TRANSCRIPTION LEVEL DETERMINATION OF CANDIDATE GENES UPON INFECTIONS OF POWDERY MILDEW ON BARLEY

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TRANSCRIPTION LEVEL DETERMINATION OF CANDIDATE GENES UPON INFECTIONS OF POWDERY MILDEW ON BARLEY

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ABSTRACT

TRANSCRIPTION LEVEL DETERMINATION OF CANDIDATE GENES UPON INFECTIONS OF POWDERY MILDEW ON BARLEY

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Immune systems are fundamentally based on the differentiation of self and non-self. Unlike mammals, plants have an innate immune system responding to the pathogen only at the site of attack. One of these pathogens is *Blumeria graminis* f. sp. *hordei* which is an obligate biotrophic pathogen causing powdery mildew disease and resulting in up to 30% yield loss for both cultivated and wild barley.

In this study, Pallas-01 (P-01) and Pallas-03 (P-03) barley lines were inoculated with powdery mildew race *Bgh*103 (64/01) resulting incompatible and compatible interactions, respectively. 6, 12, 24, 48 and 72 hour-post-inoculation (hpi) samples were used in order to define the differential gene expression of NAD malic enzyme, chloroplast lipocalin, phosphoglyceromutase (PGM), Mg chelatase and 26S protease regulatory subunit 6B homolog. In the proteomics study previously conducted in the laboratory, except for the NAD-dependent malic enzyme, the other four proteins were shown to be involved in the incompatible interaction of P-01 and *Bgh*103 at protein level, whereas NAD-dependent malic enzyme was changing in the compatible interaction. The expression level for both proteomics

and transcriptomics were assumed to be similar. However, the level of transcript would not always reflect its protein level or correlate with the level of proteins, due to complex cellular regulation processes. Post-transcriptional modifications such as synthesis, processing, degradation and post-translational modifications are changing the level of proteins expressed, thus a parallel correlation between the protein and mRNA levels can be lost. Other possible reasons for this variation can be changes in mRNA and protein stability, efficiency of translation and protein's turnover rate.

The transcription level changes of the genes investigated in this study are found to be differentially expressed, supporting the proteomics data indicating that these genes are possibly involved in resistance. For further investigations, genetic tools such as gene silencing with RNAi and knockout experiments are required in order to elucidate the mechanism of these candidate genes in the plant-pathogen interaction.

Keywords: Barley, powdery mildew, qRT-PCR, NAD-dependent malic enzyme, chloroplast lipocalin, phosphoglyceromutase (PGM), Mg chelatase, 26S protease regulatory subunit 6B homolog

ÖZ

KÜLLEME HASTALIĞINA KARŞI ARPALARDA PROTEOMİKS ADAY GENLERİN TRANSKRİPSİYON DÜZEYLERİNİN BELİRLENMESİ

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Bağışıklık sistemi kendinden olan ve olmayanı ayırt etmek üzerine kurulu bir sistemdir. Hayvanların aksine bitkiler doğuştan gelen bağışıklık sistemine sahiptirler. Bu sistemle bitkiler yalnızca patojenin hücum ettiği noktada patojene cevap verebilirler. Bu patojenlerden bir tanesi olan *Blumeria graminis* f. sp. *hordei* arpa bitkisinde külleme hastalığına sebep olmakta ve hem yabani hem kültür arpalarında % 30'a kadar verim kaybına neden olmaktadır.

Bu çalışmada külleme hastalığına neden olan *Bgh*103 (64/01) ırkı ile muamele edilen Pallas-01 (P-01) ve Pallas-03 (P-03) arpa hatları kullanılmıştır. Bu muamele sonucunda sırasıyla uyumsuz ve uyumlu ilişki gözlenmektedir. NAD malik enzim, kloroplast lipocalin, fosfogliseromutaz (PGM), Mg çelataz and 26S proteaz düzenleyici 6B homolog alt ünitesi genlerinde değişken gen anlatım düzeyini belirlemek için 6., 12., 24., 48. ve 72. saat örnekleri kullanılmıştır. NAD-malik enzim dışındaki diğer dört proteinin P-01 ve Bgh103 arasındaki uyumsuz ilişki ile ve NAD-malik enzimin ise P-03 ve Bgh103 arasındaki uyumlu ilişki ile alakalı olduğu protein düzeyinde daha önceden laboratuarımızda gerçekleştirile bir

çalışmayla gösterilmişti. Proteomik ve transkriptomik sonucunda belirlenen anlatım düzeylerinin benzer olması beklenmekteydi. Ancak hücrenin kompleks düzenleyici proseslerden dolayı transkript miktarı her zaman protein düzeyine yansıtmayabilir. Sentez, proses, yıkım gibi transkripsiyon sonrası düzenlemeler ile translasyon sonrası düzenlemeler sentezlenen protein miktarını değiştirmektedir. Hücrede mRNA miktarı ile protein miktarı arasındaki eşitsizliğin bir diğer nedeni mRNA ve proteinde meydana gelen dengesizlikler, translasyonun verimi ve protein sentezi ve yıkımı arasındaki denge olabilir.

Değişken protein miktarları daha önce gösterilmiş olan aday genler için, bu deneyde gen anlatım düzeylerinde meydana gelen değişiklikler de göz önüne alınırsa bu genlerin olası direnç ile ilişkili olabilecekleri sonucuna varılmıştır. İlerideki çalışmalarda bu aday genlerin bitki-patojen ilişkisindeki mekanizmalarını aydınlatmak için ise RNAi ile gen susturma ve knockout gibi genetik yöntemler kullanılması gerekmektedir.

Anahtar Kelimeler: Arpa, külleme, qRT-PCR, NAD malik enzim, lipocalin kloroplast, fosfogliseromutaz (PGM), Mg çelataz, 26S proteaz düzenleyici 6B homolog alt ünitesi

To my family and my beloved Onur;
For their endless support and motivation

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	: adenosine triphosphate
ABA	: abscisic acid
ADP	: adenosine diphosphate
Avr	: Avirulence
Bgh	: *B.graminis* f.sp *tritici*
^{o} C	: Degrees celcius
cm	: centimeter
cDNA	: complementary DNA
Ca^{+2}	: Calcium
CaM	: Calmodulin
CHLH	: Chelatase H subunit
DEPC	: diethylpyrocarbonate
DMSO	: dimethyl sulfoxide
DNA	: deoxyribonucleic acid
dNTP	: deoxyribonucleoside triphosphate
DTT	: dithiothreitol
EF	: Elongation factor
ETI	: effector triggered immunity
ETS	: effector-triggered susceptibility
EtBr	: ethidium bromide
g	: gram
GTP	: guanoside 5'-triphosphate
Hpi	: hour post inoculation
HR	: hypersensitive response
LRR	: leucine rich repeat
LRR-RK	: leucine rich repeat receptor kinase
M	: molar
MAPM	: microbe associated molecular pattern
mg	: milligram

min	: minute
mL	: millilitre
mM	: millimolar
mRNA	: messenger RNA
µg	: microgram
µL	: microliter
µM	: micromolar
NAD-ME	:NAD-dependent malic enzyme
NADP-ME	:NADP-dependent malic enzyme
NB-LRR	: Nucleotide binding leucine rich repeat
NO	: Nitric oxide
ng	: nanogram
nm	: nanometer
OAA	: oxaloacetate
ORF	: open reading frame
PAMP	: pathogen associated molecular pattern
PGM	: phosphoglyceromutase
PM	: powdery mildew
PPR	: pattern recognition receptors
PCD	: programmed cell death
PCR	: polymerase chain reaction
PTI	: pathogen-triggered immunity
qRT-PCR	: quantitative real time PCR
rpm	: rotation per minute
RLK	: Receptor like kinases
RLP	: Receptor like protein
TTSS	: Type III Secretion System
TIL	: Temperature induced lipocalin
Ubi	: Ubiquitin
Ubc	: Ubiquitin conjugating protein

CHAPTER I

INTRODUCTION

1.1 The Plant Immune System

Plants and mammals are differing in their respond to pathogen invasion. Unlike mammals which have an adaptive immune response via the circulatory system in order to respond the pathogen attacks; plants have innate immune response which means every single cell of the plant responds to the pathogen attack by its own effort (Jones et al., 2004).

In nature, plants should cope with several factors affecting them but disease rarely occurs due to basal defense or non-host resistance. Resistance emerges when the pathogen is not able to pass through plant barriers or plant defense system is activated in case of pathogen recognition. On the contrary, disease emerges when plants do not develop resistance responses against the matching pathogen effectors (Hückelhoven, 2005).

The battle between plant and pathogen is an evolutionary process involving four parts. In the early phase, plants induce basal defense when they recognize the broadly conserved factors secreted by the pathogen called microbe associated molecular patterns (MAMPs). This immunity is also called as pathogen-triggered immunity (PTI). Bacterial flagellins, fungal chitin, Pep-13 are several examples for the MAMPs detected by plants. MAMPs are recognized by plant's pattern recognition receptors (PRRs). For instance, a conserved *flg2* flagellin domain is recognized by a PRR located on the membrane called FLS2 which is a LRR-RK (leucine-rich repeat receptor kinase). Although the perception of the MAMPs by

PRRs provides weak immunity, it blocks further colonization (Jones et al., 2006; Bent et al., 2007).

Basal defense is not very effective against pathogen attacks since in second phase pathogen develops some adaptations towards the basal defense in order to overcome it. These modifications of the pathogen are mostly host specific. In this phase, pathogens can overcome PTI resulting in effector-triggered susceptibility (ETS). The suppression is achieved by pathogen's gaining of some functions, such as structural roles, functioning in nutrient leakage or pathogen dispersal. Pathogens also produce small molecules that are inhibiting or mimicking plant cell functions. For instance, mimicking plant hormones such as jasmonic acid, auxin, giberellin and cytokinin provides pathogen to invade host cells successfully. The delivery of these molecules is carried out by type III secretion system (TTSS) which represses PTI allowing further colonization (Jones et al., 2006; Bent et al., 2007). TTSS is mostly used for bacterial pathogens. There is no known TTSS for fungal pathogens.

The plant response process involves evolution of plants to resist the pathogens. When the specific elicitors, also named as *Avr* (avirulence) genes, are recognized by specific disease resistance *R* genes, effector-triggered immunity (ETI) is achieved. When both *Avr* gene of the pathogen and corresponding R gene of plant are present, the result is resistance against a particular pathogen race or strain. However, if one of these genes is missing, disease emerges. This R-*avr* interaction triggers the chain of signal transduction events which blocks the colonization of the pathogen. *R* gene mediated ETI is more rapid, stronger and more effective than PTI. ETI usually results in the formation of hypersensitive local cell death response (HR) at the site of infection (Dangl et al., 2001). The NB-LRR (nucleotide binding leucine rich repeat), RLKs (receptor like kinases), RLPs (receptor like protein) and Ser/ Thr kinases are four major classes of *R* gene products (Gurr et al., 2005). The largest class is NB-LRR proteins. These proteins

consist of a C-terminal LRR domain, mainly functioning in ligand binding and protein-protein interaction, and a conserved N-terminal NB domain, functioning in ATP or GTP binding. R proteins have the ability to trigger host cell death and their expression should be activated rapidly when interacts with *Avr* gene product. Therefore, their activity should be regulated by auto-inhibition of the R protein in the absence of elicitors. When ADP is bound to the protein, it is in its OFF-state while ATP-bound state is the ON-state. NB domain triggers the nucleotide hydrolysis which provides energy necessary for conformational change (Takken et al., 2009).

R genes recognize the pathogen to trigger defense responses either by directly or indirectly. Gene for gene hypothesis proposes that there is a direct interaction between *Avr* gene of the pathogen and its cognate *R* gene in the host (Flor, 1955). Whereas, guard hypothesis proposes that R proteins recognize elicitors indirectly by targeting host proteins other than R proteins triggering defense system (van der Biezen et al., 1998).

In the last phase of the process, pathogen evolves to overcome ETI. Pathogens escape from the detection by *R* gene products either by suppressing the defense induced by *R* gene products or by eliminating the effectors causing virulence. If the interaction between *R* and *Avr* genes is direct, any mutation leading to change in the shape while retaining function blocks the recognition and suppresses defense responses. On the other hand, if the interaction is indirect, pathogen escapes from recognition only by pausing the virulence activity (Bent et al., 2007).

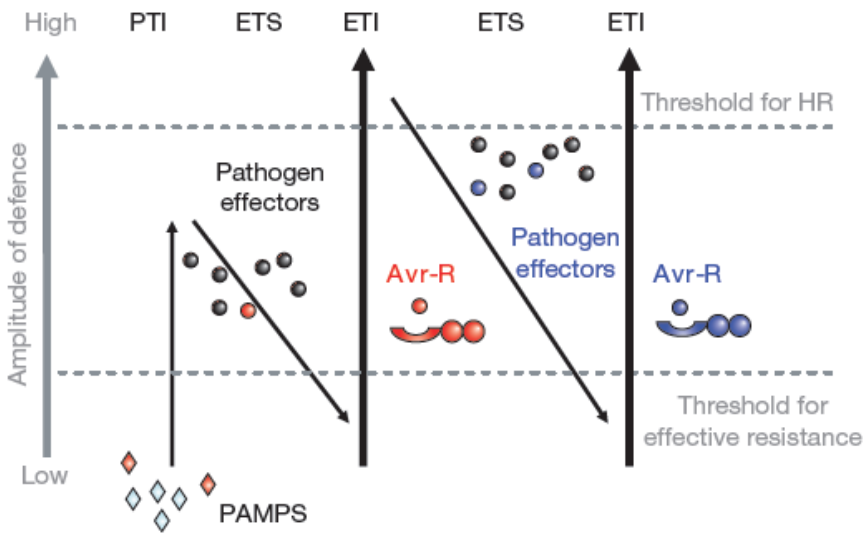


Figure 1.1 The zigzag model demonstrating the evolutionary process between plant and pathogen taken from Jones et al., (2006).

