is carotenoid that protects chlorophyll from high light radiation. But ultraviolet rays can be harmful for carotenoid pigments (Nedunchezian et al., 1996).

Swindell et al. (2007) showed that, UV-B cause some diverse negative effect on photosynthetic *Ulva fasciata*. In this study, *Ulva fasciata* was exposed to UV-B irradiation in order of 0.5, 1, 2.5, 5, 10 and 20 W m^{-2} dose . According to results, increasing of UV-B dose affect growth rate, chlorophyll *a* and carotenoid content negatively.

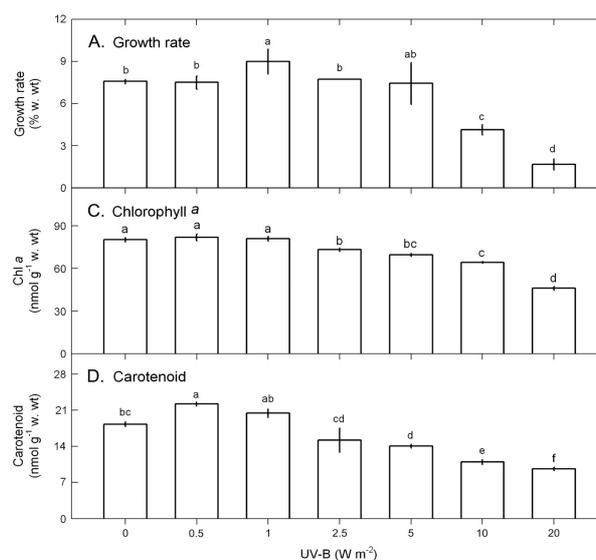


Figure 1.4 The negative effect of different doses of UV-B rays on photosynthetic *Ulva fasciata*. A) Growth rate B) Chlorophyll *a* content C) Carotenoid content

DNA replication or transcription blocking occurs because of interaction DNA and UV-B radiations. Because, absorption of UV-B by DNA cause generating of photoproducts these are cyclobutyl pyrimidine dimers and pyrimidine (6-4) pyrimidinone dimers (Britt, 1995).

Heat stress and UV-B stress results in damage of PSII, which is a major component of photosynthesis light reactions (Bredenkamp and Baker, 1994).

Expression of PR1 gene (pathogen-responsive gene) in tobacco and wound-defense genes in tomato increase by activation of certain signal transduction pathways, that condition occurs because of absorption of UV-B by tobacco and tomato plants. (Green and Fluhr, 1995; Conconi et al., 1996) In case, production of oxygen radicals may induce these pathways by UV-B radiation. UV-B-induced oxygen radicals damage the cell membrane and activate a membrane-associated signal transduction protein or through a DNA damage signal sent from the nucleus (Mount, 1996).

UV-B has also morphologic effects on plants. Decreased height, decreased leaf area, reduced numbers of stomata, leaf curling, smaller internodes and increased axillary branching are some of morphologic effects under UV-B growth condition. On the other hand, changed composition of epicuticular waxes, epidermal thickening, accumulation of screening pigments, leaf thickening and redistribution of chlorophyll are some symptoms from among visible results (Jansen et al., 1998) (Figure 1.5).

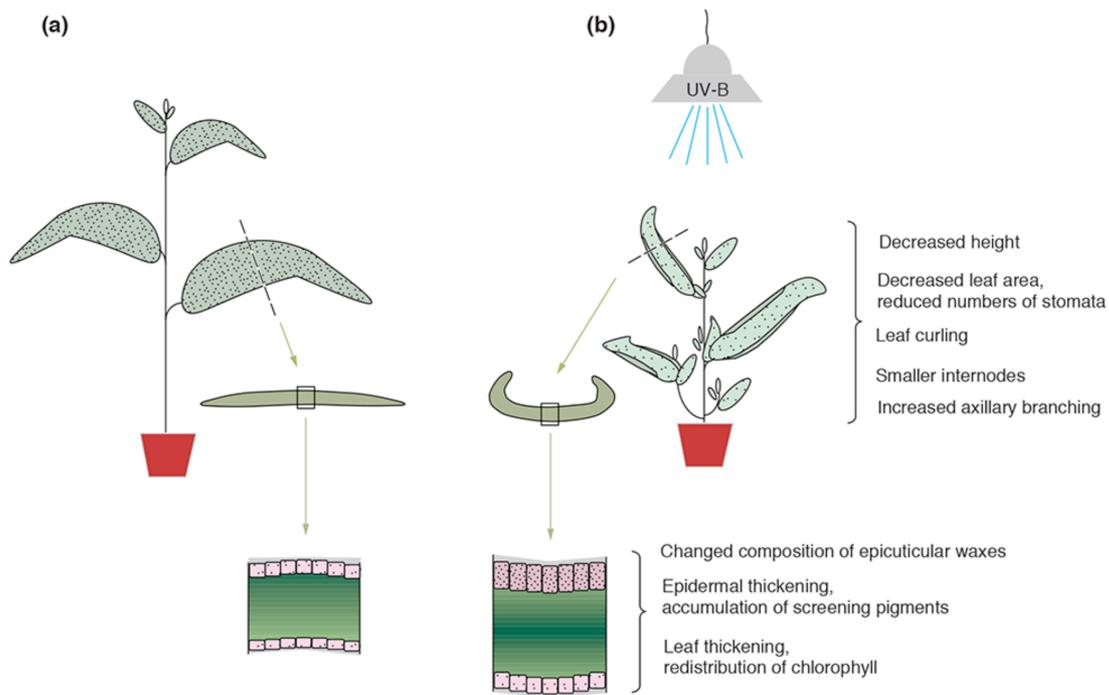


Figure 1.5 Under UV-B growth condition, changes in leaf and plant morphology. (a) Control (b) UV-B-induced effects on plant (Jansen et al., 1998)

1.2 HEAT SHOCK PROTEINS (HSPs)

Plants are exposed to several abiotic effects in their life. Deficient or excessive nutrients can be a stress factor on plants. Apart from these, pathogens, heavy metals, sudden temperature change, toxic materials, oxidants and many like these can be a stress reason on plants (Levitt, 1980; Vierling, 1991).

As with heat stress, all types of stress factors (moisture, drought, heavy metal, ultraviolet, salinity, cold, e.g.) can stimulate expression of some genes that does not express in normal conditions. In fact, all genotypic responses to the stress conditions stimulate production of the some proteins to protect living organism. These proteins are called Heat-Shock Proteins (HSPs) or Stress Proteins. Therefore, HSPs can be a research issue for all stress types outside of the heat stress (Morimoto, 1993;; Gupta et al., 2010).

1.2.1 HSP and Abiotic Stress

Abiotic stress is defined as non-living factors like drought, salinity, temperature changes, toxic materials, heavy metals and oxidative have negative effects on living organisms. Abiotic stress has most harmful effect for agricultural production on earth. Agricultural production declines about %50 due to abiotic stress factors (Wang et al., 2003).

The plants also have defense systems against stress effects like all living organisms. All other abiotic stresses like heat stress can stimulate expression of HSPs (Heat Shock Proteins) that has critical role to protect organism (Waters et al., 1996; Boston et al., 1996; Vierling, 1991).

Heat shock protein expression increment was observed with some heavy metal stress experiments. For example, high temperature and heavy metal applied rice plant encourages expression of HSP mRNAs (16-20 kDa). The other experiment with *Armeria maritima* enlarged in copper pollution soil that shows *Hsp17* expression was increased in plant roots (Tseng et al., 1993; Neumann et al., 1995; Wollgiehn and Neumann 1999).

Brassica napus was grown under different high temperature stress to observe expression of the HSPs. As a result comparison of the stressed and control plants, *Hsp17.6* (in Pollen), *Hsp70* (in leaves and pistil) and *Hsp101* were expressed highly under high temperature stress than control condition (Lester et al., 2004).

High temperature stress, osmotic, cold and salinity stress can cause HSP expression strongly. That means, HSP expression is not for only temperature stress, but also same expression can be observed under other abiotic stress conditions (Timperio et al., 2008).

Table 1.1 Cell stressors that induce Heat Shock Proteins (Macario and Conway de Macario, 2005)

| Stressors or Stressors Type | Name or Description |
|------------------------------------|---|
| Physical | Heat (including fever), cold, several types of irradiation, including ultraviolet light and magnetic fields |
| Oxygen | Oxygen-derived free radicals (reactive oxygen species), hydrogen peroxide, a shift from anaerobiosis to aerobiosis (e.g. reperfusion), hypoxia-anoxia (ischemia) |
| pH | Alkalosis, acidosis, pH shift |
| Biologic | Infection, inflammation, fever |
| Psychological | Emotions, emotional conflicts, hormonal imbalance |
| Osmotic | Changes in the concentrations of salt, sugar and other osmolytes (hyperosmotic or hypo-osmotic shock) |
| Nutritional | Starvation involving multiple nutritional components (carbon, glucose, nitrogen, phosphate and nitrate) or any one of these nutrients. |
| Antibiotics | Puromycin, tetracycline, nalidixic acid, doxorubicin |
| Alcohols | Ethanol, methanol, butanol, propanol, octanol |
| Metals | Cadmium, copper, chromium, zinc, tin, aluminium, mercury, lead, nickel |
| Mechanical | Compression, shearing, stretching |
| Other | Dessiccation, benzene and derivatives, phenol and derivatives, teratogens, carcinogens, mutagens, arsenite, arsenate, amino acid analogues, nicotine, anesthetics, insecticides, pesticides |

1.2.2 Types of HSPs and Roles of Them

Many Heat Shock Proteins can be found in all living organisms and their molecular weight is between 10kDa and 200kDa. Heat Shock Proteins have a critical role to induce signals under stress conditions of living organisms (Bharti and Nover, 2002).

Table 1.2 Families of HSPs in human beings, their site and suggested functions
(Kregel, 2002)

| HSP Families | Cellular Location | Proposed Functions |
|---------------|---|--|
| HSP27 (sHsp) | Cytosol, nucleus | Microfilaments stabilization, antiapoptotic |
| HSP60 | Mitochondria | Refolds proteins and prevent aggregation of denatured proteins, proapoptotic |
| HSP70 | | Antiapoptotic |
| HSP72(HSP70) | Cytosol, nucleus | Protein folding, cytoprotection |
| HSP73(HSC70) | Cytosol, nucleus | Molecular chaperons |
| HSP75(mHSP70) | Mitochondria | Molecular chaperons |
| HSP78(GRP78) | Endoplasmic reticulum | Cytoprotection, molecular chaperones |
| HSP90 | Cytosol, endoplasmic reticulum, nucleus | Regulation of steroid hormone receptors, protein translocation |
| HSP110/104 | Cytosol | Protein folding |

1.2.2.1 HSP60

HSP60 class is called as chaperonins in some literatures. They have major missions like assisting plastid proteins such as RUBISCO (Wang et al., 2004).

Generally, plant chaperonins HSP60 and HSP70 are known as stromal chaperonins these have important mission to attain functional conformations of new proteins are transferred into the chloroplasts (Jackson-Constan et al., 2001).

These class proteins have mission for merging and folding of proteins for transferring them to the chloroplast and mitochondria. Therewithal, HSP60 attaches to

the proteins before folding and after transcription to prevent protein merge (Parsell and Lindquist, 1993).

1.2.2.2 HSP70

Many HSP70 proteins are constitutively expressed and are required by cells under all growth conditions (Gething and Sambrook, 1992).

In the cells that are under all growth conditions, HSP70 homolog proteins have been found (Gething and Sambrook, 1992). Those homologs are located in the important cell areas such as cytoplasm, chloroplast, nucleus and mitochondria (Nover, 1991).

Heat can be very detrimental for the cells. HSP70 proteins have a function of protecting cells from the effects of heat stress (Li et al., 1991; Nover, 1991). HSP70 has thermoprotection feature by stabilizing other proteins or by acting as intermediary in the renaturation or reassembly of heat-denatured proteins (Skowyra et al., 1990). One of the situations where HSP70 protein will be needed from the cell is when proteins are to be sent into the membrane bound organelles. Translocation of pre-proteins to the mitochondria and ER need the presence of cytoplasmic HSP70 (Chirico et al., 1988; Deshaies et al., 1988). At the same time, for the finalization of the translocation process HSP70 homolog proteins are needed. Those HSP70 proteins are localized already in the ER and mitochondria (Kang et al., 1990; Baker and Schatz, 1991).

As with heat stress, other different stress types activate the HSP70. According to some experiments with maize and spinach, HSP70 mRNA and proteins accumulation was observed in the plants' injury section (Guy et al., 1985; Heikkila et al., 1984).

When maize plant exposed to water and abscisic acid that stimulated expression of HSP70 proteins. In a similar experiment, when soybean plant exposed to arsenide same result was observed (Heikkila et al., 1984; Lin et al., 1984).

1.2.2.3 HSP90

HSP90 is abundant in the cell and they need ATP to be activated (Frydman, 2001).

Cytoplasmic HSP90 has a role to protect cell against pathogens. These class proteins can be activated with resistant proteins from pathogens. At the same time, they figure to manage of cellular signals (Hubert et al., 2003).

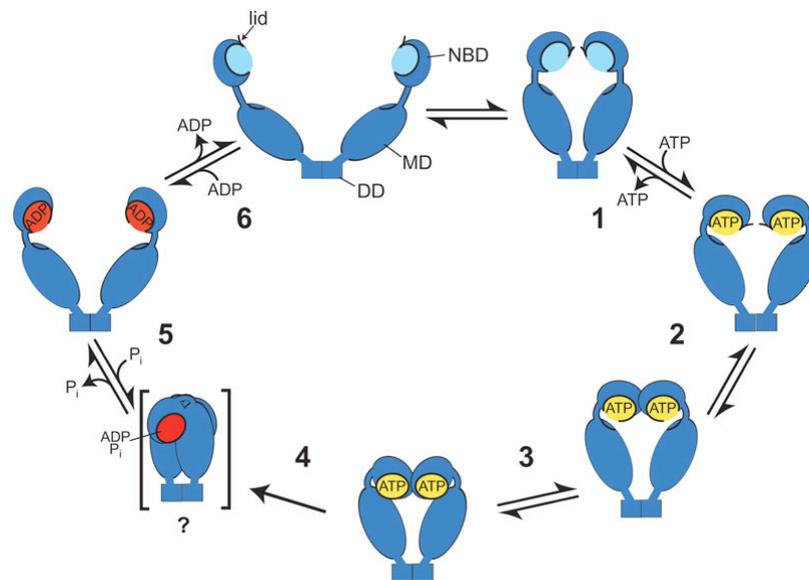


Figure 1.6 Model of the ATPase cycle of HSP90 proteins as revealed by kinetic analysis of ATP binding and/or conformational changes of yeast cytosolic Hsp82, human endoplasmic reticulum GRP94, human mitochondrial TRAP1 and *E. coli* HtpG. HSP90 proteins proceed through two distinct intermediates before reaching the γ -phosphate cleavage-competent conformation. Forward and backward reaction rates are different for the individual HSP90 proteins analyzed and may be subjected to regulation by cochaperones such as Aha1. A proposed compact post-hydrolysis state visible in EM seems to be transient and is not detectable by bulk measurements like SAXS or HX-MS (Mayer et al., 2009).

There are some studies that determined the crucial role of HSP90 in *Arabidopsis thaliana* (Queitsch et al. 2002). When the HSP90 production is without any alteration, there is no observable change in the phenotype. It seems like HSP90 suppresses genetic variation and by that protects wild type phenotype. Although, in case of damaged HSP90 production, a high number of developmental deformities have been observed. Damaged proteins require HSP90 protein under heat stress. In those situations the production of abnormal HSP90 canalized to even harsher consequences of the stress (Queitsch et al. 2002).

HSP90 in the plant cells control growth of the plants. In addition to this information, these proteins can be expressed under cold and passage of the dark luminous stresses (Krishna et al., 1995; Felsheim and Das, 1992).

The identification of an HSP90 containing chaperone complex in plant cells similar to that of animal cells suggests a role in plant cell signal transduction. A lot of genes in the *Arabidopsis thaliana* genome encode protein kinases. Many of those genes engage in control of growth and development. Those growth and development controlling genes will possibly need the HSP90 chaperone complex in the course of time (Chory and Wu, 2001).

1.2.2.4 HSP100

Hsp100 chaperone proteins are also known as Caseinolytic Protease Proteins. This family has a diverse class of chaperons, which play a role in important metabolic processes (Schirmer et al., 1996).

Hsp100 chaperon proteins have been proved to be in control of DNA binding activity of a lot of proteins (Mhammedi- Alaoui et al., 1994; Wickner et al., 1994; Lazazzera and Grossman, 1997). They also provide tolerance to heat stress (Sanchez and Lindquist, 1990; Squires et al., 1991) and salt stress (Krüger et al., 1994).

1.2.2.5 sHSP (Small Heat-Shock Protein)

In case of heat stress-induced plants produce dominantly sHSP proteins on a polyacrylamide gel electrophoresis (PAGE). Those produced proteins have 15 to 42 kDa of a molecular mass (Waters et al., 1996; DeRocher et al., 1991).

Three classes of sHsps named CI, CII and CIII are found in the nucleus (Scharf et al., 2001; Vierling, 1991) and the cytosol. The cytosolic classes CI and CII (Sun et al., 2001; Pla et al., 1998) sHsps, mitochondrial sHsp (Banzet et al., 1998) and in the chloroplast-located sHsp (Lee and Vierling, 2000) is a subset of sHsps. Oxidative stress promotes this subset of sHsps. Various sHsp-encoding genes are promoted by ozone, UV irradiation, cold and heavy metal stress. According to these studies, there may be a correlation between sHsps and general abiotic stress responses (Sebahat et al., 2001; Györgyey et al., 1991; Eckey-Kaltenbach et al., 1997; Pla et al., 1998; Banzet et al., 1998).

Table 1.3 Expression of sHsps in different conditions other than heat stress (Sun et al., 2002)

| Conditions | Plant species | sHsp gene/probes |
|------------------------------|--------------------------------|--|
| Embryo development | <i>Helianthus annuus</i> | <i>HaHsp17.6-CI</i> <i>HaHsp17.9-CII</i> |
| | <i>Pisum sativum</i> | <i>PsHsp18.1-CI</i> and three related sHsps |
| | | <i>PsHsp17.7-CII</i> and two related sHsps |
| | <i>Arabidopsis thaliana</i> | <i>AtHsp17.4-CI</i> , <i>AtHsp17.6-CI</i> <i>AtHsp17.7-CII</i> |
| Germination | <i>Lycopersicon esculentum</i> | <i>LpHsp17.7-CI</i> homologue <i>LpHsp17.3-CII</i> homologue |
| | <i>Arabidopsis thaliana</i> | <i>AtHsp17.4-CI</i> , <i>AtHsp17.6-CI</i> <i>AtHsp17.7-CII</i> , <i>AtHsp17.6-CII</i> |
| | <i>Pseudotsuga menziesii</i> | <i>cDNA DF4-5</i> (class CI) |
| | <i>Hordeum vulgare</i> | <i>HvHsp26.8-P</i> , <i>HvHsp26.9-P</i> |
| Somatic embryogenesis | <i>Medicago sativa</i> | <i>Hsp18.1</i> (class CI) <i>MsHsp18.2-CI</i> |
| | <i>Nicotiana tabacum</i> | <i>NtHsp18-CI</i> |

| | | |
|---------------------------|-----------------------------------|--|
| Pollen development | <i>Lilium bulbiferum</i> | cDNAs homologous to sHsp (class CI) |
| | <i>Zea mays</i> | <i>ZmHsp17-CII</i> (former name <i>Hsp18</i>) |
| | <i>Nicotiana tabacum</i> | <i>NtHsp18-CI</i> |
| Fruit maturation | <i>Lycopersicon esculentum</i> | <i>LpHsp17.7-CI</i> homologue <i>LpHsp17.3-CII</i> homologue <i>LeHsp23.8-P</i> (former name <i>LeHsp21</i>) <i>TOM111</i> (class P) |
| | <i>Helianthus annuus</i> | <i>HaHsp17.6-CI</i> <i>HaHsp17.9-CII</i> |
| Osmotic stress | <i>Craterostigma plantagineum</i> | <i>HaHsp17.6-CI</i> homologues <i>HaHsp17.9-CII</i> homologues |
| | <i>Quercus suber</i> | <i>QsHsp17-CI</i> |
| | <i>Arabidopsis thaliana</i> | <i>AtHsp17.7-CII</i> , <i>AtHsp17.6-CII</i> |
| Abscisic acid | <i>Helianthus annuus</i> | <i>HaHsp17.6-CI</i> |
| | <i>Craterostigma plantagineum</i> | <i>HaHsp17.6-CI</i> homologues <i>HaHsp17.9-CII</i> homologues |
| | <i>Quercus suber</i> | <i>QsHsp17-CI</i> |
| Oxidative stress | <i>Quercus suber</i> | <i>QsHsp17-CI</i> |
| | <i>Lycopersicon esculentum</i> | <i>Hsp22</i> (class M) |
| | <i>Petroselinum crispum</i> | <i>Hsp17.9</i> (class CI) |
| | <i>Oryza sativa</i> | <i>OsHsp26.6-P</i> |
| | <i>Arabidopsis thaliana</i> | <i>AtHsp17.7-CII</i> |
| Cold | <i>Solanum tuberosum</i> | <i>CI19</i> (class ER) |
| | <i>Lycopersicon esculentum</i> | <i>TOM66</i> (class CI) <i>TOM111</i> (class P)/ <i>LeHsp23.8-P</i> |
| | <i>Morus bombycis</i> | <i>WAP20</i> (class ER) |
| | <i>Castanea sativa</i> | <i>CsHsp17.5-CI</i> |
| Heavy metals | <i>Medicago sativa</i> | <i>Hsp18.1</i> (class CI) <i>MsHsp18.2-CI</i> |

| | | |
|---------------------------|-----------------------------------|--------------------------------------|
| | <i>Arabidopsis thaliana</i> | <i>AtHsp17.7-CII, AtHsp17.6-CIII</i> |
| Photoperiod | <i>Pharbitis nil</i> | <i>sHsp-1</i> (class CII) |
| Normal temperature | <i>Craterostigma plantagineum</i> | <i>HaHsp17.6-CI</i> homologues |

CHAPTER 2

MATERIALS AND METHODS

2.1 Methods

2.1.1 Preparation Plant Growth & Sampling

2.1.1.1 Sterilization of Tomato Seeds

Tomato (*Solanum lycopersicum* Mill) seeds were sterilized in %1 diluted sodium hypochlorite (NaOCl) for 5 minutes. Seeds were washed with sterilized distilled water several times to wash away all NaOCl from seed surface to keep from NaOCl reactivation.

2.1.1.2 Germination of Tomato Seeds

Suitable soil mix was prepared with sterilized baltic peat (%60) and perlite (%40). Tomato seeds were transferred into soil and grown under green house condition with 16:8h (light:dark) photoperiod, %60 humidity, 25 °C (light) and 20 °C (dark) in climate chamber for 20 days after germination of seeds.

2.1.1.3 Preparation of Hydroponic System and Hoagland Solution

After germination and first growth step, suitable seedlings were transferred to hydroponic system for beginning UV-B treatment.

Determined suitable plants were grown in hydroponic system with Hoagland solution, which contains macronutrient and micronutrient to provide tomato plants' needs.

Hoagland medium solution was prepared according to Hoagland and Arnon (1950) reference to Table 2.1.

Prepared Hoagland solution and hydroponic system parts were autoclaved.

Table 2.1 Hoagland Solution for Hydroponic Growth System

| Compound | MC ¹ g mol ⁻¹ | SSC ² mM | SSC ² g L ⁻¹ | SV ³ mL | E ⁴ | FEC ⁵ μM | FEC ⁵ ppm |
|---|--|------------------------|---------------------------------------|-----------------------|----------------|------------------------|-------------------------|
| Macro Nutrients | | | | | | | |
| KNO ³ | 101.10 | 1.00 | 101.1 | 6.0 | N | 16.00 | 224 |
| Ca(NO ₃) ₂ 4H ₂ O | 236.16 | 1.00 | 236.16 | 4.0 | K | 6.00 | 235 |
| NH ₄ H ₂ PO ₄ | 115.08 | 1.00 | 115.08 | 2.0 | Ca | 4.00 | 160 |
| MgSO ₄ 7H ₂ O | 246.48 | 1.00 | 246.49 | 1.0 | P | 2.00 | 62 |
| | | | | | S | 1.00 | 32 |
| | | | | | Mg | 1.00 | 24 |
| Micro Nutrients | | | | | | | |
| KCl | 74.55 | 25 | 1.864 | } 2.0 | Cl | 50 | 1.77 |
| H ₃ BO ₃ | 61.83 | 12.5 | 0.773 | | B | 25 | 0.27 |
| MnSO ₄ H ₂ O | 169.01 | 1.0 | 0.169 | | Mn | 2.0 | 0.11 |
| ZnSO ₄ 7H ₂ O | 287.54 | 1.0 | 0.288 | | Zn | 2.0 | 0.13 |
| CuSO ₄ 5H ₂ O | 249.68 | 0.25 | 0.062 | | Cu | 0.5 | 0.03 |
| H ₂ MoO ₄ (%85 MoO ₃) | 161.97 | 0.25 | 0.040 | Mo | 0.5 | 0.05 | |
| NaFeDTPA(%10Fe) | 468.20 | 64 | 30.0 | 1.0 | Fe | 53.7 | 3.00 |

1- MC: Molecular Weight

2- SSC: Stock Solution Concentration

3- SV: Stock Volume

4- E: Element

5- FEC: Final Element Concentration

2.1.1.4 Transferring of Seedlings to Hydroponic System

Tomato seedlings were transferred to hydroponic system carefully without damage roots of plants. After transferring of seedlings, plants were grown for 1 week for adaptation to hydroponic system. After adaptation, tomato plants were exposed to UV-B.

2.1.1.5 UV-B Treatment

In this step, Philips TL 40W/12 UV-B lamp was used with normal LED Growth Light (3:1:1 – Red-Blue-Infrared) to provide electrons needs of plants.

Each 4-tomato seedling was grown in each group under same LED growth light, but under different UV-B radiation times. All plants without control were exposed to $6 \mu\text{mol m}^{-2} \text{s}^{-1}$ UV-B radiation for 4 and 12 hours for one time (Figure 2.1).

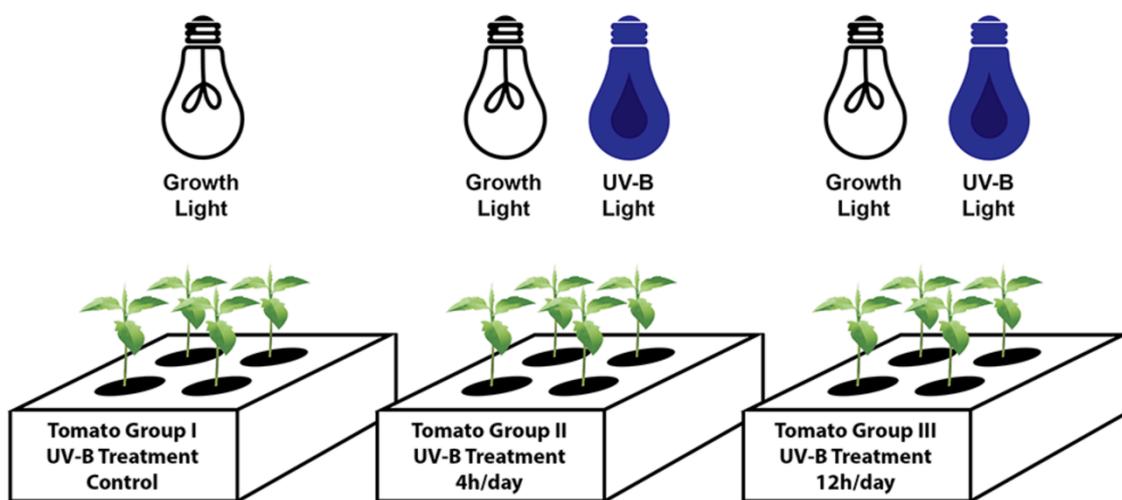


Figure 2.1 Growth conditions of tomato plants.