1.2 Powdery Mildew Disease of Barley

Barley, *Hordeum vulgare*, is an annual monocot plant and member of the Poaceae (Grass) family. It is the second most produced grain. Barley is produced 133 million tons around the world and with 7.3 million tons production in Turkey; she is the 15th barley producer of the World (FAOSTAT, 2009). Barley production increased throughout the years of 2007 to 2011 with average 6.8 million tons for 2007-2009 and estimated 7.2 million tons for 2010 and forecasted 7.3 million tons for 2011 (FAO, 2011). Barley serves as the major feed for animals as well as it is used in fermented beverages and beer production by facilitating malting.

Powdery mildew (PM) disease causes the yield loss for both wild and cultivated crops up to 30%. The causal agent for powdery mildew disease is the powdery mildew fungi which are Ascomycetes belonging to the Erysiphaceae family

(Hückelhoven, 2005). The fungus infects the aerial parts of the various monocotyledonous plants, such as barley and wheat, and dicotyledonous plants, such as Arabidopsis. Different forms of *Blumeria graminis* are specialized and host specific in order to infect different cereals; such as *B. graminis* f. sp *hordei* (*Bgh)* infects barley, *B. graminis* f. sp *tritici* infects wheat. *Bgh* is the most studied species of the powdery mildew pathogen (Both et al., 2005) so it is used as a model organism for obligate biotrophic pathogens.

Most of the pathogenic diseases usually burst in high humid areas whereas powdery mildew disease is more common in semiarid regions meaning PM spores do not need water to germinate. Conidiospores after the interaction with the plant surface produce barrel-shaped chains of one-celled conidium that are dispersed by the wind in order to start another infection cycle (Horst, 2008). Spores landing on the surface of the plant form into tangles of conidiospores that give the white velvety appearance to the pathogen. These characteristic symptoms can be observed on the leaves, stems and petals of the plant (Eichmann et al., 2008).

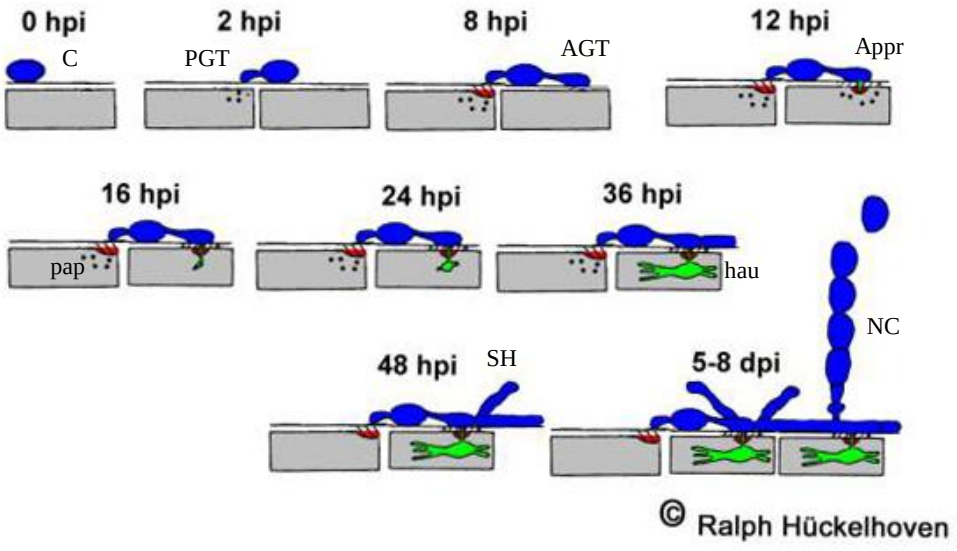


Figure 1.2 Stages of powdery mildew development of barley (modified from Hückelhoven) After dispersal of the *Bgh*'s conidia by the wind, it settles down on the plant surface. 2 hours after the conidia's landing on the surface, a primary germ tube is formed. The appressorial germ tube elongates and forms a mature appressorium at 8-10 hour post inoculation (hpi). The appressorium attempts to penetrate into cuticle and cell wall at 10-12 hpi. Around 16 hpi, appressorium differentiates into a haustorium within the epidermal cell. After 24 hpi, secondary hyphae are formed allowing the spread of the fungus on the plant surface and penetration to further epidermal cells. The appressorium forms into secondary and tertiary haustoria in the following days. About a week after inoculation, mature chains of conidia are released in order to achieve another infection cycle (Zang et al., 2005; Grell at al., 2005). (c: conidium; PGT: primary germ tube; APT: appresorial germ tube; appr: appressorium; pap: papilla; hau: haustorium; SH: secondary hyphae; NC: new conidia).

PM fungi are obligate biotrophic pathogens that are dependent on living tissues in order to survive and propagate. Biotrophic parasites use the host metabolism in

order to feed themselves with facilitating the survival of the feeding plant cell. Plant cell death is either repressed by avoiding or suppressing the host defense. Pathogenesis has several phases including spore adhesion, appressorium formation and penetration (Panstruga, 2003). Upon landing of the conidial spore on the plant's surface, it germinates within 2 hours. Following the germination, the fungus penetrates into the host's cell wall by generating a primary germ tube called appressorium germ tube. The penetration is achieved by both enzymatically or mechanically (Hückelhoven, 2005). This germ tube is responsible for the water uptake, recognition of surface of the host and derivation of signals to maintain pathogen growth (Eichmann et al., 2008). For the attachment of the pathogen to the surface of plant (Feng et al., 2009) or for the induction of PM infection structures (Hansjakob et al., 2010), epicuticular alkanes and very long chain aldehydes play important roles (Hückelhoven et al., 2011). After the penetration, colonization takes place.

Successful invasion of epidermal cell precedes the formation of a haustorium without disrupting the plasma membrane (Hückelhoven, 2005). The penetrating germ tube is slender but once it enters the epidermal cell, it becomes a pear-shaped haustorium that enables an increased surface area for nutrient absorption (Horst, 2008). Haustoria are separated from the plant cell by a newly synthesized host plasma membrane derivative called as the extrahaustorial membrane. The structure of this membrane enclosing haustoria is different from the host plasma membrane (Panstruga, 2003, Voegele et al., 2003, Zang et al., 2005, Eichmann et al., 2008). Studies show that extrahaustorial membrane lacks several proteins such as host plasma membrane resident proteins (Koh et al., 2005). The gel like and carbohydrate rich layer between the extrahaustorial membrane and the haustorial cell wall is termed as extrahaustorial matrix. This layer plays an important role for the biotrophic life of the fungus since nutrient and information exchanges are carried out here (Voegele et al., 2003). Haustorial neckband is the structure which seals the extrahaustorial membrane from the host's plasma membrane. Haustoria

are called as hidden robbers since they are the feeding organs of the PM pathogen. Haustorium uses proton-symport transporters in order for the nutrient (e.g. hexoses and amino acids) absorption from the extrahaustorial matrix (Panstruga, 2003). Nutrient uptake is not the only task for the haustorium; it serves in the suppression of the host defense responses and in management of the host's metabolic flow (Voegele et al., 2003).

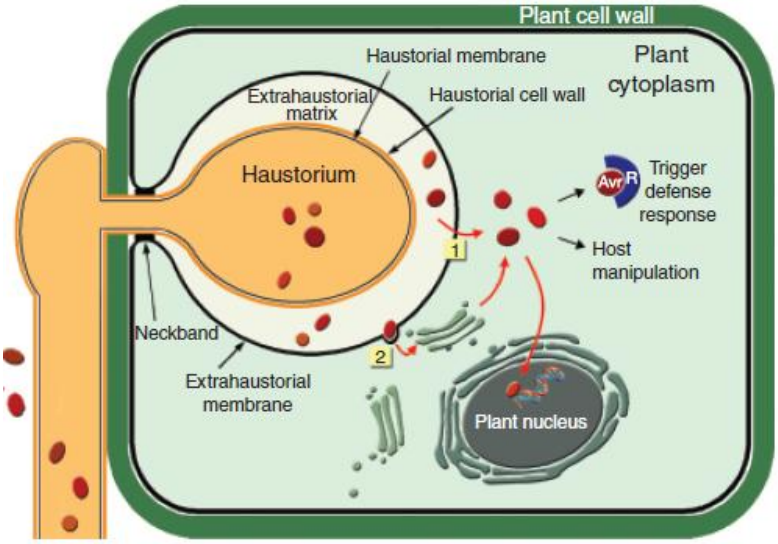


Figure 1.3 Interphase of a haustorium (Catanzariti et al., 2007): Effector proteins are secreted from the haustoria are passed through the extrahaustorial membrane. These effectors are transported through the host plant cell either by directly crossing the membrane (1) or by using vesicles (2). The effectors are important factors in suppressing host defense system. This suppression can be carried out by manipulating host metabolism or by targeting plant nucleus in order to alter the transcription level of the genes required for plant defense. The effector proteins are also termed as pathogen avirulence genes (*Avr*) which results in resistance when recognized by resistance gene products (*R*) secreted by the host plant cell.

Fungal penetration depends on the host cell types as well as the success of the individual pathogen to outstand the defense mechanism and the physiological condition of the host cell. Therefore, even for a susceptible plant, only a portion of the plant may be affected by the pathogen. During penetration, in order to facilitate successful pathogenesis, first of all pathogens should struggle with the cell-wall related defense mechanism of the host plant (Hückelhoven, 2005). Pathogens access the host cell by breaching epidermal cuticle and cell wall by both enzymatic and mechanical manner. Cell wall degrading enzymes such as cellulases, cutinases secreted by the pathogen are reported (Eichmann et al., 2008). After passing this physical barrier, effector proteins synthesized by the pathogen are recognized by the cognate resistance related proteins of the host. Pathogens cope with the plant's defense system either by suppressing the system or by masking the intrusion. Catalase is synthesized by the powdery mildew fungi from the appressorium in order to decrease H_2O_2 level which elevates at the site of infection (Hückelhoven, 2005, Eichmann et al., 2008). Mellersh et al. (2002) stated that fungal penetration is facilitated by the enzymatic removal of H_2O_2. Avirulence factors (*Avr*) secreted by the pathogen are involved in the pathogenicity unless they are not detected by the corresponding resistance (R) proteins. Ridout et al. (2006) successfully cloned two *Bgh* AVRs, AVR_{a10} and AVR_{k1}, and show these AVRs trigger the formation of haustoria in susceptible barley. When the signal of the pathogen invasion is detected by the host cell, organization of the cytoskeleton changes to facilitate the transport of the materials required for resistance to the site of penetration. Resistant barley plants show reduced or delayed actin cytoskeleton polarization (Opalski et al., 2005). Expression of the molecular chaperones and the proteins related with secretion are increased when barley is attacked by *Bgh* (Eichmann et al., 2008). Once haustoria is formed in one of the host cell, the adjacent cells become more prone to pathogenicity meaning haustoria suppress resistance by secreting some factors (Lyngkjaer et al., 1999; Eichmann et al., 2008). In barley, successful invasion of powdery mildew pathogen is achieved by a transmembrane protein called MLO protein. It is predicted that MLO protein of barley is involved

in the control cell death and senescence and it accumulates at the pathogen attack site. Loss of function mutation of *mlo* gene results in a broad spectrum powdery mildew resistance and pathogen invasion terminates at the cell wall penetration stage (Panstruga, 2003). MLO protein interacts with Calmodulin (CaM) which is a calcium (Ca^{+2}) sensor protein. Ca^{+2} concentration increases within the host cell after pathogen attack and this elevation is termed as early host response. As the Ca^{+2} concentration increases, MLO activity is increased meaning CaM is the positive regulator of MLO (Panstruga, 2003, Eichmann et al., 2008). Suppressing the host cell death is very important for successful disease formation because programmed cell death, termed as hypersensitive response (HR), at the infection site impedes the colonization of the pathogen. This cell death suppression is proved by "green island effect" in which the surrounding host cells of the infected cell remains green when compared with the rest of the leaf tissue showing senescence symptoms (Panstruga, 2003; Hückelhoven, 2005). Over-expression of bax inhibitor also suppresses the induction of plant cell death. Bax inhibitor slows down cell death by inhibiting the expressing of pro-apoptotic BAX protein (Eichmann et al., 2008).