2.1.1.6 Sampling from Plants

The end of the UV-B radiation treatment, we harvested four individual plants for each treatment. Harvested tomato leaves were stored at ultra low freezer to protect genetic structure (New Brunswick Scientific, USA)

2.1.2 Analyzes

2.1.2.1 RNA Isolation from Plant Tissue

Roughly 100 µg of tomato plant tissue was measured and placed into 1.5 ml eppendorf tube. Then, tube was thrown into liquid nitrogen. 1 min later, 400 µl of Trizol was added to the frozen plant tissue. Tissues were homogenized by electric drill and further 600 µl of Trizol reagent was added to the eppendorf tube. After 15 sec vortexing of eppendorf, plant tissues were incubated at room temperature for 5 min to uncouple of nucleoprotein complexes. 200 µl chloroform was added into the eppendorf tube and shaken by hand for 20 sec. Tubes were placed in centrifuge machine, which was set to 4°C, 12000 x g and 15mins. After centrifugation step, the aqueous-supernatant phase was transferred to a new 1.5 ml eppendorf tube, which contains 500 µl isopropyl alcohol for precipitation. Following 15mins incubation of sample in ice, samples were centrifuged under 12000 x g, 10min and 4°C conditions to obtain pelleted RNAs. After that, RNA pellet was washed with 1ml of %70 ethanol, and then centrifuged for 5 mins at 7500 x g rate. The RNA pellet was then air-dried to remove ethanol. Following removing of ethanol, pellet was re-suspended in 44 µl of nuclease free H₂O.

2.1.2.2 Monitoring of RNA

Isolated RNA was controlled with gel running method by gel electrophoresis (Bio-Rad, USA). To perform this method, %1 agarose gel was prepared with 0.4g agarose and 40ml 1xTBE, which were mixed and heated at the microwave machine till appear the pure view on the gel. Comb was placed on gel tray, which was fastened on gel caster. Liquid gel was cooled until frozen, and 5 µl SYBR Safe (Invitrogen) was added into the gel. Then, liquid gel was transferred to caster. After freezing of gel, RNA samples were loaded on gel. Electrical supply was adjusted as 90 Volt, 25 minutes. Eventually the RNA samples were screened under the GelDoc (Bio-rad, USA).

2.1.2.3 Quantitation of RNA

Absorbance values of RNA samples were measured with Nanodrop Spectrophotometer (Thermo Scientific, USA). First, 1 µl nuclease-free DEPC-treated water was used as blank. Afterwards, RNA samples were measured with 260nm and 280nm wavelength values.

Table 2.2 Measurement ratios of absorbance values and concentration of RNA samples by NanoDrop 1000 (Thermo Scientific, USA)

| UV-B Treatment | Control | 4h UV-B | 12h UV-B |
|----------------|---------|---------|----------|
| Concentration* | 102.7 | 341 | 355.4 |
| 260 | 2.568 | 8.526 | 8.884 |
| 280 | 1.181 | 3.929 | 4.079 |
| 260/280 | 2.17 | 2.17 | 2.18 |
| 260/230 | 0.82 | 2.07 | 1.31 |

* Concentration: $\mu\text{g}/\mu\text{l}$

2.1.2.4 Primer Design

Sequences of primers were found by searching database of NCBI (National Center of Biotechnology Information).

Table 2.3 Accession, Sequences and T_m (Melting Temperature) information of used Hsp primers.

| Primer | F. Primer Sequence | T _m (°C) | R. Primer Sequence | T _m (°C) | Accession |
|---------|-------------------------|---------------------|-------------------------|---------------------|--------------|
| Hsp17.4 | CTCTGCATTTGCCAACACAC | 55.75 | TCCTCATGAATTTCCAACC | 55.75 | AF090115.1 |
| Hsp17.7 | GGAACAGACTGAAAAGGGTTAGT | 58.28 | GATCAGCGGAGAGAGGAACG | 59.97 | LOC101266525 |
| Hsp17.8 | CTCTGCATTTGCCAACACAC | 57.80 | TTCTCCACGTTCTCTCTCC | 59.85 | AF123256.1 |
| Hsp20 | TTAGCGTTGGTGGAACCTCT | 57.80 | CAGTGCCTTGGTTGTTTCCT | 57.80 | U59917.1 |
| Hsp21.5 | TCCAGAACTTTCATGACTCC | 58.01 | CCAATGATAAGCAGCAATCC | 56.06 | AB026983.1 |
| Hsp23.8 | ACGCTCCTAATTGGTGGTGA | 57.80 | TCCTGTTCTGTTCTGCATCTG | 57.80 | AB239774.1 |
| Hsp70 | TACCAAGGGCCAAGTTTGAGG | 57.80 | GAAGCTCCAAGAGCAACCAC | 59.85 | EU195057.1 |
| Hsp90 | TGAAGCTCGATGACGAGAGC | 59.90 | ACGCATCTCAAAAGTCTCAACTG | 59.50 | AF123259.1 |
| Hsp100 | TTGGCAAGTGTAGTTCGTTG | 55.75 | CTCCAACAGTGCCTTCATGC | 57.56 | AB219939.1 |

2.1.2.5 1st Strand cDNA Synthesis

To degrade genomic DNA, DNase-1 recombinant (RNase-free Kit) was used. Following degradation of DNA, Total RNAs were prepared according to containing 0.75 μg RNA in mixture.

Each mixture was prepared in 0.2ml PCR tubes with 10X incubation buffer, Enzyme (Dnase I recombinant RNase free), RNase-free water and RNA. Sample mixtures were put into the 37°C water bath for 30-35 minutes. Then, 1 μ l 50mM EDTA was added to PCR tubes and put into the 60°C water bath for 10 minutes.

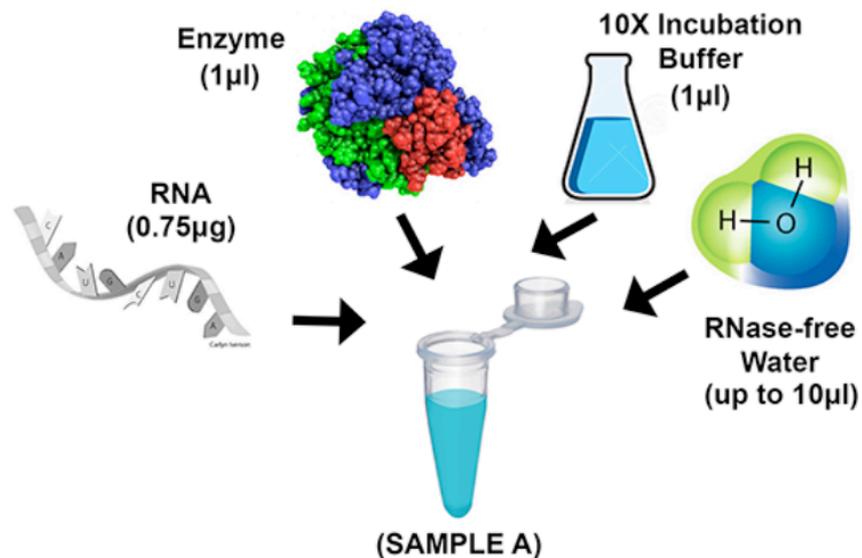


Figure 2.2 DNase-1 Treatment / Step-1

After DNase-1 treatment, Prime Script 1st Strand cDNA Synthesis Kit was used to apply second step of cDNA production.

Each 7 μ l of the mixtures were added into the new PCR tubes with 1 μ l Oligo dT Primer, 1 μ l dNTP Mixture. And finally RNase-free Water was added into the tubes to complete mixture to 10 μ l. The mixtures were placed into the 65°C water bath for 5 minutes. Then, mixtures were cooled on ice.

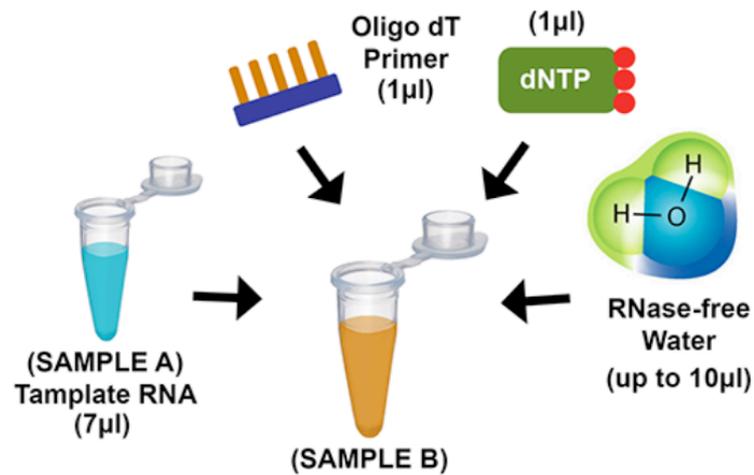


Figure 2.3 1st Strand cDNA Synthesis / Step-2

After cooling of sample, 4 µl 5X PrimeScript Buffer, 1 µl Rnase inhibitor, 1.5 µl PrimeScript Rtase was added in each PCR tubes contain 10 µl Template RNA Primer Mixture. And finally RNase-free Water was added into the tubes to complete mixture to 20 µl.

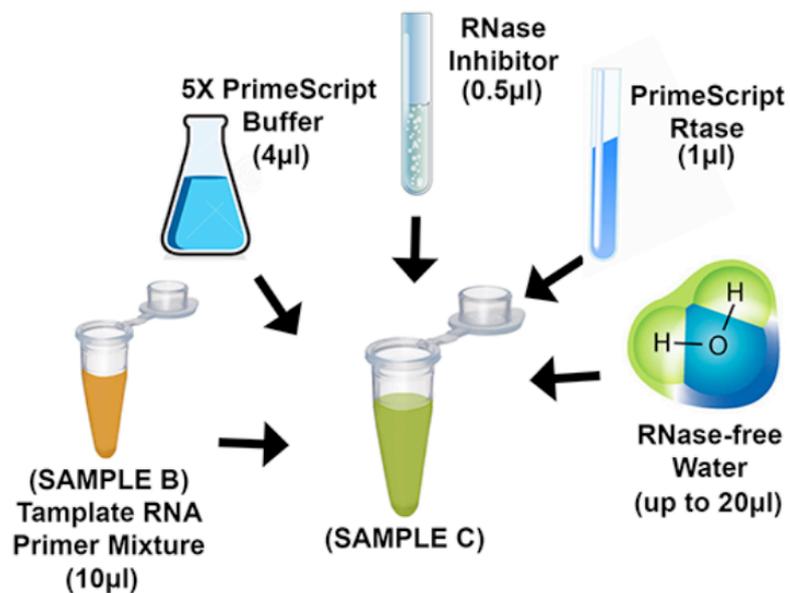


Figure 2.4 1st Strand cDNA Synthesis / Step-3

All PCR tubes were mixed quite slowly. For reaction of samples were incubated at 45 °C for 30 minutes.

2.1.2.6 Reverse-Transcriptase PCR (RT-PCR)

After 1st strand cDNA Synthesis treatment, cDNAs should be increased to a current amount. For that step, PCR was used according to following method.

2.5 mM of dNTP (Takara, Japan), 5µl 5x Prime STAR Buffer (Mg+2 plus) (Takara, Japan), 1µl (0.5pmol) Actin reverse and forward primers, 0.25 µl PrimeSTAR HS DNA Polymerase (2.5 U/µl) (Takara, Japan), 14.25 µl distilled and 1µl DNA template was prepared as 25 µl final volume. Mixture was brought down with centrifuge and performed for PCR reaction.

PCR experiment was performed with Gradient PCR (SuperCycler SC-300G) as described in Figure 2.5.

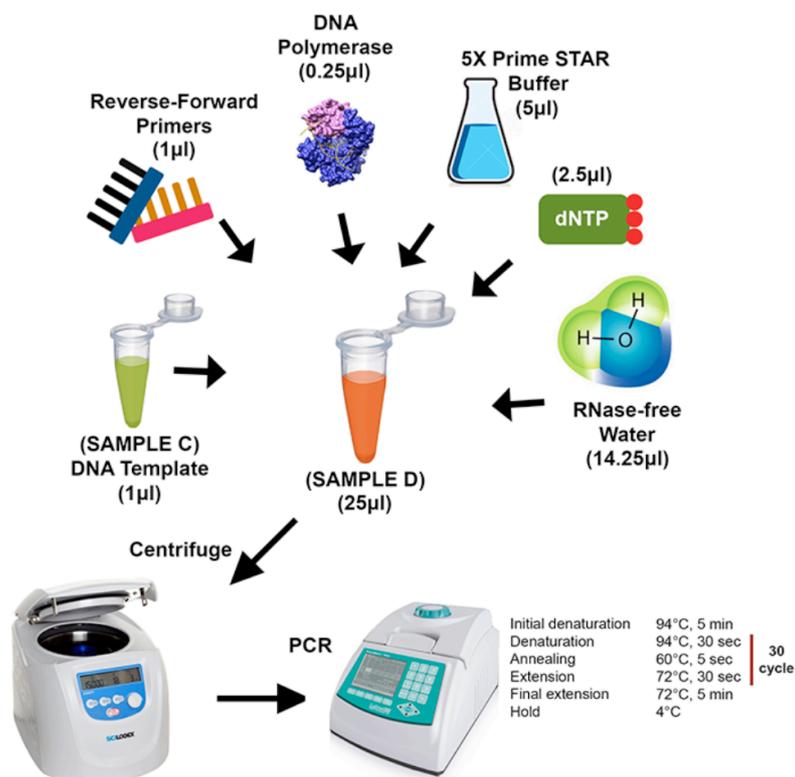


Figure 2.5 1st Strand cDNA Synthesis / Step-4

2.1.2.7 Real Time PCR

Real-Time PCR (Biosystems 7500 - Life Technologies, India) was used to measure quantitative amount of Hsp genes of UV-B induced plants.