Plants defend themselves from invasion of fungus at different stages. First step of the resistance takes place at the cell wall level. At the site of infection where the appressorium attempts to penetrate, host cell is strengthened by the formation of cell wall appositions called as papillae. Papillae protect the host cell from the mechanical and enzymatical pressure of the appressoria. Callose and other polysaccharides are deposited at the site of infection in order to form a structural scaffold. Moreover, at the site of pathogen attack, cell wall material forms cross-linking with organic and inorganic materials in order to induce defense (Hückelhoven, 2005; Eichmann et al., 2008). The orientation of the cytoskeleton is observed in order to direct the flow of antimicrobial and cell wall related materials to the site of infection and papillae formation is enhanced. H_2O_2 accumulation is observed in the effective papillae. (Thordal-Christensen et al., 1997; Hückelhoven

et al., 1999). Nitric oxide (NO) accumulation is one of the early responses to the pathogen attack and it is responsible for the initiation and development of the effective papillae. According to Prats et al (2005), NO is an important signal for cell death due to HR. Fe^{+3} accumulation at the site of infection also elicits the formation of oxidative burst (Eichmann et al., 2008). Host cell mediates the secretion of proteins that are necessary for the transport of toxic compounds to the site of infection in order to poison the apoplast. Even if the pathogen successfully penetrates the host cell, it does not always result in colonization of the PM fungus. The response that the host cell arises is called Hypersensitive Response (HR) which includes programmed cell death (PCD) of the attacked of adjacent cells. H_2O_2 accumulation leads to HR by either acting as a signal in PCD or as a fungitoxic (Thordal-Christensen et al., 1997; Hückelhoven et al., 1999). HR is formed when the effectors of the pathogen are detected by the corresponding plant R genes and this reaction is induced by the signals of haustoria (Eichmann et al., 2008).

1.3 Quantitative Real Time PCR (qRT-PCR)

Gene expression level changes under several conditions such as changes in the tissue or cell to be studied; infection with pathogen, virus or bacteria; drug or chemical treatment. Quantitative Real Time PCR (qRT-PCR) is an autonomous, reproducible and high-throughput method to identify gene expression profile. It permits the studying of multiple genes simultaneously in a test tube manner. By using this technique, post-PCR manipulations are also eliminated. qRT-PCR provides the measurement of gene expression even if the amount of the starting material is low.

There are several detection methods for the quantitative expression analysis. Using dual-labeled fluorogenic hybridization probes is one of these monitoring methods.

It is based on the 5' exonuclease activity of the Taq DNA polymerase. This assay uses fluorescent Taqman probe with a reporter dye at the 5' end and a quenching dye at the 3' end of the probe. Within the intact probe, the energy emitted by the reporter dye is absorbed by the quencher. In case of extension, 5'exonuclease activity of the DNA polymerase removes the reporter dye resulted an increase in the emission spectra at 518 nm. This increase is detected and monitored in real time by a CCD camera coupled to an instrument (Heid et al., 1996). Another monitoring method that also uses a reporter and a quencher dye is molecular beacon. In this method, single stranded probe with a reporter dye at one end and a quencher dye at the other end forms a hairpin. This structure enables the absorption of the emitted energy and no fluorescence is detected. During hybridization step, when the strands of DNA are separated, the hairpin structure of the beacon is also denatured. As the temperature falls, beacon hybridizes with the PCR strand and there is an increase in fluorescence. As a result, as the fluorescence increases, it means there is an accumulation of the product (Kramer, 1996). Although these two methods are sequence specific, SYBR Green I is the most cost effective and precise method for monitoring (Schmittgen et al., 2000). It is a fluorescent dye that binds to double-stranded DNA but does not bind to single stranded DNA (Morrison et al., 1998). SYBR Green dye does not discriminate double stranded DNA to be bound so it binds to any double stranded DNA such as primer dimers or any other contaminating DNA. In order to eliminate this misbinding, a dissociation curve is plotted by monitoring the signal within every 2^{o} C changes in the temperature. If there is only one PCR product, there will be a single peak in the dissociation curve (Schmittgen et al., 2000).

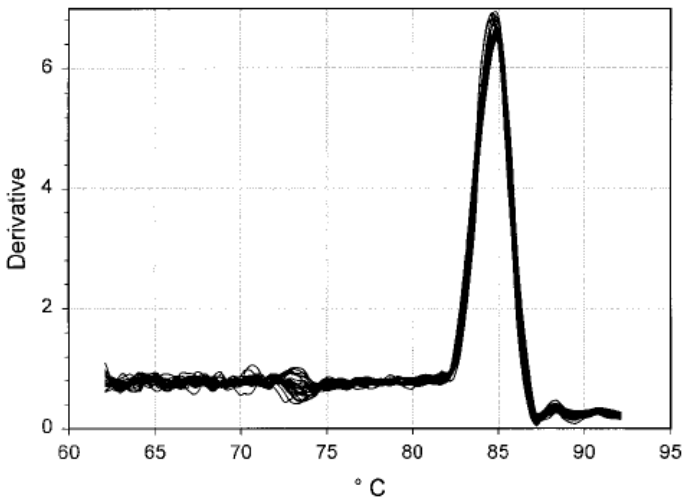


Figure 1.4 A model heat dissociation curve taken from Schmittgen et al. (2000).

The fluorescence signal is determined by an instrument and as a result an amplification curve is plotted by the instrument. y-axis of the graph represents ΔRn and the x- axis represents the cycle number. When there is no fluorescence emitted, which corresponds to the early cycles of the amplification, ΔRn value stays stable at the baseline. When sufficient fluorescence signal is determined by the instrument, which means the amount of the PCR product increases in the test tube, there will be a sudden increase in the curve. A threshold line which is calculated by the standard deviation of the ΔRn values at the baseline is plotted by the instrument. The point where the amplification curve intersects with the threshold is defined as C_T (Heid et al., 1996). C_T value is inversely proportional to the target quantity. The C_T value decreases with the increasing DNA copy number. It is expected since if amount of starting material is low, more cycle is needed to overcome the threshold line (Heid et al., 1996; Schmittgen et al., 2000). Therefore, for quantification, instead of using the amount of fluorescence emission intensity, using C_T value is more straightforward (Heid et al., 1996).

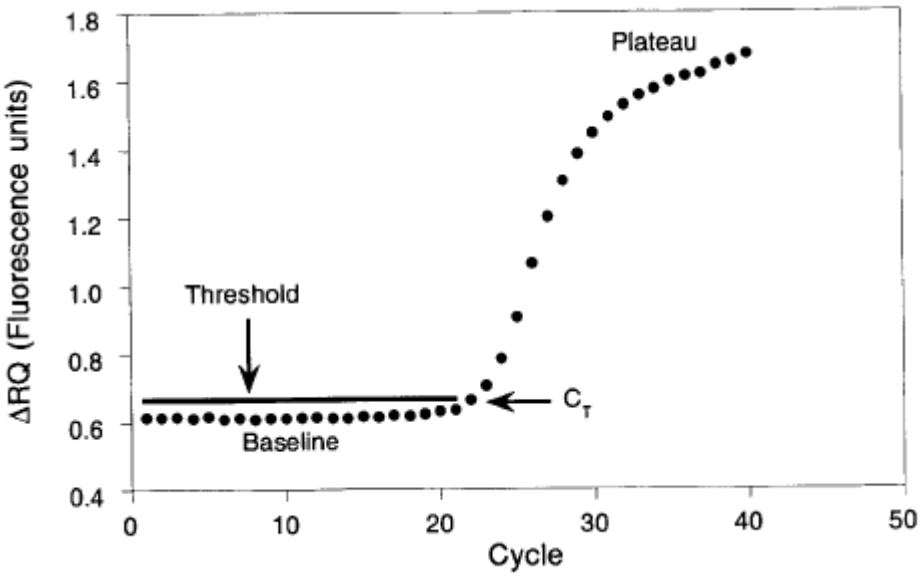


Figure 1.5 A model amplification plot taken from Heid et al. (1996).

Normalization is a very important issue in order to get more accurate and reliable results of qRT-PCR since there are several variables in gene expression analysis. An internal control such as a housekeeping gene is used for normalization. These genes are stable even if the conditions are variable. Ribosomal RNA subunits (Bas et al., 2004; Xue et al., 2010; Wang et al., 2010), ubiquitin (Olsen et al., 2010; Lund et al., 2008), elongation factor (Olsen et al., 2010; Abbal et al., 2008) and actin (Ruan and Lai, 2007; Fung et al., 2008) are commonly used reference genes for normalization. The expression of these genes are expected to be constant under different situations and in all samples; however, it is reported that their expression varies and validation of reference genes is needed in case of sampling differences (Gamm et al., 2011). Several computer based statistical methods such as geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004), Normfinder (Anderson et al., 2004) are developed to identify the appropriate reference gene for the quantification. Using these three computer based methods, the result do not show any substantial difference (Gamm et al. 2011). geNorm statistical analysis

determines the pairwise variation of a reference gene with the other reference genes tested. It is based on *M* value which is defined as the internal control of the gene stability. The lowest *M* value indicates the most stable genes. By stepwise exclusion of the genes with the highest *M* value gives the most accurate couple of stable expressed housekeeping genes for the ongoing quantifications (Gamm et al. 2011, Vandesompele et al., 2002). A freely available program termed geNorm allows the management of large number of data. The program automatically calculates the *M* value for the reference genes. According to geNorm program, the genes having *M* value higher than 1.5 indicate that they show variable gene expression (Gamm et al. 2011). A normalization factor is determined by the program and it is used to eliminate the expression differences and a more reliable and accurate normalization of the expression is achieved. Using at least two reference genes instead of one is more appropriate for normalization (Vandesompele et al., 2002). The stability of housekeeping genes used for normalization has to be determined for any study of qRT-PCR in any plant species (Gamm et al. 2011).

1.4 Aim of the Study

Plants are exposed to several biotic and abiotic factors in their natural environment which result in a yield loss. Crops are important feeding agents for both humans and animals; therefore maintenance of sustainable crops is very crucial for the continuation of life. Accurate and reliable disease diagnosis system of the plants in order to eliminate the disease emerging due to several organisms, such as fungi, bacteria, nematode or virus, is obligatory. Any technology providing the production of resistant plants is the main target for plant breeders. Genetic tools such as transcriptomics, proteomics and protein interaction studies are used to define the candidate genes.

Powdery mildew disease causes a significant yield loss of barley throughout the World. However, any protection strategy is not applied to eliminate this loss. *Bgh* is the most studied species of the powdery mildew pathogen (Both et al., 2005) so it is used as a model organism for obligate biotrophic pathogens.

In this study, transcription level differences for the selected candidate genes such as NAD malic enzyme, lipocalin chloroplast, phosphoglyceromutase (PGM), Mg chelatase and 26S protease regulatory subunit 6B homolog whose differential protein levels was documented in a previous proteomics study was aimed. The selected time points for the determination of the expression profile were 6, 12, 24, 48 and 72 hpi. In 6 hpi, early plant responses against pathogen could be observed. At 12 hpi, the genes responsible for penetration of the pathogen and suppression of the plant defense are expected to be observed. In 24 hai, since the formation of the haustorium is achieved, the genes related with this formation and feeding from haustorium would be elevated. In resistant plant of view, the genes responsible for the plant defense suppression are expected to be observed. In 48 hai, the fungus starts feeding on the host plant so we expected to obtain pathogen development related genes. For the case of resistance, the level of defense-related genes would be elevated. 72 hpi was selected to see the late defense responses.

CHAPTER II

MATERIALS AND METHODS

2.1 Preparation of Plant Materials

2.1.1 Maintenance of Powdery Mildew

Barley seeds were planted in soil and placed in a growth chamber at the 20 ^{o}C for 16-hour light and at 15 ^{o}C for 8-hour dark periods. The seedlings were watered in every 2 days. Powdery mildew spores were maintained on susceptible Bulbul 89 leaves detached from seedlings and placed on agar media. The agar medium was prepared by dissolving 7 g agar in 450 mL of ddH_2O and sterilized. Into which 50 mL of 1g/L Benzimidazole in 0.1 % DMSO was added. The media were divided into culture plates. When the second leaves have just emerged, they were cut into about 5 cm long pieces and placed on the media. For the maintenance, powdery mildew spores were blown on plant leaves. Plates were incubated in a growth chamber (Zeybek et al., 2008). The process was repeated continuously to keep the spore alive throughout the thesis.

2.1.2 Inoculation of Plants with Powdery Mildew Spores

Both barley lines, P-01(R) and P-03 (S), were inoculated with spores of powdery mildew pathotype *Bgh*103 (64/01), obtained from Prof. Dr. Mogens Støvring Hovmøller of Aarhus University, Denmark. One leaf was cut into two pieces; a piece was kept as control and the other one was used for infection. Control plants were blown with air and incubated under the same conditions as infected plants.

Table 2.1 The biologic material used throughout the thesis. Infection was carried out by spores of powdery mildew pathotype *Bgh*103 (64/01).

Barley line	**R-gene**	**Infection type**	**Plant Response**
Pallas-01	Mla1	Incompatible	Resistant
Pallas-03	Mla6, Mla14	Compatible	Susceptible
Bulbul-89	-	Compatible	Susceptible

2.1.3 Collection of Leaf Samples

Samples were collected at 6^{th}, 12^{th}, 24^{th}, 48^{th} and 72^{nd} hour after inoculation for both control and infected ones. The edges of the leaves were cut by clean scissors and frozen quickly in liquid nitrogen. Then samples were stored at - 80 ^{o}C. One set of leaves was left on the agar media confirm the successful infecting to observe the formation of disease symptoms.