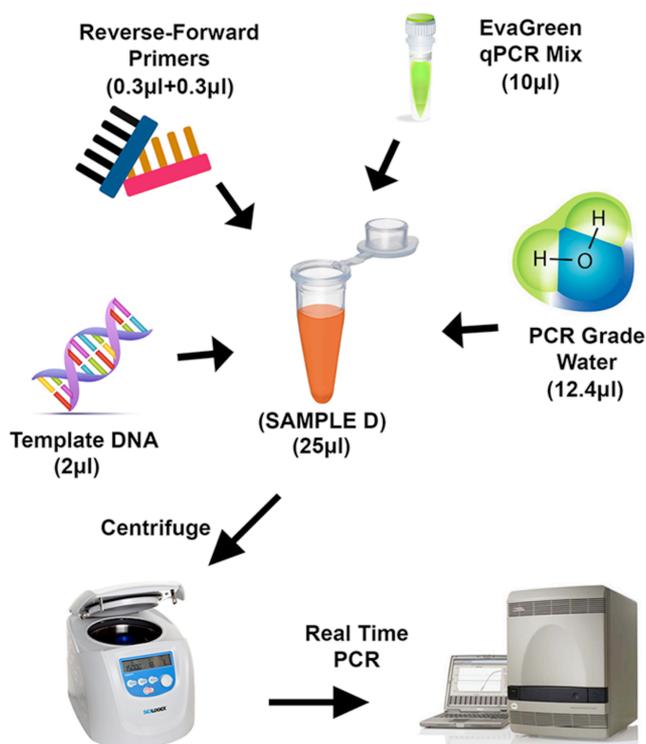


Figure 2.6 Real-time PCR experiment steps

1 µl Template were transferred into PCR tube contains 12.4 µl PCR Grade Water, 0.3 µl Forward Primer, 0.3 µl Reverse Primer, 10 µl EvaGreen qPCR Mix (Biotium Inc). Total value should be 25 µl for beginning Real Time PCR analysis. This step was applied one by one for all samples.

After mixtures were ready which were span with centrifuge to start Real Time-PCR step.

Real Time PCR conditions were adjusted according to used specific primers in Figure 2.7. First step (95 °C – 10 mins) was performed one time. Step 2 (95 °C – 15 secs) and step 3 (60 °C – 1 min) was performed as 40 repeats respectively. To increase the precision, Real-time PCR conditions were performed in two replicates.

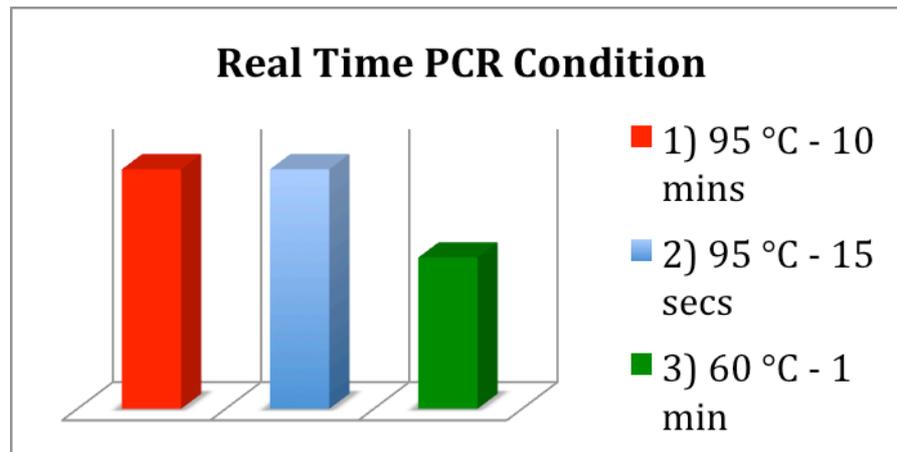


Figure 2.7 Real Time PCR condition. 40 repeats (cycle) were performed for all primers in order of step 2 and step 3

2.1.2.8 Calculation of Relative Quantitation Data

Relative quantitation data was calculated by a mathematical model. Ct (Control) is the housekeeping gene value. We have used actin as housekeeping gene. All Hsp expression results were calculated by reference of housekeeping gene.

Relative quantification determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample (Livak and Schmittgen, 2001). Relative quantification was used to compare expression levels of control plant with UV-B-induced plants.

CHAPTER 3

RESULTS

3.1 Morphological Effects of UV-B

After growing plants under normal light condition, they were exposed UV-B radiation for 0:4:12 hours (Density: $6 \mu\text{mol m}^{-2} \text{s}^{-1}$).

As shown in the images, UV-B radiation has destructive effect on living organisms. Leaf curling can be seen on Figure 3.1 and this result is an effect of UV-B. We have exposed plants to high dose UV-B for 0, 4 and 12 hours for once. If plants were grown for a long time under diminished UV-B light, they should have several diversity results (Figure 1.5).

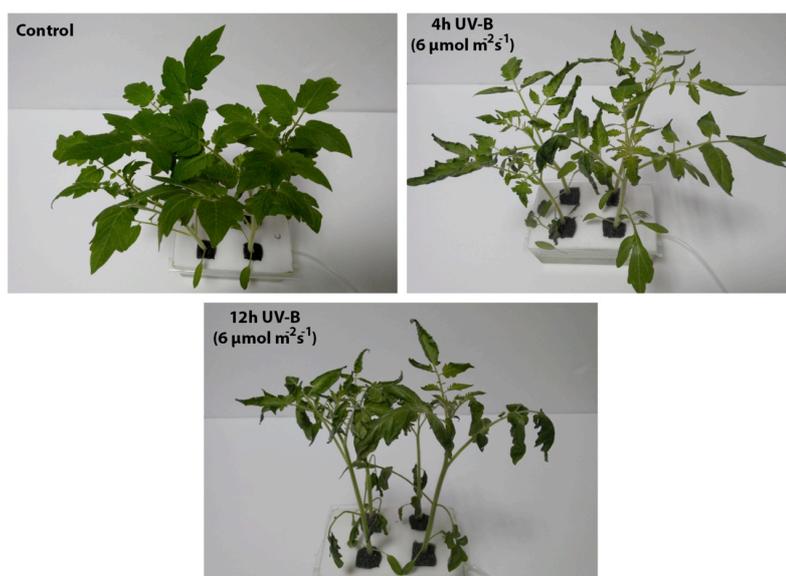


Figure 3.1 Morphological conditions of tomato plants after UV-B treatment (0:4:12 Hours).

3.2 Screening of Isolated RNAs

Isolated RNAs of UV-B exposed plants were shown in Figure 3.2. 5S, 18S and 28S RNAs are visible on %1 agarose gel clearly.

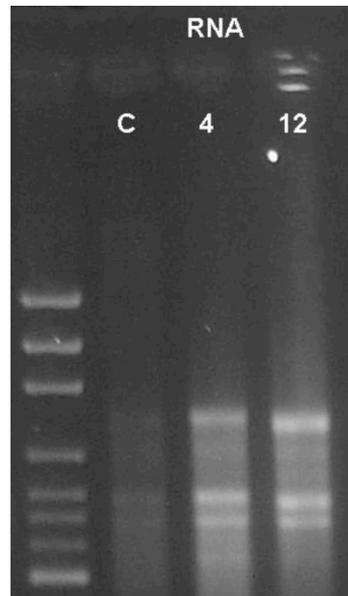


Figure 3.2 Total isolated RNAs of UV-B-exposed tomato plants were checked by using Gel Electrophoresis method (%1 agarose gel)

3.3 Real-Time PCR and Relative Quantitative Data

Real Time PCR concluded 3 types results to interpret of Hsp expressions relation with UV-B treatment. First result type is Melting Curve Analyses to determine dissociation-characteristics of double-strand DNA during heating (Figure 3.3). Second result type is Amplification Plot is need for assessment of fluorescence signal values versus cycle number (Figure 3.4). Third result type is Gene Expression Plot to compare expression level of HSP genes according to UV-B exposed level (Figure 3.5).

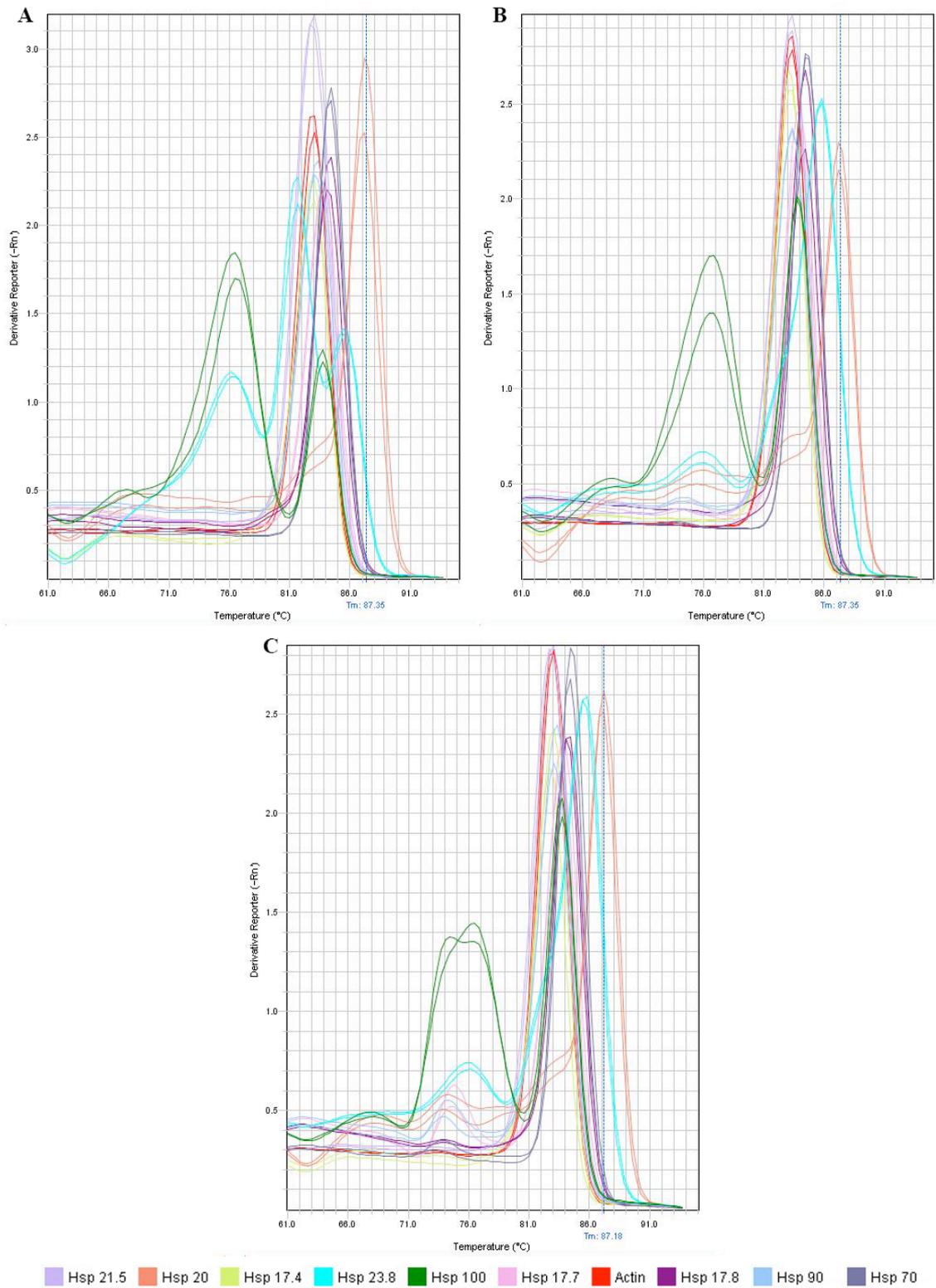


Figure 3.3 Melting curve analysis of Control, 4h exposed UV-B and 12h exposed UV-B plants.