2.2 Staining Barley Leaves with Trypan Blue

Trypan blue staining was performed in order to visualize hyphael growth of powdery mildew spores. Both P-01 and P-03 leaves infected with *Bgh*103 pathogen were harvested at 24 hour after infection and stained according to Vogel and Sommerville (2000). First of all, plant leaves were immersed in absolute ethanol overnight and then stained with Trypan Blue Solution (250 µg/mL trypan blue in lactic acid: glycerol: water (1:1:1) mixture) for 15 minutes. After rinsing the leaves in rinsing solution (lactic acid: glycerol: water (1:1:1) mixture) for 2 min, leaves were mounted on glass slide for visualization under the light microscopy (Leica DM4000B).

2.3 Total RNA Isolation

2.3.1 RNA Handling

Tubes, mortars, pestles and spoons were immersed in 0.1 % diethylpyrocarbonate (DEPC) for at least 1 hour. Plasticware were autoclaved and then baked at about 50 ^{o}C. Mortars, pestles and spoons were incubated in a 200 ^{o}C oven overnight after DEPC treatment. RNase-free water was prepared as 0.1 % DEPC ddH_2O. After overnight incubation, it was autoclaved. Before the experiment, workbench, micropipettes were cleaned with 70 % ethanol and then RNase-off solution. Disposable gloves were always worn during RNA isolation.

2.3.2 Isolation of Total RNA

Plant leaf samples of both compatible and incompatible interaction were collected at 6, 12, 24, 48 and 72 hour after inoculation for both control and infected plant material were used to isolate total RNA. Invitrogen Trizol reagent was used for this purpose according to instructors' manual. Every material to be used was cooled down in liquid nitrogen. Leaf samples (100 mg) were homogenized by using mortar and pestle. The homogenized plant powder was collected into 2 mL eppendorf tubes and 1 mL Trizol reagent (1 mL Trizol reagent for each 100 mg of plant sample) was added immediately. The mixture was vortexed briefly and homogenized samples were incubated for 5 min at 15-30 ^{o}C for the dissociation of nucleoprotein complexes completely. Then, 200 µL of chloroform was added to the homogenates and shaken vigorously for 15 seconds. The mixture was let stand for 15 min at 15-30 ^{o}C. For phase separation, samples were centrifuged at no more than 12000 x g for 15 min at 4 ^{o}C. After centrifugation, the upper clear phase was transferred to a clean 1.5 mL eppendorf tube. For 1 mL of Trizol reagent, 0,5 mL of isopropanol was added onto this organic phase for the precipitation of RNA and

inverted the tube 2-3 times. The samples were incubated for 10 min at 15-30 ^{o}C and then centrifuged at no more than 12000 x g for 10 min at 4 ^{o}C. The supernatant was removed and the RNA pellet was washed with 1 mL of 75 % ethanol prepared with DEPC treated water. The sample was mixed by vortexing and centrifuged at no more than 7500 x g for 5 min at 4 ^{o}C. After the ethanol was discarded, the tubes were stood for air drying until ethanol smell was disappeared. Finally, the RNA precipitates were dissolved in 50-100 µL of nuclease free water by holding the tubes in a 65 ^{o}C water bath.

2.3.3 Analyses of RNA Samples

RNA samples were separated in 1 % agarose gel in 1X (10 mM) phosphate buffer pH 6.8 in the presence of 0.5 µg/mL EtBr. The gel was applied and run at about 80 volt for about an hour and it was visualized under UV transilluminator. Total RNA concentration was determined by using NanoDrop 1000 RNA settings.

2.4 cDNA Synthesis

2.4.1 DNase and LiCl Treatment

The total RNA samples were incubated at 70 ^{o}C for 2 min to eliminate the secondary structures and immediately cooled on ice before starting the experiment. DNase treatment was carried out by adding 10 µg total RNA, 10 µL 5x first strand buffer 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 µL Ambion Turbo DNase (2 u/µL) into DEPC treated eppendorf tubes and complete their volume to 50 µL with nuclease free water. The samples were incubated at 37 ^{o}C for 30 min and then at room temperature for 2 min. Ice cold 50 µL 7.5 M LiCl was added to the incubated samples and let stand at – 20 ^{o}C for 30 min. The tubes were centrifuged at 15300 rpm for 30 min at 4 ^{o}C and discarded the supernatant. The pellets were washed with 1 mL of 70% ethanol and centrifuged at 15300 rpm for

30 min at 4 ^{o}C. After centrifugation, the pellets were air-dried until they became transparent. Finally, the pellet was suspended in 10 mL nuclease free water and the concentration values were determined on NanoDrop 1000.

2.4.2 First Strand cDNA Synthesis

For cDNA synthesis, SuperScript III reverse transcriptase (Invitrogen) was used as in the instructor's manual. The following reaction components were added into nuclease free eppendorf tubes; 1 µg DNase and LiCl treated total RNA, 1 µL dNTP mix, 0,2 µL oligo $(dT)_{32}$ primer (10 pM). The reaction volume was completed to 14 µL with nuclease free water. The mixture was heated to 65 ^{o}C for 5 min and the incubated on ice for at least 1 min. The tubes were spun down briefly. Onto these components, 4 µL of 5x first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 µL 0,1 M DTT, 0,2 µL RNase out and 0,8 µL RT enzyme (200 u/ µL) were added. The mixture were incubated at 55 ^{o}C for 45 min and then at 70 ^{o}C for 15 min to inactivate the enzyme.

2.5 PCR

2.5.1 Control PCR to Test Primers

As template for the PCR reactions, cDNA of 24 hour infected P1 samples were used for the amplification of NAD malic enzyme, lipocalin chloroplast, 26S regulatory protein and phosphoglyceromutase (PGM). For the amplification of Mg chelatase and 26S protease regulatory subunit 6B, cDNA of 12 hour infected P1 samples were used. There were also a negative and a positive control for the reaction; non-template control and 18S ribosomal RNA, respectively. The template for the positive control was cDNA of 12 hour infected P3 samples. Reaction components and their amounts are listed in Table 2.2. After the master mix was divided into the tubes, template and primers (Table 2.3) were added. The PCR

reaction was carried out by a master cycler (Eppendorf Mastercycler Gradient) under conditions; at 94 ^{o}C for 3 min in 1 cycle; 94 ^{o}C for 1 min, 53 ^{o} C for 1 min, 72 ^{o}C for 1 min in 35 cycles; 72 ^{o}C for 5 min in last cycle.

Table 2.2 The composition and preparation of PCR master mix.

Reaction Components	**Per Reaction (µL) (1x)**	**Master Mix (µL) (13x)**
Template	2	-
10X PCR Reaction Buffer	2,5	32,5
dNTP Mix (10mM)	0,5	6,5
$MgCl_2$ (25mM Mg^{2+})	1,5	19,5
Forward Primer (10 µM)	1,5	-
Reverse Primer (10 µM)	1,5	-
Taq DNA polymerase (5 u/µL)	0,2	2,6
H_2O (sterile-dd)	15,3	198,9
TOTAL	25	260

Table 2.3 The primer sequences of candidate genes.

NCBI identifier	**PrimerNames**	**Seq 5' to 3'**	**Amplicon Size (bp)**
AK376837	26S-regulatory-F	cacttaattaagtcatcggccagttcatgga	218
	26S-regulatory-R	catgcggccgcagcgagatgctggagtcggcc	
AK359167	Lipocalin-F	cacttaattaagtgtcctcggaccagctagc	223
	Lipocalin-R	catgcggccgcttacttgaaatacacttcggc	
AK353754	Mgchetalase-F	cacttaattaatcggttctgtgcagctcgacc	227
	Mgchetalase-R	catgcggccgcctagccaaagacttcatagaa	
AK366262	PGM-F	cacttaattaatcctcagtgggaagtttgacc	222
	PGM-R	catgcggccgccttaaccatatcctctgcgtt	
AK360961	Nadmalic-F	cacttaattaagcccaatgtgattgtacagtt	286
	Nadmalic-R	catgcggccgccccagcatccttgccatggtc	

2.5.2 Analyses of Amplified Gene Products

Amplified DNA fragments were analyzed in 1 % agarose gel prepared by 1x TBE buffer in the presence of 0.5 µg/mL EtBr. The gel was applied and electrophoresed at 80 volt for an hour and it was visualized under UV transilluminator.

2.6 qRT-PCR

2.6.1 Reference Gene Determination

cDNA synthesized from the isolated RNA of P-01 and P-03 leaf tissues inoculated by *Bgh*103 at 6, 12, 24, 48 and 72 hours post inoculation (hpi) as well as the mock-inoculated control plants at each of the corresponding time points were used as templates for the qRT-PCR reaction. Ubiquitin (Ubi), ubiquitin conjugating protein (Ubc), α-tubulin, elongation factor (EF) (Table 2.4) were the candidate housekeeping genes to determine the reference gene. The reactions were carried out as a duplicate, as well as for non-template control. First of all, cDNA samples were distributed to all wells and then 15 µL master mix (Table 2.5) prepared for each primer set was added. The plate setup was as follows;

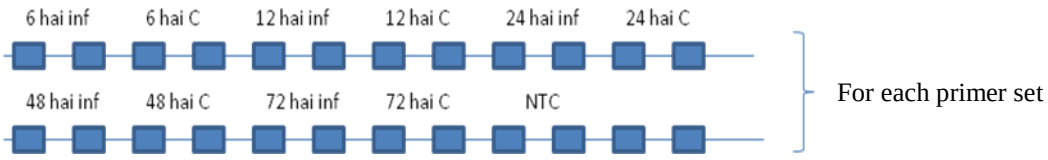


Figure 2.1 The distribution of samples throughout the wells.

Table 2.4 The sequences of primers designed for reference genes.

Primer Name	Sequence (5' to 3')
Ubi Fwd	gccgcaccctcgccgactac
Ubi Rev	cggcgttggggcactccttc
Ubc Fwd	ggttctgctttcaatctgctcgctg
Ubc Rev	gggagacacacgcaaccgacaagta
alpha Fwd	agtgtcctgtccacccactc
alpha Rev	agcatgaagtggatccttgg
EF Fwd	atgattcccaccaagcccat
EF Rev	acaccaacagccacagtttgc

Table 2.5 The composition and preparation of qRT-PCR master mix.

Reaction Components	Per Reaction (µL) (1x)	Master Mix (µL) for each primer (24x)
Template cDNA (1/20 diluted)	5	-
Forward Primer (10 µM)	0,5	12
Reverse Primer (10µM)	0,5	12
ddH_2O(sterile)	4	96
SYBR Green Mix (Stratagene Brilliant SYBR Green QPCR Mastermix)	10	240
TOTAL	20	360

The tubes were spinned down. Immediately after the preparation of reactions, the plate was put on Stratagene Mx3005P Real-Time PCR System. The program for SYBR green dye with melting point determination was selected and the wells were marked as unknown. The cycling conditions for qRT-PCR reaction begun with a denaturation step including 10 min incubation at 95 ^{o}C. A 40 cycle amplification and quantification step; 95 ^{o}C for 30 sec, 55 ^{o}C for 1 min and 72 ^{o}C for 1min with a fluorescence determination once at every 55 ^{o}C, followed by the denaturation step. The final cycle was the melting curve step starting with incubation at 95 ^{o}C for 1 min and then heating from 55 ^{o}C to 95 ^{o}C with a fluorescence determination at all points.

2.6.2 qRT-PCR for Candidate Genes

The reaction was carried out as described in section 2.6.1. The only difference was the primers used. The primers mentioned in section 2.5.1. Designed for the amplification of NAD malic enzyme, lipocalin chloroplast, phosphoglyceromutase (PGM), Mg chelatase and 26S protease regulatory subunit 6B homolog were used for the expression profile analyses. The calculation of expression ratios was performed according to Pfaffl (2001).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Powdery Mildew Disease

Barley leaves, both Pallas-01 (P-01) and Pallas-03 (P-03), were inoculated with powdery mildew *Bgh*103 as described in section 2.1.2 and 10 days after inoculation, as expected white velvety pustules were observed on the surface of susceptible P-03 leaves; whereas, on the resistant P-01 leaves, no disease symptoms were observed. No disease symptom was observed in mock inoculated plants, as expected, which proved the absence of involuntary contamination.

P-01 leaves 24 hour post inoculation (hpi) with *Bgh*103 were analyzed under light microscopy after stained with Trypan Blue. It is a diazo dye generally used to stain dead cells blue. Trypan blue also selectively stains chitin in fungi cell walls (Virant et al., 1988) so it is used for the visualization of fungal hyphae. In Figure 3.1 (a), the spore pointed out by the red arrow was not germinated as expected. For the susceptible interaction (Figure 3.1 b), 24 hpi corresponds to the penetration stage by the hyphae and the formation of haustorium is achieved.

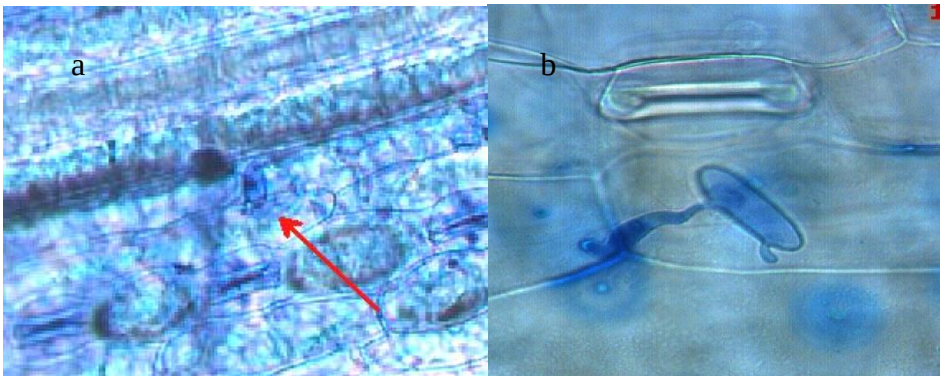


Figure 3.1 Imaging of trypan blue staining of P-01 leaves (24 hpi) at 10X (a) and P-03 leaves (24 hpi) at 40X under light microscopy.

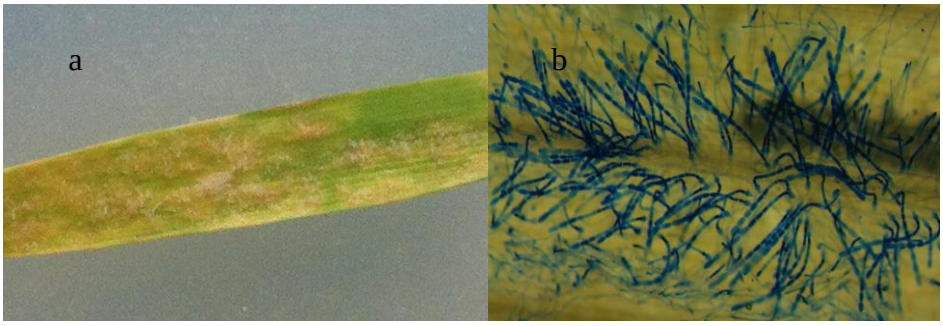


Figure 3.2 Successful infections of P-03 leaves at 10 days post inoculation (a). White velvety pustules can be easily recognized. Susceptible plant stained with trypan blue. Chains of conidia can be observed in blue. Imaging at 10X under light microscopy.

3.2 Gel Analyses and Concentration Determination of Total RNA Samples

The parameters important for the isolated RNA are its quantity, integrity and purity. Isolated RNA samples were analyzed in 1% agarose gel in order to detect their integrity and their concentrations were determined on Nanodrop. As seen in Figure 3.3 and Figure 3.4, the total RNA samples isolated from P-01 and P-03 leaves are intact for all the time-points and for both infected and control samples.

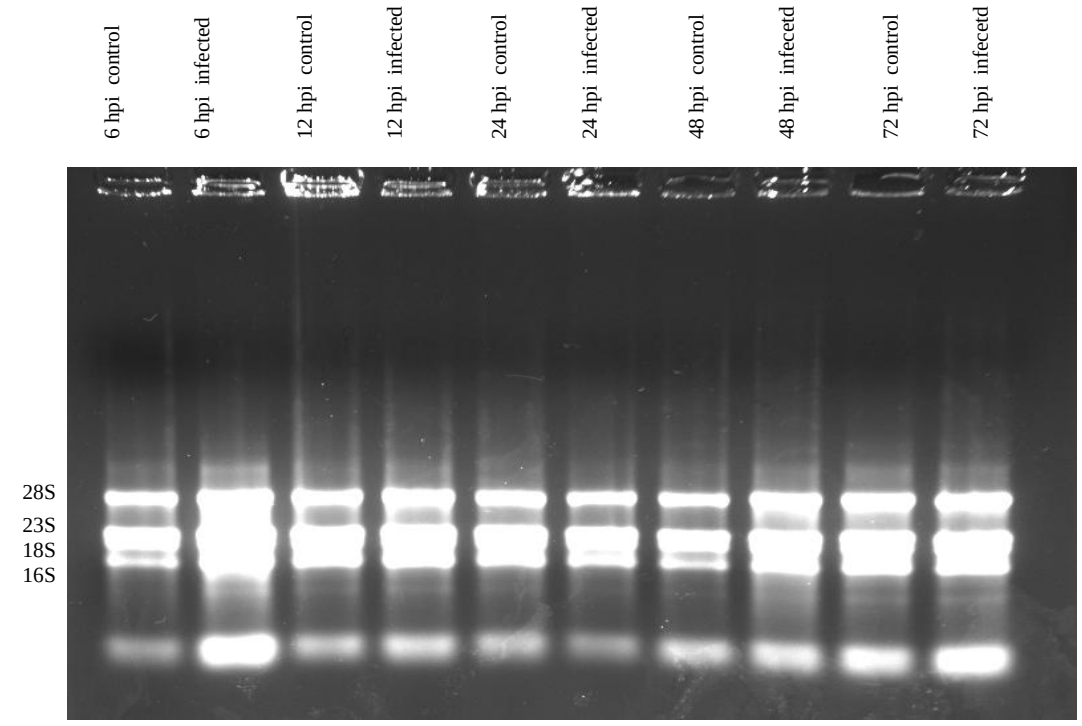


Figure 3.3 Total RNA samples isolated from barley P-01.

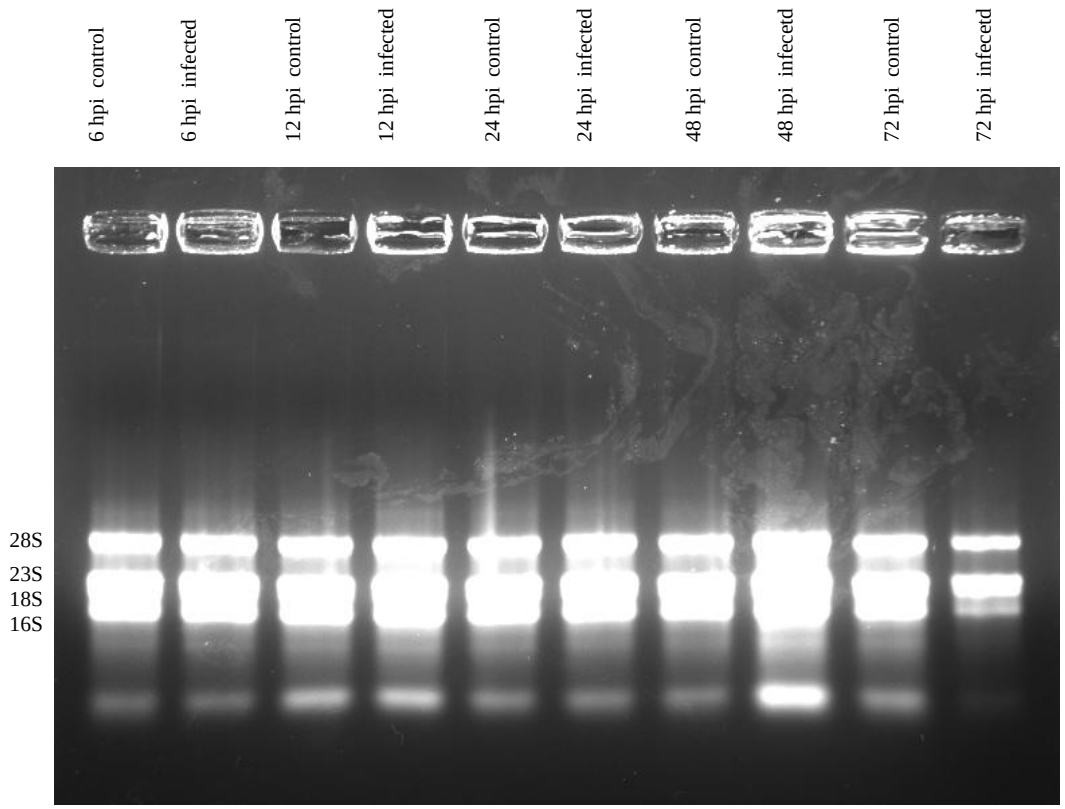


Figure 3.4 Total RNA samples isolated from barley P-03.

The concentrations measured on NanoDrop were satisfactory (Table 3.1 and Table 3.2). NanoDrop gives the concentration in ng/µL for quantification as well as the A260nm/A280nm ratio and A260nm/A230nm ratio which are used for the determination of purity. The A260nm/A280nm ratio should be above 1.8 while the A260nm/A230nm ratio should be above 1.0; otherwise, it indicates phenol, protein or salt contamination of isolated RNA. The RNA samples isolated were said to be pure when the A260nm/A280nm and A230nm/A280nm ratios were in consideration.

Table 3.1 Concentrations of total RNA isolated from P-01 leaf samples.

	Total RNA	Concentration (ng / µL)
P-01 + B103	6 hpi C	1058.3
	6 hpi	1161.3
	12 hpi C	506.2
	12 hpi	555.4
	24 hpi C	565.9
	24 hpi	378.9
	48 hpi C	430.2
	48 hpi	531.7
	72 hpi C	761.6
	72 hpi	620.2

hpi: hour post inoculation
C: Control (mock inoculated)

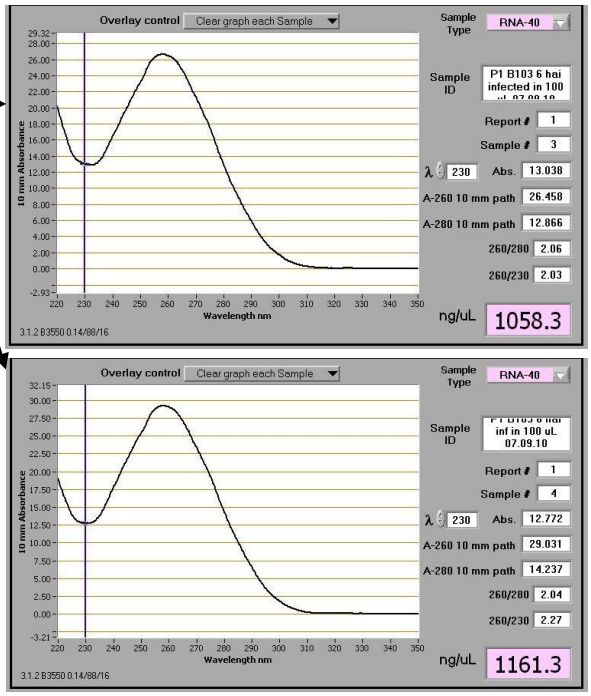
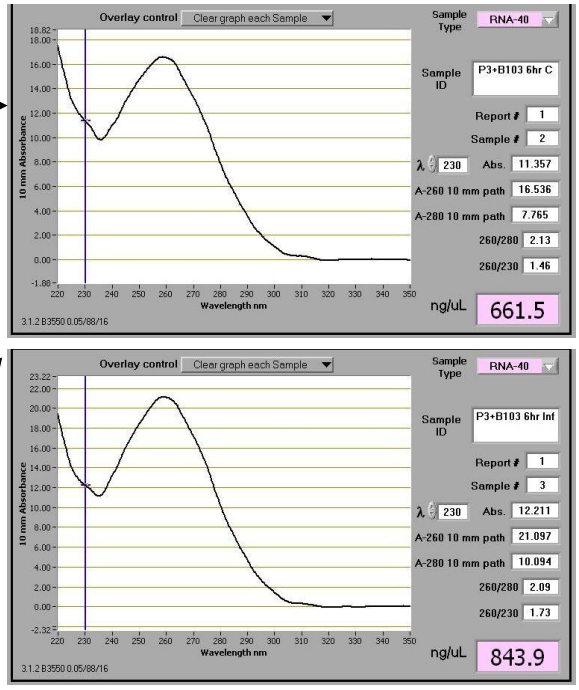


Table 3.2 Concentrations of total RNA isolated from P-03 leaf samples.

	Total RNA	Concentration (ng / µL)
P-03 + B103	6 hpi C	661.5
	6 hpi	843.9
	12 hpi C	556.1
	12 hpi	645.0
	24 hpi C	522.9
	24 hpi	670.1
	48 hpi C	486.7
	48 hpi	1128.7
	72 hpi C	682.6
	72 hpi	875.8

hpi: hour post inoculation
C: Control (mock inoculated)



3.3 Amplification of Candidate Genes

Candidate genes whose expression profiles were detected in this study were determined according to the MSc. study conducted by Özgazi (2009). Özgazi (2009) was also used P-01 and P-03 barley lines infected with *Bgh*103 pathogen. The samples investigated were 12, 24 and 48 hour after *Bgh*103 inoculated and mock inoculated P-01 and P-03 leaves. In order to define the proteins that are differentially expressed in control and infected barley plants, the samples were subjected to 2D-PAGE and following nano-LC-ESI-MS/MS analysis. The MASCOT algorithm was used in order to define the corresponding proteins to the spots identified. According to this proteomics study, 36 proteins were determined to be differing in their expression levels, 18 of which was up-regulated while 8 of which were down-regulated (Table 3.3 and Table 3.4).