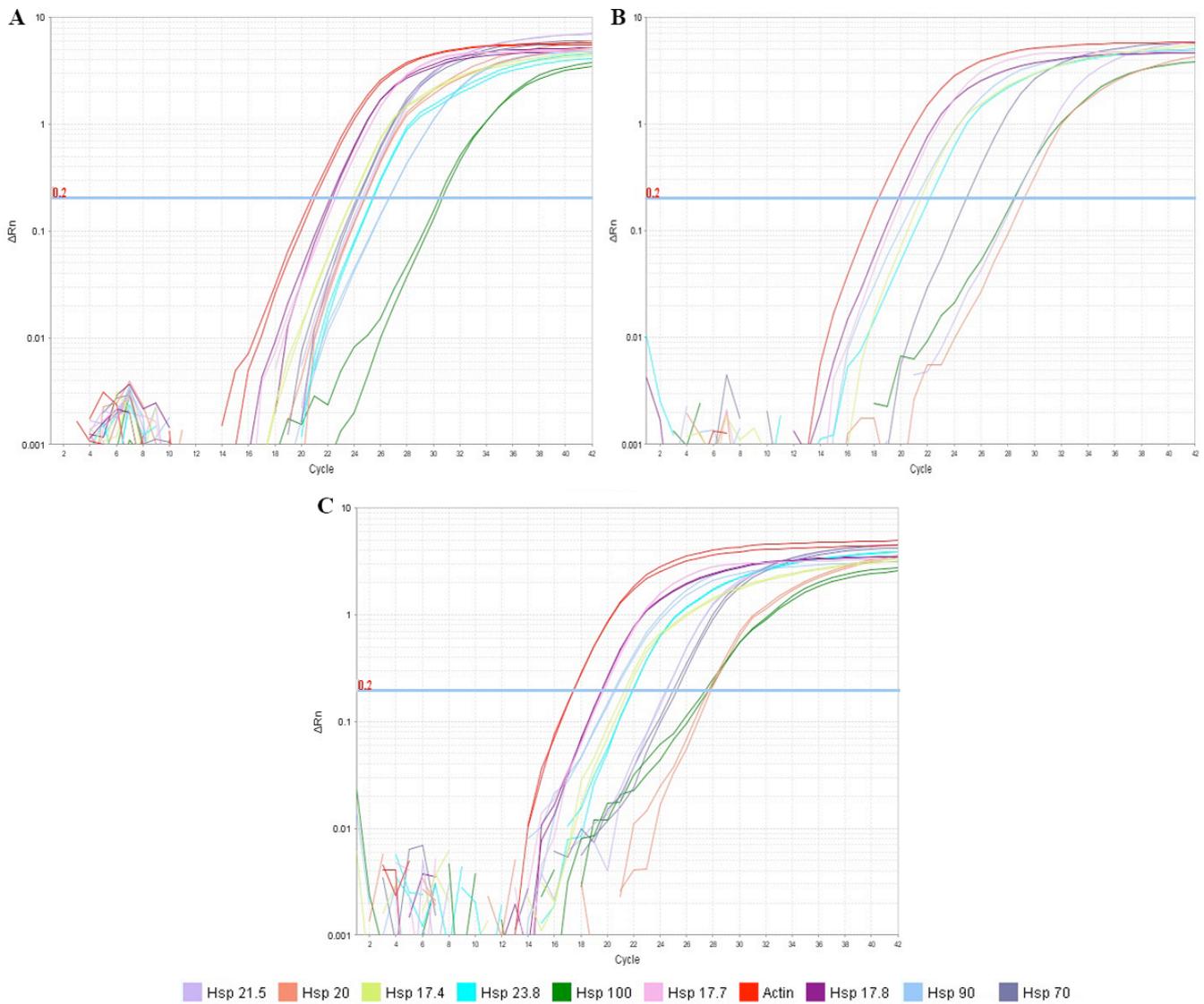


Figure 3.4 Amplification Plot of Control (A), 4h exposed UV-B (B) and 12h exposed UV-B (C) plants.

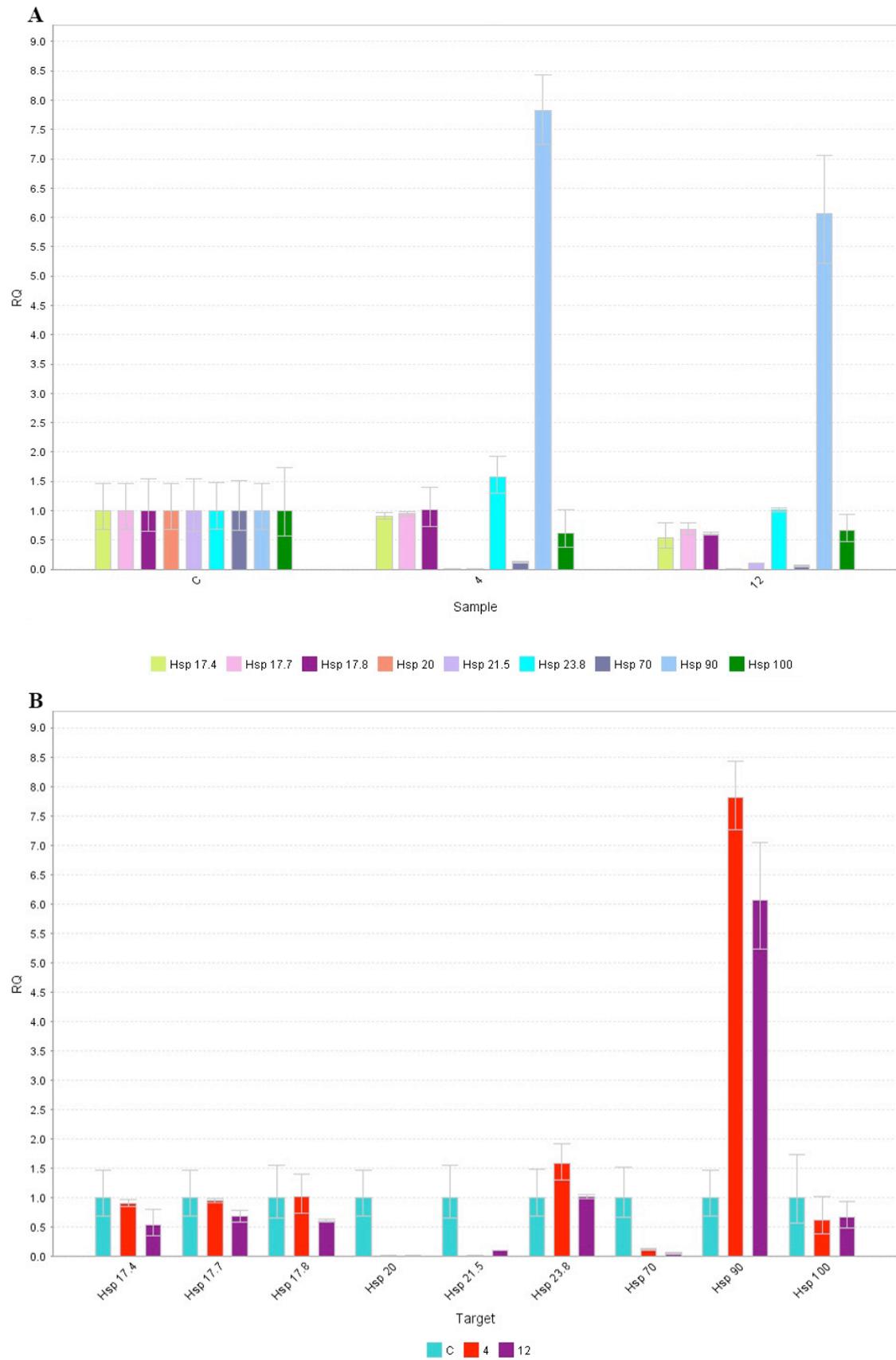


Figure 3.5 Comparing of Gene Expression according to (A) Samples (UV-B Exposing Duration) and (B) Targets (HSPs).

3.3.1 Hsp17.4

Hsp17.4 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. According to result, *Hsp17.4* expression of 4h UV-B and 12h UV-B exposed tomato plants was decreased when compared to control plant.

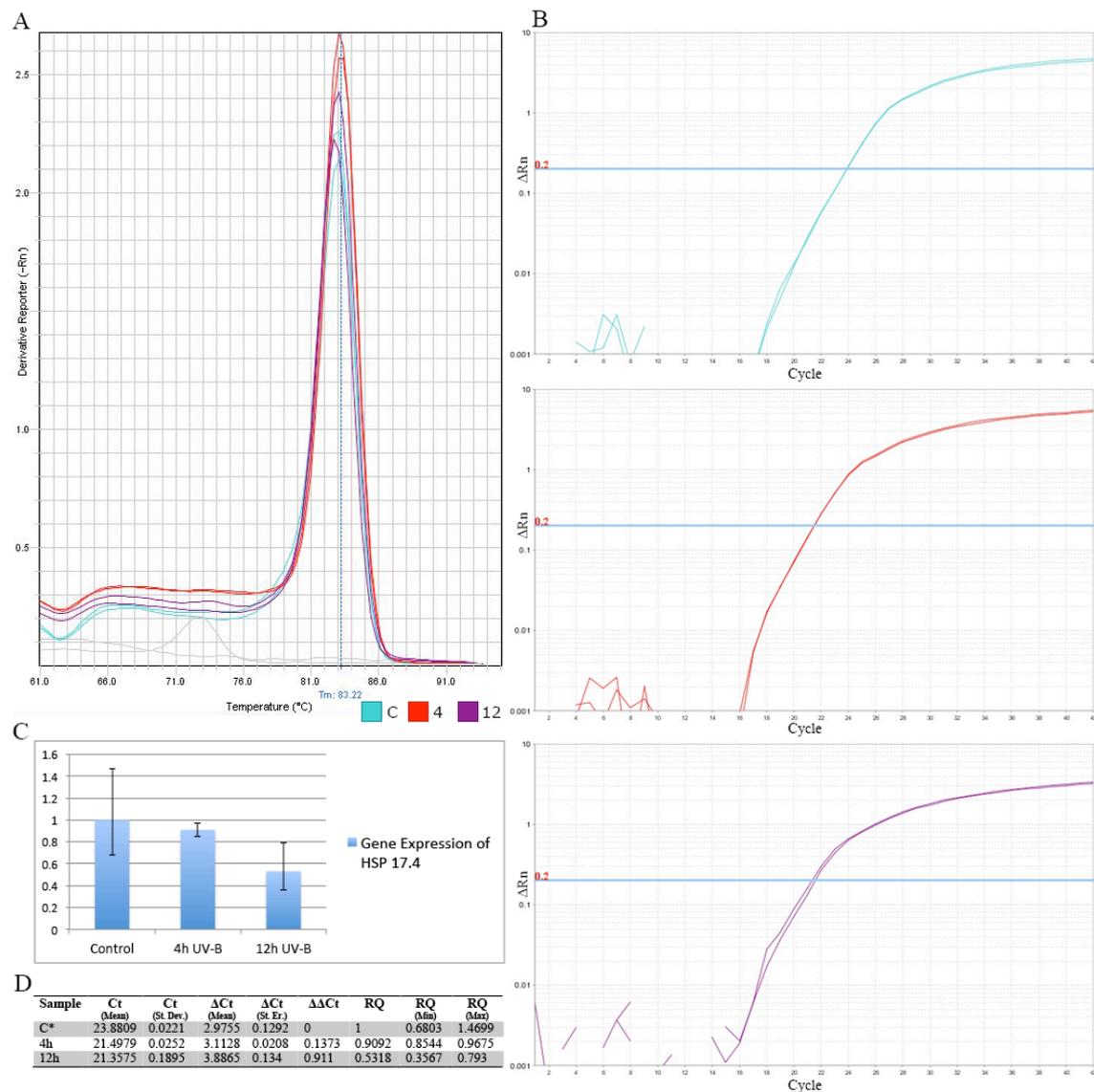


Figure 3.6 Real Time PCR results of *Hsp17.4* expression analysis. A) Melting curve plot of transcription phase of *Hsp17.4* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp17.4* (Relative quantities of *Hsp17.4* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.2 Hsp17.7

Hsp17.7 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. Based on the gene expression chart, *Hsp17.7* expression of 4h UV-B and 12h UV-B exposed tomato plants was decreased when compared to control plant.

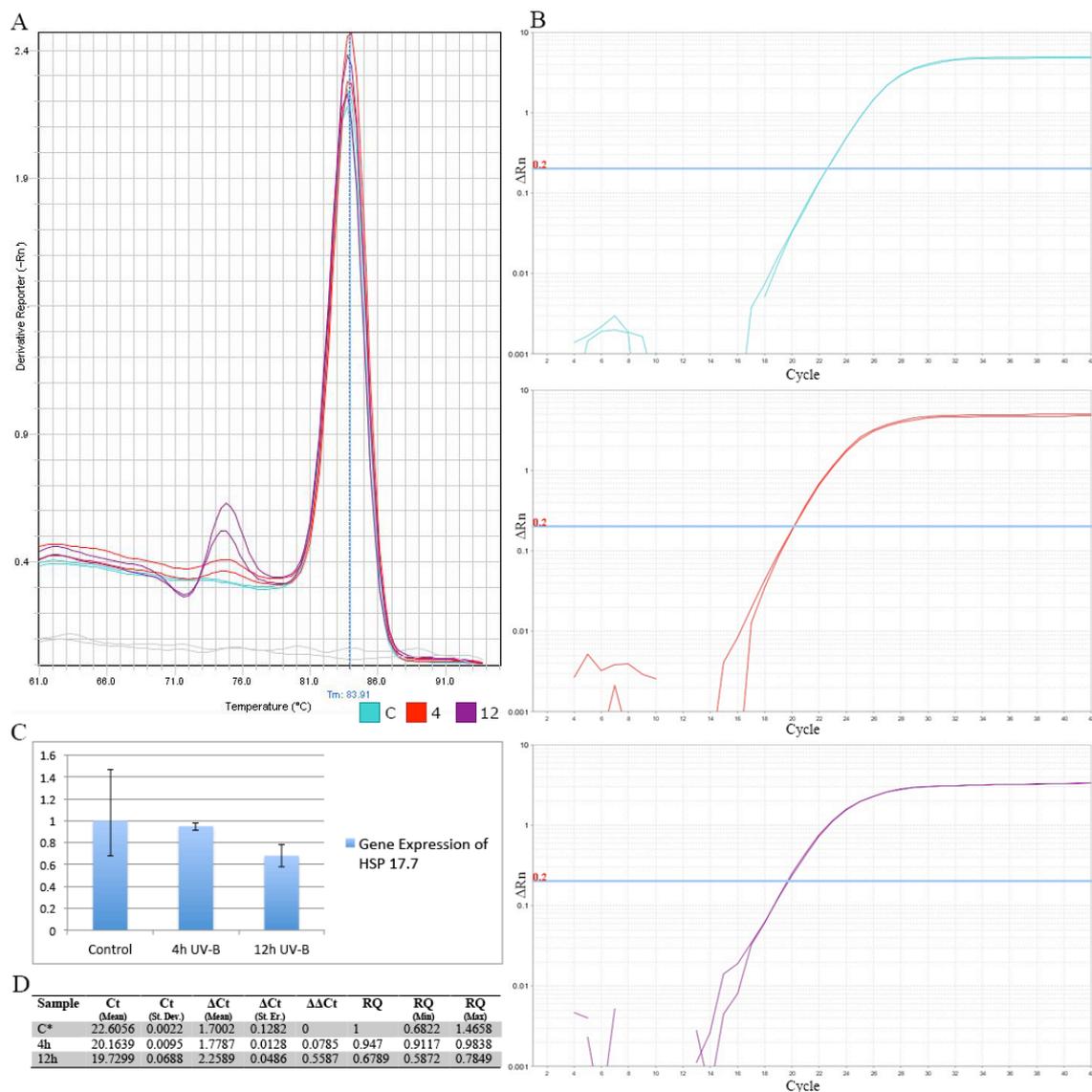


Figure 3.7 Real Time PCR results of *Hsp17.7* expression analysis. A) Melting curve plot of transcription phase of *Hsp17.7* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp17.7* (Relative quantities of *Hsp17.7* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.3 Hsp17.8

Hsp17.8 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. When compared results, 4h UV-B expression was firstly increased, but with more UV-B exposing, 12h UV-B expression was decreased highly according to each other.