Table 3.3 Differentially expressed proteins of *Bgh*103 treated P-01. (Modified from MSc. thesis of Neşe Özgazi "Proteome analysis of *Blumeria graminis* f. sp. *hordei* inoculated barley" (2009)).

Spot #	Prot ID	12 hpi	24 hpi	48 hpi
1	Mg-chelatase subunit	2.33 ↓	-	-
2	Light-harvesting complex	1.51 ↓	+*Bgh*103	-
3	Os10g0416500	1.41 ↑	4.11 ↓	-
4	Os08g0447000	+ *Bgh*103	-	-
5	ATP synthase CF1 alpha subunit	1.5 ↑		
6	Mitochondrial aldehyde dehydrogenase ALDH2	2.70 ↑	-	-
7	Putative transketolase	2.06 ↑	-	-
8	Porphobilinogen deaminase	3.39 ↑	-	-
9	26S protease regulatory subunit 6B homolog	+*Bgh*103	2.33 ↓	
17	Glutamine synthetase leaf isozyme	-	6.84 ↓	-
18	Phosphoglycerate mutase	-	2.52 ↑	-
19	BP2A	-	2.51 ↑	-
20	Unnamed protein product (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit)	-	2.22 ↑	2.22 ↑
21	Chlorophyll a-b binding protein of LHCII type III	2.76 ↑	4.14 ↑	-
22	Chloroplast lipocalin	-	2.06 ↑	-
27	SGT1	-	-	2.78 ↑
28	Laminin receptor homologue	4.53 ↓	-	3.75 ↑
29	RuBisCO large subunit-binding protein subunit beta	-	-	1.44 ↓
30	Isopentenyl pyrophosphate isomerase	2.056 ↑	-	1.55 ↑
31	Os02g0634500	-	-	+*Bgh*103
32	Conserved hypothetical protein	-	-	+*Bgh*103

± *Bgh*: present in infected and absent in control, ↑: up-regulated, ↓: down-regulated

Table 3.4 Differentially expressed proteins of *Bgh*103 treated P-03. (Modified from MSc. thesis of Neşe Özgazi "Proteome analysis of *Blumeria graminis* f. sp. *hordei* inoculated barley" (2009)).

Spot #	Prot ID	12 hpi	24 hpi	48 hpi
10	SAL1 phosphatase	+*Bgh*103	-	-
11	3-phosphoglycerate kinase	+*Bgh*103	-	-
12	Os06g0264300	4.04 ↑	-	-
13	Os12g0183300	1.94 ↑	-	-
14	60 kDa chaperonin subunit alpha	+*Bgh*103	-	-
15	Putative ribosomal protein S5	+*Bgh*103	-	-
16	NADP-dependant malate dehydrogenase	2.81 ↑	1.52 ↑	-
23	Prohibitin 2	-	4.11 ↓	-
24	Putative 40S Ribosomal protein	-	2.33 ↓	-
25	Actin-97	-	1.5 ↑	-
26	No hit	-	-*Bgh*103	-
33	Phosphoribulokinase	-	-	1.83 ↑
34	Betaine aldehyde dehydrogenase	-	-	1.71 ↑
35	No hit	-	-	1.50 ↑
36	Actin	2.13 ↓	-	1.56 ↑

± *Bgh*: present in infected and absent in control, ↑: up-regulated, ↓: down-regulated

From these differentially expressed proteins NAD-dependent malic enzyme, lipocalin chloroplast, phosphoglycero mutase, 26S protease regulatory subunit 6B homolog and magnesium chelatase were chosen for further transcription level determination experiments in this study. The reason for selecting these genes for qRT-PCR was the fact that we could obtain the sequences as provided in the databanks with appropriate accession (according to gene ontology data mentioned

in UniProt (http://www.uniprot.org/), these proteins were predicted to be potentially involved in plant-pathogen interaction.)

Table 3.5 General information about the candidate proteins (Modified from MSc. thesis of Neşe Özgazi "Proteome analysis of *Blumeria graminis* f. sp. *hordei* inoculated barley" (2009)).

2D-PAGE Spot #	Protein Name	Molecular Function	Accession number (NCBI)
9	26S protease regulatory subunit 6B homolog	Protein metabolism	gi|1709798
22	Chloroplast lipocalin	Intracellular protein trafficking	gi|77744909
1	Mg-chelatase subunit	Porfirin biosynthesis	gi|847873
18	Phosphoglycerate mutase	Carbohydrate metabolism	gi|551288
16	NAD-dependent malic enzyme	Electron Transport	gi|24370966

After designing appropriate primers, they were tested whether the primers were successfully able to amplify the gene of interest or not. This confirmation is carried out according to 2.5.1 section. The resulting PCR products were analyzed in 1 % agarose gel in order to get one band for each primer set implying there was only one gene product for each primer set.

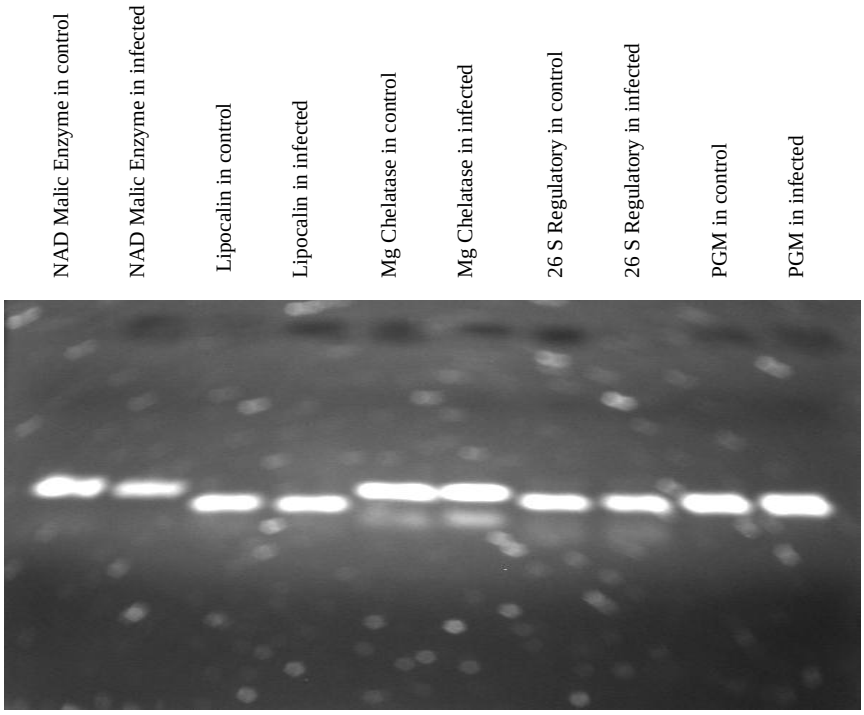


Figure 3.5 The amplification of candidate genes NAD-dependent malic enzyme, lipocalin chloroplast, 26S protease regulatory subunit 6B homolog, magnesium chelatase and phosphoglycero mutase analyzed in 1 % agarose gel.

3.4 Reference Gene Determination for Further qRT-PCR Experiments

In order to normalize the gene expression levels, the use of stable internal control gene is obligatory. The reference genes to be used should be systematically determined for any study of gene expression. For this purpose, 4 different housekeeping genes, ubiquitin (Ubi), ubiquitin conjugating protein (Ubc), α-tubulin and elongation factor (EF), were used in this experiment. Generally, the ribosomal RNA subunits, especially 18S rRNA, were used as an internal control. However, it was obviously seen from the proteomics data that ribosomal subunits, 40S and 26S rRNAs, were changing for the Barley-*Bgh* interaction.

An MS Excel program, geNorm was used in order to define the best couple of reference genes for normalizations. The program relies on the idea that even if the experimental conditions or samples used were changed, the expression level for the internal control genes would stay identical for a given cDNA value. For all of the candidate reference genes, the *M* value corresponding to lower than 1.5 meaning none of them shows variable gene expression. The α-tubulin qRT-PCR product having the highest *M* value (Table 3.6), previously defined in Section 1.3, was excluded from the study which also resulted in more than one peak in its dissociation curve indicating that there was more than one PCR product for this primer pair (Figure 3.6). In this study, the combination of EF and ubi, couple having the minimum *M* value, were used as the reference genes. As a result, geNorm also calculated the normalization factor in order to eliminate the expression differences providing more accurate and reliable normalization.

Table 3.6 Expression stability of reference genes according to geNorm program.

Gene Name	***M* value**
Ubi	0.533
EF	0.539
Ubc	0.551
Alpha	0.676

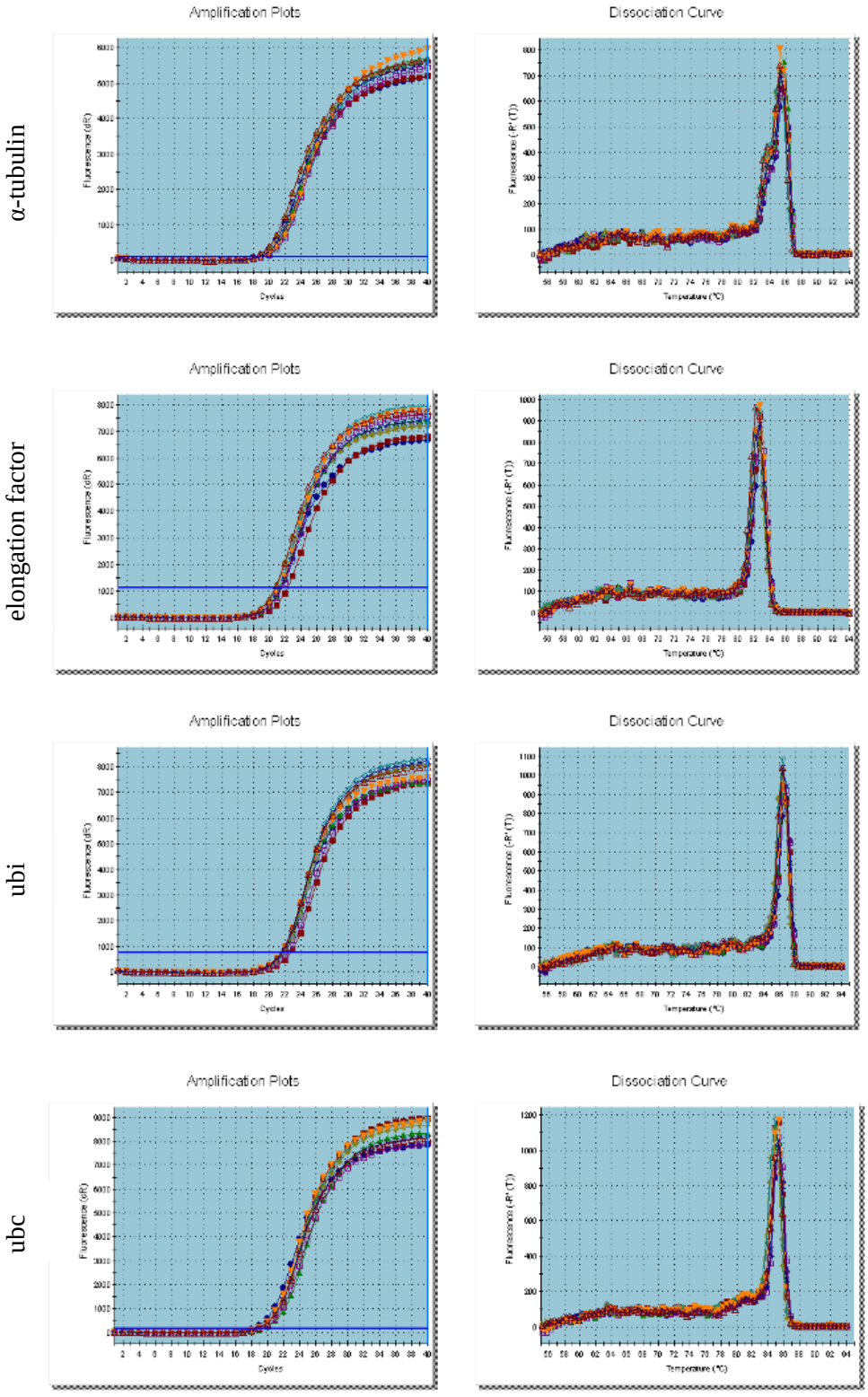


Figure 3.6 Amplification plots (left) and dissociation curves (right) of the genes used as reference genes.

3.5 Transcription Level Determination of Selected Candidate Genes

The relative expression of the selected candidate genes were calculated according to Pfaffl (2001). For the precise quantification, first all the amplification efficiency should be calculated. Efficiency is calculated according to the equation;

Efficiency (E) = 10^(-1/slope)

PCR efficiency for the genes examined was shown in Figure 3.7 and it was 1.95 for NAD malic enzyme; 2.40 for lipocalin; 2.11 for Mg chelatase; 2.04 for 26S regulatory Subunit 6B and 2.02 for PGM with high linearity (Pearson correlation coefficient r > 0.95).

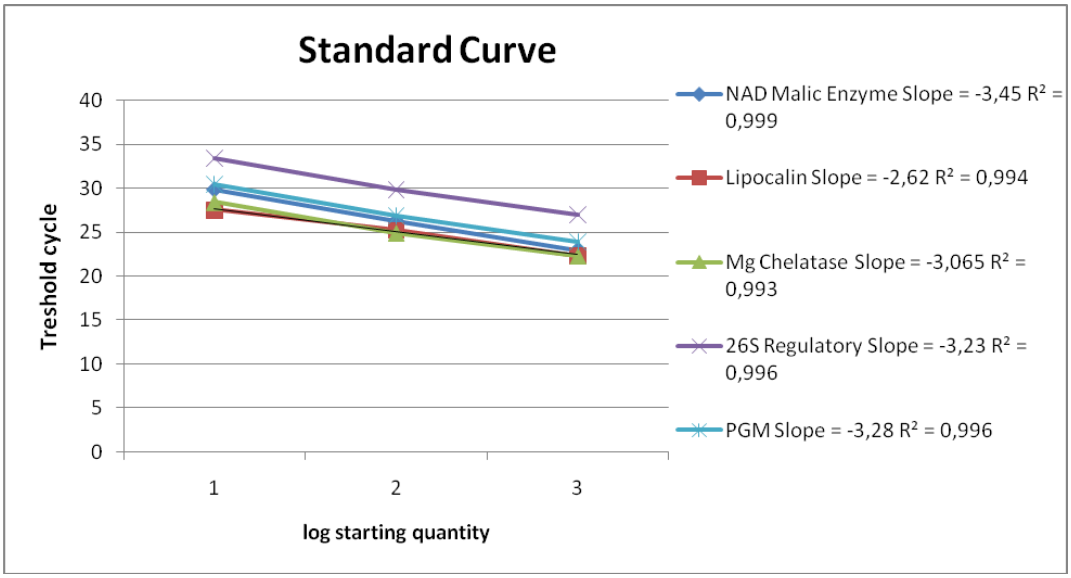


Figure 3.7: qRT-PCR amplification efficiency and linearity

Relative expression of a target gene is calculated according to the equation given below of Pfaffl (2001) which enables the determination relative expression ratio based on the efficiency, average C_t difference of infected for this study and control for both gene of interest and reference gene. ΔCP represents the mean C_t values.

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control-sample)}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(control-sample)}}$$

When the normalization factor for reference genes were taken into consideration by using geNorm program, the C_t difference for the reference genes would be 0. Therefore, the bottom line would be equal to 1 and the equation is simplified to;

$$\text{ratio} = (E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(control-sample)}$$

For each gene of interest, the intra-assay precision was carried out in two repeats within one run to confirm the accuracy of the quantification by qRT-PCR. Also, the inter-assay precision was performed for each gene in two different qRT-PCR runs. As a result, there was 4 data for each condition. The mean of these data and the error rates for them are listed in Appendix A.

Table 3.7: Comparison of the gene expression analysis of candidate genes by qRT-PCR and their protein expression profile detected in P-01and P-03 infected with *Bgh*103.

	Proteomic Analysis				**Gene Expression Analysis**				
	Protein Name	**Fold Change Infected vs. Control**			**Fold Change Infected vs. Control**				
		12 hpi	**24 hpi**	**48 hpi**	**6 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
P-01 (resistant/incompatible)	26S protease regulatory subunit 6B homolog	*+Bgh*	2,33↓	_	1.5↓	1.5↓	1.4↑	1.1↓	NA
	Chloroplast lipocalin	_	2,06↑	_	1.4↓	1.7↓	1.5↓	NC	1.1↓
	Mg-chelatase subunit	2,33↓	_	_	1.2↑	1.1↑	1.2↓	1.1↓	1.2↓
	Phospho-glycerate mutase	_	2,52↑	_	1.4↓	1.6↑	NC	1.1↓	NA
	NAD-dependent malic enzyme	_	_	_	2.0↓	1.9↓	1.1↑	1.2↓	1.1↓
P-03 (susceptible/compatible)	26S protease regulatory subunit 6B homolog	_	_	_	3.5↓	1.8↓	2,0↑	1.4↓	1.7↑
	Chloroplast lipocalin	_	_	_	2.0↓	1.5↓	1.1↓	NC	NA
	Mg-chelatase subunit	_	_	_	2.3↓	1.4↓	3.0↓	1.3↑	1.4↑
	Phospho-glycerate mutase	_	_	_	1.7↓	1.3↓	1.1↓	NC	1.6↑
	NAD-dependent malic enzyme	2,81↑	1,52↑	_	2.3↓	1.9↓	1.3↑	NC	1.2↑

NA: not applicable, NC: no change, + *Bgh*: present in infected and absent in control, ↑: up-regulated, ↓: down-regulated

3.6 Roles of Studied Candidate Genes in Plant Defense Mechanisms

The ubiquitin/26S proteasome system (UPS) is the key element in eukaryotic protein degradation processes. Regardless of the affecting pathogen, UPS plays a central role in plant-pathogen interactions by involving in the every step of the defense mechanism. UPS is not only involved in the defense system by changing the proteome in order to increase the survival rate but also being a target for the invaded pathogens. Several studies showed that UPS is also involved in hormone signaling pathways, such as jasmonate, salicylic acid and ethylene signaling pathways as response of plants against pathogens (Dielen et al., 2010). UPS is essential for the defense against powdery mildew invasion of barley by regulating the protein turnover. The silencing of 19S regulatory particle of 26S proteasome system meaning the depletion of cellular ubiquitin resulted in the induction of susceptibility for *Bgh* strain which is resistant under normal conditions (Dong et al., 2006). As well as playing a role in basal defense responses, ubiquitination pathway also activates the downstream signaling of some R gene-mediated responses (Dielen et al., 2010, Goritschnig et al., 2007). The subunits of the 20S core particle of the 26S proteasome system are rapidly elevated at the transcription level in the case of a fungal elicitor –cryptogenin- recognition. These subunits are thought to be involved in the induction of systemic acquired resistance (SAR) which is defined as global resistance in the whole plant upon pathogen attack (Dielen et al., 2010, Dahan et al., 2001). In proteomics data, the presence of 26S regulatory protein only in resistant P-01 lines at 12 hpi supports the involvement of this protein in resistance mechanism against pathogen. At 24 hpi, the level of protein declines which supports the suppression of resistance by the pathogen. However, at transcription level, the relative expression of the gene encoding this protein seems to be decreased for both resistant and susceptible strains.

Mg-chelatase H subunit (CHLH) has several functions such as participating in chlorophyll biosynthesis, signaling and abscisic acid (ABA) synthesis pathway.

ABA plays an important role in development and in adaptation of the plant to the changing environmental conditions. When plant is under drought stress, ABA forces the closure of stomata in order to prevent water loss. CHLH functions as a receptor in the ABA-induced stomatal closure in *A. thaliana* (Shen et al., 2006). ABA is known to be involved in the improvement of plant disease susceptibility. Anderson et al. (2004) stated the mechanism of ABA in plant defense system in that jasmonic acid-ethylene signaling pathway and ABA signaling pathway are coordinated to each other in an antagonistic manner. When ABA becomes deficient by mutating the genes expressing the synthesis of enzymes involved in ABA biosynthesis, the transcription of the genes for both basal and JA-ethylene activated defense were up-regulated. By contrast, ABA suppressed the basal and JA-ethylene–activated defenses. In the 2D PAGE findings, Mg chelatase protein level at 12 hpi was dropped for the resistant barley strain which designates the involvement of Mg chelatase in the suppression of resistance upon the recognition of pathogen. As seen in Table 3.7, the relative expression of Mg chelatase gene was elevated in resistant barley while increasing in susceptible one.

Phosphoglycerate mutase (PGM) is involved in carbohydrate metabolism. It catalyzes the conversion of 3-phosphoglycerate into 2-phosphoglycerate in the glycolysis pathway. Mazarei et al. (2003) designated a PGM homolog in *A. thaliana* as *AtPGM* whose promoter was down-regulated by ABA. In another study, PGM was shown to be down-regulated in *Bgh*-susceptible barley (Gjetting et al., 2007). Down-regulation in susceptible plants means that the protein is involved in resistance and in order to make the plant intolerant to the invading pathogen, the level of this protein should be decreased. At protein level, the elevation of PGM level at 24 hpi for *Bgh*-resistant P-01 plants was consistent. In transcription level (Table 3.8), PGM transcript was down-regulated at 6 hpi for in the susceptible P-03 plants.

In plants, there are two types of lipocalins; choloroplast lipocalins (CHLs) and

temperature induced lipocalins (TILs). Plant lipocalins are thought to be functioning in protection of photosynthetic system against temperature stress (Charron et al., 2005). Under stress conditions, TILs are responsible for the biogenesis and repair of the membrane by transporting sterols to the membrane at high temperatures (Charron et al., 2002; Wan et al., 2008). The expression of TILs, especially in leaves marks the role of TILs in the chloroplast function in case of temperature stress (Charron et al., 2002). When rice leaves treated with H_2O_2, a putative TIL was decreased in abundance (Wan et al., 2008). This decline in TIL level is unexpected since Charron et al. (2008) stated that lipocalins are involved in the regulation of the plant's tolerance to oxidative stress. In order to define the potential role of lipocalins in disease resistance, knockout (KO) studies carried out in Arabidopsis are enlightening. Several genes related to defense were over-expressed in *AtTIL* KO plant. One of these genes is the WRKY54 gene which associates with defense response by increasing SA level; thus, leads to the induction of defense related genes (Charron et al., 2008). From these studies, it is easily understood that the level of this protein should be increased for pathogen susceptible interactions, vice versa, increased in resistance. However, it was decreased in protein level at 24 hpi for resistant barley. Transcription level experiments (Table 3.8) showed that its expression level was decreased for resistant P-01-*Bgh* interaction.

Malic enzymes catalyze the decarboxylation of L-malate into pyruvate in all organisms. There are three classes identified for this enzyme. NADP-dependent malic enzyme (NADP-ME) generates NADP and is able to decarboxylate oxaloacetate (OAA). This enzyme is found in mitochondria, chloroplast and cytosol. The difference of the second malic enzyme from this one is its presence in bacteria and insects. The third class of malic enzymes is NAD generating NAD-dependent malic enzyme (NAD-ME) which is unable to decarboxylate OAA. It is specifically found in mitochondrial matrix of many plant species. In most plants, NAD-ME functions in the conversion of C4 acids into acetyl-CoA of the TCA

cycle. In NAD-ME type C4 plants, in which NAD-ME is present abundantly, the enzyme plays a central role in photosynthetic carbon fixation (Tecsi et al., 1994). In a study investigating the compatible interaction between marrow plant and cucumber mosaic virus and elevation in the activity of NAD-ME was reported when infected regions of the plant are compared with the healthy tissues (László et al., 1996). The other type of malic enzyme, NADP-ME, is involved in phytoalexin and lignin biosynthesis of the phenylpropanoid pathway and it was shown to be induced after pathogen infection (Fujiware et al., 2006). At the protein level, NAD-malic enzyme as expected was elevated at susceptible barley line P-03 for both 12 and 24 hpi. However, its transcript level was declined (Table 3.8). The gene expression level also decreased for the resistant barley.

For Mg chelatase, the mRNA level found to be high whereas its corresponding protein level is low at the same condition. This situation can be explained by the different half-lives of the protein with differing synthesis and degradation ratios. Proteins with similar functions may have different turnover rates meaning their synthesis rate is lower than its degradation rate which results in the lowering of the protein level. Another reason might be the presence of mRNAs with different secondary structures which also affects the protein translation efficiencies (Guo et el., 2008).

For the situations in which the protein level is high whereas its transcription level is low can be explained by the presence of a complementary sequence to a small interfering RNA called microRNA. In this case, the synthesized mRNA is targeted and destructed *via* the RNA interference pathway which resulting in reduction of the mRNA level. Another reason might be the synthesis of the protein also from a different pathway since the protein synthesis mechanisms are complex in plants. For instance, although NADP-malic enzyme is involved only in glycolysis pathway in humans, however in plants; it is also involved in TCA cycle (Nakane et al., 2003).

The translation of mRNA into protein results in the assumption of the presence of correlation between mRNA and protein. The expression level for both protein and mRNA are expected to be in accordance. However, due to dynamic and complex cellular events, the correlation can be observed and consistent for only some gene products. The main reason for this inconsistency between transcript and protein levels in this study is although use of the same samples and same sample preparation except were conducted at different times.

There are several studies carried out in yeast and human tissues showing that there is an uncertainty in the correlation between mRNA and its corresponding protein levels (Guo et el., 2008). mRNAs having similar expression patterns could result in up to 20 fold difference in protein abundance in yeast and vice versa (Gygi et al., 1999). In different biological categories, when comparing the transcription and translation levels of a gene, there is a relatively large expression divergence. In case of the difference in cell type, the same gene would result in different mRNA-protein correlation (Guo et el., 2008). For this reason, while studying whole genome, gene expression level changes for development or diseases, the measurements at the transcription level is considered to be insufficient and it must be supported by protein level studies. Since proteins may reflect the real time gene function than mRNA does. Although both transcription and translation level studies are important for gene function studies, mRNA level is generally informative but not predictive for the further protein level studies.