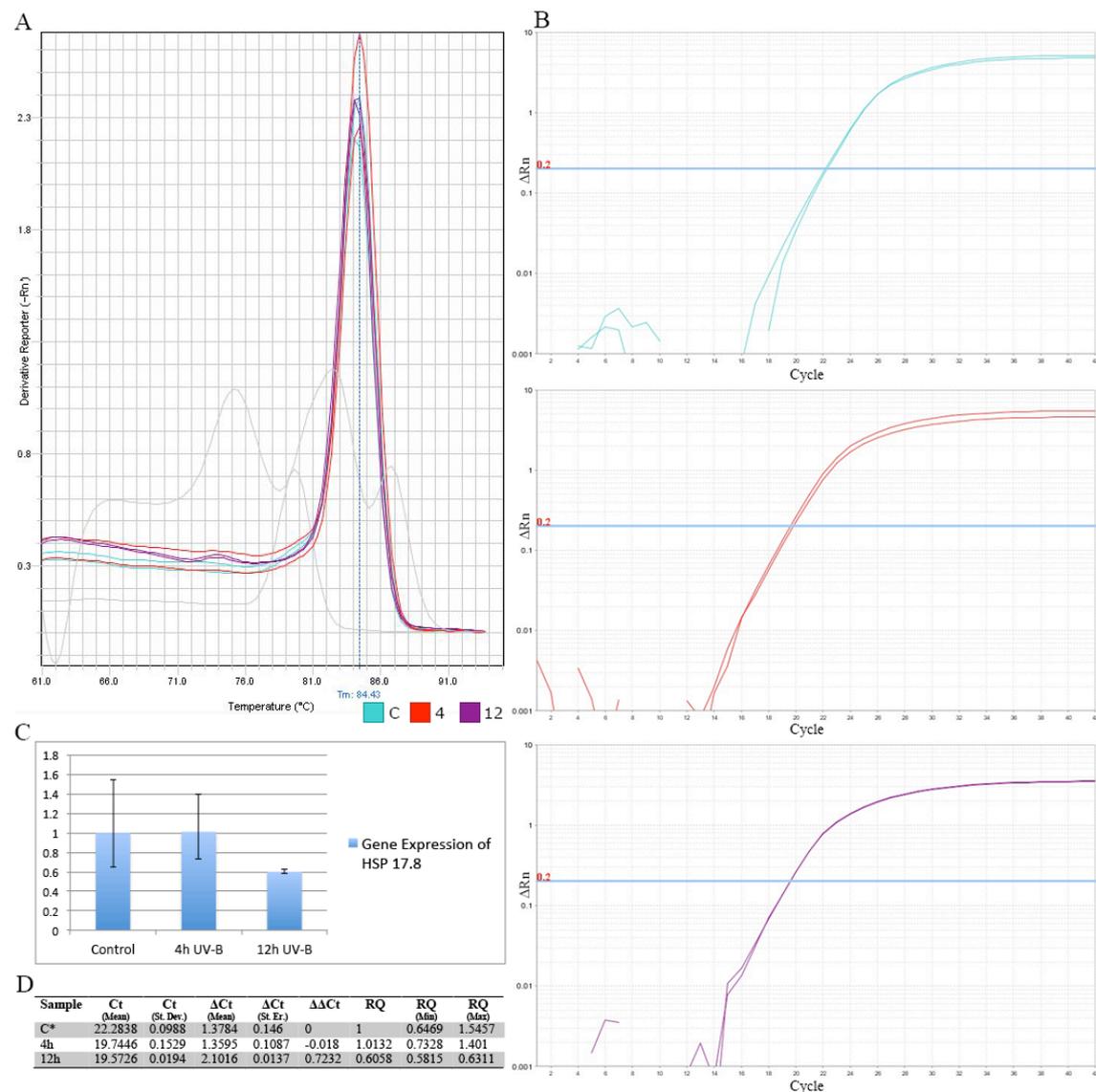


Figure 3.8 Real Time PCR results of *Hsp17.8* expression analysis. A) Melting curve plot of transcription phase of *Hsp17.8* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp17.8* (Relative quantities of *Hsp17.8* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.4 Hsp20

Hsp20 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. Two different UV-B condition affected *Hsp20* expression seriously. According to results, *Hsp20* expression was decreased to nearly zero value in both 4h UV-B and 12h UV-B.

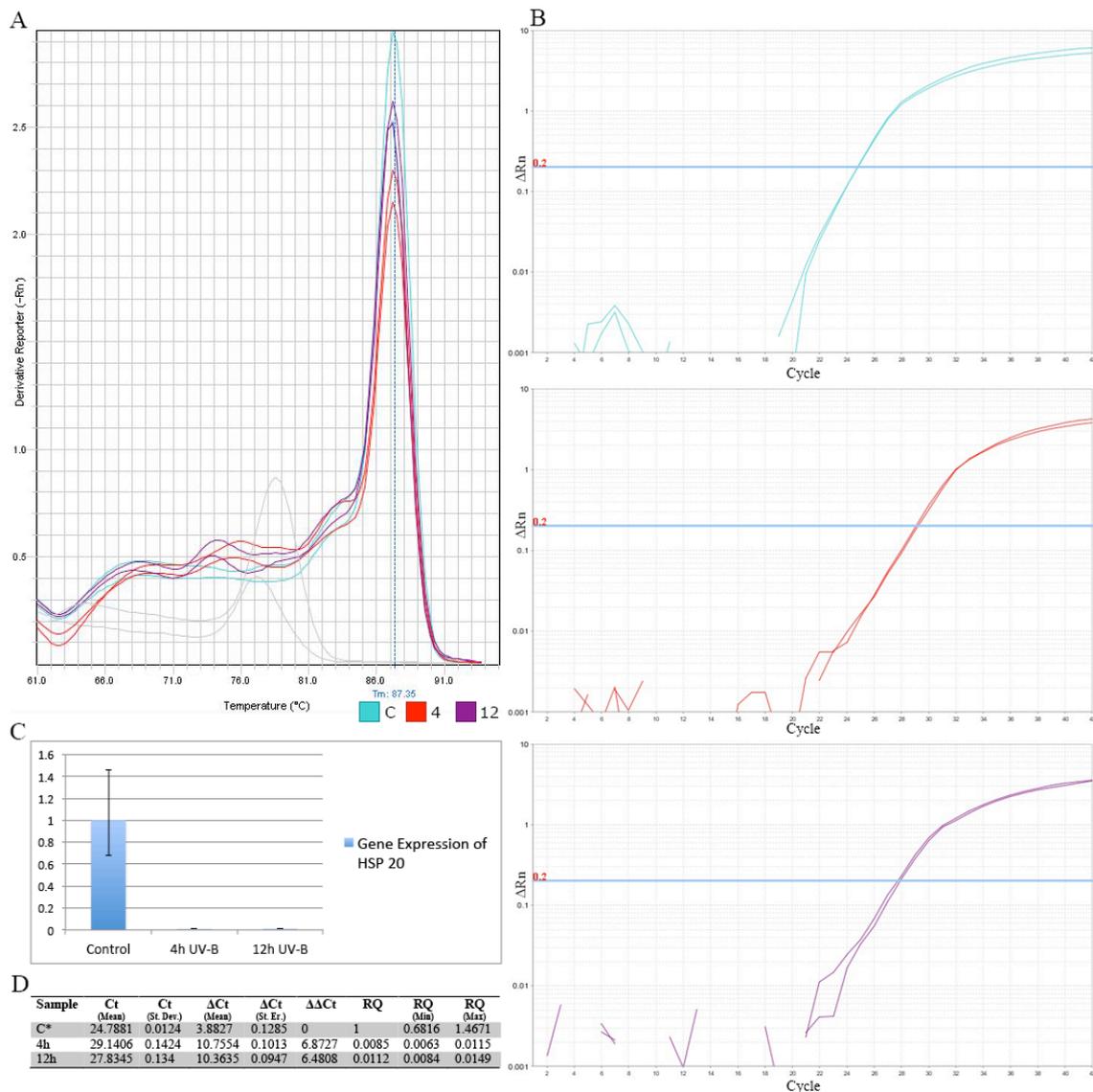


Figure 3.9 Real Time PCR results of *Hsp20* expression analysis. A) Melting curve plot of transcription phase of *Hsp20* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp20* (Relative quantities of *Hsp20* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.5 Hsp21.5

Hsp21.5 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. When compared control plant gene expression with each other, 4h UV-B and 12h UV-B gene expression was highly decreased.

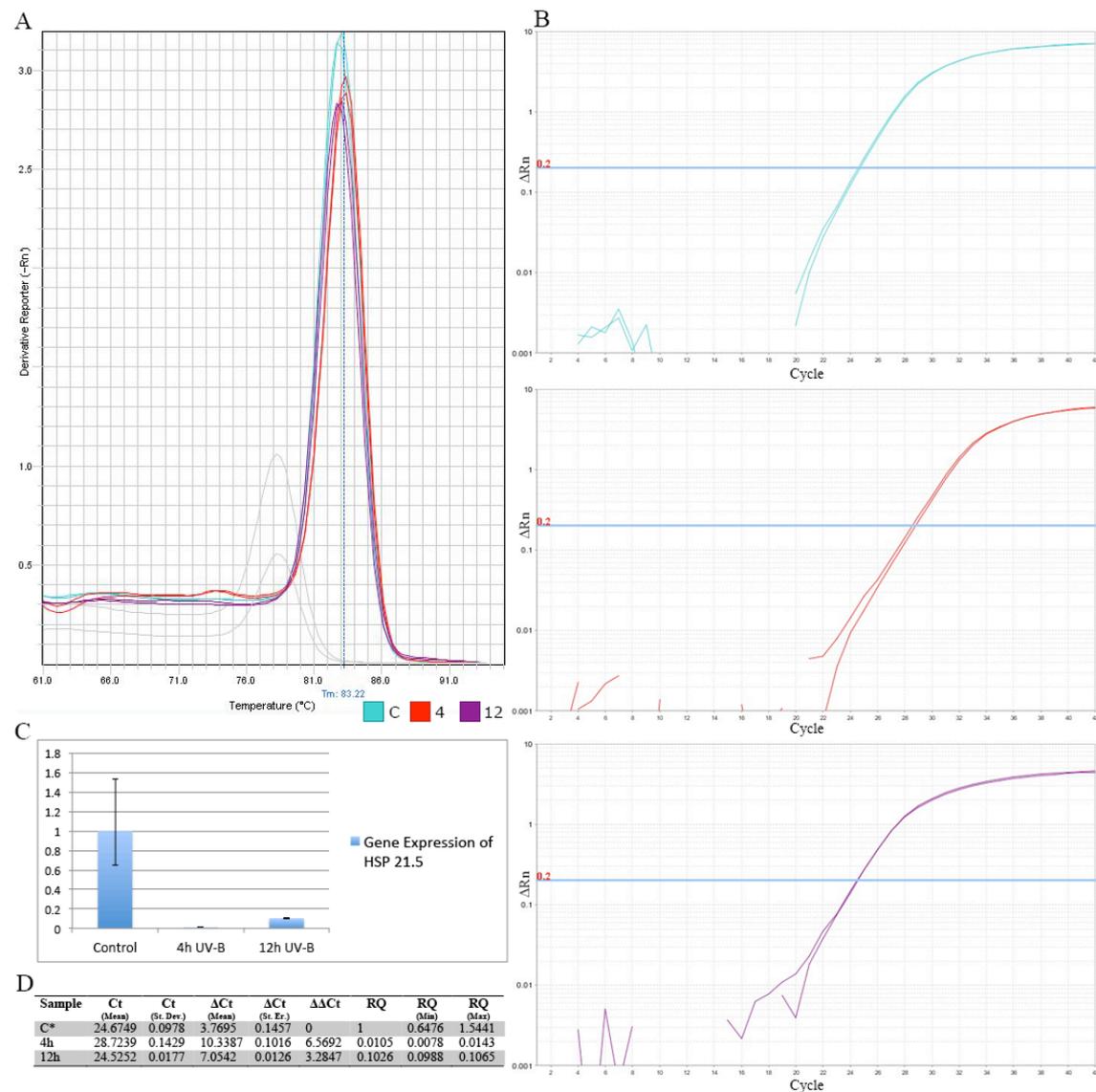


Figure 3.10 Real Time PCR results of *Hsp21.5* expression analysis. A) Melting curve plot of transcription phase of *Hsp21.5* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp21.5* (Relative quantities of *Hsp21.5* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.6 Hsp23.8

Hsp23.8 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. Gene expression of 4h UV-B was increased about 1.5-fold, then this value has decreased to about control plant expression level.

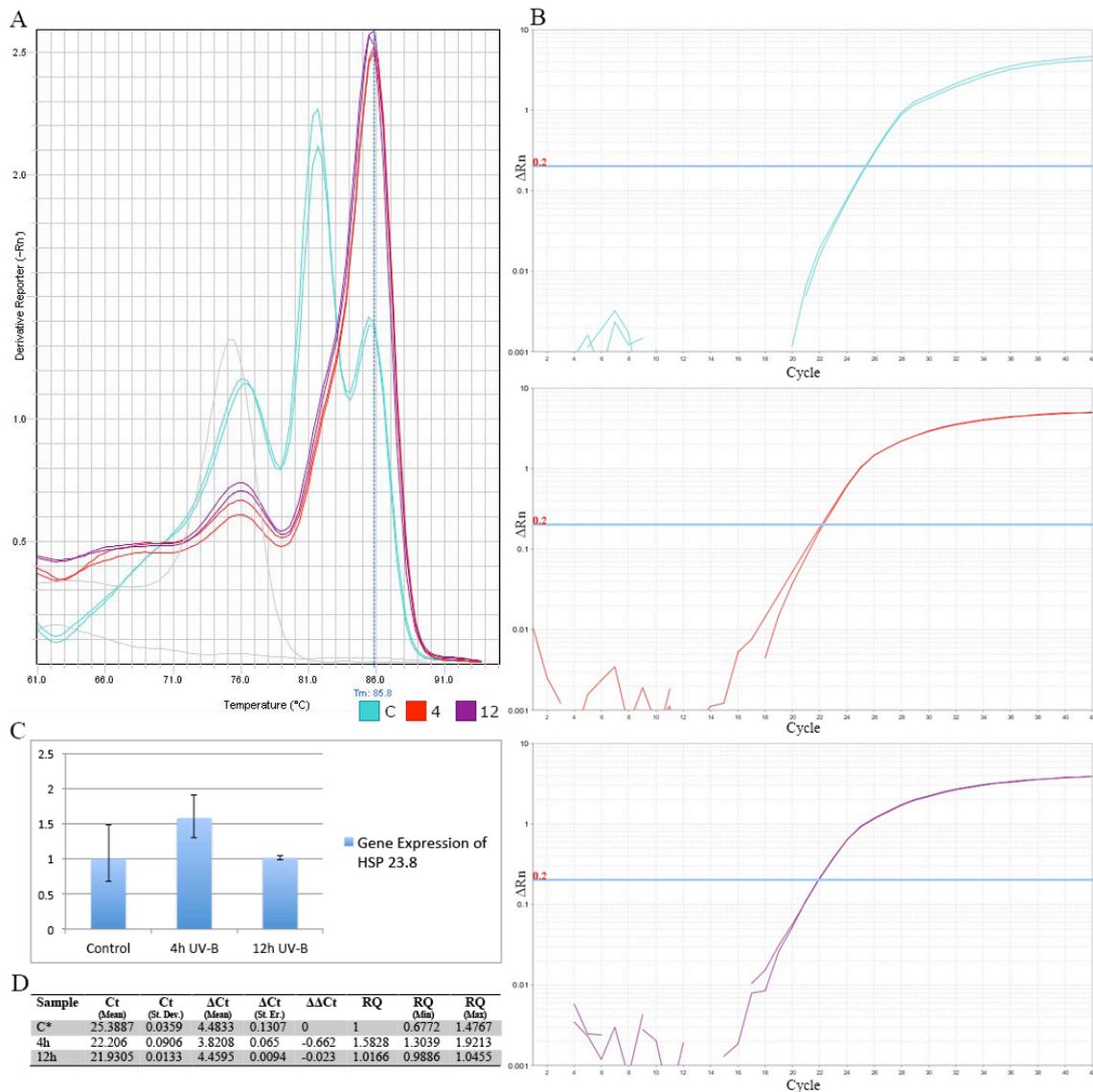


Figure 3.11 Real Time PCR results of *Hsp23.8* expression analysis. A) Melting curve plot of transcription phase of *Hsp23.8* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp23.8* (Relative quantities of *Hsp23.8* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.7 Hsp70

Hsp70 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. Based on the gene expression chart, *Hsp70* expression of 4h UV-B and 12h UV-B exposed tomato plants has decreased highly when compared to control plant.

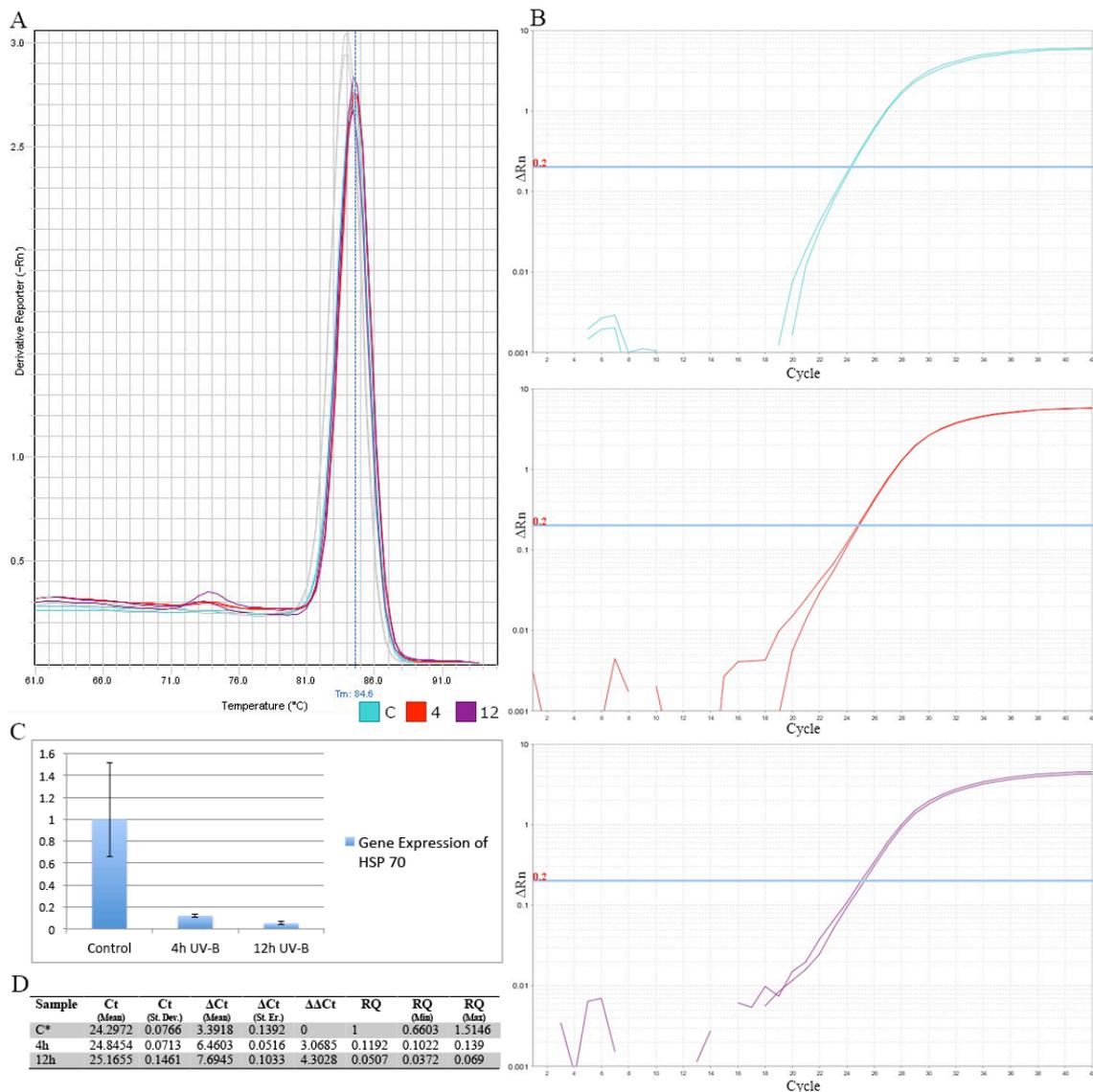


Figure 3.12 Real Time PCR results of *Hsp70* expression analysis. A) Melting curve plot of transcription phase of *Hsp70* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp70* (Relative quantities of *Hsp70* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.8 Hsp90

Hsp90 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. *Hsp90* expression level of 4h UV-B and 12h UV-B plants was increased about 6-8-fold level according to control plant. On the other hand, 12h UV-B expression level was decreased slightly when compared to 4h UV-B plant.

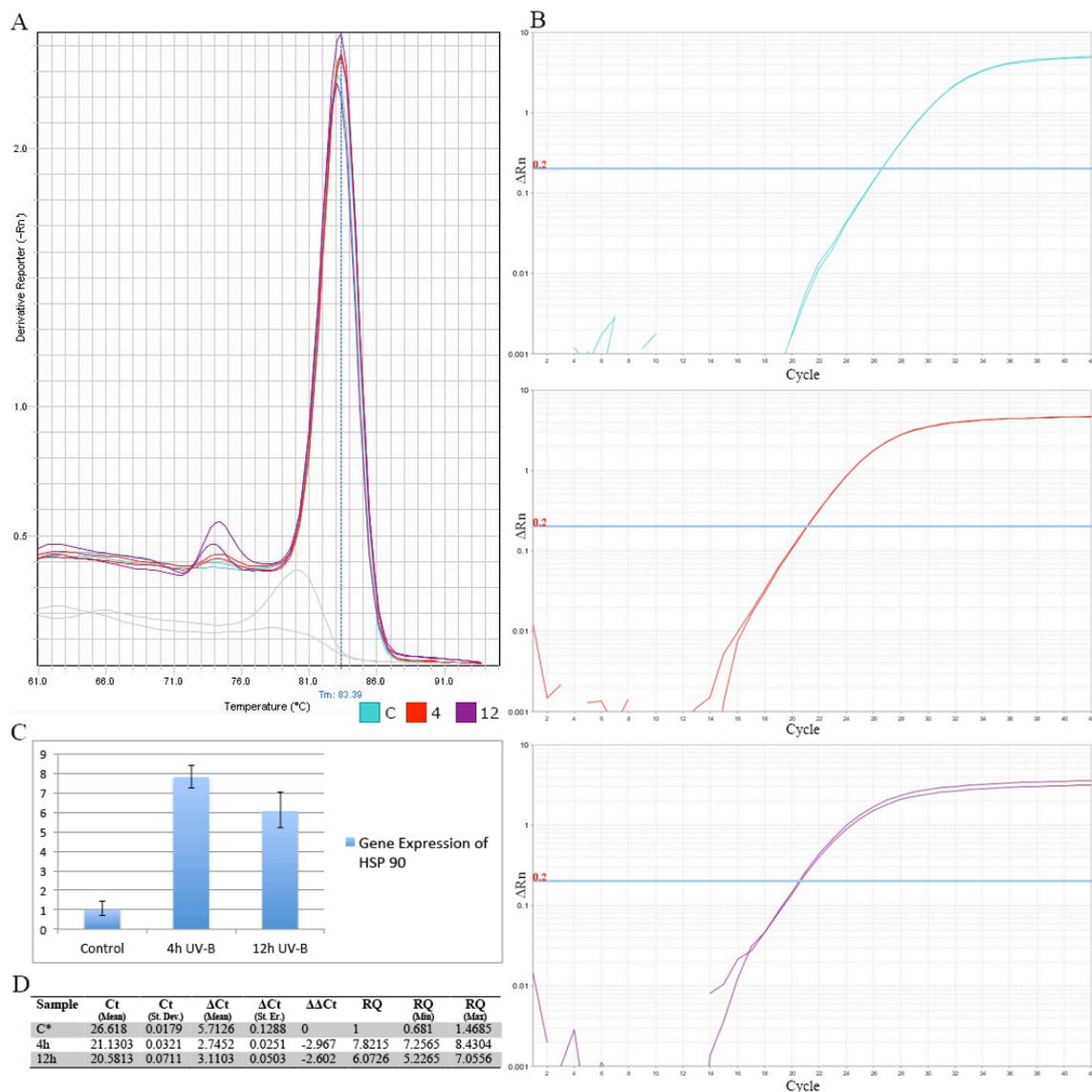


Figure 3.13 Real Time PCR results of *Hsp90* expression analysis. A) Melting curve plot of transcription phase of *Hsp90* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp90* (Relative quantities of *Hsp90* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.9 Hsp100

Hsp100 expression results of 0h UV-B (Control), 4h UV-B and 12h UV-B exposed tomato plants. 4h UV-B and 12h UV-B expression levels of *Hsp100* gene was decreased if compared with control plant. Expression level of 12h UV-B was increased slightly when compared to 4h UV-B.

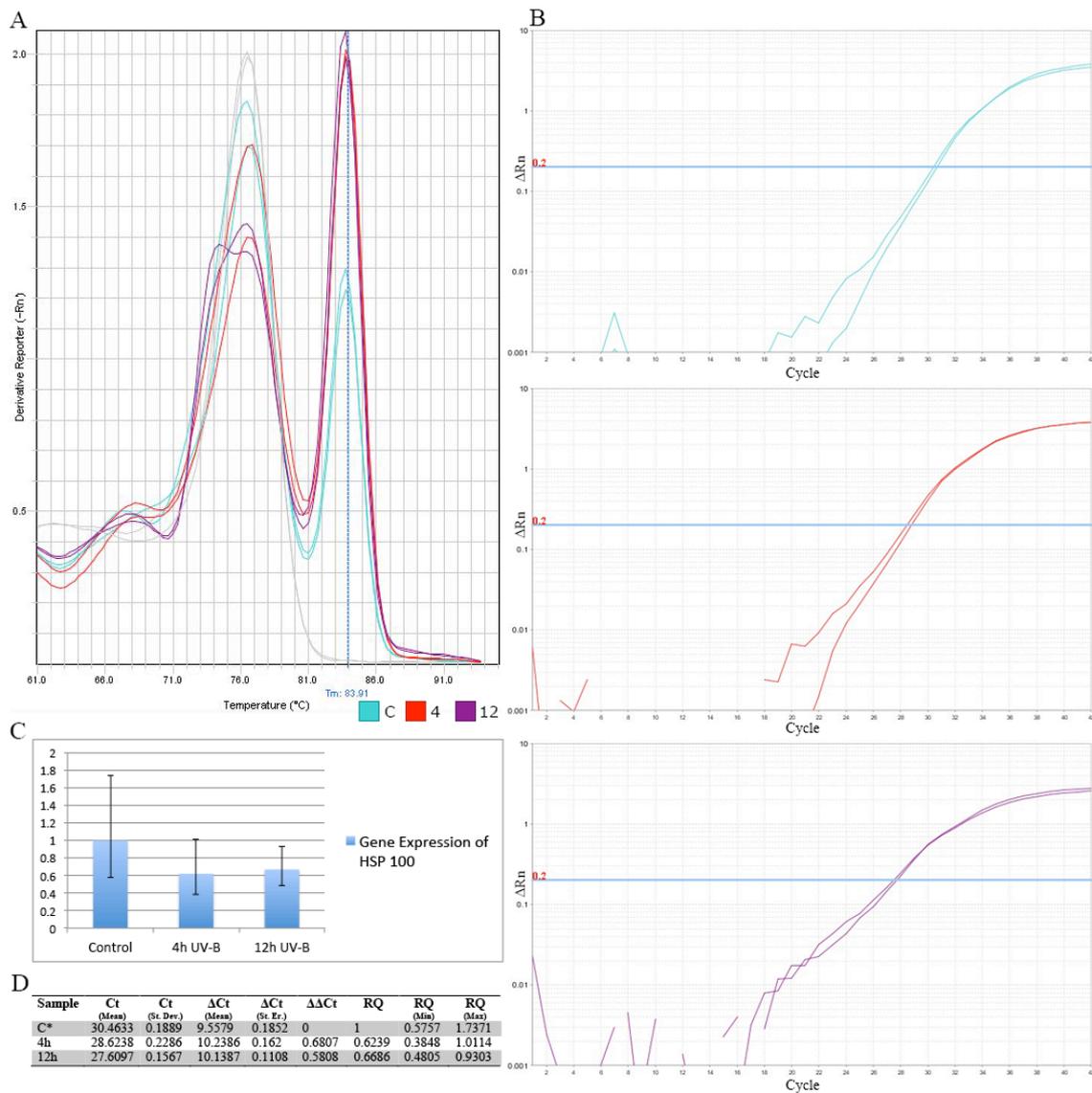


Figure 3.14 Real Time PCR results of *Hsp100* expression analysis. A) Melting curve plot of transcription phase of *Hsp100* genes. B) Amplification plot of fluorescence signal versus cycle number. C) Gene expression of *Hsp100* (Relative quantities of *Hsp100* transcriptions.) Gene expression value of control (C*) plant was fastened to 1 value to compare with other plant expressions. D) Detailed results of Real Time PCR experiment.

3.3.10 Actin

Housekeeping genes are expressed in all cells of an organism under normal and other (stress, patho-physiological) conditions. In this experiment, *Actin* was used as housekeeping gene.

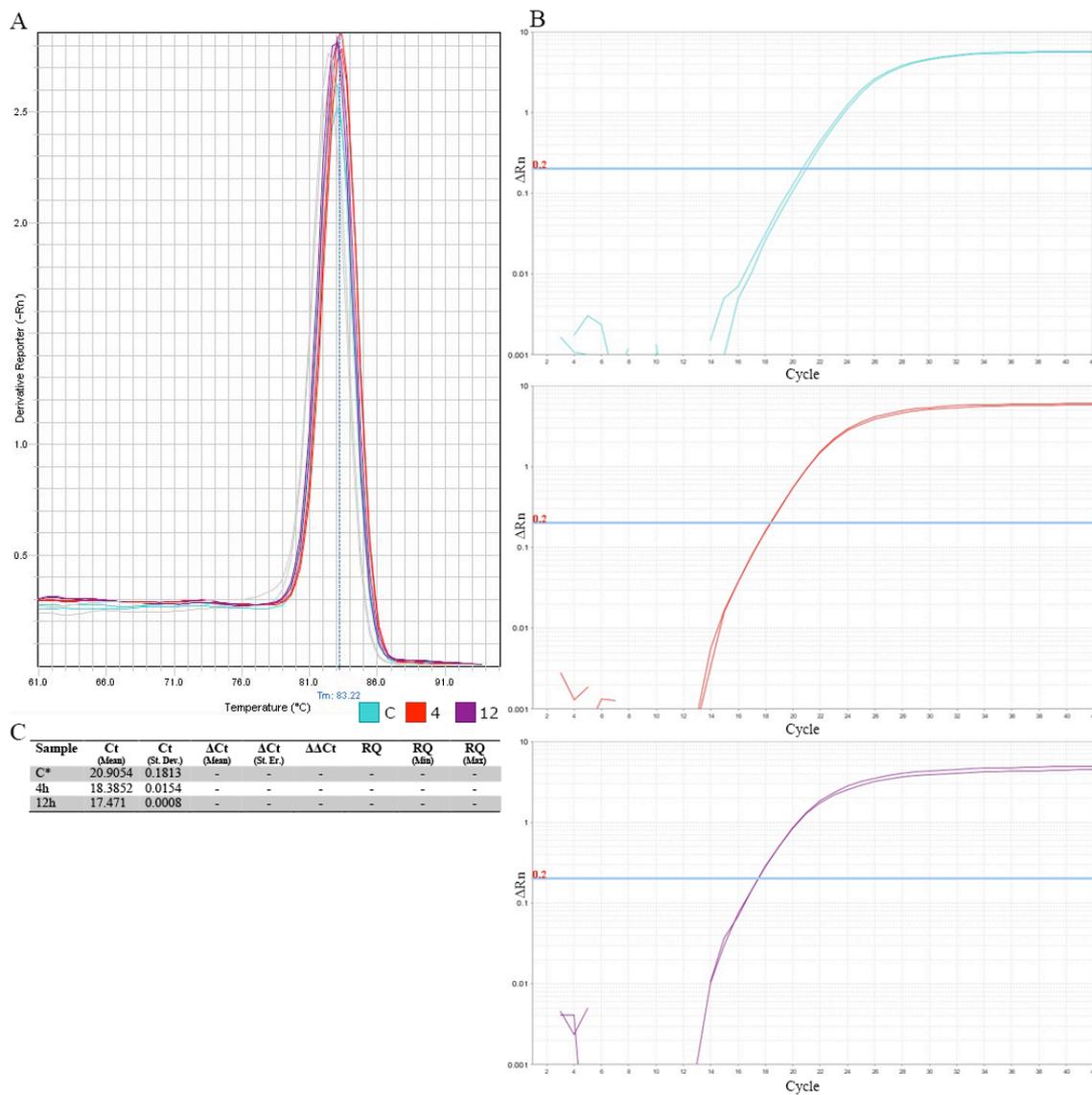


Figure 3.15 Real Time PCR results of *Actin* expression analysis. A) Melting curve plot of transcription phase of *Actin* gene. B) Amplification plot of fluorescence signal versus cycle number. C) Detailed results of Real Time PCR experiment.

CHAPTER 4

DISCUSSION & CONCLUSION

NASA briefly explained ozone depletion in the following way. “*The term "ozone depletion" means more than just the natural destruction of ozone, it means that ozone loss is exceeding ozone creation. Think again of the "leaky bucket." Putting additional ozone-destroying compounds such as CFCs into the atmosphere is like increasing the size of the holes in our "bucket" of ozone. The larger holes cause ozone to leak out at a faster rate than ozone is being created. Consequently, the level of ozone protecting us from ultraviolet radiation decreases (Shelley Canright, 2004).*”

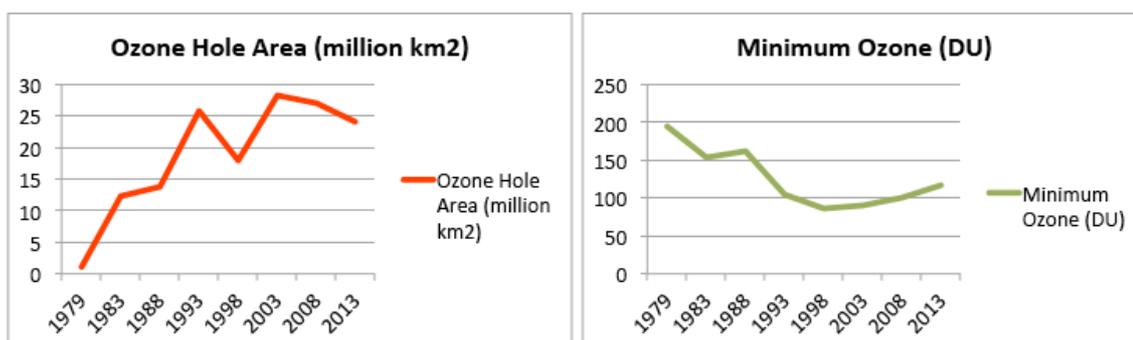


Figure 4.1 Ozone hole area and minimum ozone for each year. The maximum of the daily ozone hole size and the minimum of the daily ozone minimums for each year and the date of occurrence (Eric R. Nash, NASA Annual Records)

All plants need to absorb light energy coming from Sun to survive. They use this energy type for photosynthesis. By photosynthesis green plants transform solar energy

to chemical energy, which is used to synthesis food, wood and the biomass. Human eye will not respond to wavelengths 400 nm and below. At the same time, these rays (ultraviolet) are not be absorbed by plant pigments. 330 nm and below wavelengths have serious harmful effects on living organism cells. Most of them are filtered by the ozone layer virtually (Vermaas, 2007).

Plants are very susceptible to all kinds of abiotic stress such as ultraviolet-B radiation in their natural life. The outcome of such stress can vary between subgroups of the same species. This variation is largely dependent on the molecular response of plant cells to implement a UV-B protection mechanism. The implement of a UV-B protection mechanism is related to molecular response of plant cell of this variation. The Sun emits a full-spectrum of UV radiation; one of them is ultraviolet-B, which is the most important factor in controlling plant development and growth. Light is one of the most important needs of plants to survive and support plant photosynthesis. Different wavelengths affect developmental processes as diverse as de-etiolating, phototropism and flowering of plants. At the molecular level, light is first perceived by plants and in turn regulates gene expression through a signal transduction pathway. Up-regulation and down-regulation of gene expressions are altered by the UV-B radiation. Many defence-related genes become active by UV-B radiation. On the other hand, several photosynthetic genes (*Lhcb*, *RuBisco*, etc.) are down regulated. UV-B stress conditions can affect gene regulation level of plants with specific and diverse responses. Ultraviolet-B-induced morphological and molecular responses can differ according to several factors such as dose and exposure time to UV-B, plant variants or species, etc. (Jordan, 1996).

In brief, solar energy has some different wavelengths, which can be useful or harmful for living organisms such as photosynthetic plants. Stratosphere layer has a critical role in filtering harmful lights to prevent them from reaching on the Earth. With the depletion of ozone layer, harmful ultraviolet-B rays cannot be filtered in stratosphere and more UVB reaches living organisms. They can be subject of harmful effects of UV-B, which is one of the abiotic stress factors for plants. As known, all living organisms have defence mechanisms to prevent harmful effects of abiotic stress.

We exposed the plants to high dose UV-B in this treatment. In some literatures, a high and low dose of UV-B was used to determine *Hsp* expression. For example, Kim et al. (2010) used several UV-B conditions (2-4-6-8-10 kJ/m²) to determine *Hsp* expression rate. Different results can be seen in these literatures. As mentioned earlier, results can be different not only in various types of plants, but also in the same species of a plant. Given that, a comparison of our results with other researches may not be accurate. We have used 6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (0h, 4h and 12h) to obtain Heat Shock Protein expression level under these UV-B conditions, which were performed for the first time for tomato plant.

The melting curve results of *Hsp23.8* and *Hsp100* show us that, during Real Time PCR experiment, formation of primer dimer and non-specific amplifications were observed. *Hsp23.8* and *Hsp100* results may not be reliable for comparison with other results. Reanalysis of those two genes may give more accurate results.

According to the results, UV-B radiation causes up- or down-regulation of some important genes. Growth retardation is observed as a result of up- and down regulation of those genes. Previous researches show that, 8 kJ/m² (4.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ – PPF) and higher UV-B radiation induces down-regulation of several *Hsp* genes (Kim et al., 2010). In this study, Real-time PCR experiments demonstrated the down-regulation of *Hsp17.4*, *Hsp17.7*, *Hsp17.8*, *Hsp20*, *Hsp21.5*, *Hsp70* and *Hsp100* genes. On the other hand, an increase at the expression level of *Hsp90* was observed. However, there was no significant change at the expression rate of *Hsp23.8* in UV-B-exposed plants compared to control plant. The result of *Hsp23.8* is not sufficient enough to interpret the correlation between UV-B radiation and expression rate.

The expression level of *Hsp90* increased about 8-fold after 0h and 4h UVB compared to control plant. However, the expression rate demonstrated a 3/4 fold of decreased expression level after 12h UV-B. In this instance, two significant reasons may have occurred during UV-B treatment. Plants may have developed a tolerance against UV-B stress after 4h UV-B exposure or a damage in biological system and loss of defence mechanism against UV-B of plants resulted in incapability in stress-tolerance of high dose of UV-B. For more accurate results, this experiment may be repeated under several UV-B conditions. For example, plants can be grown under growth light with natural UV-B dose to simulate solar light.

Eventually, *Hsp90* genes may have a critical role in providing tolerance against enhanced UV-B stress in tomato plant. This study can be important source for future studies to obtain a UV-B tolerant plant by using gene-cloning method.

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