There are several reasons explaining the inconsistencies observed between protein and mRNA levels. As stated by Greenbaum et al. (2003) the first reason for this inconsistency is the complex post-transcriptional mechanisms leading the turning of mRNA into its corresponding protein. Open reading frames (ORFs) may lead to steady state or varying levels of mRNA expression into protein. For the genes showing little difference in mRNA expression throughout the cell cycle, the control of these ORFs are carried out in translational or post-translational level.

This would result mRNA levels independent of the protein meaning little or no correlation between mRNA and its final protein product. For the ORFs that have low levels of ribosomal occupancy, which is the percentage of concentration of mRNA being translated and ribosomes, the ORF again would not tightly controlled and results in uncorrelated mRNA and protein level (Greenbaum et al., 2003).

Yet another reason for the inconsistency between mRNA and protein level is the significant error rate and noise for both mRNA and protein experiments. The methods used for the quantification of mRNA and protein level are not perfectly accurate (Greenbaum et al., 2003). The reason for the negative expression correlation might be artifacts resulted from technical noise or a novel mRNA to protein regulation mechanism such as negative feedback (Guo et el., 2008).

CHAPTER IV

CONCLUSION

The battle between plant and pathogen has been progressing throughout the history. For the sake of human beings, the winner of this war needed to be plants because, especially the crops are the main food for human and animals. For this purpose, the main approach for the breeders is the identification of the resistant genes from the varieties and the integration of these properties into cultivated ones (Hidalgo, 2004). At this stage, genetic engineering comes to the rescue which enables the transformation of the genes playing central role in plant resistance against pathogens. Finding genes for all organisms that provide durable broad spectrum resistance is an important issue. Although, the loci of the genes and the gene sequence of the few conferring resistance are determined, and they are being introgressed by breeding, it is difficult to maintain the resistance since pathogen evolve against the resistance genes. In many cases, the over-expression of resistance related genes results in dwarf and/or seedless plants (Gurr et al., 2005). The solution to overcome these consequences is the selective expression of these genes only at the infection site by the use of promoters induced upon pathogen recognition. The candidate defense-related genes can be determined by transcriptomics, proteomics and protein interaction studies. Over-expression, knockouts or silencing experiments are used to determine the probable roles of these candidate genes in plant-pathogen interaction (Gurr et al., 2005).

In this study, changes in the expression level of five candidate genes upon infection of barley with the fungus *Blumeria graminis* f. sp *hordei* for both compatible and incompatible interaction was aimed. These candidate genes, named as NAD malic enzyme, lipocalin chloroplast, phosphoglyceromutase (PGM), Mg

chelatase and 26S protease regulatory subunit 6B homolog, were shown to be differentially expressed in the previous proteomics study. Although there found to be proteins with 6 to 7 fold difference in the proteomics data, we have selected the genes with available the cDNA sequences in the databanks for barley (Table 2.3).

The expressions at the protein and transcription levels are considered to be similar. However, the level of transcript would not always correlate or reflect its protein level. Post-transcriptional modifications such as synthesis, processing, degradation and post-translational modifications are changing the level of protein expressed. The mRNA level would not reflect its corresponding protein in the cell due to complex regulations in cellular processes. The possible reasons for this inequality are changes in mRNA and protein stability, efficiency of translation and protein's turnover rate (Mehta et al., 2008).

The real time PCR analysis are conducted in this study revealed the fact that the genes investigated in the analysis are indeed regulated with a complex and antagonistic means due to the lack of correlation found between the protein and mRNA levels. In fact, the outcome of the thesis study is crucial for this respect, it will entail further analysis for understanding the details of these regulations.

For the genes investigated, differential expression in the protein level was previously argued. In this study, also differential expression of these genes in the transcription level was supported. This differentiation in both protein and transcript levels makes the genes possible candidates for the research of disease resistance mechanisms. For further investigations, genetic tools such as RNAi and knockout experiments are required in order to elucidate the mechanism of these candidate genes in the plant-pathogen interaction. We were unable to conduct western blot analysis since there are no antibodies commercially available against the proteins investigated. Thus, for the future studies antibodies can be raised against the proteins worked out in this study.

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APPENDIX A

Average C_t Values for Each Gene of Interest

	NAD malic enzyme			
	P-01		P-03	
	Mean C_t	Error rate (±)	Mean C_t	Error rate (±)
6 I	21.94	0.016	23.44	0.144
6 C	22.05	0.212	23.28	0.385
12 I	21.47	0.180	24.31	1.278
12 C	20.90	0.244	23.22	0.975
24 I	22.61	0.361	24.15	1.546
24 C	22.69	0.483	24.51	0.778
48 I	23.66	0.257	24.87	0.943
48 C	23.01	0.221	23.98	0.643
72 I	23.02	0.298	24.30	0.524
72 C	23.35	0.378	23.75	0.705

6 I: 6 hour post inoculation infected sample, 6 C, 6 hour-post inoculation control

	Lipocalin			
	P-01		P-03	
	Mean C_t	Error rate (±)	Mean C_t	Error rate (±)
6 I	25.00	0.142	22.99	0.062
6 C	25.32	0.685	22.88	0.122
12 I	25.91	1.126	22.46	0.207
12 C	24.95	0.739	21.74	0.101
24 I	25.54	1.793	23.54	0.198
24 C	25.19	1.358	23.76	0.067
48 I	25.60	1.464	23.91	0.220
48 C	25.03	1.728	23.34	0.209
72 I	25.03	1.533	NA	NA
72 C	23.34	0.209	NA	NA

6 I: 6 hour post inoculation infected sample, 6 C, 6 hour-post inoculation control

	Mg Chelatase			
	P-01		P-03	
	Mean C_t	Error rate (±)	Mean C_t	Error rate (±)
6 I	27.87	1.102	23.54	3.030
6 C	28.43	0.543	23.20	2.569
12 I	27.91	1.387	22.54	2.689
12 C	27.22	0.725	21.83	2.720
24 I	26.89	0.807	23.56	3.560
24 C	27.36	1.601	22.25	2.369
48 I	27.74	1.500	23.00	2.331
48 C	26.73	1.171	22.82	2.959
72 I	27.16	2.032	22.38	2.278
72 C	26.57	2.109	22.47	2.245

6 I: 6 hour post inoculation infected sample, 6 C, 6 hour-post inoculation control

	26S Regulatory Subunit B			
	P-01		P-03	
	Mean C_t	Error rate (±)	Mean C_t	Error rate (±)
6 I	24.76	2.893	27.03	3.213
6 C	24.99	2.495	26.06	7.332
12 I	25.09	2.895	26.61	2.823
12 C	24.30	2.772	25.60	2.452
24 I	24.26	3.050	26.75	2.141
24 C	24.99	3.309	27.94	2.312
48 I	25.62	3.164	28.43	3.468
48 C	24.95	2.783	27.40	3.146
72 I	24.08	2.866	27.58	3.135
72 C	22.41	0.028	27.94	3.043

6 I: 6 hour post inoculation infected sample, 6 C, 6 hour-post inoculation control

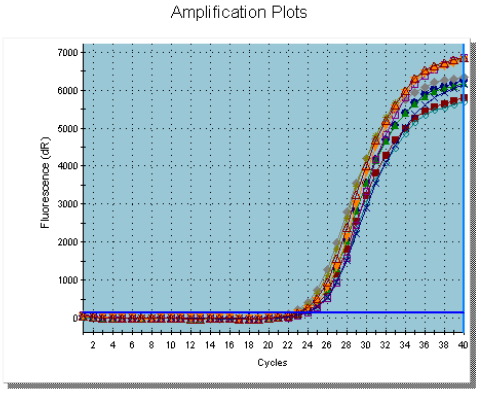
	Phosphoglycerate mutase			
	P-01		P-03	
	Mean C_t	Error rate (±)	Mean C_t	Error rate (±)
6 I	21.14	1.58	21.94	0.016
6 C	21.50	0.46	22.05	0.212
12 I	21.17	1.315	21.47	0.180
12 C	21.65	1.62	20.90	0.244
24 I	20.31	1.44	22.61	0.361
24 C	20.48	1.78	22.69	0.483
48 I	21.07	1.05	23.66	0.257
48 C	20.35	0.58	23.01	0.221
72 I	NA	NA	23.02	0.298
72 C	NA	NA	23.35	0.378

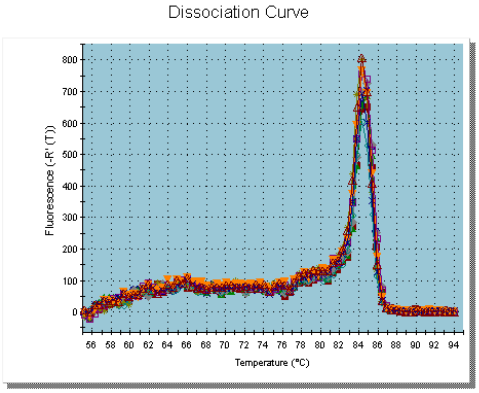
6 I: 6 hour post inoculation infected sample, 6 C, 6 hour-post inoculation control

APPENDIX B

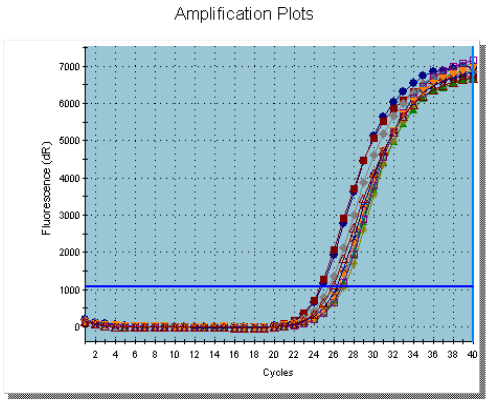
Amplification Plots and Dissociation Curves of Genes of Interest

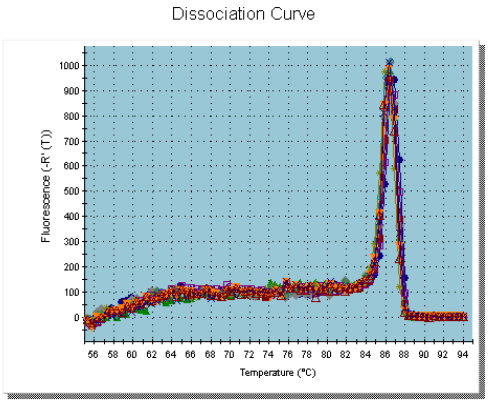
NAD Malic Enzyme:



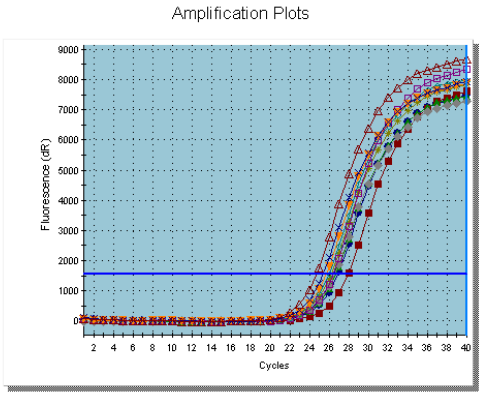


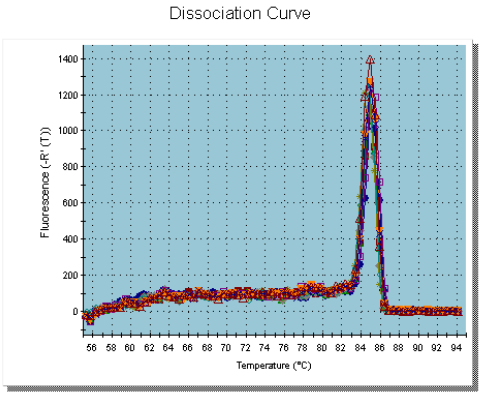
Lipocalin:



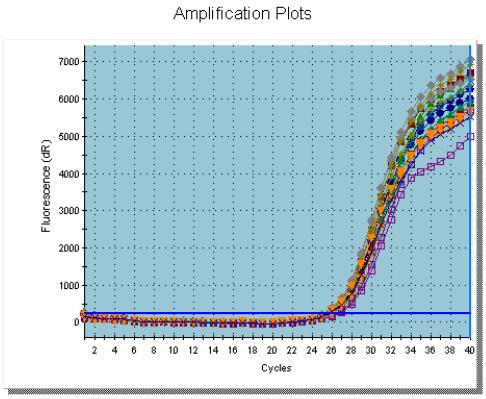


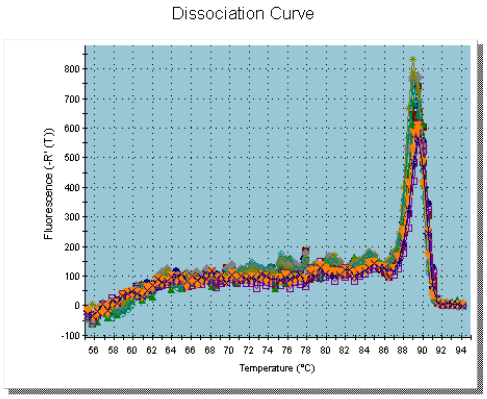
Mg Chelatase:





26 S Regulatory Subunit 6B:





PGM: