PROTEOME ANALYSIS OF BLUMERIA GRAMINIS F. SP. HORDEI INOCULATED BARLEY

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PROTEOME ANALYSIS OF *BLUMERIA GRAMINIS F. SP. HORDEI* INOCULATED BARLEY

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ABSTRACT

PROTEOME ANALYSIS OF *BLUMERIA GRAMINIS* F. SP. *HORDEI* INOCULATED BARLEY

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Blumeria graminis f. sp. *hordei* is a biotroph pathogen that causes powdery mildew disease in barley. In this study, Pallas01 and Pallas03 barley lines having *Mla1, Ml (Al2)* and *Mla6, Mla14* R-genes were inoculated with Bgh103(64/01) race of the *Blumeria graminis* f. sp. *hordei* having avirulence and virulence to Pallas01 and Pallas03, respectively.

The proteins were isolated from the three biological replicates of 12, 24, and 48 hpi samples following the method in Rampitsch et al., 2006. These there biological replicates of three time points together with the mock inoculated plant proteins were separated on 2D-PAGE using IPG strips of 4-7 pH values as three technical replicates, resulting 108 gels.

The gels were analyzed using PdQuest (Bio Rad) in order to assess up- or down-regulated protein spots by comparing against controls and the samples having resistance or susceptible responses with each other. According to the analysis, 36 proteins were found to be differentiated and among them 18 proteins were found up-regulated and 8 proteins were found down-regulated. The spots were manually

excised and subjected to the nano-LC-ESI-MS/MS analysis (Proteome Factory, Germany). The MASCOT algorithm was used for identification of the possible proteins. The experimental pI and MW values were used for selecting the differentiated proteins from the mass results.

The relative abundance of each of the 38 identified polypeptides was calculated in terms of spot intensity. The majority of the most abundant proteins were found to be carbohydrate metabolism related. The relative distribution of the proteins into four main functional categories was taken into consideration.

Statistical tests (Students' T-test) were carried among the identified proteins in order to reveal statistically significant proteins throughout the study.

By making a WoLF PSORT search, subcellular localization of the proteins was predicted. Accordingly, most of the proteins were found to be located in cytoplasm or chloroplast.

Key Words: Proteomics, plant proteomics, plant disease proteomics, powdery mildew, *Blumeria graminis f.spp. hordei*, nano-LC-ESI-MS/MS

ÖZ

BLUMERİA GRAMİNİS F. SP. HORDEİ İLE ENFEKTE EDİLMİŞ ARPANIN PROTEOMİK ANALİZİ

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Biotrof patojen olan Blumeria graminis f. sp. hordei arpada külleme hastalığının etmenidir. Bu çalışmamızda Pallas01 ve Pallas03 arpa ırkları Blumeria graminis f. sp. hordei nin Bgh103(64/01) ırkı ile enfekte edilmesiyle 12, 24, 48 inci saatlerde toplanan örekler Rampitsch ve arkadaşları nın kullandığı protein izolasyonu yontemi kullanılarak izole edildi.

Çalışmada üç biyolojik ve her biyolojik replika için üç teknik replika kullanılarak yapılan 2D-PAGE esnasında pH 4-7 IPG stripler kullanıldı. Elde edilen jellerin görüntü analizi PdQuest (Bio Rad) ile yapılarak artan yada azalan proteinler tespit edildi. Bu analiz sonucunda farklılaşması tespit edilen 38 proteinden 18 tanesinin anlatım seviyesinin yükseldiği, 8 tanesinin ise anlatım seviyesinin düştüğü gözlenmiştir.

Tespit edilen proteinler el ile kesildikten sonra nano-LC-ESI-MS/MS ile kütle analizi yapılması için Proteome Factory'e gönderilmiştir. Analiz sonuçları MASCOT algoritması kullanılarak proteinlerin tanımlanmasında kullanılmıştır.

Eksperimental moleküler agırlık ve pI değerleri kullanılarak imaj analizi ile tespit ettiğimiz 38 farklılaşmış proteinin identifikasyonu sağlanmış oldu. Identifiye edilmiş proteinler arasından karbonhidrat metabolizması sınıfından olan proteinlerin külleme hastalığı ile ekspresyon seviyesi değişmiş proteinler arasında en çok farklılaşma gösteren protein sınıfı olduğu tespit edilmiştir.

Ekspresyon seviyelerindeki değişimler istatistik testleri kullanılarak birkez daha doğrulanarak sonuçlarımızın tutarlılığı gösterilmiştir.

WoLF PSORT kullanılarak proteinlerin subselüler lokalizasyonu tahmin edilmiştir. Bu doğrultuda farklılaşan proteinlerin birçoğunun sitoplazma ya da kloroplastta bulunduğu tespit edilmiştir.

Anahtar kelimeler: proteomik, bitki proteomiği, bitki hastalık proteomiği, külleme, *Blumeria graminis* f.spp. *hordei*, nano-LC-ESI-MS/MS

To my family, who offered me unconditional love, support and respect throughout the thesis study.

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LIST OF ABBREVIATIONS

AGT: Appresorial Germ Tube

App: Apical Appresorium

APS: Ammonium Per Sulphate

Avr: Avirulance

Bgh: *Blumeria graminis f. sp. hordei*

BHT: Butylated Hydroxytoluene

BSA: Bovine Serum albumin

C: Conidia

CBB: Coomassie Brilliant Blue

CCD: Charged Coupled Device

CH: Chain Apex

Cm: Centimeter

DAB: 3,3-diaminobenzidine

dai: Days After Inoculation

dd: Double Distilled

DP: Digitate Processes

dpi: Dots Per Inch

DTT: Dithiothreitol

F: Haustorium

FAO: Food and Agriculture Organization

FAOSTAT: Food and Agriculture Organization Corporate Statistical Database

h: Hour

HA: Hypheal Appresoria

hai: Hours After Inoculation

HR: hypersensitive Response

IEF: Iso Electric Focusing

M: Molar

mA: Milliamper

MAMP: Microbe Associated Molecular patterns

METU: Middle East Technical University

mL: Milliliter

mM: Nanomolar

NBLRR: Nucleotide Bind Leucine Rich Repeats

NCBI: National Center for Biotechnology Information

nm: Nanometer

PAMP: Microbe Associated Molecular patterns

PCA: Principle Component Analysis

PCD: Programmed Cell Death

PGT: Primary Germ Tube

PMF: Peptide Mass Fingerprints

R: Resistance

RLK: Receptor Like Kinases

RLP: Receptor Like Proteins

ROS: Reactive Oxygen Species

SDS: Sodium Dodecyl Sulphate

S: Septum

SA: Salicylic Acid

SAR: Systemic Acquired Resistance

SH: Secondary Hyphea

TGS: Tris Glycine SDS

µg: Micro Gram

µL: Micro Liter

µM: Micro Molar

CHAPTER 1

INTRODUCTION

1.1 Powdery mildew and barley

1.1.1 Barley (*Hordeum vulgare*)

Barley Hordeum vulgare is an annual monocot plant and member of the Poaceae (Grass) family. It is cllasified as (USDA, 2009);

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Liliopsida – Monocotyledons

Subclass: Commelinidae

Order: Cyperales

Family: Poaceae – Grass family

Genus: *Hordeum L.* – barley

Species: *Hordeum* vulgare L. – common barley

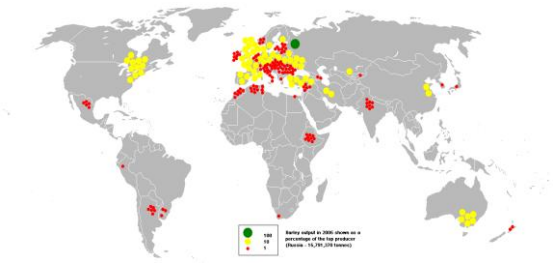


Figure 1.1 Barley output in 2006 (FAOSTAT, 2006)

Barley is producing 138 million metric tons around the world and Turkey is the sixth barley producer across the world. Barley is mainly used as animal feed crop and malting. Malting can be used for beer production and malt beverages which are rich in vitamin B.

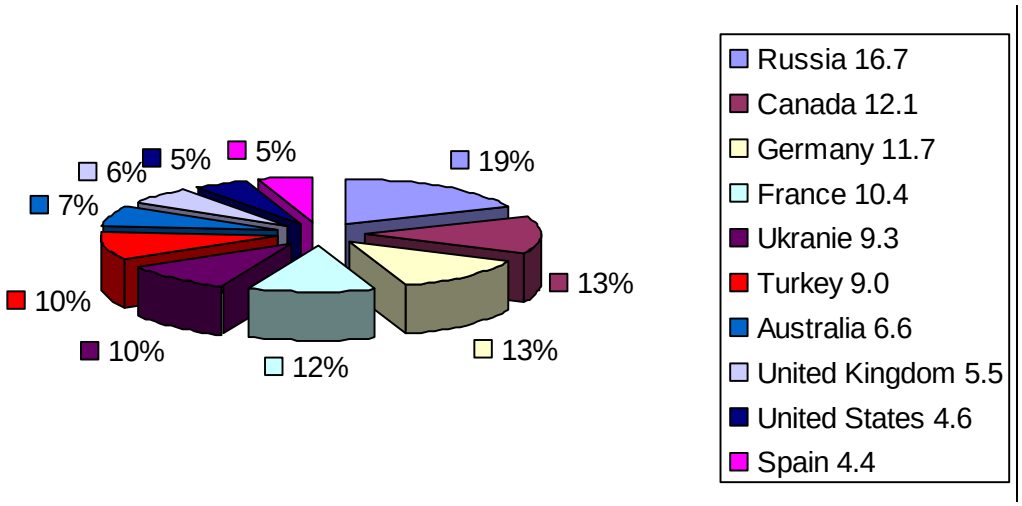


Figure 1.2 Top ten barley producers 2006 (million metric tons) (FAO, 2006)

1.1.2 *Blumeria graminis f.sp. hordei*

Powdery mildew is a serious fungal disease which lowers the quality and yield seriously. It can be characterized by the whitish velvety pustules that cover leaves, stems and also flowers (Eichmann and Huckelhoven 2008). The pathogen that causes the disease is *Blumeria graminis f.sp. hordei* (Bgh). *Blumeria graminis* belongs to the ascomycota phylum of the kingdom fungi. Ascomycota are the biggest phylum of fungi and the name of ascomycota comes from the ascus cells that cover the spores named ascospores during their sexual life cycle. It is an obligate biotrophic ecto-parasite that has two life cycles. It has to pass its asexual life cycle on the plant in order to obtain food by forming an intracellular organ called haustorium. The infection is caused by the air borne asexual conidia which are released by conidiophore infects the epidermal leaf layers within 2 hours. After

the infection cleistothecia develop on the lower leaves. Powdery mildew can be seen in monocotyledonous and dicotyledonous plants such as apple, tobacco, grape, wheat, *Arabidopsis* and barley. There are several forms of the disease which are specific to individual crops and do not cross-infect meaning that they are host specific pathogens. For example *B. graminis f. sp tritici* infects wheat, *B. graminis f. sp hordei* infects barley, *B. graminis f. sp avenae* infects oats and *B. graminis f. sp secalis* infects rye.

1.1.2.1 The asexual life cycle of *B. graminis*

Bgh is the fungus which causes powdery mildew on plants that were mentioned above. After the maturation, asexual conidiophores can produce over 200.000 air borne conidia. The conidia are responsible for the epidemic spread. Figure 1.2 explains how fungal development takes place on epidermal tissues of the barley. The spreading process starts with the air born mature conidia (C) which is present at the chain apex (CH) of the conidia. After the arrival of the conidia to the leaf epidermis primary germ tube (PGT) emerges within 2 hours. The PGT is present in the contact site between the leaf surface and the conidia, so that we can say that the spore is attached to the host body and the differentiation of the spore has been started. After the PGT formation the spore can take water from the plant and also can activate the perception transduction of leaf-derived signals to induce fungal development. In 8-10 h, appresorial germ tube (AGT) can be seen. Between the AGT and the spore body septum (S) form and the differentiation of the AGT it continues to form a hooked apparatus which ends with the formation of the apical appresorium (APP). This apparatus forms the penetration peg and the penetration to epidermis and the initiation of haustorium (F) starts. (15-18 h) and in 24 h secondary hyphea (SH) is seen. In 48 h, hypheal appresoria (HA) differentiation from SH is seen. After that we can see the newly formed haustoria (F) (Eichmann and Huckelhoven 2008). Bgh can take nutrients from the host by haustorium. A haustoria can be described by the digitate processes (DP) at each side of the

haustorium body and the total length is 120-150 μm (Zhang et al., 2005) (Gurr et al., 2005).

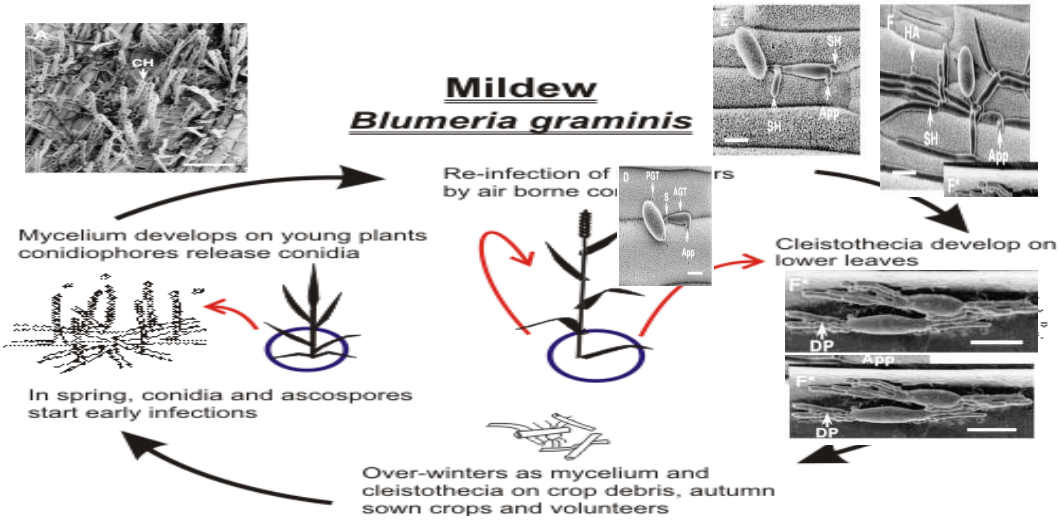


Figure 1.3 *Blumeria graminis* life cycle (Modified from Zhang et al., 2005)
Abbreviations: conidium (C), chain apex (CH), primary germ tube (PGT), appresorial germ tube (AGT), septum (S), apical appresorium (App), haustorium (F), secondary hyphea (SH), hypheal appresoria (HA), digitate processes (DP)

1.2 Plant disease mechanisms

Immunity system of plants differs from the animals in lacking of the somatic adaptive immunity and the defender cells which migrate to the place of infection. Plants have innate immunity on each cell and the communication between them depends on the signals that are initiated at the infected site (Ausubel et al., 2005; Chisholm et al., 2006; Jones and Dangl et al., 2006. Plants can recognize broad range of conserved molecular patterns and activate the defense system. These patterns were called pathogen associated molecular patterns (PAMP) but then they

were changed into microbe associated molecular patterns (MAMP) in order to make them more specific to the plant innate immunity system (Bent et al., 2007).

Pathogens have effectors that are devoted to suppress the basal defense system of the plant. Those effectors sometimes are host specific meaning that they have ability to suppress the basal defense only on the specific host. The effectors are delivered into the plant by different mechanisms. For example gram-negative bacteria deliver the proteins inside the host cell by the specialized secretion system such as T3SS (Abramovitch et al., 2006; Hogenhout et al., 2009) biotrophic fungi have haustoria for delivery (Panstruga et al., 2003).

On the plant point of view, the defense against the pathogen is activated by recognition of the pathogen by the elicitors. Plants have R genes (Resistance genes) which directly or indirectly recognize the pathogen in order to activate the defense system. R genes were classified as NB-LRR (Nucleotide bindinmg Leucine Rich Repeats) genes, Ser/Thr kinases, receptor like kinases (RLKs) and receptor like proteins (RLPs). Pathogens have avirulace (*Avr*) genes that were recognized by the host cell. The disease occurs when the plant is lacking the R or the pathogen is lacking the *Avr* genes or suppression of the plant immunity by the pathogen and the plant becomes susceptible.

The disease is rare in the nature and plants carry different types of defense systems against pathogens. If the resistance is controlled by one or a few major genes, the race specific resistance is observed (Bonas et al., 2002). Resistance can be overcome by one or few mutations in the *Avr* genes of the pathogen. The response of the host is called hypersensitive response (HR) by which plant slows pathogens reproduction rate and stops the development of the epidemics. The powdery mildew HR response is characterized by localized areas of green living tissues (green islands) surrounded by the senescent leaf tissues (Rushton et al., 2002). If the resistance is controlled by many genes, it is called race nonspecific resistance. This type of resistance is generally a durable one. It slows the development of the

infection, so that the spreading slows down too. The last type of resistance is non-host resistance which is observed when all individuals in a plant species exhibit resistance to all members of a pathogen species. It is a durable resistance (Rushton et al., 2002).

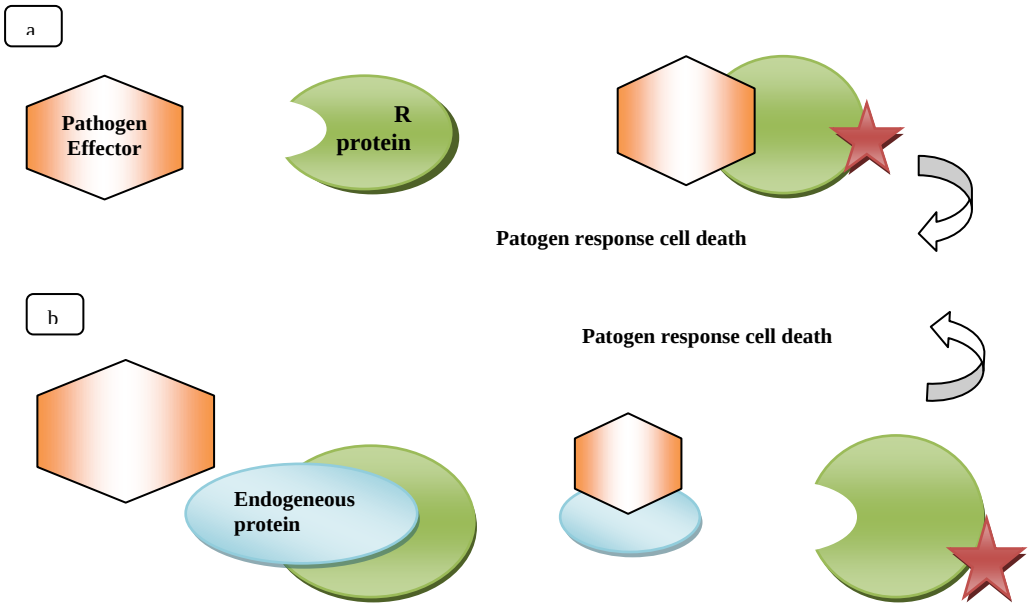


Figure 1.4 (a) Gene-for-gene, (b) Guard hypothesis
(Modified from Bomblies et al., 2007)

Two models of pathogen recognition in plant disease resistance, namely 'gene-for-gene' (Flor et al., 1971) and 'guard' hypothesis (Dangl and Jones, 2001), are the accepted current basic models of the plant pathogen studies (Figure 1.3). If this interaction is a direct recognition of *Avr* gene by corresponding R gene, this is called "gene-for-gene" interaction and lacking of either R gene or *Avr* gene leads to disease (Flor, 1971). Recognition of *Avr* factor by R gene generally activates a local response, which is followed by cell death in infected area. *Avr* recognition

also immunizes plant by spreading of signal molecules from the infection site, this result in a global response (systemic acquired resistance (SAR)) (Heath et al., 2000; Durrant et al., 2004) "Gene-for-gene" interaction is supported by discovery of interaction of Flax R gene "L" with flax rust *Avr* gene "AvrL" (Ellis et al., 1999).

In addition to direct interaction, indirect interactions between *R* genes and *Avr* genes were also discovered. This type of resistance mechanism was explained by "guard hypothesis" (Dangl and Jones, 2006). According to guard hypothesis, R gene recognizes modified *Avr* proteins such as *Avr* protein complexed with host proteins such as RIN4 protein of *Arabidopsis* (Van der Hoorn et al., 2002; Mackey et al., 2003). *Avr* proteins (also known as effectors) interact with host target proteins and this interaction results in susceptibility. In order to have a resistance response, R genes must recognize host target molecules that are modified by *Avr* molecules (Dangl and Jones, 2006).

1.2.1 Powdery mildew disease and resistance mechanisms in barley

Figure 1.4 explains the general disease mechanisms between *Hordeum vulgare* and Bgh. The interaction between Bgh and barley depends on gene-for-gene concept. Gene-for-gene type resistance is race specific resistance. The recognition of the isolate specific pathogen effectors by *R* protein is fundamental for race specific resistance (Dangl and Jones, 2006; Ridout et al., 2006; Eichmann et al., 2008). In powdery mildew over 20 *Avr* genes were discovered but only AVR_{k1} and AVR_{a10} were successfully cloned and characterized (Ridout et al., 2006). Unlike the other fungal *Avr*s, they lack the signal peptides.

On the resistance point of view, the resistance of barley to powdery mildew was first discovered at the cell wall. It was discovered that callose and cross linking materials and polysaccharides accumulate to the place where pathogen tries to penetrate. During the pathogen attack cell wall phenolics become resistant to the saponification caused by the papilla.

When the plant is attacked by the fungus, plant forms papillae. Papillae are defined as “cell wall appositions deposited on the inner surface of epidermal cell walls directly beneath appressoria” (Prats et al., 2005). Plants that formed effective papillae survive against the pathogen. During papilla formation plant cell accumulates hydrogen peroxide to the site of attack with vesicles (Huckelhoven et al., 1999). By those modifications plant tries to defend itself by creating a barrier against the fungus (von Ropenack et al., 1998).

Nitric oxide is the signal of cell death. It can elicit (Clarke et al.,2000) and propagate (Zhang et al., 2003) the cell death. There is a synergism between the nitric oxide and hydrogen peroxide accumulation in barley. During papilla formation there is trafficking of hydrogen peroxides with vesicles. In barley ROR1 targets the vesicles to the plasma membrane (Collins et al., 2003). The nitric oxide may affect the distribution of hydrogen peroxide and papilla components by influencing vesicle formation (Prats et al., 2005).

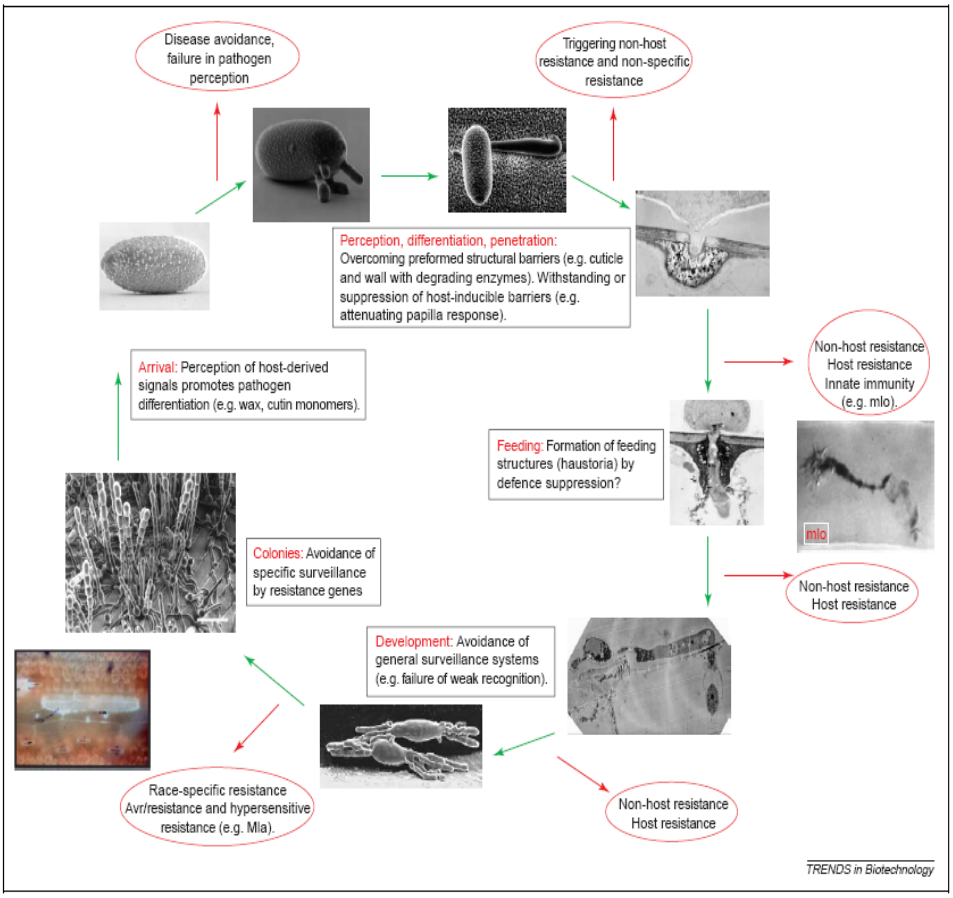


Figure 1.5 General disease mechanisms between *Hordeum vulgare* and Bgh (Gurr et al., 2005).

1.3 Proteomics

Proteome is the whole of proteins expressed in an organism, at a given tissue or cells in a given condition or time (ex. biotic or abiotic stress). The extensive study of the proteom is called proteomics. In 1994, Wilkins had developed the concept of the proteome and coined the term called proteomics. “From protein maps to genomes" Siena, Italy. He has also co-developed many of the protein analysis tools available on the ExPASy web server. By the help of growing DNA sequence information proteomics has became a useful tool for characterizing gene function,

for building functional linkages between protein molecules, and for providing insight into the mechanisms of biological processes in a high-throughput mode. There is also need to understand the dynamic processes within the cells, e.g. during cell differentiation and regulation or during disease development, it is mandatory to study the proteins involved. Their presence, actual concentrations, and possible modifications must be known in order to gain clues about their functional activities. This means that the entire protein extracts of cells or tissues have to be analyzed in order to learn about the expression rates of the proteins and their various species under certain conditions, in certain cells, tissues or organs. Because of that reasons proteomics has its sub-disciplines in order to study the systemic analysis of protein abundance, post translational modifications and protein-protein interaction (Baginsky 2009).

The proteomic study can differ according to the usage of the gel systems (gel based) or direct treatment of the protein samples with mass spectroscopic tools (gel free). The study of the proteome can be structural or functional. In structural proteomics the atomic resolution of 3-D protein structures is determined on a genome-wide scale in order to ascribe biochemical functions to gene products and to better understand the relationship between protein sequence, structure and function. In functional proteomics, the changes in protein expression during differentiation of cells, tissues or organs are studied. X-Ray crystallography and NMR spectroscopy are the common methods in structural proteomics. The usage of yeast two hybrid, affinity tags, western blotting and blue native PAGE followed by the western blotting is giving us dozens of protein-protein interaction data.

In this thesis work, we focused on expression-proteomics which is the branch of functional proteomics. Expression-proteomics defines patterns and the level of proteins expressed in different biological samples. By proteomics, we can examine the expression levels of 10000 proteins in high throughput manner. Number of proteins can change according to the gel size and pI range. After the isolation of the proteins they were first separated on 2D-PAGE according to their mass to charge

ratios. The gels were then visualized using various staining methods and then compared by image analysis software. By the help of this software expression level changed spots were chosen according to the spot color density. The selected spots were re-analyzed for statistically significant different expression, then excised from the gel and subjected to MS analysis to match the masses of fragments to the either amino acid deduced sequences of the DNA sequences or protein sequence data available in the GeneBank data in order to identify the proteins. If a given fragment does not match with any known sequence information in the data banks, then the fragment is subjected to "de novo" sequencing to amino acid sequence of the unknown fragment.

1.3.1 Plant proteomics

When we take a deeper look into the plant proteomics, we can see that proteomics has become a powerful tool in the functional characterizations of plant biology. We can see increase amount of papers about plant proteomics. When we wrote "plant proteomics" as a query to the "ISI Web of Knowledge" 594 papers are returned. Most of the papers were about Arabidopsis or Rice. Scientists study them because of their complete genome sequence. Compared to past years we notice a huge increase in the number of manuscripts on plant proteomics, we can also see papers about whet or barley because of their importance in our daily life. In the past years, scientists were suffering from the lack of the review papers. Now we can see the growing number of reviews (162). Most of them are about new extraction methods (Wang, Tai et al. 2008), staining methods (Miller, Crawford et al. 2006), plant sub-proteomics (Ephritikhine, Ferro et al. 2004; Lilley and Dupree 2007), general concepts about plant proteome analysis (Baginsky 2009) (Timms and Cramer 2008), or plant vs. biotic stress (Bestel-Corre et al. 2004) or plant vs. abiotic stress (Timperio, et al. 2008).

During the study of plant proteomics, it is important to obtain all the plant materials in the same conditions, a control group, a biological and also technical replicates.

In this thesis study, whole proteomic analysis approach was used and focused on the aspects of the total proteomics. Total proteomic studies were mostly specific to the plant organ (leaf or root) (Rampitsch, Bykova et al. 2006; Mehta, Magalhaes et al. 2008) and mostly TCA/Acetone extraction methods with little modification has been used (Carpentier, Witters et al. 2005; Wang, Tai et al. 2008). Some modifications were made because the source of the tissue is always limited and plant organs are composed of different cell types, each having its own protein signature (Rossignol, Peltier et al. 2006). In some cases, especially working with disease, only restricted numbers of cells play an important role in disease and mostly they were the low abundant proteins. In order to solve this problem sometimes laser microdissections were made from the site of pathogen or vascular bundle isolations were made (Ball et al., 2004; Schad et al., 2005; von Eggeling et al., 2007). However, in those years some scientists continue working with the whole extracts but they get rid of the high abundant proteins for example RubisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase) by the removal of the RubisCo low abundant proteins are made visible and take a deeper look to the disease or resistance proteins (Xi et al. 2006; Widjaja et al. 2009).

On the other hand, bioinformatic techniques have been updated to handle high-throughput data and get rid of highly abundant proteins. These studies can be named as “targeted proteomic surveys” that focus on the previously defined subset of proteins. Also by the help of mass filter option of the mass spectrometers, the scans can include the probable marker peptides (Malmstrom et al., 2007; Baginsky et al., 2009).

1.3.1.1 Plant disease proteomics

The studies among proteomics; plant-virus, plant-bacterium, plant-fungus, plant-nematode interactions proteomics on disease response or resistance in plants can be mentioned. According to these studies the identified proteins belongs to two main

categories: defense or stress related protein, enzymes associated with C and N metabolism and secondary metabolism (Figure 1.5).

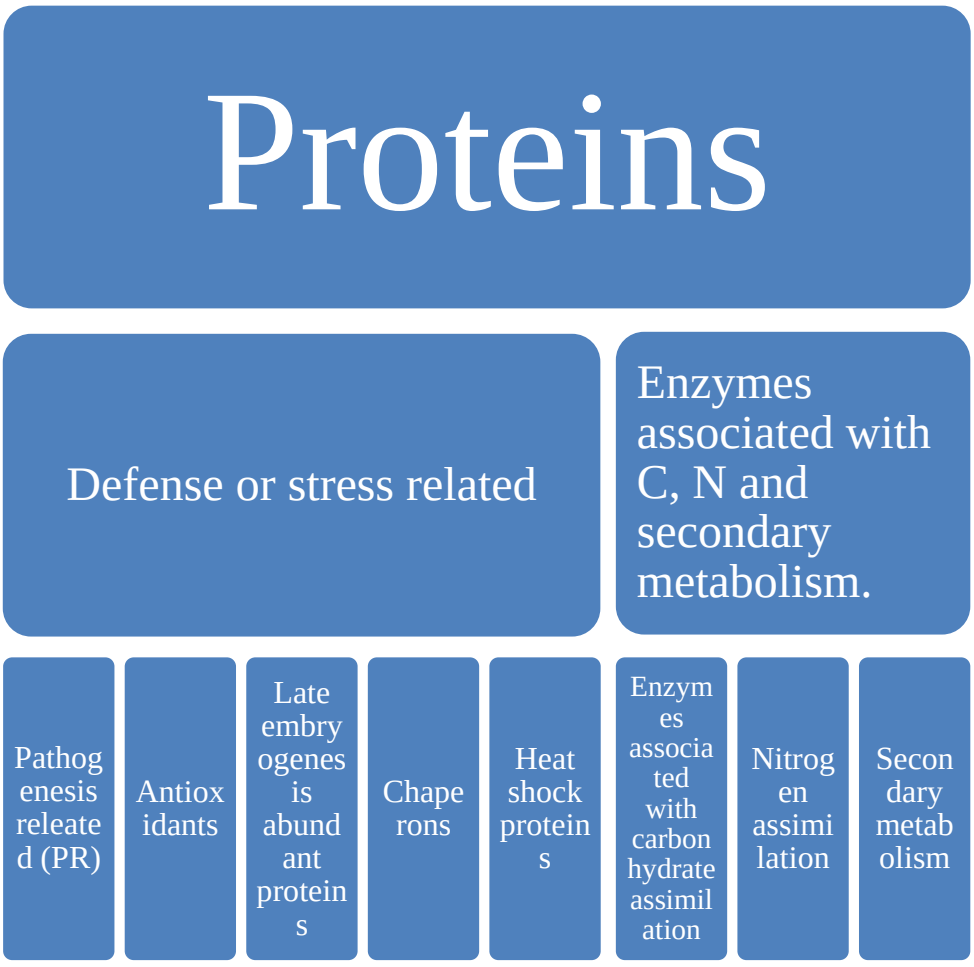


Figure 1.6 Proteins present in resistant and susceptible phenotypes

The other identified proteins are signaling pathway proteins which can change according to the pathogen attacks or environmental stresses. Scientists that are studying these proteins mainly focus on phosphorylation cascades or defense

related signaling molecules such as salicylic acid (SA), hydrogen peroxide and nitric oxide.

Reactive oxygen species (ROS) are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and can cause DNA damage. ROS releted studies can be divided into two parts. In first, plants or other compounds were studied to find out the antioxidant activities against ROS. The results of such studies can be utilized preventive agents. It was reported that Barley includes many phenolic compounds such as vanillicacid, p-coumaricacid, p-hydroxylbenzoicacid, p-hydroxy-benzaldehyde, quercetin and 3,4-dihydroxybenzaldehyde (Jeong et al,. 2009). Those compounds have an effect on scavenging ROS (Etoh et al., 2004). Especially quercetindehydrate and butylated hydroxytoluene (BHT) has inhibitory effects on oxidative DNA damage (Yen et al., 2000; Wilms et al., 2007). Studying with plants we focuses especially on ROS, the defense responses in resistant phenotypes are investigated; such as apoptosis. Programmed cell death (PCD) is similar to apoptosis but differs due to differences in plant cell and animal cell. Plants have cell wall and they lack immune system; therefore PCD is preferred definition in plants. Instead of the immune response, dying cell synthesize or secrete some substances to kill itself only or to awake the other cells against the pathogen. In Arabidopsis, an extracellular glycoprotein (EP1 like protein) has been identified that may serve to transmit a “death signal” from cell-to-cell (Swidzinski et al., 2004). In rice, by proteome comparisons of mutants displaying spontaneous cell death lesions and constitutive resistance features suggested that the metabolic changes underlie the PCD phenotype (Tsunezuka et al., 2005).

Treatment with some pathogen-derived elicitors induced changes in the phosphorylation status of extracellular matrix of maize cell proteins and the apparent recruitment of cytosolic proteins into the cell wall (Chivasa et al., 2005).

In soybean cells, elicitor (syringolide) treatment induced an oxidative burst and protein phosphorylation. Both phosphorylation and protein translocation events are important in signal modulation during induced defense responses (Slaymaker and Keen, 2004).

The constitutive expression of resistance related response lead to a reduction in plant growth and fitness as a consequence of "metabolic competition" directed toward the synthesis of defense elements (Heil and Baldwin, 2002; Rossignol et al., 2006) were also reported.

1.3.2 Gel staining

Gel staining refers to the detection. Detection is described as the reversible or irreversible binding of the colored organic or inorganic chemicals to the proteins. During the gel staining processes the most important thing is the sensitivity and reproducibility of the staining technique. Of course in 2DE, we have to consider the compatibility of the procedure for the mass analysis. For example, in silver nitrate staining, resolution is the best among all the staining techniques, but most of the silver nitrate procedures interferes with mass and makes identification impossible. Because of the irreversibility of this type of staining, once performed mass analysis cannot be performed. Among the most useful techniques we can mention, that is coomassie brilliant blue staining (CBB). Coomassie brilliant blue staining was first discovered in 1963 (Fazekas et al., 1963). It is a textile stain and two forms of stain is present; G-250 (greenish tint) and R-250 (reddish tint). In acidic solutions, the dye sticks to the amino groups of the proteins by electrostatic and hydrophobic interactions. R-250 was first used in proteomic studies. In our study, we used G-250 staining procedure, which is more sensitive than R-250 with solvents used. Phosphoric acid stains with clear background and by ammonium sulphate in solution reversible denaturates, precipitates and also fixates the proteins in the gels. By adding methanol, the equilibrium is shifted from colloidal form to the

molecularly dispersed form of the CBB which also speeds up the procedure (Neuhoff et al., 1998; Miller et al., 2006).

1.3.3 Imaging

1.3.3.1 Image capturing

Like in all the steps of proteomic studies, in image analysis too everything must be kept constant during each gel scanning. In most proteomic studies, image capturing is carried out by laser-based detectors, charged coupled device (CCD) cameras or flatbed scanners.

Laser based detectors are mostly used for recently developed stains such as spyro ruby stains. In this technique, the scanning is done by a powerful laser and then the signal is converted to pixels. On the other hand CCD cameras can be used for visible or fluorescent stains. In this systems, visible or UV illumination is achieved and then the emitted light is collected by high sensitive cooled area array CCD sensors and converted into digital signals. However, commercial office scanners, which are sealed well, can also be used for scanning.

During the image capturing, bit depth and dynamic range of all images must be the same. Bit depth means nothing if the investigation is performed by eye, since our eyes can only see 8-bit image depth. As the depth increases, there will more range of tones to be represented, so that we can easily detect low abundant spots on gels. Dynamic range represents the actual range of grayscales that are utilized within a digital image. If it is not limited for low abundant proteins during the experiment, we do not have chance to look into the whole image (Miller et al., 2006).

Image resolution represents the number of pixels displayed per unit length of the image and mostly represents dpi (dots per inch). Better resolution means better image quality, but if we exceed the limit, there will be noise in the image. Better

resolution also increases the processing time and can cause problems in storage of the images, too. (Miller et al., 2006). Table 1.2 represents the scanning recommendations for image capturing.

Table 1.1 Scanning recommendations for image analysis
(Miller et al., 2006).

Recommendation	Reason
Scan at the best resolution for your images	The active area of the gel (the area of spot material) should fall in the range 1000–1800 pixels in both horizontal and vertical directions. This range provides a good trade-off in information content and analysis performance. For most images, this will correspond to 300 dpi or 100 μm. If your gels are small, then you may need to increase the resolution to achieve this
Scan at 16-bit rather than 8-bit	The bit depth of a 16-bit image compared to an 8-bit image results in enhanced sensitivity and accuracy of quantification for less abundant proteins
Optimize the dynamic range to maximize use of available grayscale values	Aim for the maximum gray levels in the image to be 5–10% less than the maximum available by adjusting the exposure time for a CCD camera, or altering the PMT voltage
Avoid signal saturation when scanning	Saturated spots cannot be accurately measured and have the potential to bias normalization
Try to only scan the active area of the gel	Crop during scanning to remove blank parts of the scanner plate, labels etc. These areas provide no useful information, can 'steal' dynamic range, distort image statistics and increase storage requirements
Try to scan gel images using the same orientation	If you do need to rotate, flip or mirror images after scanning use the tools provided within your 2-D software package. Other packages may alter the integrity of the original data
Do not perform any post-processing of 2-D gel images in any general image processing software	These do not maintain the integrity of your original data, and you will almost certainly lose any calibration information contained in the image file
If possible, choose GEL or IMG/INF files formats, rather than generating TIFF files	The former often contain additional grayscale calibration information, which will not be included in the TIFF version
Do not use JPEG files for image analysis	The JPEG format is what is called a "lossy" compression system; while the images may look the same they are not. A great deal of smoothing and averaging may have taken place within the compression process and this will affect the underlying raw pixel data. Converting a JPEG image back to a TIFF is not a solution; once the image has been compressed in this way, the data have been lost and cannot be retrieved

1.3.3.2 Image analysis

After making proteins visible with the staining tools the most important step of the proteomics starts: determination of the differentiated spots, which is a complicated and tough process. As the technology improves, comparisons of the images are being more complicated. However, by the help of softwares, image analysis can produce high resolution image data. There has been grooving number of softwares (PDQuest, Delta2DE, Melanie, Nonlinear dynamics, Ludesi, ImageMaster 2D Platinum) that compares and performes statistical analyses of the spots.

1.3.4 Bioinformatic tools

Proteomic studies are usually high-throughput studies that yield large data sets to work on. Including image analysis tools there are dozens of bioinformatics tools that proteomics studies uses. (Lubec et al., 2005).

There are lots of protein databases (NCBI, Swiss-Prot/UniProt) that contain hundreds and thousands of proteins. In proteomics studies "database selection depends more on the studied organism than the status of the complete genome availability." (Lisacek 2006).

After the image analysis, the detection of up- or down-regulated proteins according to their intensities can be analyzed by used image analysis program, internet based statistical tools or by traditional statistical calculations (Student T Test).

In MS/MS computer algorithms identify proteins based on peptide mass fingerprints (PMF) and fragmentation (MS/MS) information by searching protein databases. Algorithms rank peptides according to their decreasing number of matching peptides (Lubec et al., 2005). There has been lots of programs (MASCOT, Phenyx, X!Tandem) that analyses huge data obtained from MS that collects and identifies high number of peptide mass fragments in order to reveal the identity of the protein.

Gathered data from the analyzed proteins can be further analyzed among the amino acid sequences of the proteins. General approach is to build in silico proteome map using the available genome sequence and comparing it with the experimental data. Bioinformatics can predict subcellular localization (Dönnes et al., 2004), signal peptides (Nielsen et al., 1997), function (Watson et al, 2005) and pI/MW values (Bjellqvist et al.1993).

1.4 Aim of the study

Genomic studies reveal what could theoretically happen, where as proteomics provides insight to the actual players involved in mediating specific cellular processes. There have been growing numbers of studies dealing with genes. Those studies may cover gene sequencing and functional analysis. Due to limited functional analysis studies and the truth of not covering the whole process by genomics, the functions of the some identified gene sequences are still waiting to be studied. Main aim is shifted to the functional characterization of proteins that are encoded by the cellular genetic machinery (Patterson et al., 2003)

Using proteomic tools to study the cellular machinery leads us to study the cellular proteome, which is extremely dynamic, complex, and underpinning cellular behavior and function (Pandey et al., 2000). Protein studies' covering regulatory networks also leads us to reveal the expression and the modification of new identified proteins. Expression studies mainly identify under- or over-expressed proteins that are separated by 2-DE PAGE.

There are several biotic and abiotic factors that lowers the yield in corps. Accelerated increase in global population brought the needs of maintaining sustainable crop production. In 2007 ranking of cereal crops in the world, barley was fourth both in terms of quantity produced (136 million tons) and in area of cultivation (566.000km^2). (Faostat,2007) In turkey barley was second in terms of area of cultivation. As years pass barleys yield have been decreasing and Turkey is below the limits of the worlds barley yield (TEAE, 2007).

Reliable and accurate disease diagnosis and pathogen detection are very important for optimizing crop yield and quality. Due to the numerous organisms (nematode, bacteria, fungus) and their subgroups that can cause serious limitations to crop production, any technology that is specialized for plant disease detection should serve as strong tool of agriculture, to produce resistant plants for the increasing world population.

BGH causes one of the most significant diseases of barley in worldwide, unless it is not controlled by fungicides and resistant varieties. Due to barleys economical importance and BGH's well known developmental biology (Zhang et al., 2005) it is the most studied among the other varieties of powdey mildew fungi and it is also a model organism for research on obligate biotrophic fungal pathogens. As it was mentioned in previous parts, barley Mla genes are rapidly overcome by BGH genotypes which have lost *Avr* activity, resulting in race-specific virulence (Brown et al., 2000; Ridout et al., 2006).

As mentioned above BGH is responsible for important yield looses in economically important crop, barley. 2 DE PAGE focuses on detection and characterization of plant pathogens we used proteomics to investigate the various processes that occur during the recognition of pathogens by resistant or susceptible plants. Wishing to give important results to be used genetically engineered crops for enhanced disease tolerance and other beneficial responses.

Knowing BGH's dynamic developmental stages well, lead us to study this host-pathogen interaction by dynamic proteomics. We used 12^{th}, 24th, and 48^{th} hai plants to reveal differentiated proteins. In 12^{th} h we expected to the differentiation process which is started by the arrival of asexual conidia. The proteins can be related to penetration (cuticula or wall degrading enzymes), differentiation or suppression of host inducible barrels (related to attenuating palilla response). In 24^{th} h as defense is already suppressed we aim to see proteins related to the feeding of the fungus. In 48^{th} h pathogen starts to feed from haustoria. In this time point we wish to see developmental proteins and their impact on the host. On the resistant plant point we aim to see proteins related to race-specific and hypersensitive resistance.

In order to give results to be used in the forthcoming studies we also aim to categorize the proteins according to their function, structure and subcellular localization. We also aim to find (in-silico) signal peptides to serve for the studies on elicitors and effectors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Maintenance of the spores and powdery mildew infection

2.1.1 Spore maintenance

Spores were maintained on Bülbül variety of barley. Leaves were cut to small pieces and placed on the plates containing 15 % agar and 0.1 % benzimidazol. (Zeybek et al., 2008) B103 isolate of the *Blumeria graminis* f.sp. *hordei* (Bgh) was blown onto the plant material in order to obtain heavily infected stock fungus material, which is obtained from Prof. Dr. Mogens Støvring Hovmøller (Aarhus University, Faculty of Agricultural Sciences, Denmark).The plates were incubated in 18-22 ^{0}C 16/8 light / dark periods with 60 % humidity in the growth chamber. Conidiaspores were ready to reinoculate after 10-15 days of growth.

2.1.2 Powdery mildew infection

Bulbul, Pallas01 and Pallas03 lines of *Hordeum vulgare* as described in Table 2.1 were planted in soil for 16 hour light and 8 hour dark periods at 22 ^{0}C and 18 ^{0}C, respectively. Planting of bülbül was repeated for 3 weeks periods in order to obtain fresh leaves for the maintenance of Bgh isolates. After infection 15 - 20 seedlings of Pallas01 and Pallas03 plants were used for the proteomics study. The plants were grown for 12 days in 22 - 18 ^{0}C 16 / 8 light / dark periods with 60 % humidity in the growth chamber and infected with the B103 isolate of the Bgh. Infected plant materials were collected after 12, 24, 48 hour after inoculation (hai) and immediately placed in liquid nitrogen. The collected plant materials were stored at -80 ^{0}C for protein extraction for the same period of time for each sample.

Table 2.1 Biologic material that was used during the study

Plant Line	R-gene	Scoring with B103 (64/01)	Pathogenity	Plant Response
Pallas01	Mla1, Ml (Al2)	0	Avirulent	Resistant
Pallas03	Mla6, Mla14	4	Virulent	Susceptible
Bülbül 89 / control	-	4	Virulent	Susceptible
0-4: 4 : (compatible) disease occurs, 0: (resistant) no disease occurs. (Kolster, Munk et al. 1986)				

2.2 Staining of the plant material

2.2.1 Trypan blue staining

Trypan blue staining was made in order to detect death cells and fungal growth. (Vogel and Sommerville, 2000). Infected and uninfected leaves at different timepoints were incubated in Methanol-Acetic Acid solution (Methanol (Merck, Germany): acetic acid (Applichem, Germany) [1:1, v/v]) for 1 day and then they were put into the Chloral Hydrate Solution (2.5 % (w/v) chloral hydrate in ddH_2O) for 1 day. Then, the leaf samples were incubated in Trypan Blue Solution (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL water and 0.02 g of trypan blue 1:2 v/v ethanol) overnight and the imaging carried out by light microscopy (Leica DM4000B).

2.2.2 DAB (3,3-diaminobenzidine) Staining

In order to detect H_2O_2 accumulation in resistant plant samples fallowed by fungal inoculation, endogenous peroxidase-dependent in situ histochemical staining was conducted using DAB chromogen (Huckelhoven et al., 1999). Plant leaves were cut and placed into DAB Substrate (1:9 (v/v) DAB liquid chromogen (Sigma-Aldrich, Germany No. D7554) was mixed with the buffer (Sigma-Aldrich, Germany No.

7429) for 8 hours and than they were placed into Clearance Solution (0.15 % TCA [w/v] in ethyl-alcohol:chloroform [4:1, v/v]) for overnight and the imaging carried out by light microscopy (Leica DM4000B).

2.3 General scheme of the study

For clarity of the study the experimental steps are represented in Figure 2.1.

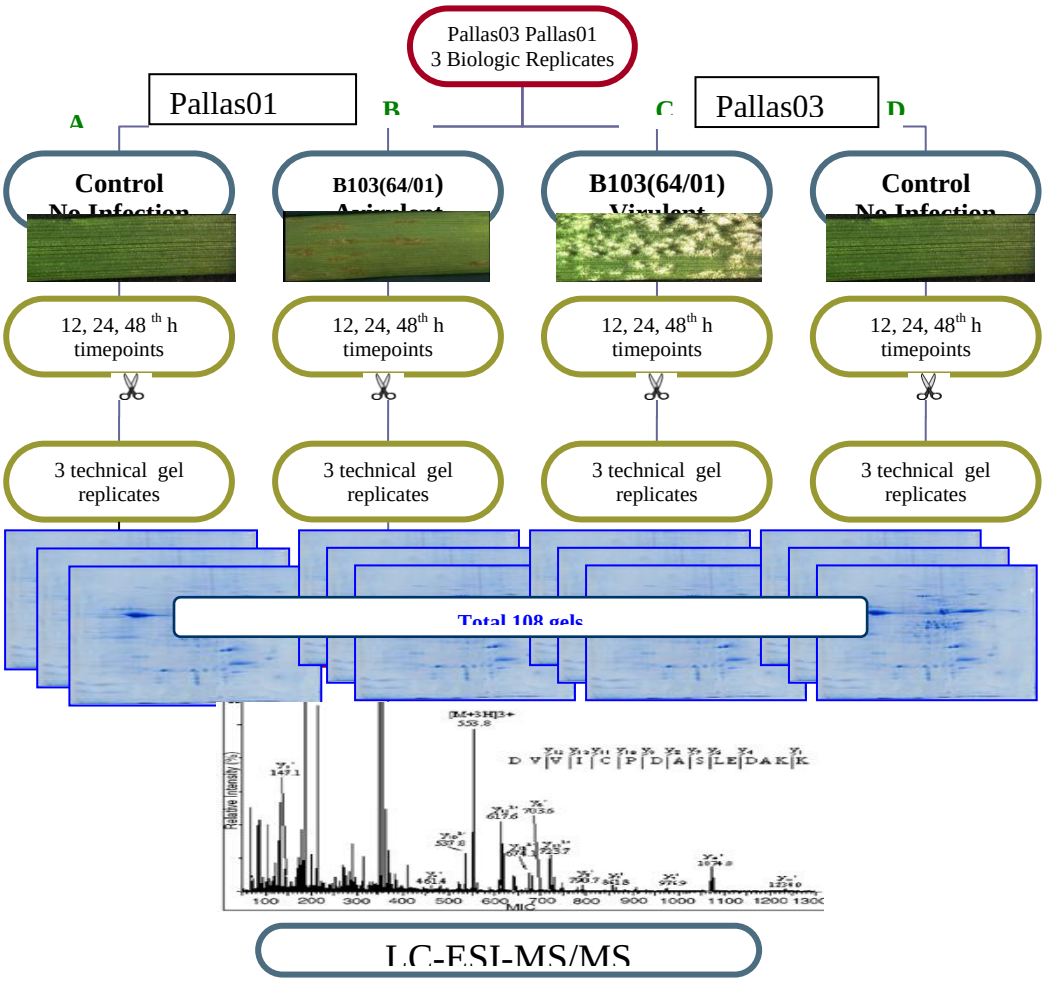


Figure 2.1 General experimental stages during the study

2.4 Total protein extraction

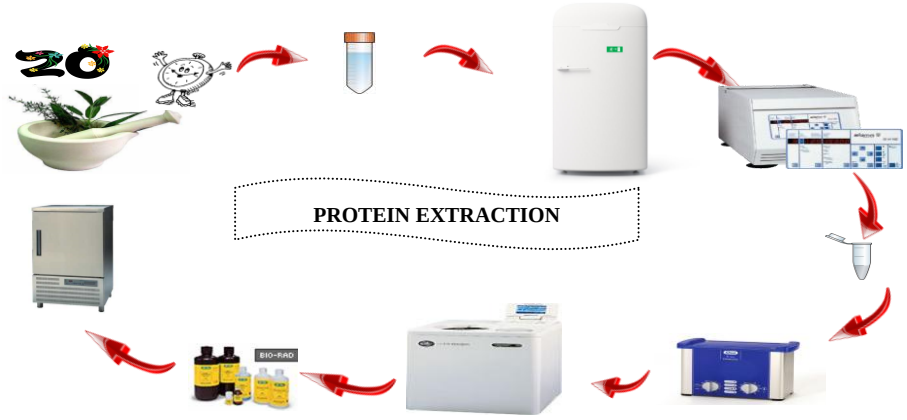


Figure 2.2 The scheme of protein extraction.

Plant protein extraction was made as it was presented in Figure 2.2 according to Rampitsch et al. (2006) with little modifications. About 1 g of plant material was grinded by mortar and pestle in liquid nitrogen for 20 minutes. The homogenized frozen powder was mixed with 15 mL of Precipitation Solution (Ice cold acetone containing 10 % TCA (Sigma-Aldrich, Germany), 0.07 % DTT) was added and put at -20 ^{0}C and left overnight to let the proteins precipitate. Next morning, it was centrifuged (SIGMA 3K15, Germany) at 12000 × g for 20 min, at 0 ^{0}C. After the removal of the supernatant the pellets were washed 6 times with Wash Solution (0.07 % DTT in ice cold acetone). After the last wash the protein pellets were stabilized with 90 % acetone for the storage. After the wash steps, the pellet were dried at room temperature until no acetone odor remains. The pellets were dissolved in Solubilization Buffer (7 M urea (Sigma-Aldrich, Germany), 2 M thiourea (Sigma-Aldrich, Germany), 4 % (w/v) CHAPS (Sigma-Aldrich, Germany), 20 mM DTT (Sigma-Aldrich, Germany) and 0.5 % (w/v) ampholyte (Serva, USA), pH 3–10). The sample then was sonicated five times for 5 s

(Elmasonic S, Germany) at RT. The sample was then centrifuged, first at 30.000 × g for 30 min and then at 90.000 × g for 1 h at RT. The final protein content was determined using a Bradford Microassay Procedure (Bio-Rad Laboratories) the volume was adjusted by rehydration buffer (7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 20 mM DTT and 0.5 % (w/v) ampholyte, pH 3–10) to obtain 500 μg protein / 350 μL volume, keeping the chaps concentration below 4% in all the samples. Samples were stored in aliquots at -80 ^{0}C to be used in the next steps.

2.5 Protein assay (Bradford micro assay)

Four dilutions (2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL) of protein standard (BSA) were prepared from the BSA stock solution (1 mg/mL in ddH_2O) as it was described in Table 2.2. The samples and standards were incubated at room temperature for at least 5 minutes avoiding to incubate at room temperature no more than 1 hour due to increase of the absorbance of the samples over time. The absorbance is then measured at 595 nm by spectrophotometer (Shimadzu UV-1601).

Table 2.2 Bradford micro assay

Protein	**20 μL (BSAStock)**	**40 μL from (BSA Stock)**	**60 μL from (BSA Stock)**	**80 μL from (BSA Stock)**	**2 μL from total plant protein**
dd water	780 μL	760 μL	740 μL	720 μL	798 μL
Bradford R.	200 μL	200 μL	200 μL	200 μL	200 μL
Total	1000 μL	1000 μL	1000 μL	1000 μL	1000 μL

2.6 Two dimensional gel electrophoresis (2D-PAGE)

Isoelectric focusing was conducted according to the instructor's manual (Bio-Rad ReadyStrip IPG Strip Instruction Manual Catalogue 163-2099, PROTEAN IEF Cell Instruction Manual Catalogue 165-4000). After the protein extraction and determination of the protein concentration, the second dimension was carried out. In the second dimension, proteins were separated according to molecular weight by SDS-PAGE (Laemmli, 1970).

2.6.1 Sample loading and adsorption of the proteins to the strips

After the removal of the samples from -80 ^{0}C, they were kept at room temperature in order to defrost. Sample loading is depicted in Figure 2.3.

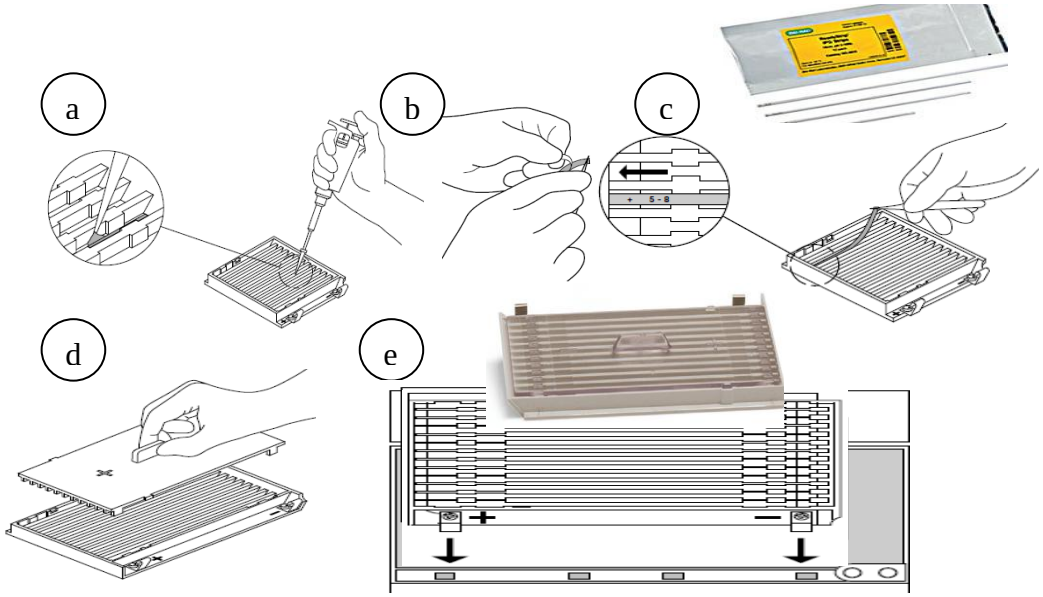


Figure 2.3 Sample loading

Sample was pipetted to IEF focusing tray (Bio-Rad, USA) as a line along the edge of a channel leaving 1cm from each end. Left bubbles were avoided because they can interfere with the distribution of the samples during rehydration and isoelectric focusing (a). IPG strips (ReadyStrip IPG strips, Biorad, USA) were taken from -20 ^{0}C and used immediately. The cover sheet was peeled from the IPG strip using forceps (b). The IPG strip (gel side down) was placed into the IEF focusing tray by putting the acidic (marked with "+") end at the anode (red/+) side of the IEF focusing tray making sure that the gel is making contact with the electrodes and no bubble is left. The strips were kept at room temperature for about 45-60 minutes in order to let the strips to absorb the protein sample (c). 2 mL of gel cover fluid (Ammersham, Germany) is overlaid. The lid was placed on the focusing tray so that the lid pressure tabs press on the IPG strips directly over the electrodes to insure good contact between the strips and the electrodes (d). IEF focusing tray was placed in the Protean IEF cell (e) (Bio-Rad, USA).

2.6.2 Active rehydration of the IPG strips

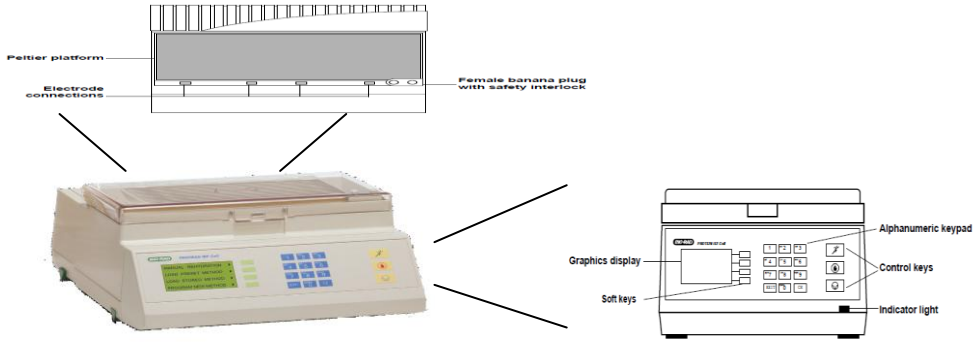


Figure 2.4 Protean IEF Cell (Bio-Rad, USA).

During IEF Protean, IEF Cell (Bio-Rad, USA) was used (Figure 2.4). The IEF Cell was programmed to two major steps, which were active rehydration and isoelectric focusing (IEF), respectively. The strips were rehydrated by adding active rehydration step before the standard IEF program of the protean IEF cell to the default cell temperature of 20 ^{0}C, with a maximum current of 50 μA/IPG strip. The isoelectric focusing steps were programmed according to the table 2.3 and focused for a total of 67.0 kVh (The current was monitored for not exceeding 50 μA /strip during the run). After the last step of the program, hold step was added but the strips were taken as soon as the fifth step ends.

At the end of the focusing, the IPG strips were removed from the focusing tray and they were transferred into a new clean, dry disposable rehydration/equilibration tray in the position of gel side up by maintaining the IPG strips in the same order as in the focusing tray (Two of them were placed in one tray in order to make the further studies easier). Prior to placing the gels into the tray, the IPG strips were hold vertically with forceps and let the mineral oil drain from the IPG strip for ~5 sec. If the next step was not preceded immediately, the strips were placed into the passive rehydration tray and stored at -80 ^{0}C till the equilibrium step.

Table 2.3 IEF and Active rehydration steps

	Hour	**kV**	**kVh**	**Temperature**	**Mode**
Rehydration	12	0.05		20ºC	
Step 1	5	300	1500	20 ºC	Rapid
Step 2	3	1000	3000	20ºC	Rapid
Step 3	1	3000	3000	20ºC	Rapid
Step 4	2	5000	10000	20ºC	Rapid
Step 5		10000	49500	20ºC	Rapid
Hold	12	500	-	20ºC	Rapid
Total			67000	20ºC	Rapid

2.6.3 Equilibration of the strips

The IPG strips and Equilibration Buffers (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2 % (w/v) SDS (Applichem, Germany), 20 % (w/v) glycerol (Applichem, Germany). Was weighed and completed to 500 mL with ddH_2O, aliquated to 12 mL and stored at -80 ºC) were removed from the freezer and placed onto the bench to thaw. 6 mL of Equilibration Buffer 1 (2 % (w/v) DTT was added to Equilibration buffer) was added to the each lane of the equilibration/rehydration tray. The trays were placed on an orbital shaker and gently shaked for 15 min at 100 rpm. At the end of the 15 min incubation, the used Equilibration Buffer 1 was discarded by carefully decanting the liquid from the tray. Then 6 mL of Equilibration Buffer 2 (2.5 % iodoacetamide (Sigma-Aldrich, Germany), was added to Equilibration buffer.) was added to the each lane of the equilibration/rehydration tray. The trays were placed on an orbital shaker and gently shake for 15 min at 100 rpm. At the end of the 15 min incubation, the used Equilibration Buffer 2 was discarded by carefully decanting the liquid from the tray. The IPG strip was removed from the disposable rehydration/equilibration tray dipped briefly into the graduated cylinder containing

1x Tris Glycine SDS running buffer. Then the strip was laid, with the gel side towards us, onto the back plate of the SDS-PAGE gel. This process is repeated for the second IPG strip.

2.6.4 Second dimension SDS-PAGE

Table 2.4 Gel composition

Gel content for 60 mL 12.5% gel composition	**Volume**
Acrylamide/Bisacrylamide (30 % T, 0.8 % C)	24 mL
Tris-HCl 1.5 M pH 8.8	15 mL
10% (w/v) SDS	0,6 mL
Ddwater	20,1 mL
APS (just before pouring the gel)	275 µL
TEMED (just before pouring the gel)	30 µL

After the polymerization of the gels for 1.5-2 h equilibrated strips were placed on top of the gels and agar solution (1 % agar in TGS with trace amount of brom phenol blue) was added on the strips. Second dimension separation based on the molecular mass was carried out in 12.5% gels (20cm×20cm×1.5cm) by applying 10mA/gel for 1 h then 20mA/gel till blue dye runs off from the gel in the Bio-Rad Protean 2xi Cell after adding Tris Glycine SDS running buffer (0.25 M Tris Base, 1.92 M Glycine and 1 % Sodium Dodecyl Sulfate (w/v). For electrophoresis, 1x buffer was used).

2.6.5 Gel Staining

After the removal of the gels from the cassette the gels were washed with ddH_2O and put into 200 mL of staining solution (Stock Staining Solution A: 10 % (w/v) ammonium sulphate (Merck, Germany), 2 % w/v phosphoric acid in 100 mL ddH_2O. Stock Staining Solution B: 5 % (w/v) Coomassie Brilliant Blue G-250 in ddH_2O. Staining Solution: 2 mL of stock staining solution B, 80 mL of stock staining solution A and 20 mL methanol was added into the mix.) for overnight. Next morning gel was transferred into the Neutralization Buffer (0.1 M Tris-phosphate, pH 6.5.) for 1-3 minutes. Then the gel was washed with 25 % (w/w) Methanol less than 1 minute. The gel was transferred into the fixation buffer (20 % (w/v) ammonium sulphate in water) for overnight. During the whole study the staining was repeated for 3 times. There was no problem during the stay of the gel in staining or fixation solution for weekend (Neuhoff et al., 1985; Neuhoff et al., 1988).

2.7 Image Analysis

All the gels were scanned on GE Healthcare scanner by using lab scan software at a resolution of 300 dpi with green filter as it was recommended in scanner manual. Spot comparison was performed by PDQuest version 8.0.1 in order to measure spot intensity differences. Most of the spots were matched by using automatic matching tool of the program. Some spots were matched by manual matching tool. All the spots were detected using spot detection wizard by setting removing horizontal and vertical streaks, applying Gaussian model during the test and trying to find the same number of spots on each gel options. Master gel was automatically selected and normalization was made according to the total density in gel image. Analysis set was selected from the spots that have 1.5 fold change and that are statistically significant to a level of 95 % per group. Protein spots that were consistently differentially expressed in mean normalized spot volume by at least 1.5 fold (50 %) in three independent experiments were selected for excision and subsequent nano

LC-ESI-MSMS analysis. Statistical analysis was performed by 2-tailed student's t-test.

2.8 Mass spectroscopy

After the identification of the spots by image analysis spots were excised from the gel by pipette tips and sent to Proteome Factory (Proteome Factory AG, Berlin, Germany; http://www.proteomefactory.com) for nano LC-ESI-MS/MS.

2.8.1 Tryptic in-gel digestion

Before in-gel trypsin digestion the spots were briefly washed three times with Washing Solution (100 mM $(NH_4)_2CO_3$, 50% acetonitrile and 50 mM $(NH_4)_2CO_3$) till there is no blue color remained. After the last wash, the protein spots were overlaid by Trypsin Digestion solution (200 ng trypsin (Promega, Mannheim, Germany) in 50 mM $(NH_4)_2CO_3$, 10% acetonitrile in ddH_2O). Incubation was carried out overnight at 37°C and was stopped by adding 0.5 volumes of Stopping Solution (2% formic acid.) and waited for 1 h. The supernatant was transferred to a new reaction tube or directly applied to nano LC-ESI-MS/MS analysis.

2.8.2 Nano LC-ESI-MS/MS

The MS system consists of an Agilent 1100 Nano LC system (Agilent, Germany), PicoTip emitter (New Objective, USA) and Esquire 3000 plus ion trap MS (Bruker, Bremen, Germany). Protein spots were in-gel digested by trypsin and applied to nanoLC-ESI-MSMS. MS process starts by trapping and desalting of the peptides on enrichment column (Zorbax SB C18, 0.3 x 5 mm, Agilent) using desalting solution (1% acetonitrile, 0.1% formic acid.) for five minutes. Peptides were separated on Zorbax 300 SB C18, 75 μm x 150 mm column (Agilent) using Mobile phase solution (0.1% formic acid gradient from 5% to 40% acetonitrile) within 40

minutes. MS spectra were automatically taken by Esquire 3000 plus according to manufacturer's instrument settings for nano LS-ESI-MS/MS analyses.

2.9 Bioinformatic tools

In MS step proteins were identified using MS/MS ion search of MASCOT search engine (Matrix Science, London) and “nr” (NCBI) protein database (National Center for Biotechnology Information, Bethesda, MD, USA) (Proteome Factory).

After identification protein subcellular localization predictions were obtained from PSORT web server (http://wolfpsort.seq.cbrc.jp/) by submitting amino acid sequences of the identified proteins to this server. The existence of signal peptide sequences was checked in the public web server of signal peptide prediction program SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

2.10 Statistical Analysis

Student T-test and Principle Component Analysis (PCA) were made in order to find differentiated spots.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Staining of the plant material

3.1.1 DAB (3,3-diaminobenzidine) staining

DAB staining was made in order to see H_2O_2 accumulation in resistant plants. In resistant plant, recognition of *Avr* factor by *R* gene generally activates a strong local response, named hypersensitive response (HR), which is followed by cell death in infected area. H_2O_2 accumulation is strong evidence in those sites of the attacked plant. Figure 3.1 shows H_2O_2 accumulation in resistant plant. It was also shown that there is also fluorescence in the place of H_2O_2 accumulated sites.

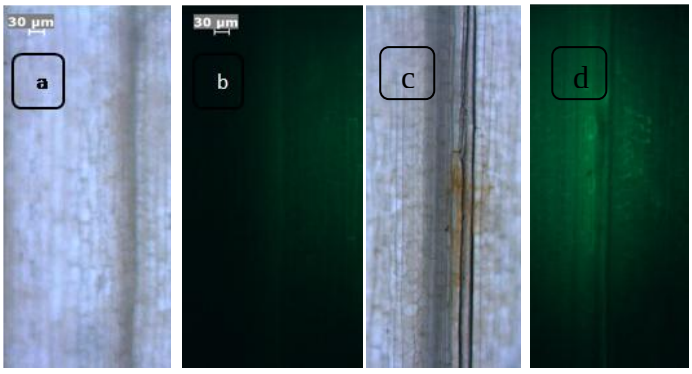


Figure 3.1 Pallas01 and Pallas03 infected with B 103 detected by DAB staining Pallas03 infected with B103 (a) under light microscopy. (b) under fluorescence light microscopy. Pallas01 infected with B103 under visible light microscopy (c). H_2O_2 accumulated sites gives fluorescent under fluorescence light microscope (d).

3.1.2 Trypan blue staining

Prior to collecting large number of samples, the infections were confirmed by trypan blue staining. Hypersensitive response (HR) is seen as expected since Pallas01 is resistant against B103 (Figure 3.2). In trypan staining with susceptible plant Pallas0 different developmental stages of the pathogen was confirmed (Figure 3.1). Although the time points were strictly the same in each biologic replicate, staining was repeated and the same developmental stages were confirmed.

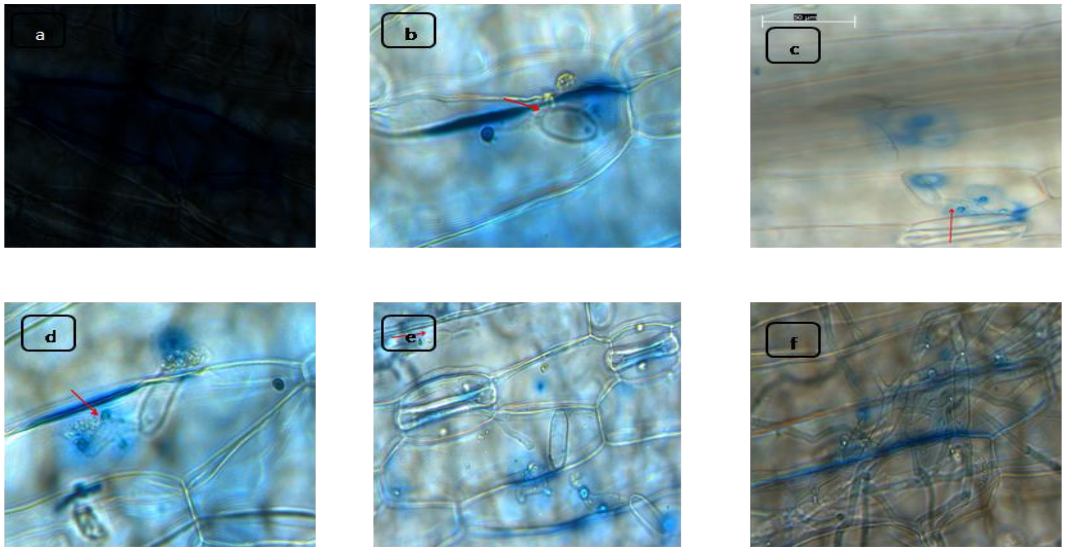


Figure 3.2 Pallas01 and Pallas03 plants infected with B 103 trypan blue staining Pallas01 infection with B 103 and no spore germination is seen (a). Pallas03 12 hai, germinated conidia; appresorium (arrow) delimited from secondary hyphae by a septum was observed (b). 24 hai, penetration by hyphae (c). 30 hai, penetration (arrow) region seems shiny due to host cell depositions (d,e). 48 hai, feeding organ haustorium (arrow); branched haustorium was formed, epihpytic mycelium formation was observed along epidermal tissue (f). (All images were captured under visible light microscopy 40x).

3.2 Characterization of the time points

The objective of this research was to focus on protein expression profiles of virulent and avirulent *Blumeria gramınıs* f. sp. *hordei* inoculated barley leaves. Due to BGH's well known developmental biology (Zhang et al., 2005) and the staining results, 12^{th} 24^{th} 48^{th} h Pallas01 and Pallas03 plants infected with B103 was used for further studies.

3.3 2DE PAGE results of the gels

To better solve the protein expression profiles of virulent and avirulent *Blumeria gramınıs* f. sp. *hordeı* inoculated barley leaves by 2-DE PAGE, protein expression patterns was obtained by 2-DE with 17 cm pH 3-10 and pH 4-7 gel strips. Eye comparison revealed that, highly accumulation of the spots in the middle gives poor resolution in pH 3-10 strips and most of the proteins in barley leaves were distributed around pH 4-7. Therefore, pH 4-7 gel strips were used to obtain protein expression profiles of virulent and avirulent *Blumeria gramınıs* f. sp. *hordei* inoculated barley leaves.

2-DE separation in the pH 4-7 gels was resolved more than 1,100 protein spots from barley leaves soluble proteins at 12^{th}, 24^{th}, 48^{th} h hai (Figure 3.3). In order to get more reproducible results, the experiment was carried on tree biological samples, each containing 3 technical replicates resulting 108 gels (All biologic and technical replicates are presented in appendix A).

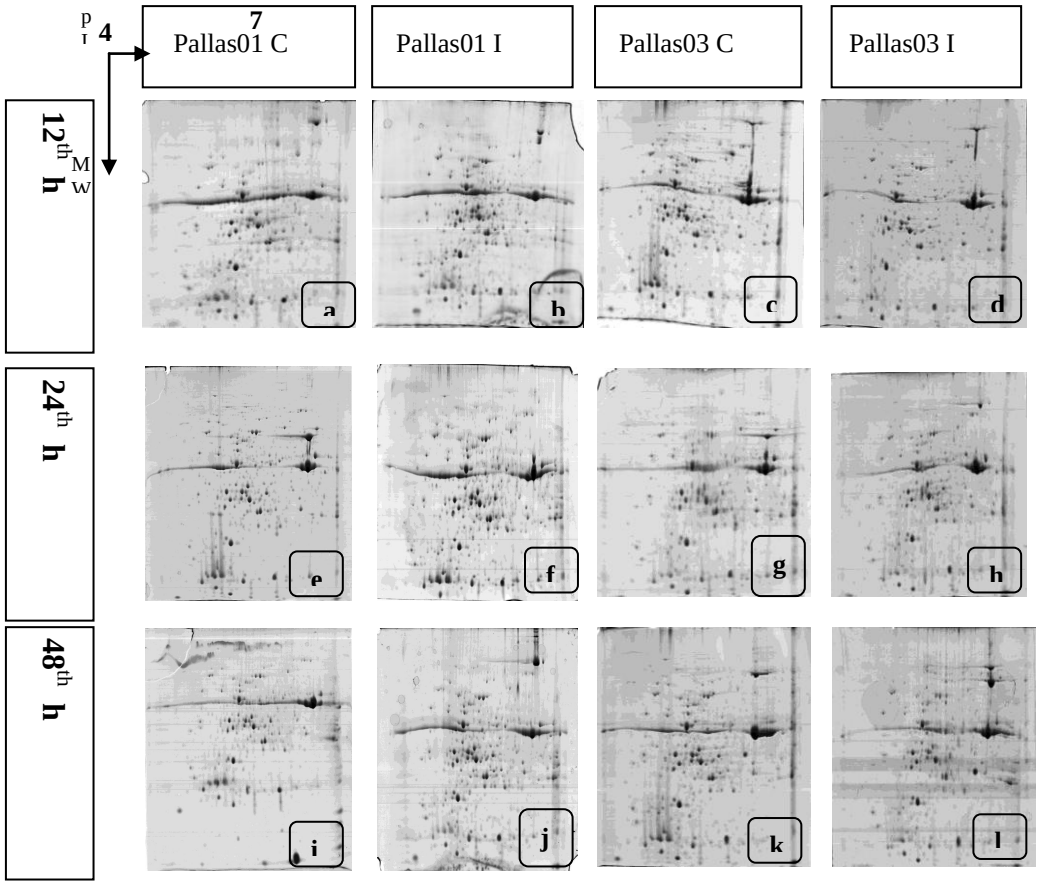


Figure 3.3 Representative 2-DE images for 12 protein samples.
Pallas01 Control (C) 12th H (a). Pallas01 Infected (I) 12thH (b). Pallas03 C 12th H (c). Pallas03 I 12thH (d). Pallas01 C 24th H (e). Pallas01 I 24th H (f). Pallas03 C 24th H (g). Pallas03 I 24th H (h). Pallas01 C 48th H (i). Pallas01 I 48th H (j). Pallas03 C 48th H (k). Pallas03 Infected (I) 48th H (l).

3.4 Image analysis

After 2-DE PAGE image scanning for image analysis with BioRad PDQuest ver 8.0.1 was performed. Some weak spots with low relative density on 2-DE gels are usually highly variable in different samples, even in different biologic replicate of

the same sample; this variation affects the identification of differentially expressed proteins throughout multiple biological developmental stages or treatments. Therefore, to identify differentially expressed proteins in different developmental stages the image analysis experiments were designed. The experiments depend on the different time points and different plant lines representing resistant and susceptible genotypes including control groups (Table 3.1). To identify differentially expressed proteins in each experiment groups, a group of proteins comparable throughout 6 distinct experimental groups was established. The analysis involved evaluation of reproducible spots in triplicate biological replicates of each sample and qualification of these proteins present in at least two distinct samples by comparing reproducible spots in each sample. The qualification comparison was made with BioRad PDQuest ver 8.0.1 to compose master gel by adding each selected spots on the master gel. With these analyzes, the program detect 600-750 spots on each experiment group. By further quantitive and comparative analysis of the spots, 40 proteins were found (Figure 3.4, Figure3.5, Figure3.6, Figure3.7, Figure 3.8, Figure 3.9) with at least 1.5-fold change in expression (Appendix B, Table 3.1, Table 3.2, Table 3.3, Table3.4, Table3.5, Table3.6, Table3.7)

Table 3.1 Experiment groups of the image analysis

Experiment number	**Description**	**Up-regulated**	**Down-regulated**
Experiment 1 (E1)	Pallas01 24^{th} H	5	1
Experiment 2 (E2)	Pallas03 24^{th} H	1	4
Experiment 3 (E3)	Pallas01 12^{th} H	7	2
Experiment 4 (E4)	Pallas03 12^{th} H	7	-
Experiment 5 (E5)	Pallas01 48^{th} H	4	1
Experiment 6 (E6)	Pallas03 48^{th} H	4	1

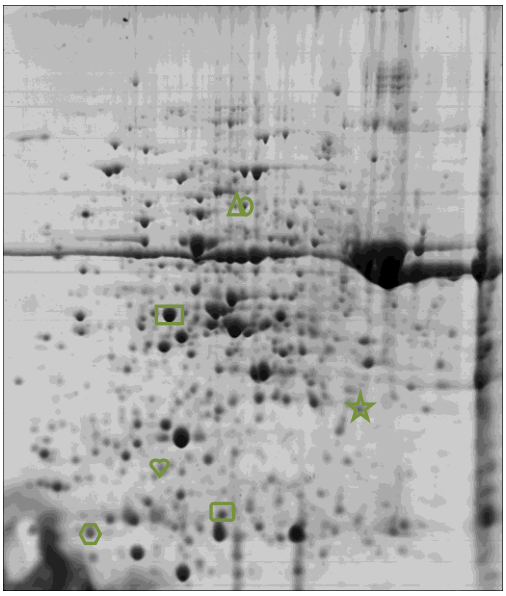


Figure 3.4 Selected spots from E1

Table 3.2 Spot identities of E1

Figure	SSP Number	Up- (↑), down (↓)- regulation
□	SSP 2302➔ 1	↓
▢	SSP 3106 ➔ 2	↑
○	SSP 4604➔3	↑
☆	SSP 4603➔ 4	↑
△	SSP 8101➔5	↑
⬡	SSP 0002➔6	↑
♡	SSP 1108➔7	↑

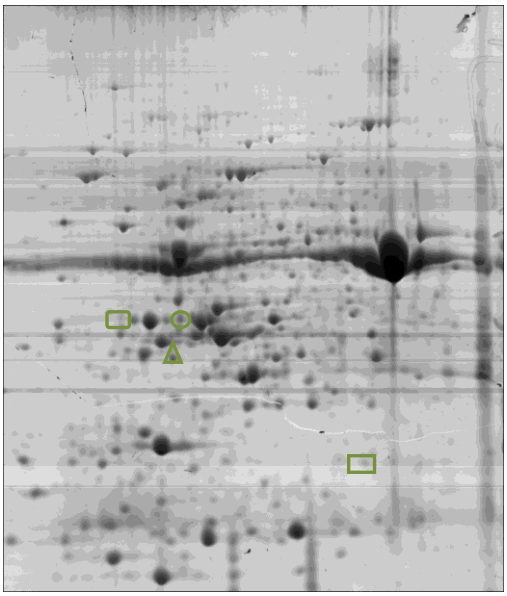


Figure 3.5 Selected spots from E2

Table 3.3 Spot identities of E2

Figure	SSP Number	Up-, down- regulation
▭	SSP 7202➔8	↓
▢	SSP 1402 ➔9	↓
○	SSP 3401➔10	↑
△	SSP 2406➔29	↑

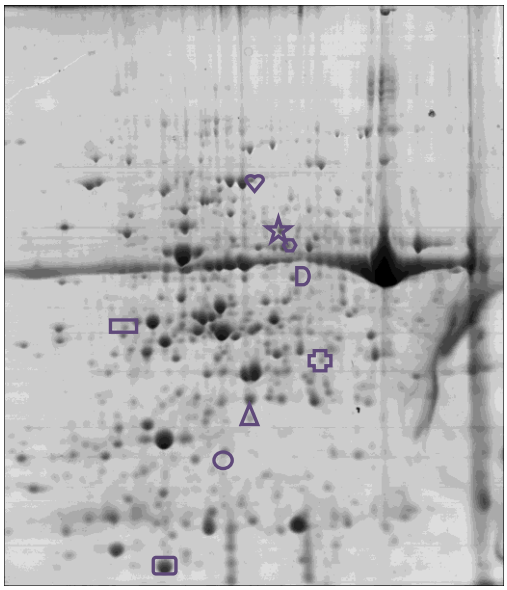


Figure 3.6 Selected spots from E3

Table 3.4 Spot identities of E3

Figure	SSP Number	Up-, down-regulation
□	SSP1304➔ 12	↓
▢	SSP2009➔ 13	↓
○	SSP4106➔14	↑
△	SSP5703➔15	↑
☆	SSP5611➔ 16	↑
⬡	SSP5612➔17	↑
♡	SSP5701➔18	↑
⯃	SSP6206➔19	↑
D	SSP6505➔39	↑

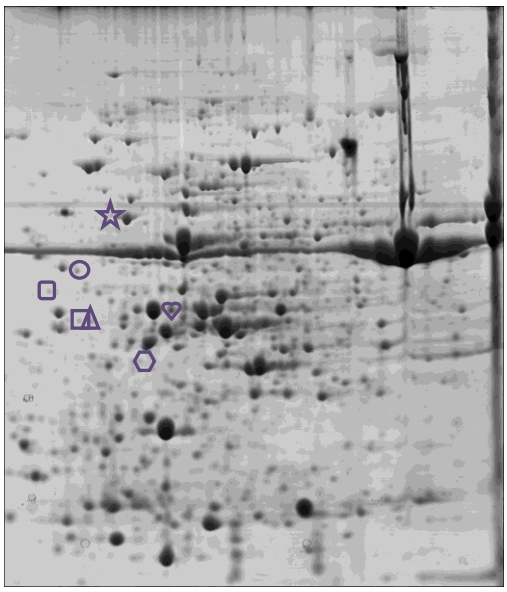


Figure 3.7 Selected spots from E4

Table 3.5 Spot identities of E4

Figure	SSP Number	Up-, down-regulation
□	SSP0306➔20	↑
▢	SSP0401➔21	↑
○	SSP0403➔22	↑
△	SSP1301➔23	↑
☆	SSP1601➔	↑
⬡	SSP2201➔	↑
♡	SSP2305➔	↑

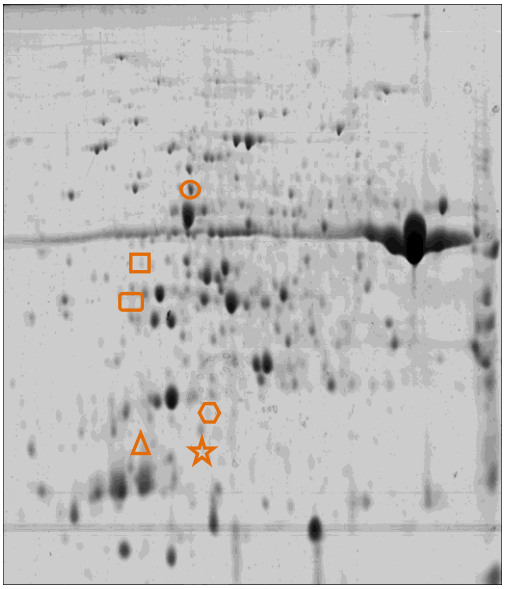


Figure 3.8 Selected spots from E5

Table 3.6 Spot identities of E5

Figure	SSP Number	Up-, down-regulation
▭	SSP2401➔27	↑
▢	SSP1304➔28	↓
○	SSP3603➔30	↓
△	SSP2102➔31	↑
☆	SSP3105➔ 32	↑
⬡	SSP4101➔33	↑

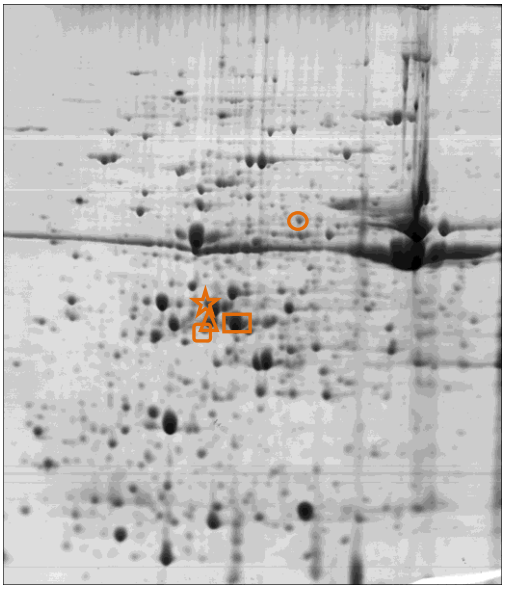


Figure 3.9 Selected spots from E6

Table 3.7 Spot identities of E6

Figure	SSP Number	Up-, down-regulation
▭	SSP5402➔34	↓
▢	SSP4401➔35	↑
○	SSP7701➔36	↑
△	SSP4502➔37	↓
☆	SSP4501➔38	↑

3.4 Statistical Tests

Two tailed student-T test (Appendix D) and PCA was used in order to characterize differentiated spots. Table 3.8 shows calculated fold changes by two tailed student's T-test and PCA analysis of differentiated spots that were identified by image analysis.

In order to identify the most potential marker proteins among the selected spots from image analysis for powdery mildew infected barley leaves, PCA was used. PCA was used as an exploratory data analysis method to quickly detect the most important markers in a huge data set. PCA also combines multiple signals that increase or decrease (multivariate) simultaneously and therefore gives more information about the possible relevance of differentially expressed proteins as markers for powdery mildew disease resistance proteins. (Figure 3.10) Based on the PCA results potential marker proteins were selected for further studies.

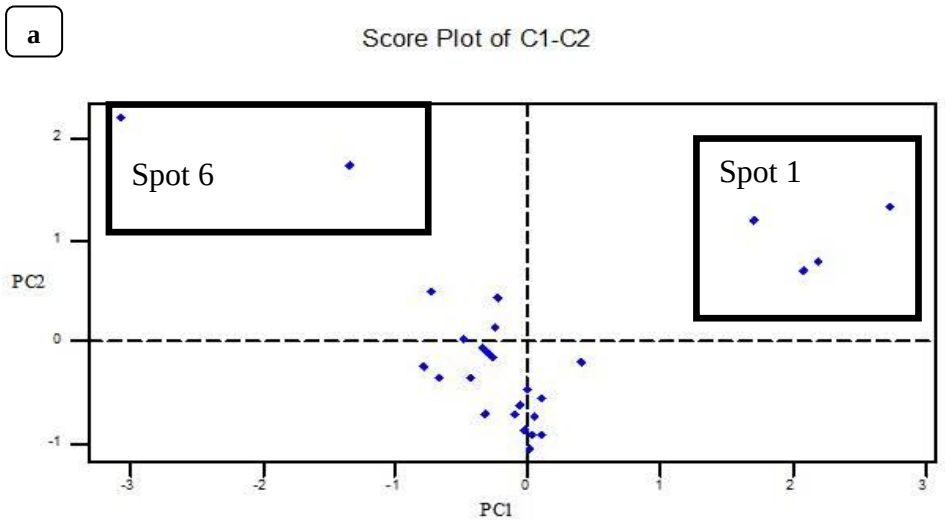


Figure 3.10 **PCA results for the identified proteins from image analysis.**
E1, Pallas01 24th h (a). E2, Pallas03 24th h (b). E3, Pallas01 12th h (c). E4, Pallas03 12th h (d). E5, Pallas01 48th h (e). E6, Pallas03 48th h (f).

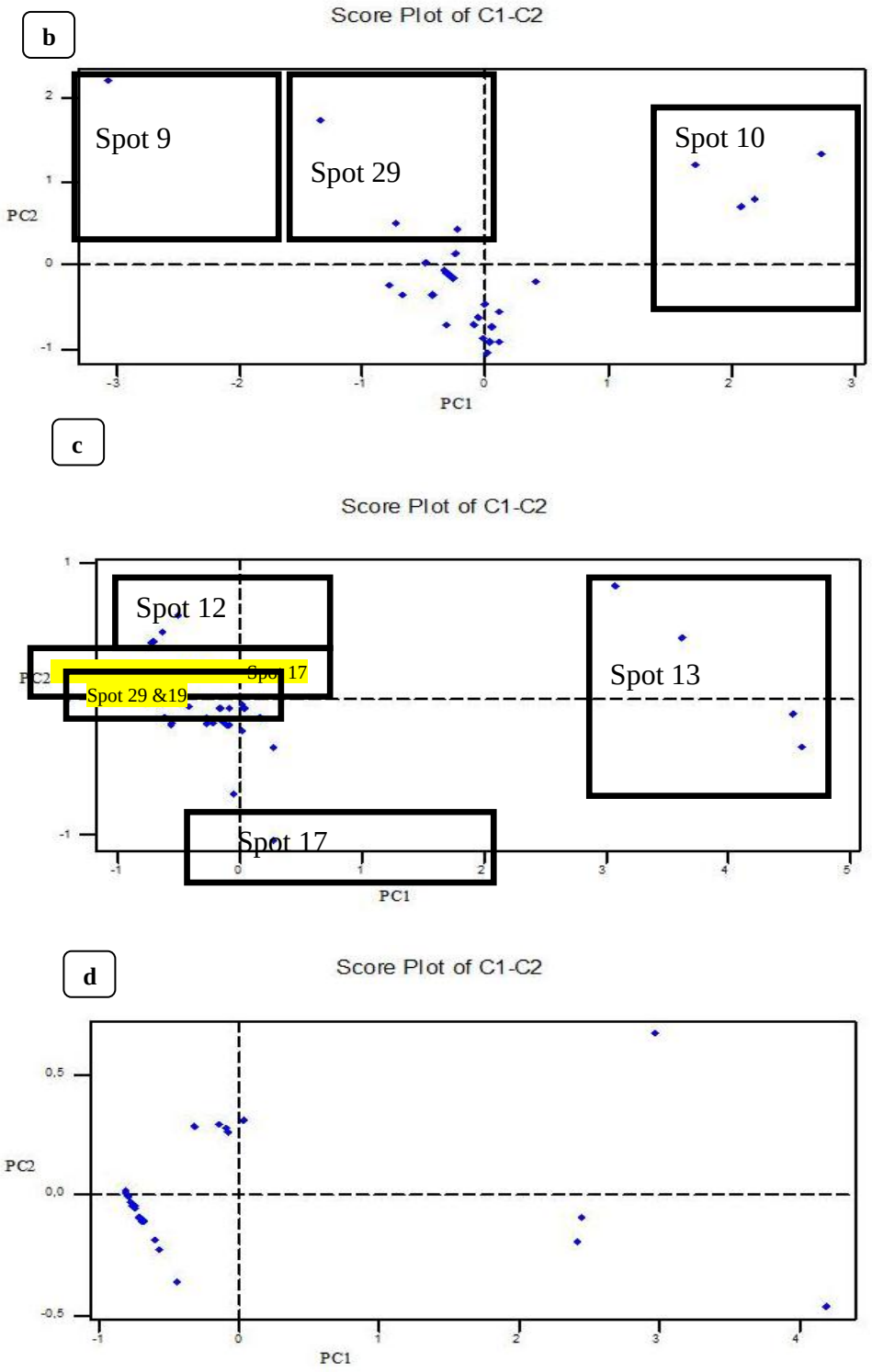


Figure 3.10 Continued

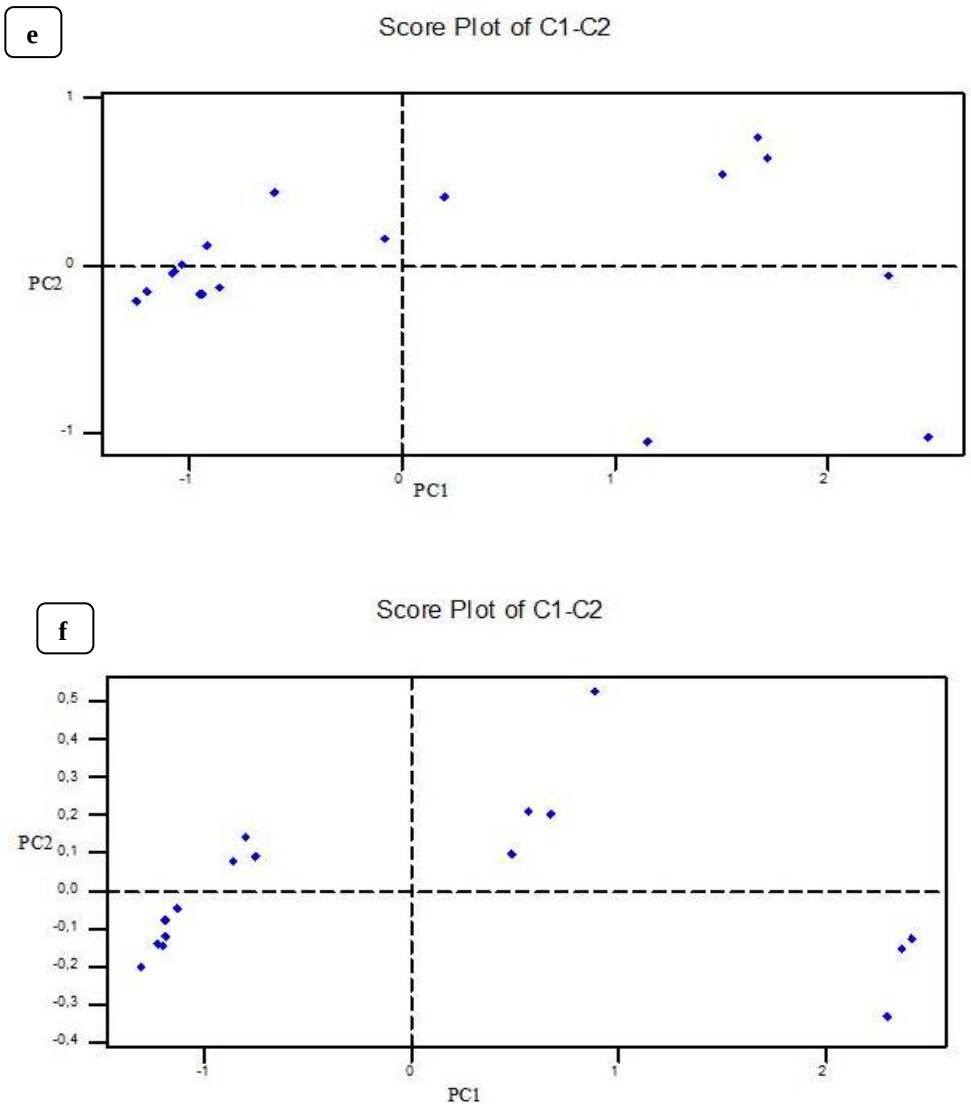


Figure 3.10 Continued

According to PCA analysis 2 spots from experiment1, 3 spots from experiment2, 5 spots from experiment3, 7 spots from experiment4, 6 spots from experiment5, 4 spots from experiment6 were selected as potential marker proteins.

Table 3.8 Summary of differentially expressed proteins during image analysis

	Spot numbers	SSP Numbers	Control	Infected	t-test P value	PCA
EXP1	Spot1	SSP 2302	6.84 fold±0,73		0.000	✓
	Spot 3	SSP 4604		2.52 fold±0,32	0.016	
	Spot 4	SSP 4603		2.51 fold±0,61	0.018	
	Spot 5	SSP 8101		2.22 fold±0,29	0.048	
	Spot 6	SSP 0002		4.14 fold±0,09	0.018	✓
	Spot 7	SSP 1108		2.06 fold± 1,52	0.025	
EXP2	Spot 8	SSP 7202	4.11 fold±0,15		0.000	
	Spot 9	SSP 1402	2.33 fold±0,51		0.019	✓
	Spot10	SSP 3401		1.5 fold± 0,48	0.025	✓
	Spot 29	SSP 2406	Present in cont	Absent in Inf	0.020	✓
EXP3	Spot 12	SSP 1304	4.53 fold±1,03		0.002	✓
	Spot 13	SSP 2009	1.51 fold±		0.005	✓
	Spot 14	SSP 4106		1.41 fold±0,092	0.026	
	Spot 15	SSP 5703	Absent in cont	Present in Inf	0.046	
	Spot16	SSP 5611		1.50 fold±0,294	-	
	Spot 17	SSP 5612		2.70 fold±0,019	0.011	✓
	Spot 19	SSP 6206		3.39 fold±0,00078	0.022	✓
	Spot 39	SSP 6505	Absent in cont	Present in Inf	0.003	✓
EXP4	Spot 20	SSP 0306	Absent in cont	Present in Inf	0.016	✓
	Spot 21	SSP 0401	Absent in cont	Present in Inf	0.001	✓
	Spot 22	SSP 0403		4.04 fold±0,0000039	0.002	✓
	Spot 23	SSP 1301		1.94 fold±0,053	0.009	✓
	Spot 24	SSP 1601	Absent in cont	Present in Inf	0.000	✓
	Spot 25	SSP 2201	Absent in cont	Present in Inf	0.000	✓
	Spot 26	SSP 2305		2.81 fold±0,23	0.012	✓
EXP5	Spot 27	SSP 2401		2.78 fold±0,00062	0.008	✓
	Spot 28	SSP 1304		3.75±0,095	0.016	✓
	Spot 30	SSP 3603	1.44 fold±0,18		0.097	✓
	Spot 31	SSP 2102		1.55 fold±0,0021	0.002	✓
	Spot 32	SSP 3105	Absent in cont	Present in Inf	0.002	✓
	Spot 33	SSP 4101	Absent in cont	Present in Inf	0.000	✓
EXP6	Spot 34	SSP 5402	2.37 fold±0,019		0.000	
	Spot 35	SSP 4401		1.83 fold±0,15	0.028	✓
	Spot36	SSP 7701		1.71 fold±0,105	0.015	✓
	Spot 37	SSP 4502		1.50 fold±0,018	0.003	✓
	Spot 38	SSP 4501		1.56 fold±0,036	0.020	✓

Intensity changes of the selected spots in each time point was examined in order to get some information about expression change of each spot in every time point. In Table 3.9, intensity changes in different time points of the selected spots from E1 was examined; in Table3.10 intensity changes in different time points of the selected spots from E2 was examined, in Table 3.11 intensity changes in different time points of the selected spots from E3 was examined, in Table 3.12 intensity changes in different time points of the selected spots from E4 was examined, in Table 3.13 intensity changes in different time points of the selected spots from E5 was examined, in Table3.14 intensity changes in different time points of the selected spots from E6 was examined. By examining the expression changes of all time points fallowed by spot identification by MS/MS possible pathways for some differentiated proteins of powdery mildew disease resistance mechanism was aimed to shown.

Table 3.9 Intensity changes in different time points of the selected spots from E1

E1		E2		E3		E4		E5		E6	
SSP	**Spot #**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**
2302	1↓		0.293		0.422		0.145		0.640		0.787
3106	2↑		0.736		0.661	1.25↓	0.011		0.119		0.330
4604	3↑		0.266		0.197		0.399		0.683		0.219
4603	4↑				0.692		0.887	2.22↑	0.020		
8101	5↑		0.382	2.76 ↑	0.012		0.290		0.250		0.537
2	6↑		0.719		0.130		0.849		0.891		0.671
1108	7↑				0.492		0.067				

Table 3.10 Intensity changes in different time points of the selected spots from E2

E2		E1		E3		E4		E5		E6	
SSP	**Spot #**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**
7202	8↓	5.63↑	0.006		0.027		0.475		0.145		
1402	9↓			4.53↓	0.002		0.975		0.167		
3401	10↑		0.350		0.294		0.860		0.536		
2406	29↓		0.796							↓	0.027

Table 3.11 Intensity changes in different time points of the selected spots from E3

E3		E1		E2		E4		E5		E6	
SSP	**Spot #**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**
1304	12↓			2.33↓	0.019				0.163		
2009	13↓	↑	0.004		0.761				0.643		0.460
4106	14↑							↑	0.004		
5703	15↑		0.094		0.487				0.053		0.402
5611	16↑		0.296	1.85↑	0.008				0.596		0.745
5612	17↑		0.392		0.657				0.519		
5701	18↑		0.697		0.070				0.840		0.886
6206	19↑				0.493				0.114		
6505	39↑	↓	0.024		0.852				0.129		

Table 3.12 Intensity changes in different timepoints of the selected spots from E4

E4		E1		E2		E3		E5		E6	
SSP	**Spot #**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**
306	20↑										
401	21↑								0.024		
403	22↑				0.924		0.327		0.909		
1301	23↑				0.649		0.673		0.724		
1601	24↑		0.297				0.717		0.988		
2201	25↑						0.960		0.086		
2305	26↑		0.414	1.52↑	0.032		0.842		0.324		0.354

Table 3.13 Intensity changes in different time points of the selected spots from E5

E5		E1		E2		E3		E4		E6	
SSP	**Spot #**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**
2401	27↑		0.987				0.408		0.234		
1304	28↑			2.33↓	0.019	4.53↓	0.002		0.975		
3603	30↓		0.015	3.77↑	0.008		0.483		0.204		0.616
2102	31↑	2.056↑	0.019				0.985		0.067		0.730
3105	32↑								0.067		0.691
4101	33↑						0.871				

Table 3.14 Intensity changes in different time points of the selected spots from E6.

E6		E1		E2		E3		E4		E5	
SSP	**Spot #**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**	**Intensity changes**	**t-test P value**
4305	34↓		0.144		0.324		0.972		0.923		0.530
3308	35↑		0.684		0.855		0.749		0.215		0.577
6605	36↑		0.296	1.85↑	0.008		0.248		0.244		0.596
3309	37↑		0.404		0.103		0.360		0.204		0.477
	38↑				0.540	2.13↓	0.030				

3.5 Bioinformatics studies

3.5.1 Identification of the spots by nano-LC ESIMS/MS

For each spot, in gel digestion with trypsin was performed and the spots were processed for MALDI TOF MS analysis. After MALDI-TOF MS analysis, the peak list of each protein spot, i.e. PMF spectra was analyzed with the aid of MASCOT Search engine for Viridiplantae (Green Plants) database.

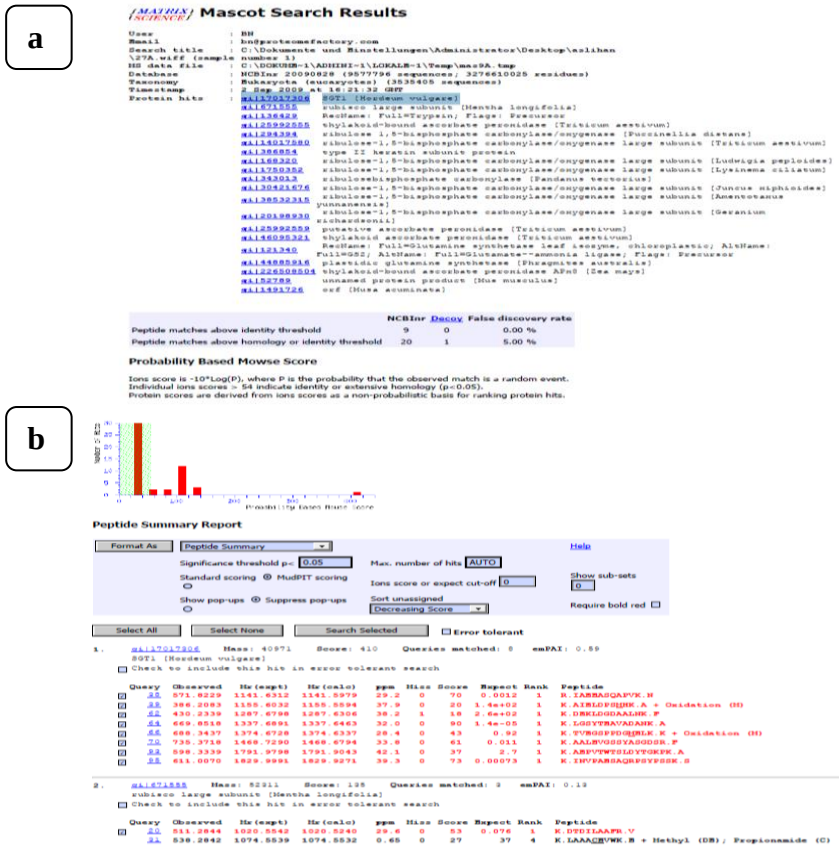
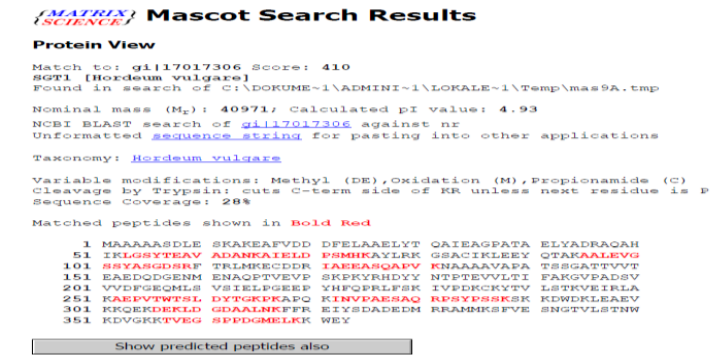


Figure 3.11 Representative figure of spot identification process by bioinformatic tools. Mascot output (a,b,c) NCBI output according to the accession number (d). SwissProt output according to the fasta sequence obtained from NCBI (e).

c



MATRIX SCIENCE Mascot Search Results

Protein View

Match to: gi|17017306 Score: 410
SGT1 [Hordeum vulgare]
Found in search of C:\DOKUME~1\ADMINI~1\LOKALE~1\Temp\mas9A.tmp

Nominal mass (M_r): 40971; Calculated pI value: 4.93
NCBI BLAST search of gi|17017306 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Hordeum vulgare

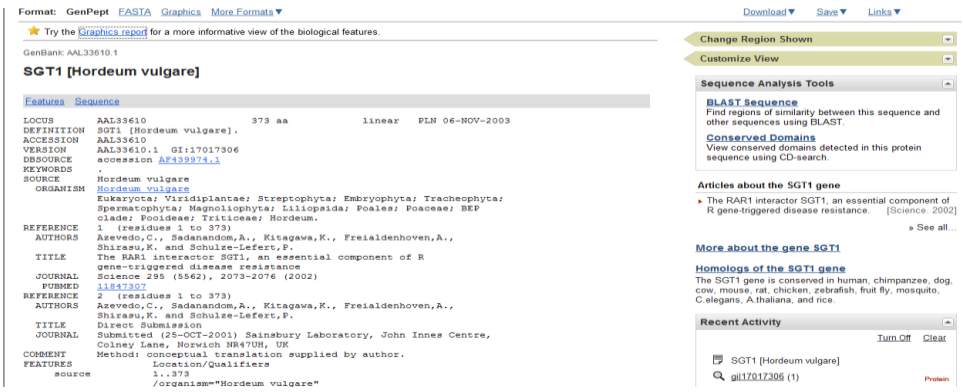
Variable modifications: Methyl (DE),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 28%

Matched peptides shown in Bold Red

```
    1 MAAAAASDLE SKAKEAFVDD DFELAAELYT QAIEAGPATA ELYADRAQAH
   51 IKLGSYTEAV ADAKKAIELD PSMHKAYLRK GSACIKLEEY QTAKAALEVG
  101 SSYASGDSRF TRLMKECDDR IAEEASQAFV KNAAAAVAPA TSSGATTVVT
  151 EAEDQDGENM ENAQPTVEVP SKPKYRHDYY NTPTEVVLTI FAKGVPADSV
  201 VVDFGEQMLS VSIELPGEEP YHFQPRLFSK IVPDKCKYTV LSTKVEIRLA
  251 KAEPVTWTSL DYTGKPKAPQ KINVPAESAQ RPSYPSSKSK KDWDKLEAEV
  301 KKQEKDEKLD GDAALNKFFR EIYSDADEDM RRAMMKSFVE SNGTVLSTNW
  351 KDVGKKTVEG SPPDGMELKK WEY
```

Show predicted peptides also

d



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GenBank: AAL33610.1

SGT1 [Hordeum vulgare]

Features Sequence

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LOCUS       AAL33610               373 aa            linear   PLN 06-NOV-2003
DEFINITION  SGT1 [Hordeum vulgare].
ACCESSION   AAL33610
VERSION     AAL33610.1  GI:17017306
DBSOURCE    accession AF439974.1
KEYWORDS    .
SOURCE      Hordeum vulgare
  ORGANISM  Hordeum vulgare
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Hordeum.
REFERENCE   1  (residues 1 to 373)
  AUTHORS   Azevedo,C., Sadanandom,A., Kitagawa,K., Freialdenhoven,A.,
            Shirasu,K. and Schulze-Lefert,P.
  TITLE     The RAR1 interactor SGT1, an essential component of R
            gene-triggered disease resistance
  JOURNAL   Science 295 (5562), 2073-2076 (2002)
   PUBMED   11847307
REFERENCE   2  (residues 1 to 373)
  AUTHORS   Azevedo,C., Sadanandom,A., Kitagawa,K., Freialdenhoven,A.,
            Shirasu,K. and Schulze-Lefert,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-OCT-2001) Sainsbury Laboratory, John Innes Centre,
            Colney Lane, Norwich NR47UH, UK
COMMENT     Method: conceptual translation supplied by author.
FEATURES             Location/Qualifiers
     source          1..373
                     /organism="Hordeum vulgare"
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Sequence Analysis Tools

BLAST Sequence
Find regions of similarity between this sequence and other sequences using BLAST.

Conserved Domains
View conserved domains detected in this protein sequence using CD-search.

Articles about the SGT1 gene

- The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. [Science. 2002]

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Homologs of the SGT1 gene
The SGT1 gene is conserved in human, chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, C.elegans, A.thaliana, and rice.

Recent Activity

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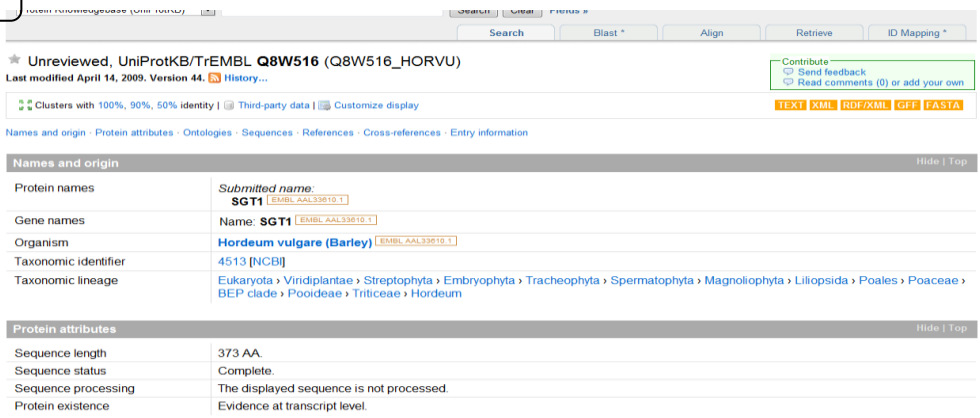
SGT1 [Hordeum vulgare]

gi|17017306 (1) Protein

e



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Unreviewed, UniProtKB/TrEMBL **Q8W516** (Q8W516_HORVU)

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Names and origin · Protein attributes · Ontologies · Sequences · References · Cross-references · Entry information

Names and origin Hide | Top

Protein names	*Submitted name:* **SGT1** EMBL AAL33610.1
Gene names	Name: **SGT1** EMBL AAL33610.1
Organism	**Hordeum vulgare (Barley)** EMBL AAL33610.1
Taxonomic identifier	4513 [NCBI]
Taxonomic lineage	Eukaryota › Viridiplantae › Streptophyta › Embryophyta › Tracheophyta › Spermatophyta › Magnoliophyta › Liliopsida › Poales › Poaceae › BEP clade › Pooideae › Triticeae › Hordeum

Protein attributes Hide | Top

Sequence length	373 AA.
Sequence status	Complete.
Sequence processing	The displayed sequence is not processed.
Protein existence	Evidence at transcript level.

Figure 3.11 Continued

3.5.2 Functional categorization of proteins

The distinct proteins identified by their accession numbers from NCBI were then searched in NCBI and SwissProt database for specific functions. Figure 3.11 represents the MASCOT search result showing the respective protein accession number along with its probability score and the output of Swiss-prot, NCBI database search for assignment of function. As it was represented, the protein was identified with the accession number of gi|17017306 and defined as SGT1. Table 3.15 shows predicted functions and identities of the peptides.

In table 3.8 all proteins were shown. According to the results and protein identification some proteins were discussed to reveal some unidentified proteins related to powdery mildew.

It was shown that glutamine synthetase root isozyme was down-regulated in 24th h of resistant condition. In previous studies (Thompson et al., 2005) it was shown that this protein was up-regulated in trascript level due to induction of plant defence by phloem-feeding insects in resistant rice plants.

Expression level of triose phosphate isomerase was up-regulated in resistant condition of 24th h plant. In previous studies (Fujita et al., 2004) it was shown that the expression of this gene was irrespective of infection by the powdery mildew pathogen.

Another carbohydrate metabolism related protein was phosphoglycerate mutase. This protein was previously related to the powdery mildew diseases. It was shown that phosphoglycerate mutase was down-regulated in susceptible phenotype of powdery mildew infected barley (Gjetting et al., 2007).

As expected we identified some oxidative-stress related proteins. NAD-dependent malic enzyme involves in in the lignin and phytoalexin biosynthesis of the

phenylpropanoid pathway and known to be highly induced after infection of pathogens (Fujiware et al., 2006). In table 3.8 it was shown that NAD-dependent malic enzyme was up-regulated in resistant phenotype.

In resistant conditions lipocalin up-regulation was shown in table 3.8. Previous study about lipocalin had showed that its level was decreasing unexpectedly in stres condition (Wan et al., 2008). Lipocalins play a possible biological role in membrane biogenesis and repair as well as in the transport of sterol molecules to the membrane under adverse stress conditions. Another study was presented lipocalins expression changes under cold stres conditions. It was shown that plasma membrane-associated AtTIL (temperature induced) lipocalin plays a role in protecting plants against the oxidative stress induced by freezing, paraquat treatment and light (Charron et al., 2008). According to these results we hypothesize that during oxidative stress, lipocalins may bind and scavenge peroxidated lipids, and thus help restore membrane integrity.

One of the identified protein metabolism related protein was 40S ribosomal protein. It was found to be down-regulated in susceptible conditions. 40S ribosomal protein was also identified as BGH conidia protein (Noir et al., 2009).

In susceptible phenotype actin1 was found up-regulated. Actin is a structural protein which contributes to the establishment of effective barriers at the cell periphery against fungal pathogen. It was shown that barley (Hordeum vulgare) epidermal cells require actin cytoskeleton function for basal defense to the appropriate powdery mildew pathogen Blumeria graminis f. sp. hordei and for mlo-mediated resistance at the cell wall, but not for several tested race-specific immune responses (Miklis, 2007).

It has been shown that CPN-60 was up regulated during the infection. Previous studies could only cover the expression changes during the abiotic stress. We can

say that the up-regulated rubisco binding protein may lead us to understand that the carbon fixation could be more efficient during the disease. (Kieffer, 2009)

Carbohydrate metabolism related NADP-dependent malate dehydrogenase mostly increase in infected leaves during the early stages of infection and later decreases. The pathogen is not directly responsible for increase in respiratory in green leaves rather that this is response in the host cells to a loss of photosynthetic capacity. (Scott, 1966)

Putative ribosomal protein s5 involves in initiation and elongation of the newly growing peptide chains. This protein was also identified in powdery mildew haustorias and grouped into the protein biosynthesis group which contains the most abundant proteins of haustoria proteome. (Godfrey, 2009)

Ubiquitin ligase-associated protein SGT1 are required for disease resistance in plants mediated by nucleotide- binding site leucine-rich repeat (NBS-LRR) proteins (Peart, 2002). SGT1 up- regulation in resistant phenotype was also shown in this study.

Table 3.15 Predicted functions and identities of the proteins.

SSP	Protein id	Accesion Number	PM/Score	Sequence coverage	Molecular Weight Ex/The	pI Ex/The	Subcellular Localization	Signal Peptide	Molecular Function
2302	Glutamine synthetase root isozyme	585201	4/87	14	39.162/39.226	5.2/5.6	Cytoplasmic	Yes	Protein synthesis
3106	Triosephosphate isomerase	1174745	25/207	19	21.872/31.613	5.4/6	Chloroplastic	Yes	Carbohydrate metabolism
4604	Phosphoglycerate mutase	551288	6/292	13	52.113/60.592	5.61/5.3	Cytoplasmic	No	Carbohydrate metabolism
4603	NAD-dependent malic enzyme	10834736	1/65	8	27.006/18.271	6.28/4.9	Cytoplasmic	No	Electron transport
8101	rbcL	1389564	6/196	13	52.763/52.793	5.52/6.09	Chloroplast		Intracellular protein trafficking
0002	-	-	-	-	-	-	-	-	-
1108	chloroplast lipocalin	77744909	10/358	27	23.271/36.822	5.17/5.75	Cytoplasmic	No	Intracellular protein trafficking
7202	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	61378666	13/398	19	23.855/53.101	6.36/6.22	Cytoplasmic	No	Intracellular protein trafficking
1402	40S ribosomal protein SA	3334320	2/77	6	38.696/33.885	5.0/5.1	Cytoplasmic	No	Protein metabolism
3401	actin 1	7211789	3/126	10	39.179/41.762	5.40/5.22	Cytoskeletal	No	Other (Structural)

Table 3.15 Continued

1304	Mg-chelatase subunit	847873	5/246	17	37.282/36.416	5.08/4.89	Cytoplasmic	No	Other (porphyrin biosynthesis)
2009	light-harvesting complex I	544700	9/177	8	17.698/24.21	5.29/8.11	Chloroplastic	Yes	Carbohydrate metabolism
4106	light-harvesting complex I	544700	16/145	8	24.944/24.2	5.51/8.11	Chloroplastic	Yes	Carbohydrate metabolism
5703	Os08g0447000	255678485	4/114	10	29.01/23.68	5.72/6.95		No	Carbohydrate metabolism
5611	ATP synthase CF1 alpha subunit	14017569	14/577	28	49.763/55.261	5.84/6.11	Cytoplasmic	No	Carbohydrate metabolism
5701	putative transketolase	28190676	7/246	7	59.141/79.978	5.74/6.12	Chloroplastic	Yes	Carbohydrate metabolism
6206	porphobilinogen deaminase	19849543	3/131	13	32.529/32.226	6.09/5.5	Nuclear	No	Intracellular protein trafficking
6505	26S protease regulatory subunit 6B homolog	1709798	7/254	17	43.717/46.503	5.93/5.5	Cytoplasmic	No	Protein metabolism
0306	-	-	-	-	-	-	-	-	
0401	3-phosphoglycerate kinase	21396683	7/213	24	40.2/31.318	4.61/4.88	Cytoplasmic/	No	Carbohydrate metabolism
0403	RAD23-like protein	164665688	3/67	3	43.8 /39.258	4.8/4.84	Cytoplasmic	No	Protein targeting localization
1301	Os12g0183300	115487678	3/81	7	38.49/37.457	4.8/4.61	Chloroplastic	No	Direk kromozom
1601	60 kDa chaperonin subunit alpha	134107	10/433	17	49.9/57.485	5.00/4.83	Cytoplasmic	No	Protein metabolism
2201	putative ribosomal protein S5	29244648	3/178	9	33.8/34.951	5.1/4.97	Chloroplastic	Yes	Protein biosynthesis
2305	NADP-dependant malate dehydrogenase	24370917	2/77	5	39.27/38.159	5.3/5.24	Cytoplasmic	No	Carbohydrate metabolism
2401	SGT1	17017306	3/135	6	40.978/52.311	5.35/6.23	Nuclear	No	Intracellular protein trafficking
1304	laminin receptor homologue	16380	2/118	10	37.282/32.282	5.12/5.13	Cytoplasmic	No	
3603	RuBisCO large subunit-binding protein subunit beta	2493650	28/745	34	50.836/53.379	5.4/4.88	Cytoplasmic	No	Carbohydrate metabolism

3.5.3 Prediction of subcellular Localizations and signal peptides

Prediction of subcellular localization of the proteins was accomplished by submitting the amino acid sequences of the 38 identified proteins to the WoLF PSORT Protein Subcellular Localization Prediction Program (http://wolfpsort.seq.cbrc.jp). According the results obtained from the WoLF PSORT search, 15 proteins were predicted to be located in cytoplasm, 1 found to be either chloroplastic or mitochondrial, 17 proteins were predicted as chloroplastic and 2 of them were found to be nuclear and 1 of them found to be cytoskeletal (Table 3.15). Among those results most of tha proteins were found cytoplasmic (Figure3.12).

Out of the 38 selected proteins that were selected previously, 6 of them were predicted to have a signal peptide sequence according to the SignalP algorithm (http://www.cbs.dtu.dk/services/SignalP) (Table 3.15). We think that these 6 signal peptide-containing proteins can be elicitors or effectors or related proteins.

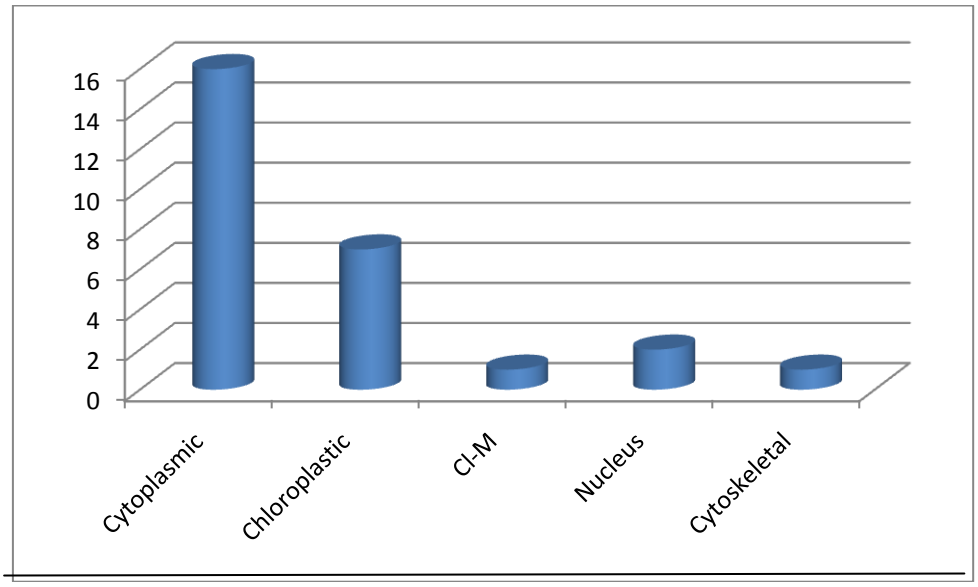

Figure3.12 Distribution of proteins according to their subcellular localization

CHAPTER 4

CONCLUSION

In this research, we identified differentiated spots in resistance and susceptible phenotypes. Following the image analysis by PdQuest we identified 36 spots. In order to see expression changes students T-test was made using the intensity values that were obtained by the image analysis program.

According to the statistical study 28 spots were found up-regulated and 8 spots were found down-regulated. We also made PCA analysis in order to make second confirmation and find possible markers among the differentiated spots. According to the PCA 27 spots were confirmed.

Selected spots were sent to the Proteome Factory for identification. Identified spots were subjected to WolfPSORT for sub cellular localization. 16 proteins were predicted to be located in cytoplasm, 1 found to be either chloroplastic or mitochondrial, 17 proteins were predicted as chloroplastic and 2 of them were found to be nuclear. Among those results most of the proteins were found cytoplasmic.

Out of the 38 selected proteins that were selected previously, 6 of them were predicted to have a signal peptide sequence according to the SignalP algorithm.

Proteins were also classified according to their function. Most of the proteins were found carbohydrate metabolism related proteins. Identified proteins were discussed in disease point of view.

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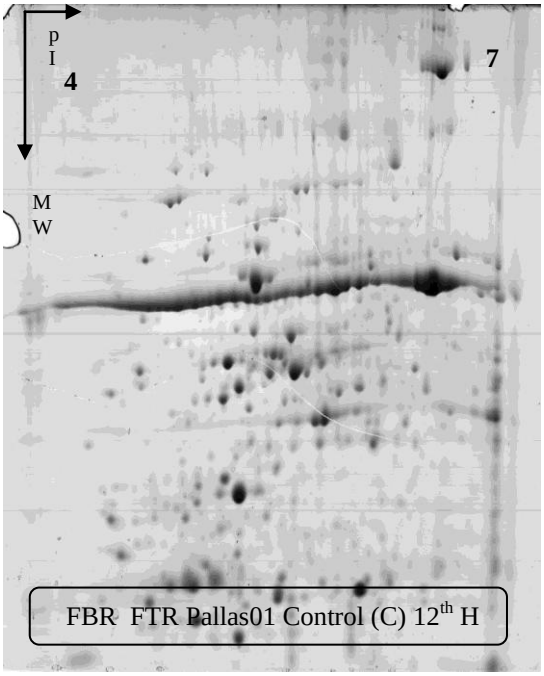
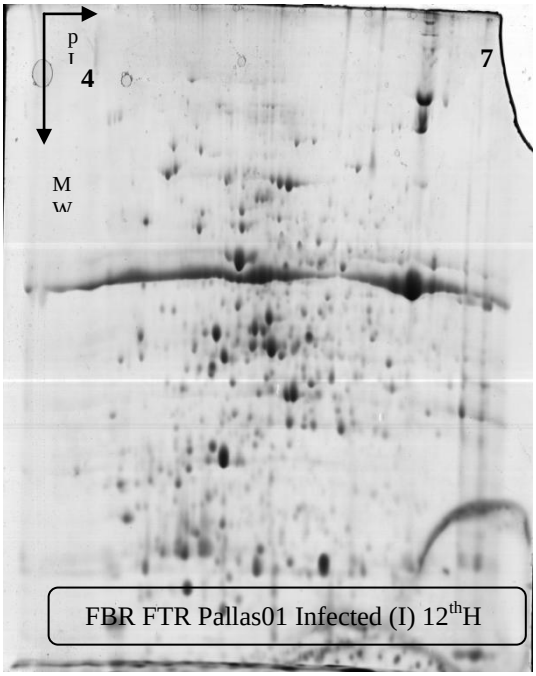
APPENDIX A

Pictures of 2 DE PAGE Gels

First Biologic Replicate (FBR) First Technical Replicate (FTR) Gel Pictures



FBR FTR Pallas01 Control (C) 12th H



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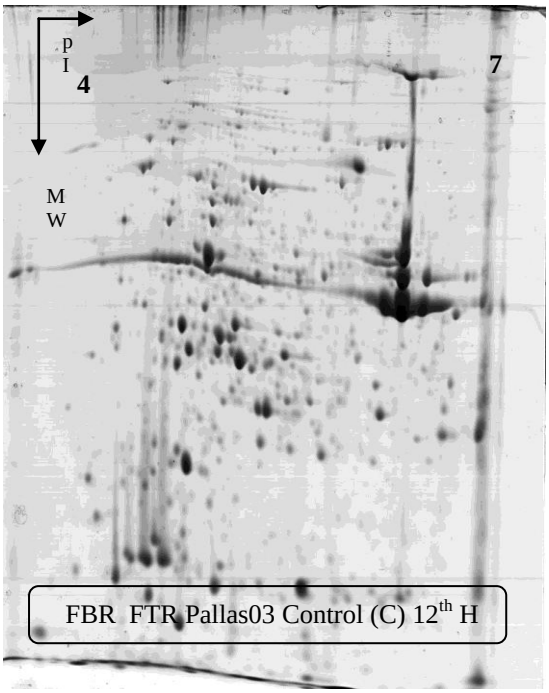

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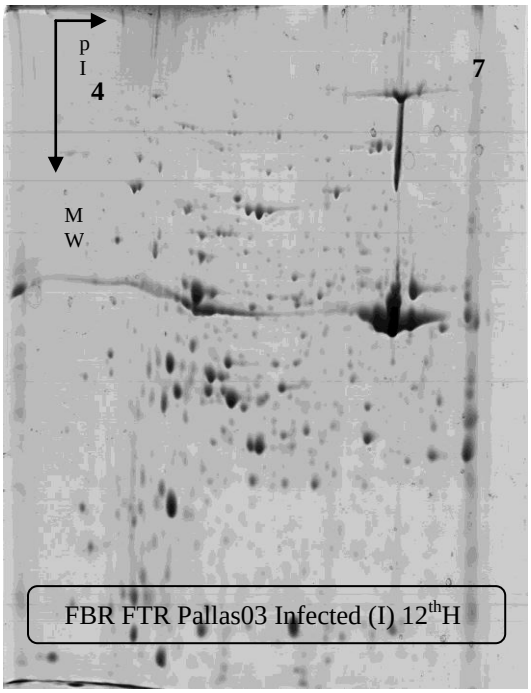

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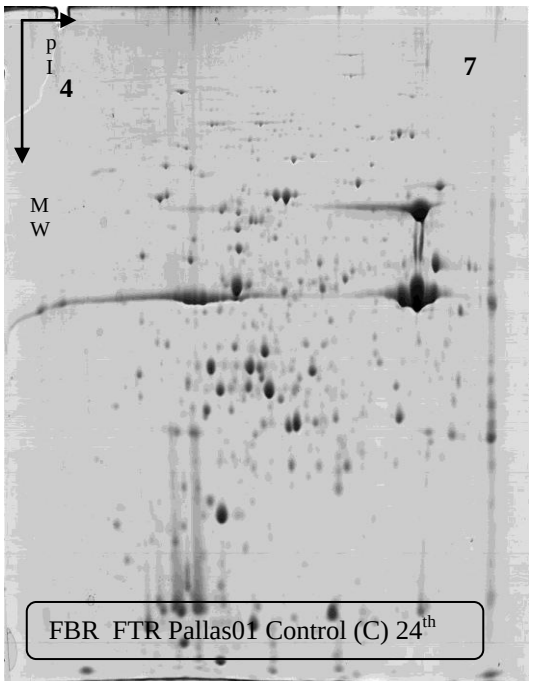
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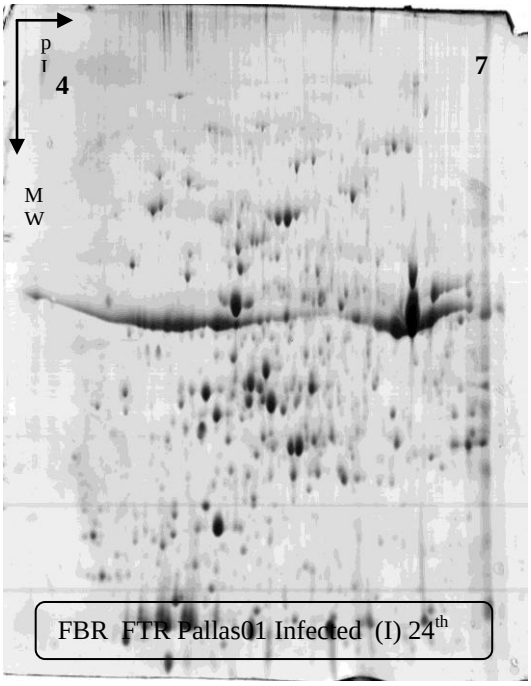
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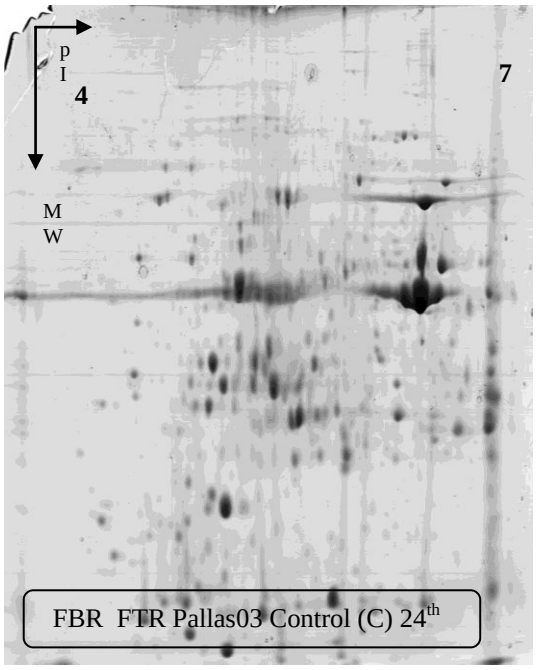
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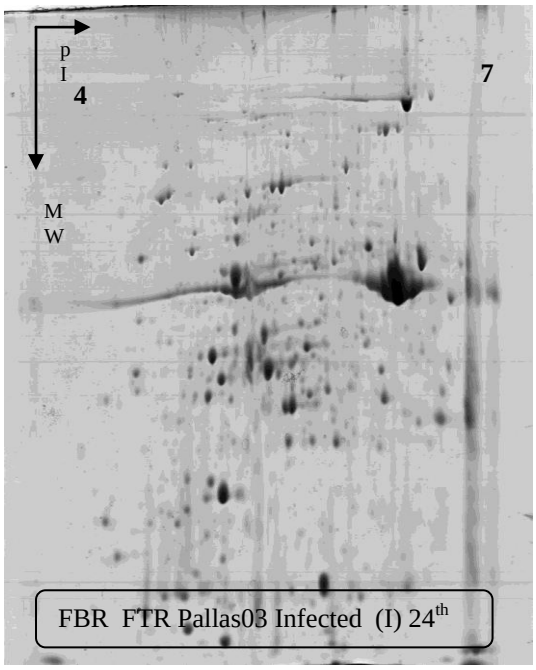
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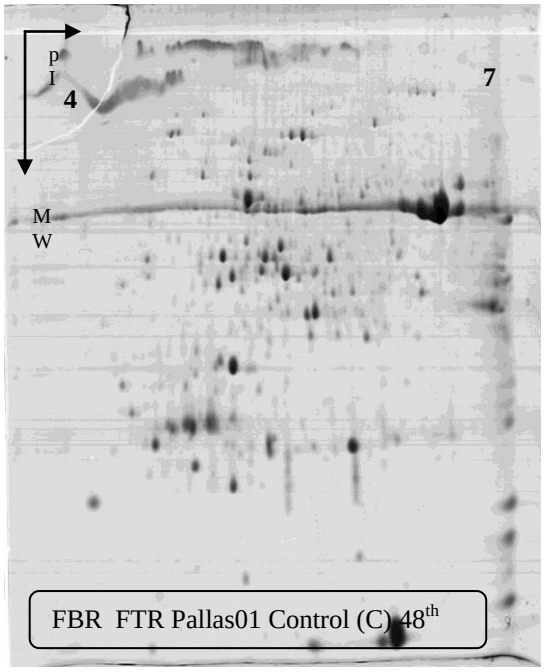
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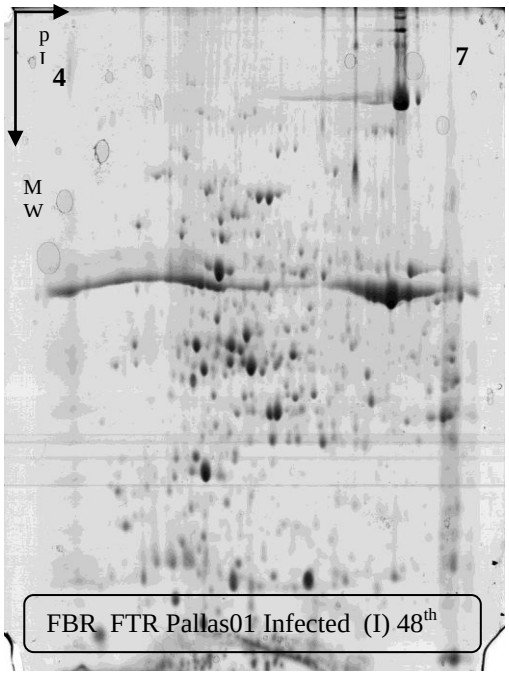
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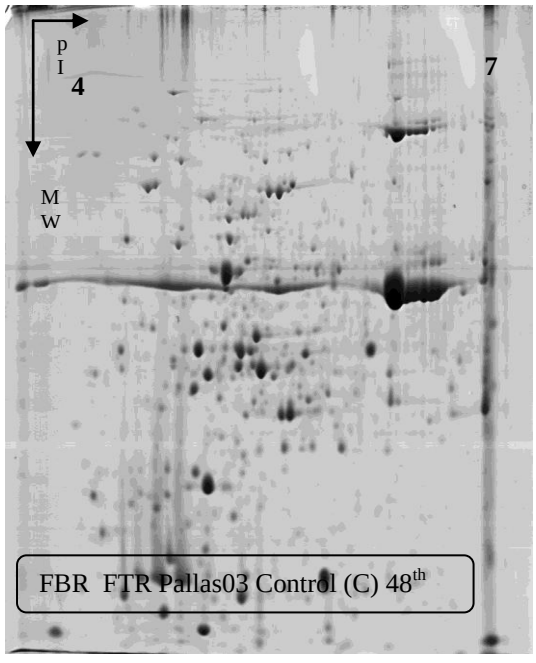
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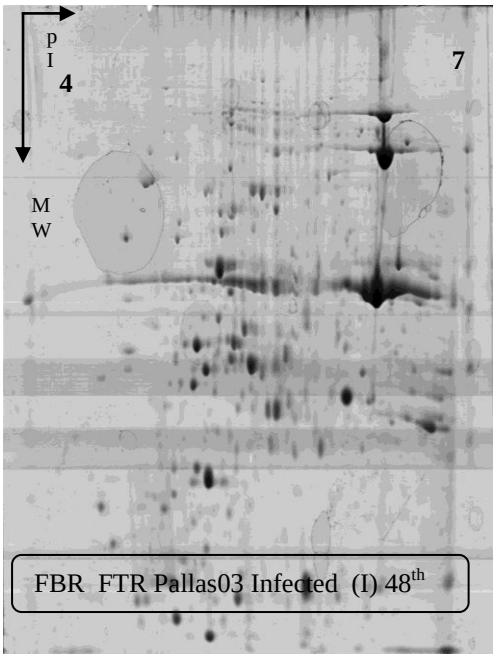
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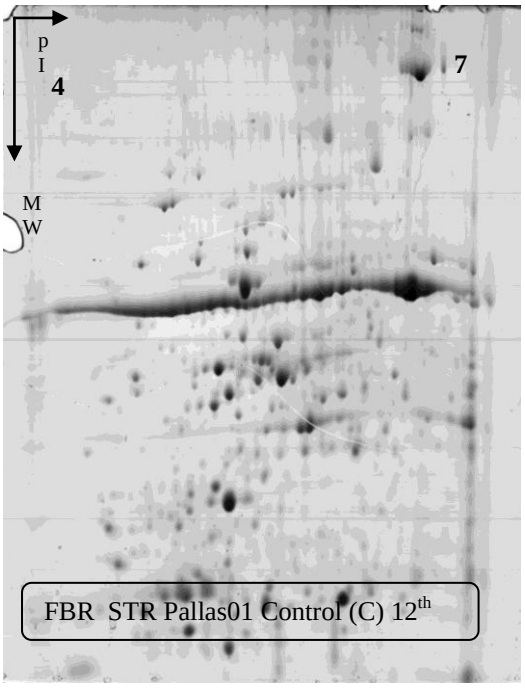
First Biologic Replicate (FBR) Second Technical Replicate (STR) Gel Pictures



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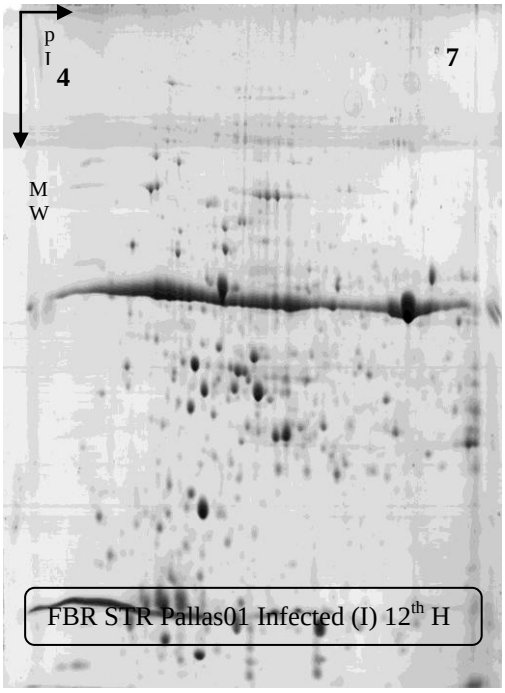

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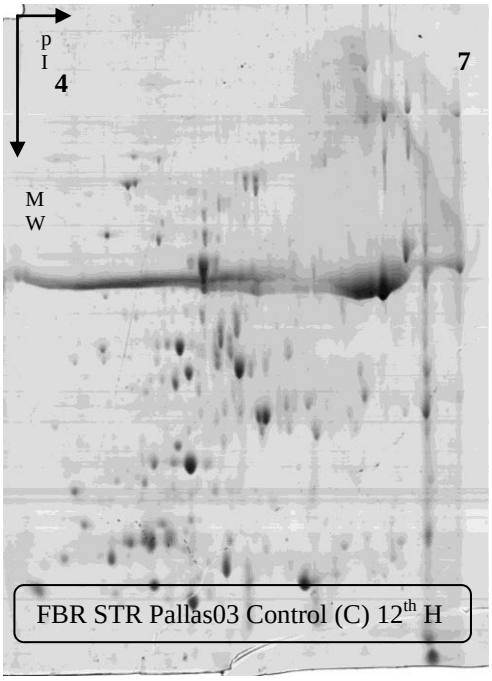

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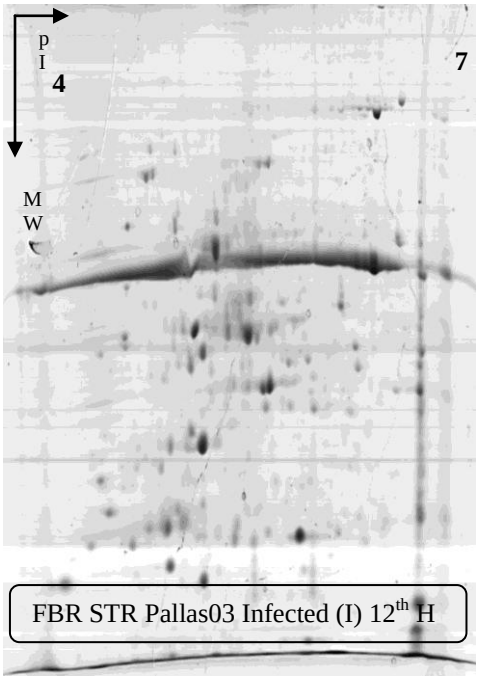

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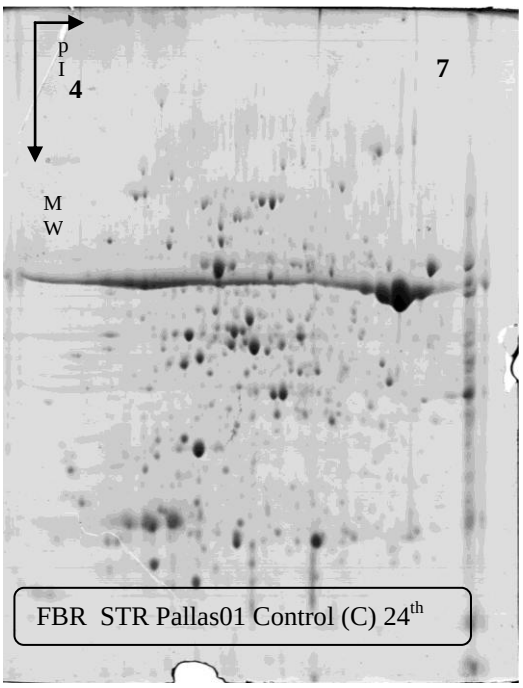

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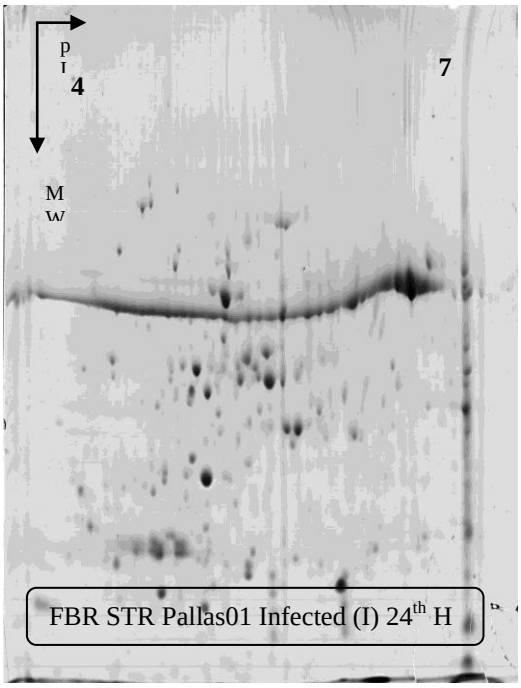

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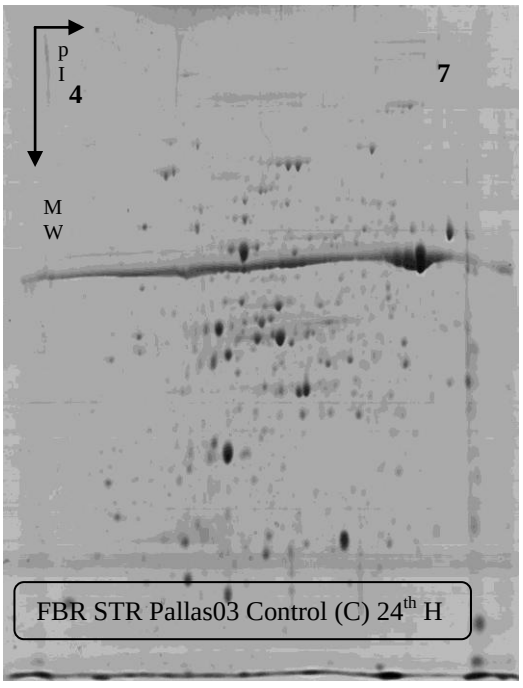

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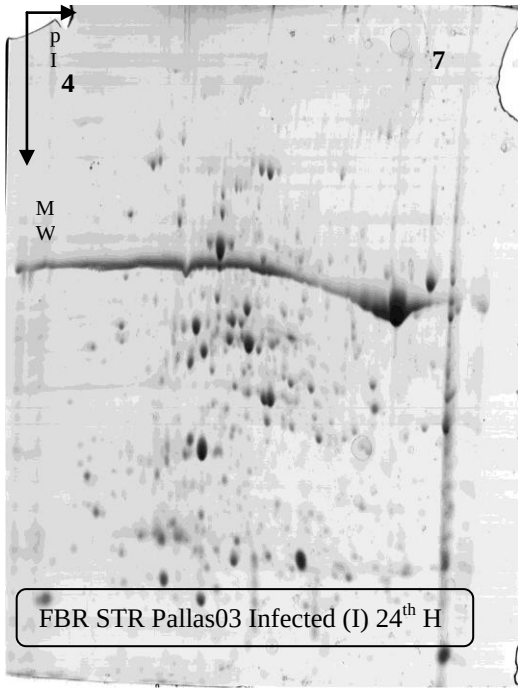

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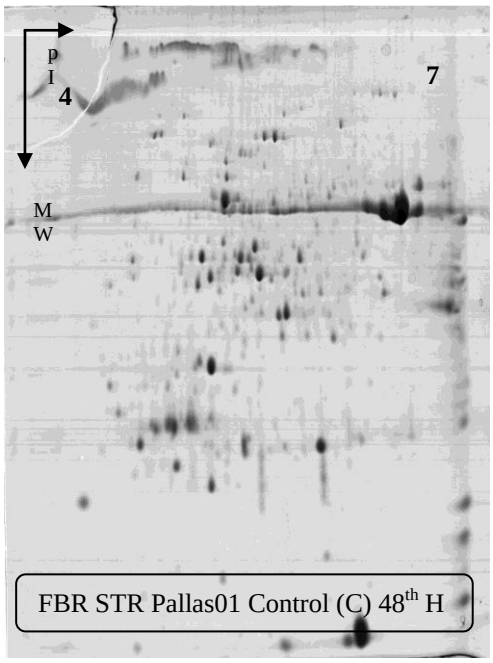

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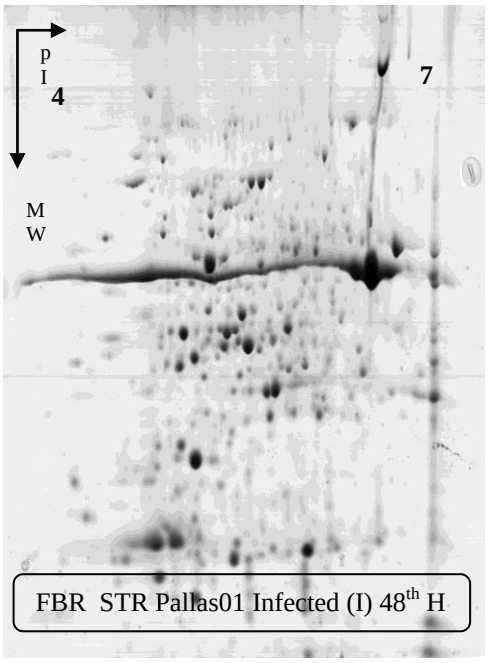

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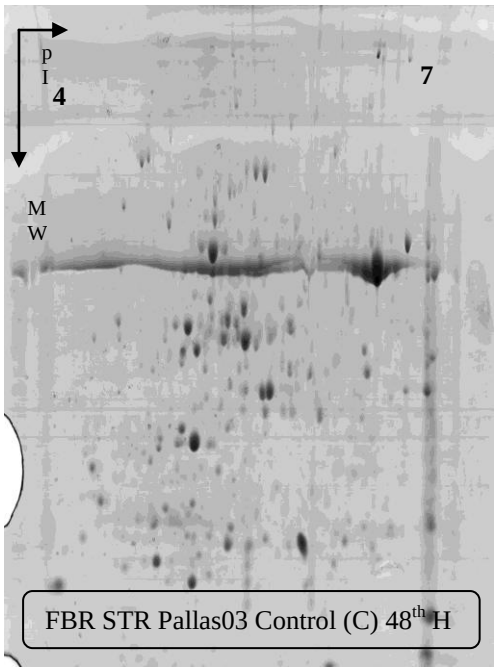

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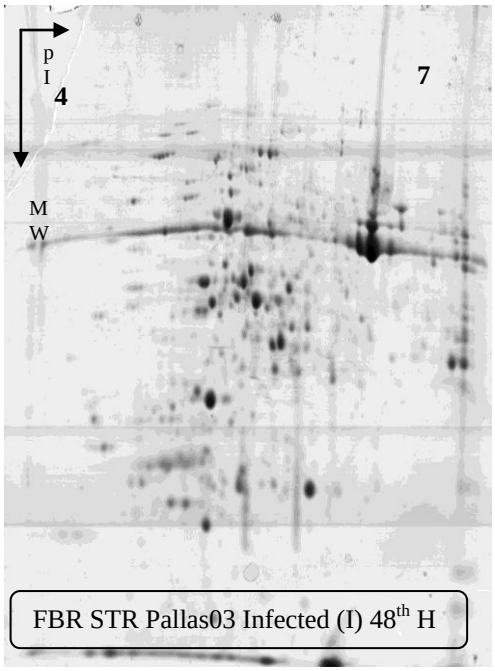

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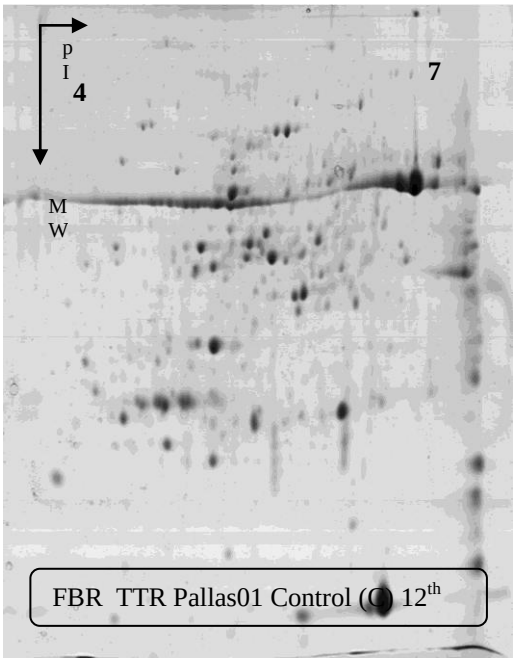
First Biologic Replicate (FBR) Third Technical Replicate (TTR) Gel Pictures



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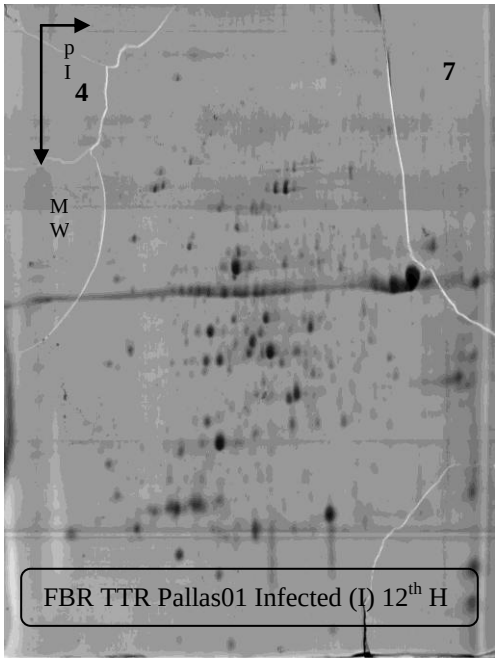

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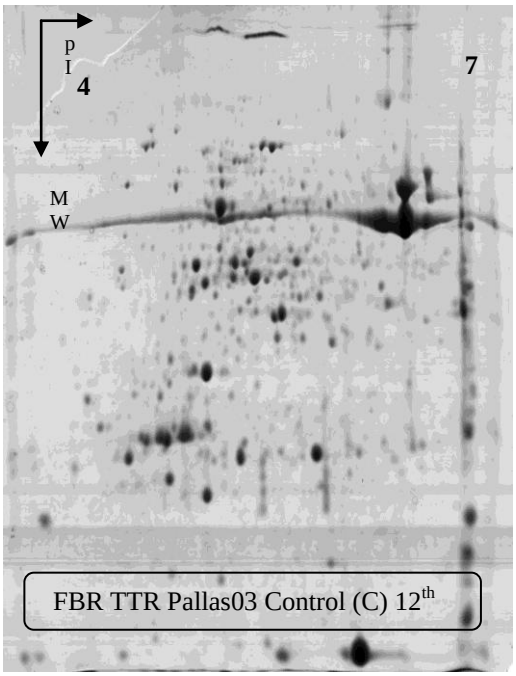

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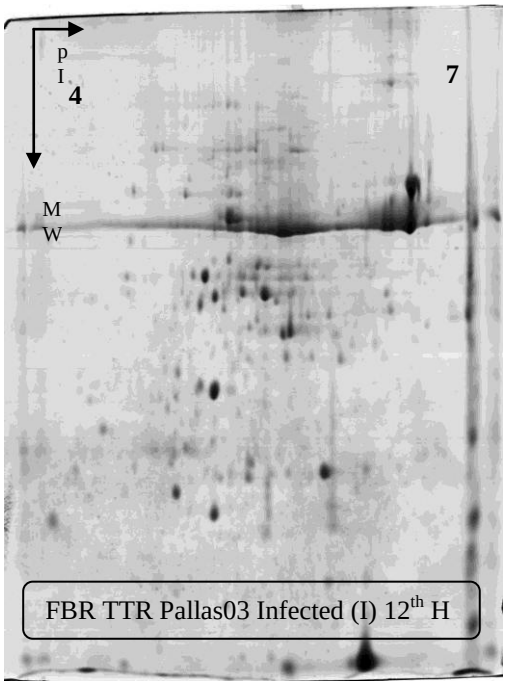

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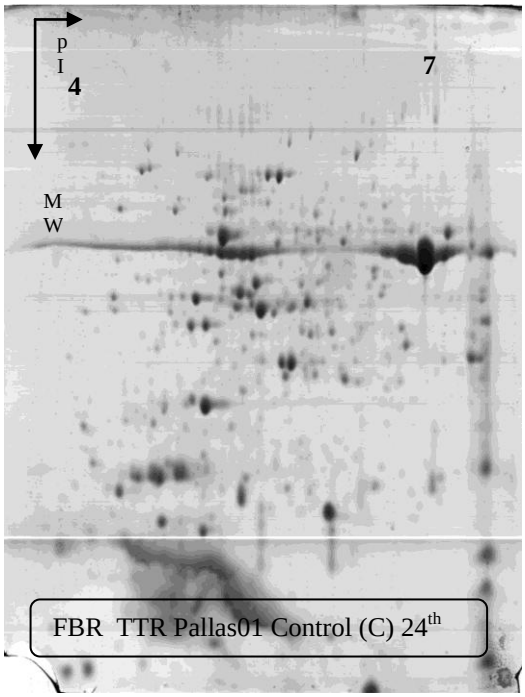

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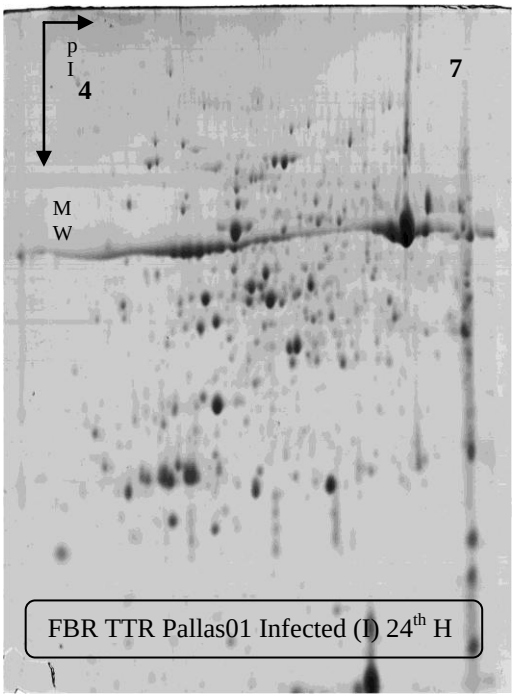

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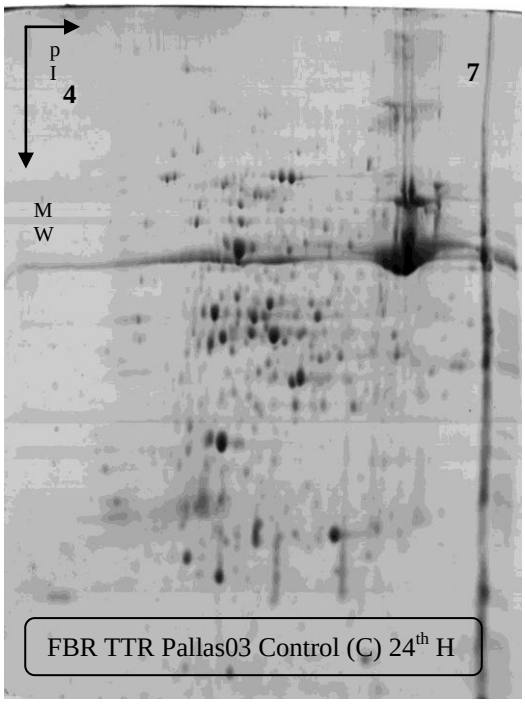

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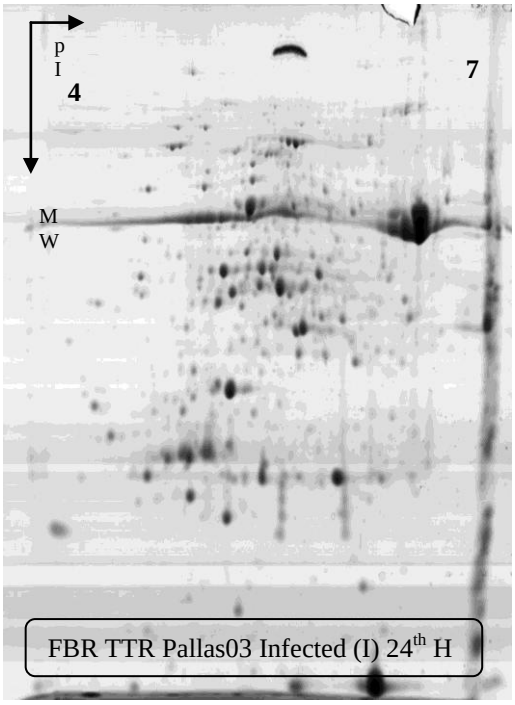

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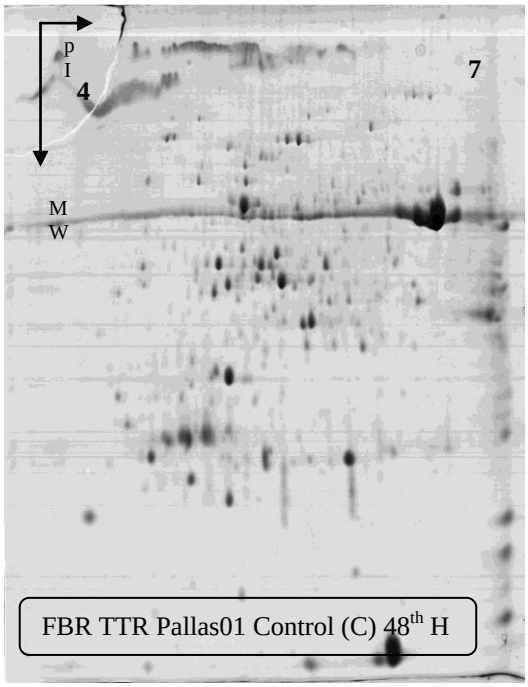

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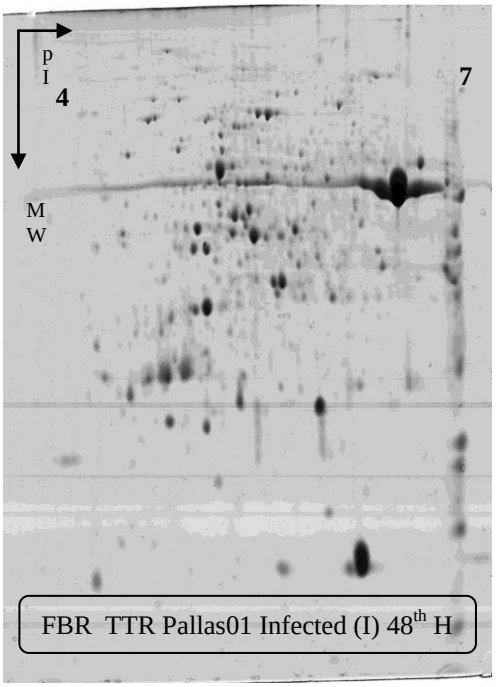

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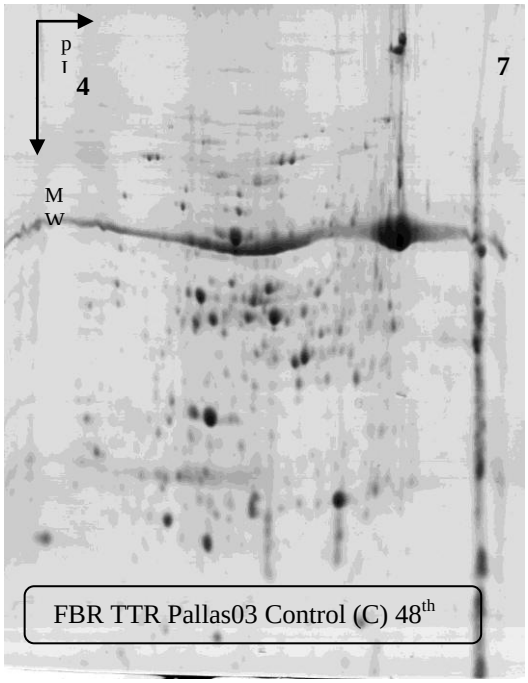

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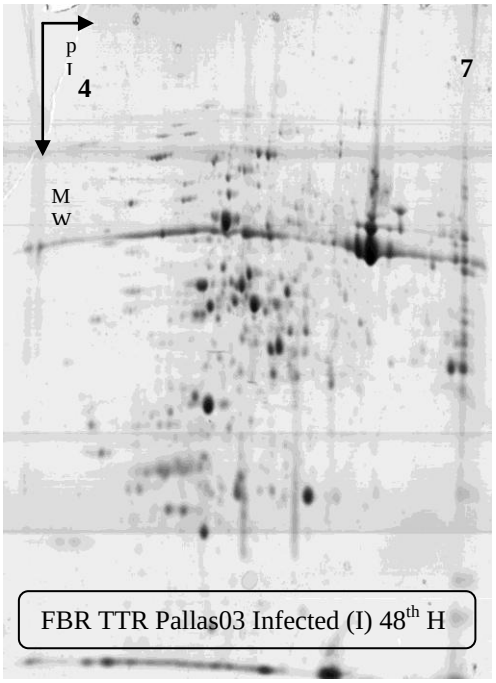

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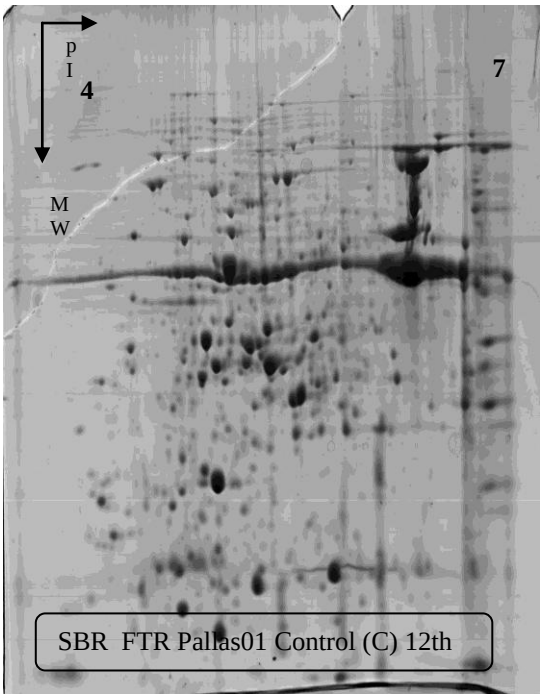
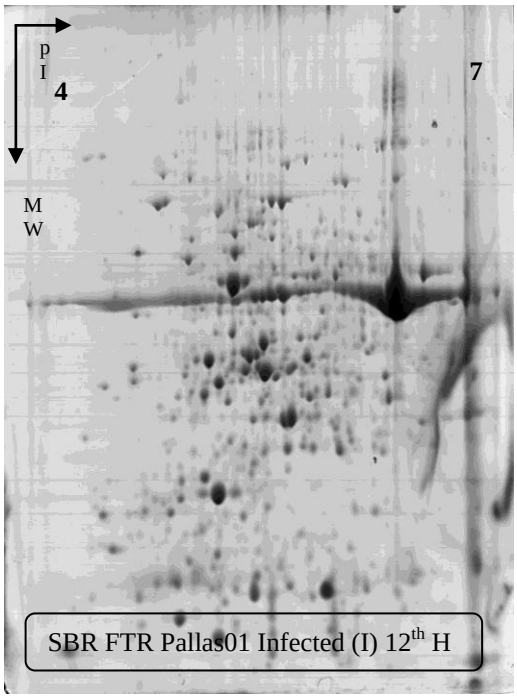
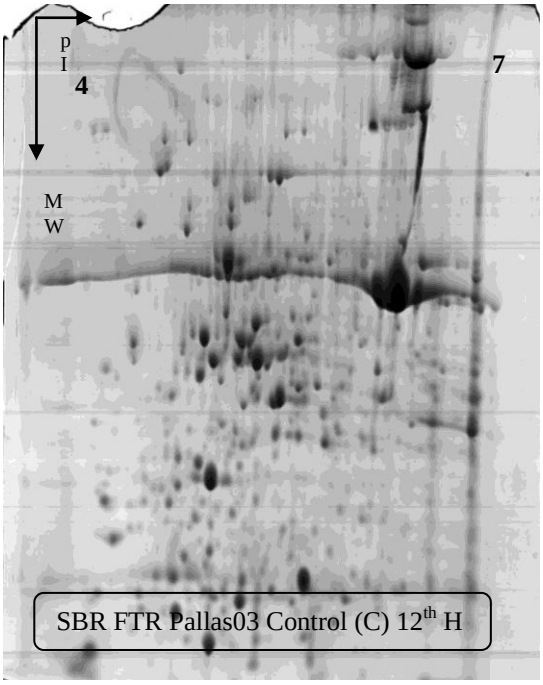
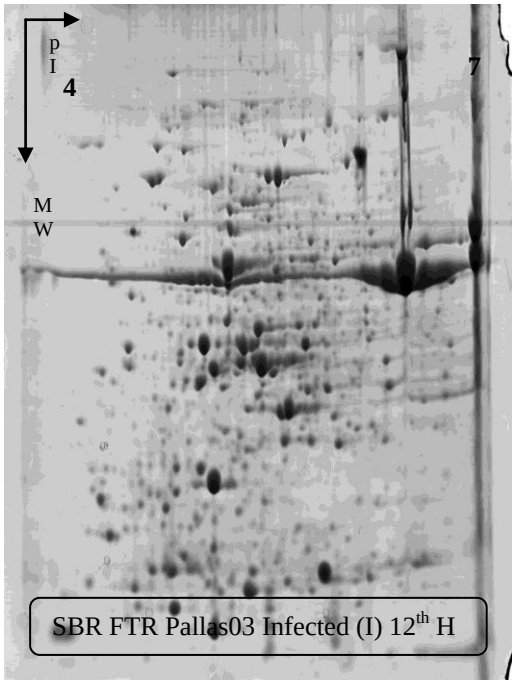
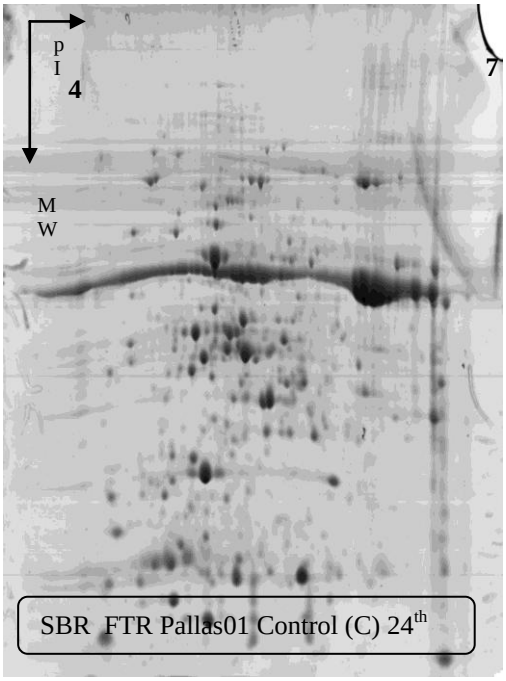
Second Biologic Replicate (SBR) First Technical Replicate (FTR) Gel Pictures







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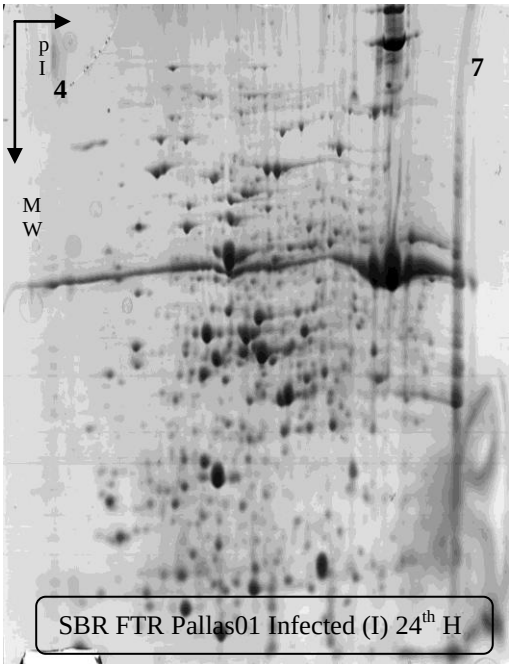
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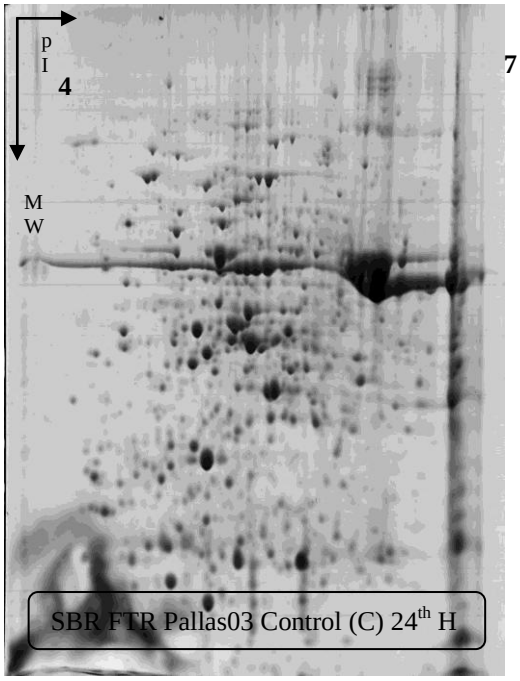
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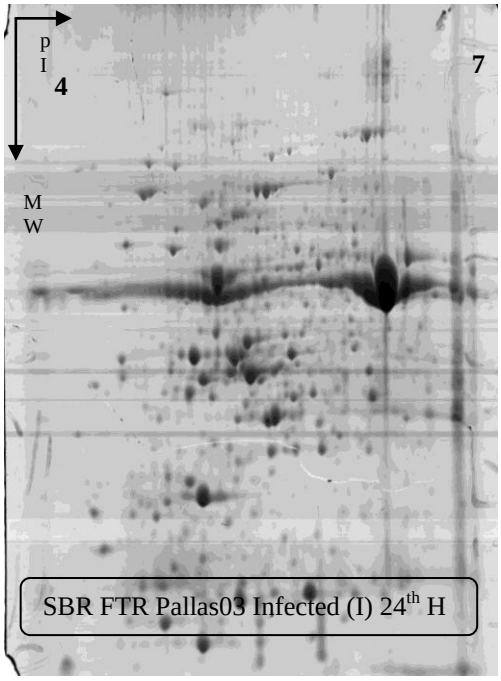
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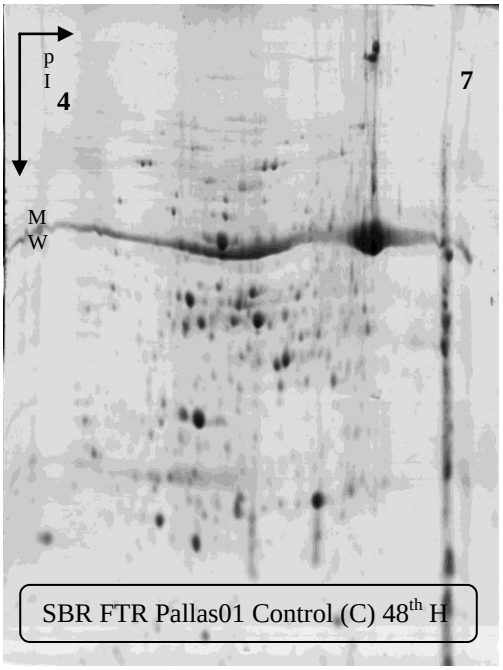
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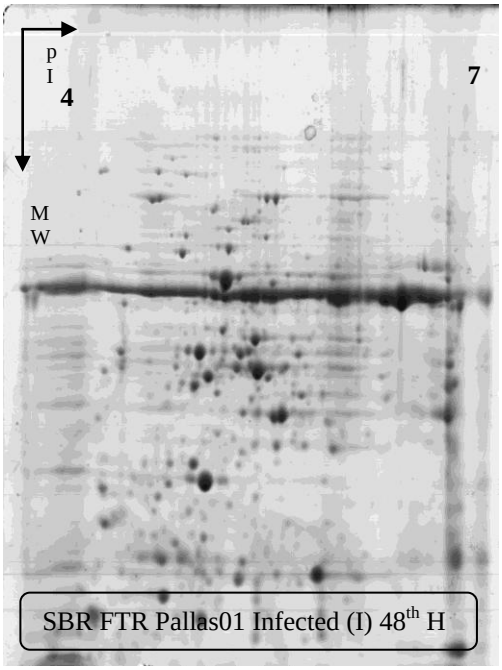
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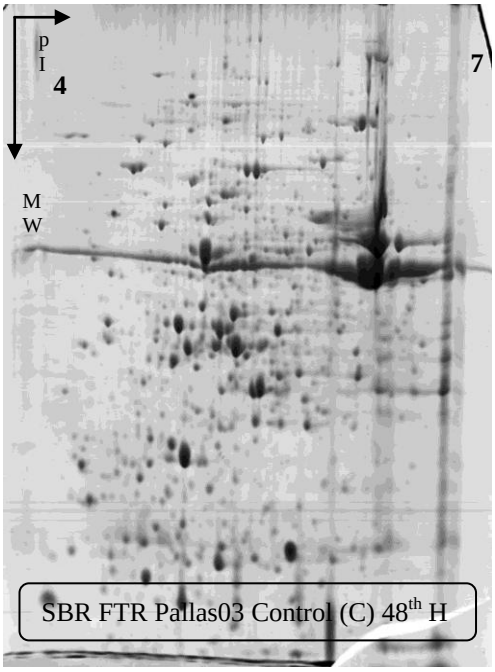
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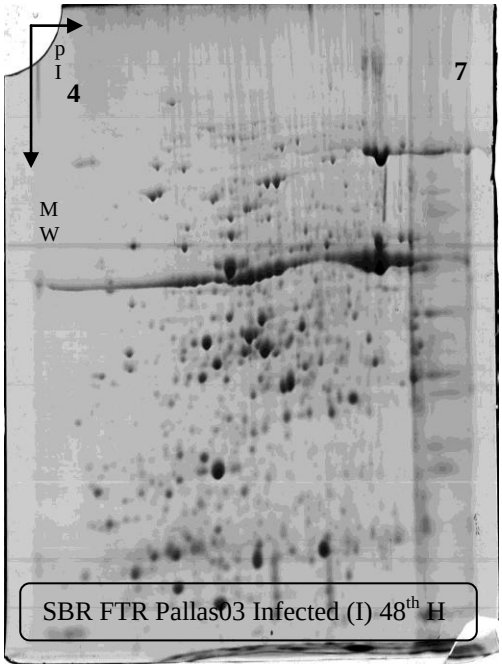
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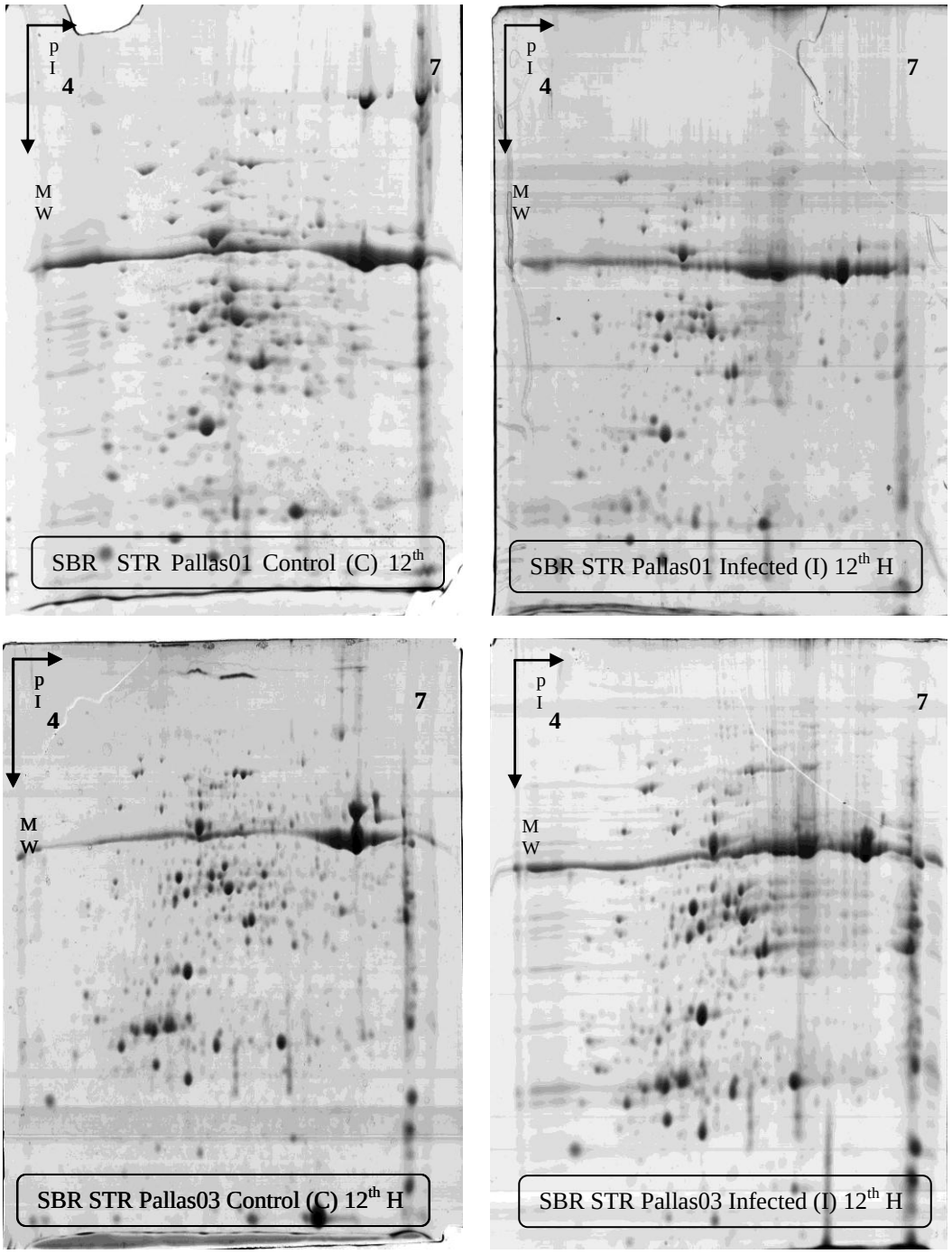
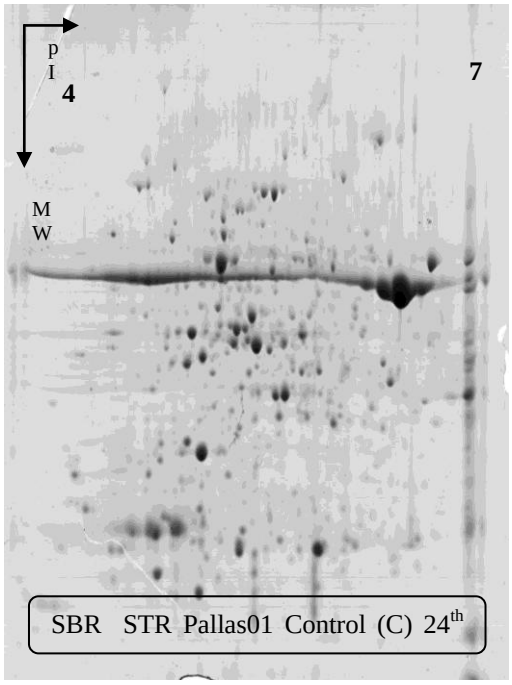
SBR FTR Pallas03 Infected (I) 48th H

Second Biologic Replicate (SBR) Second Technical Replicate (STR) Gel Pictures

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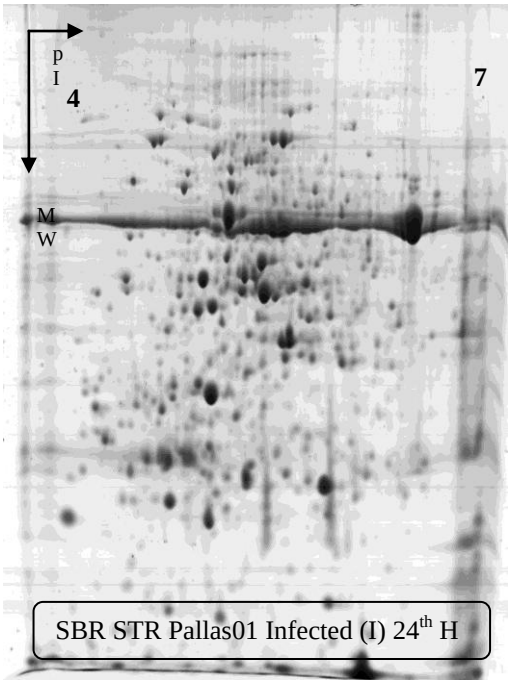
SBR STR Pallas01 Control (C) 24th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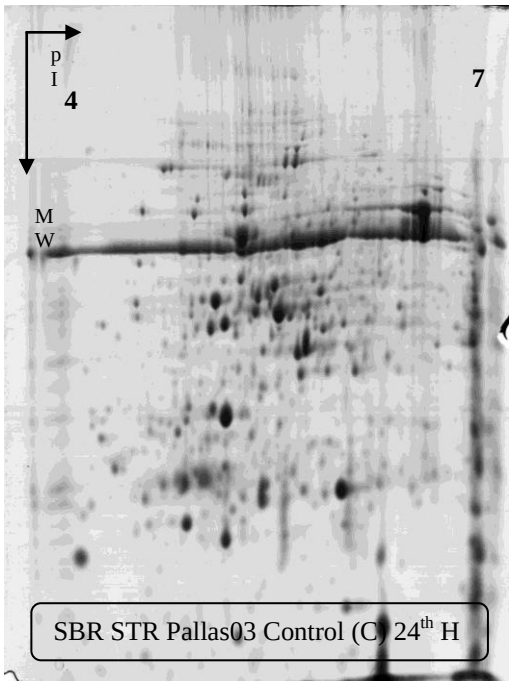
SBR STR Pallas01 Infected (I) 24th H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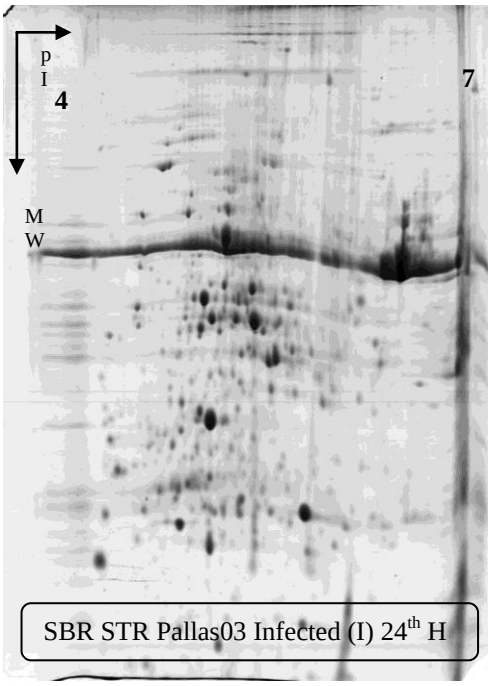
SBR STR Pallas03 Control (C) 24th H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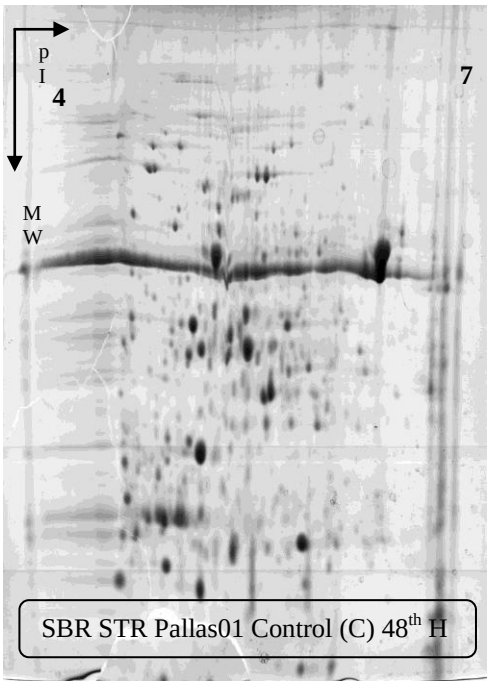
SBR STR Pallas03 Infected (I) 24th H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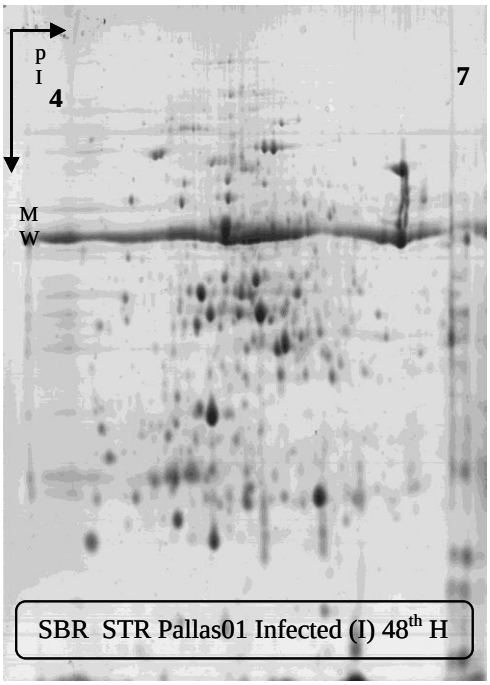
SBR STR Pallas01 Control (C) 48th H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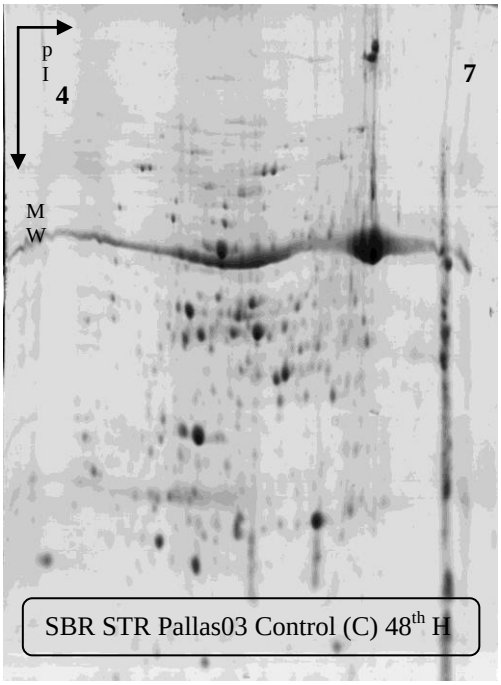
SBR STR Pallas01 Infected (I) 48th H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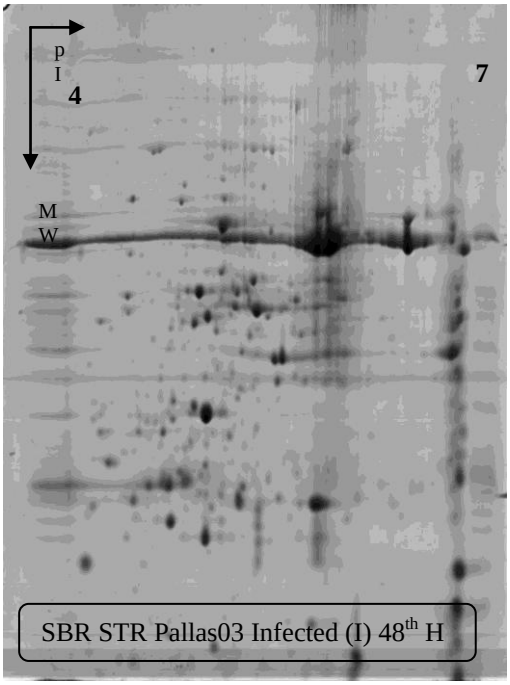
SBR STR Pallas03 Control (C) 48th H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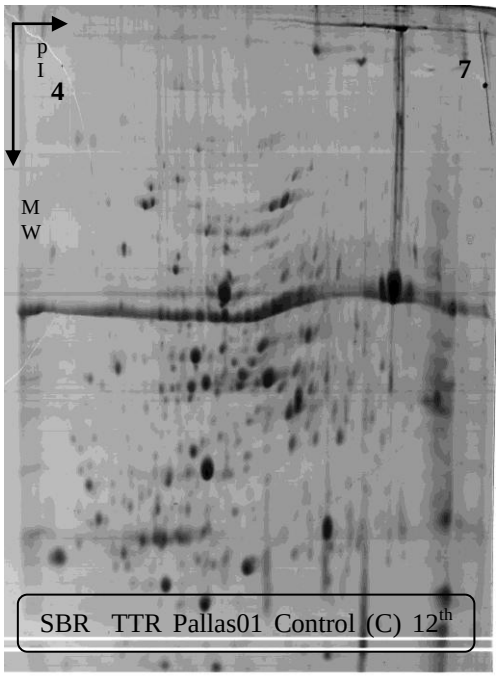
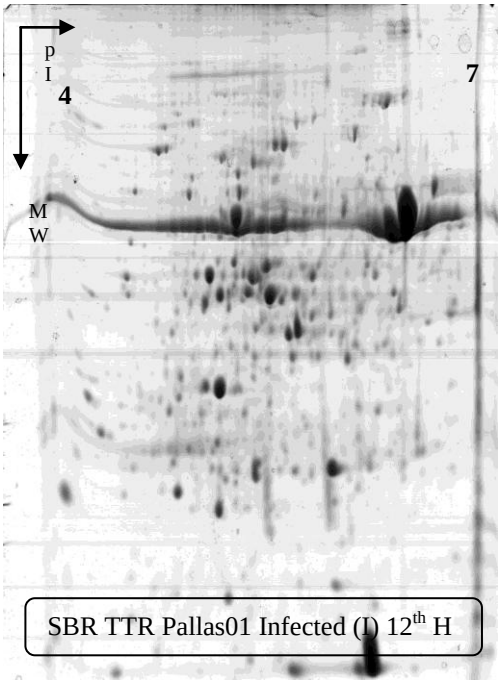
SBR STR Pallas03 Infected (I) 48th H

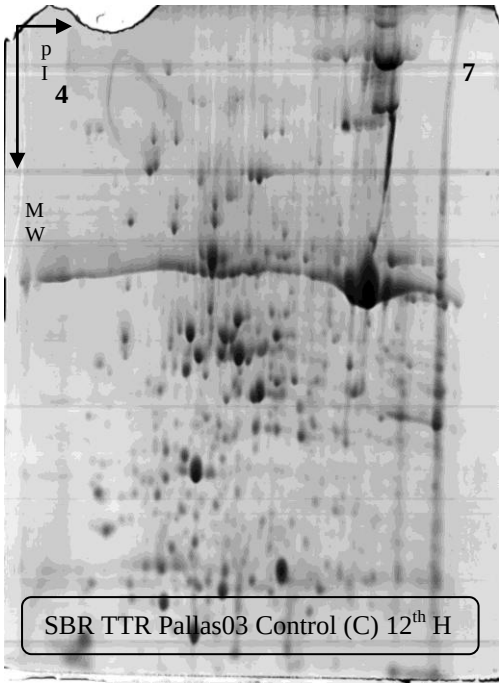
Second Biologic Replicate (SBR) Third Technical Replicate (TTR) Gel Pictures



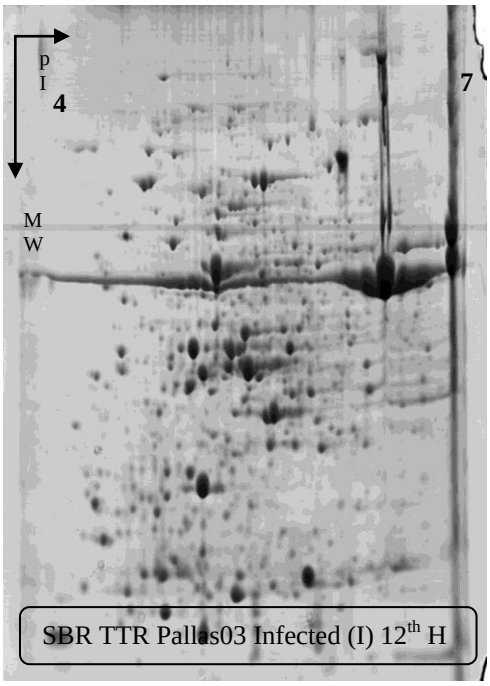
SBR TTR Pallas01 Control (C) 12th



SBR TTR Pallas01 Infected (I) 12th H



SBR TTR Pallas03 Control (C) 12th H



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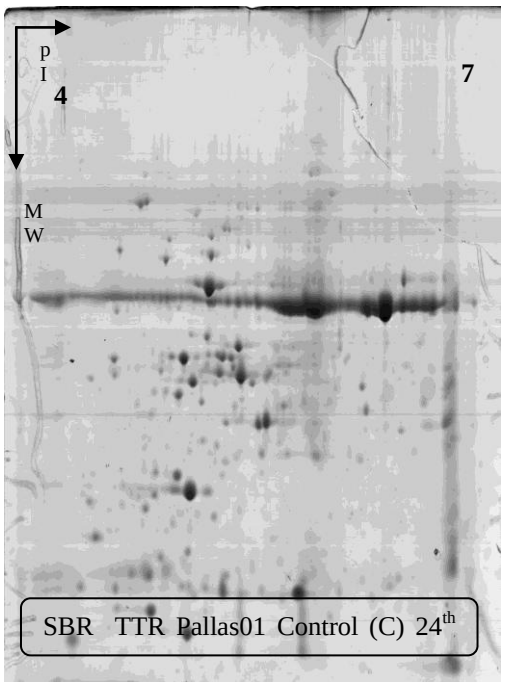
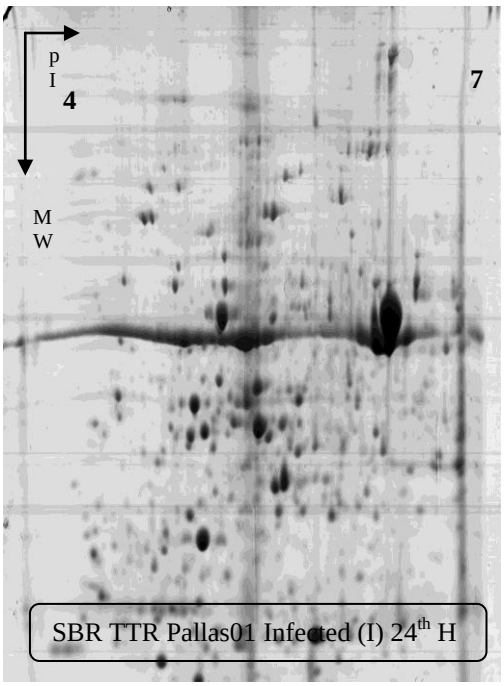


SBR TTR Pallas01 Control (C) 24th



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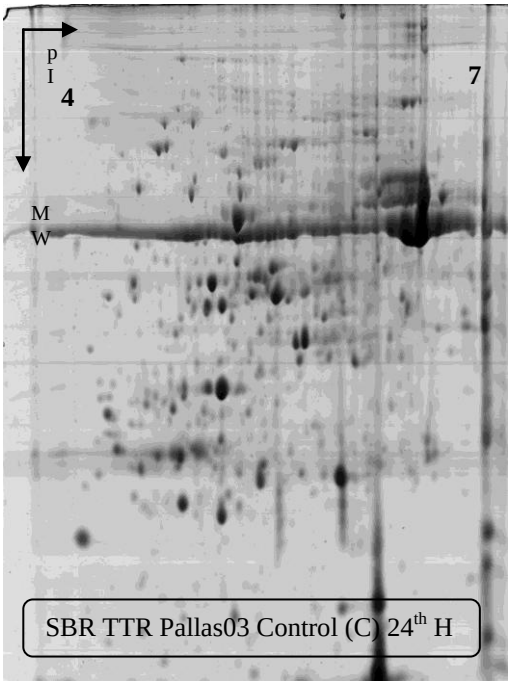

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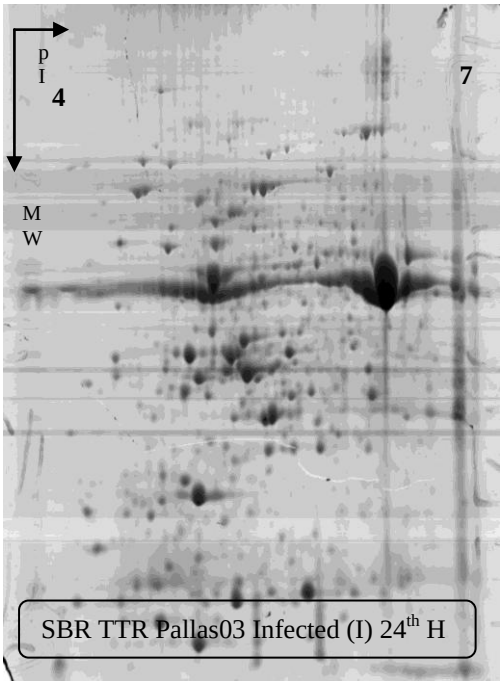

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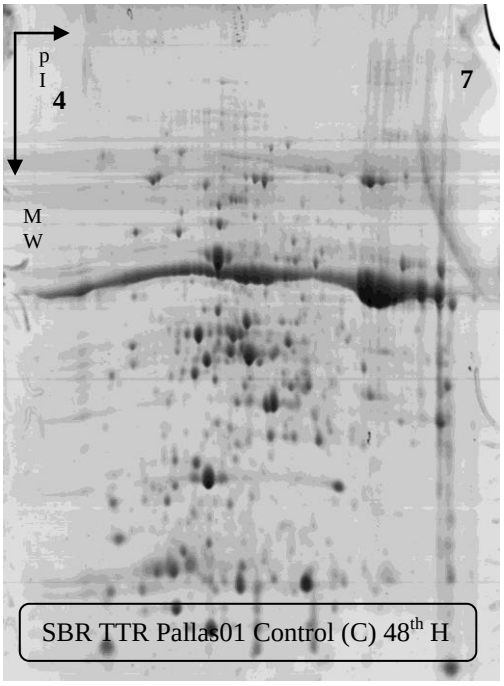
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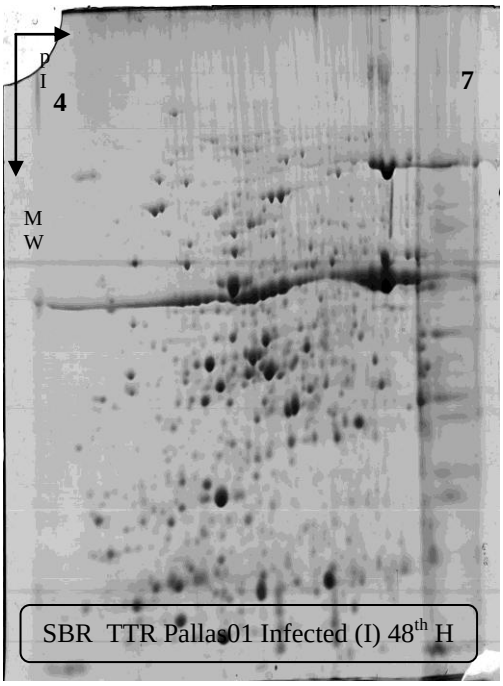
SBR TTR Pallas01 Control (C) 48th H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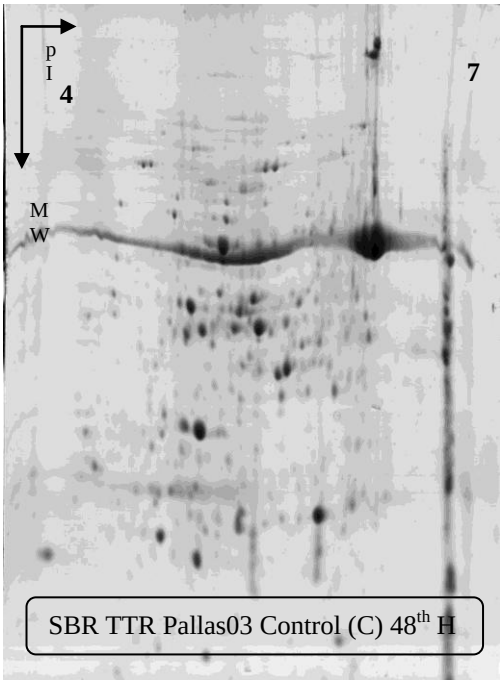
SBR TTR Pallas01 Infected (I) 48th H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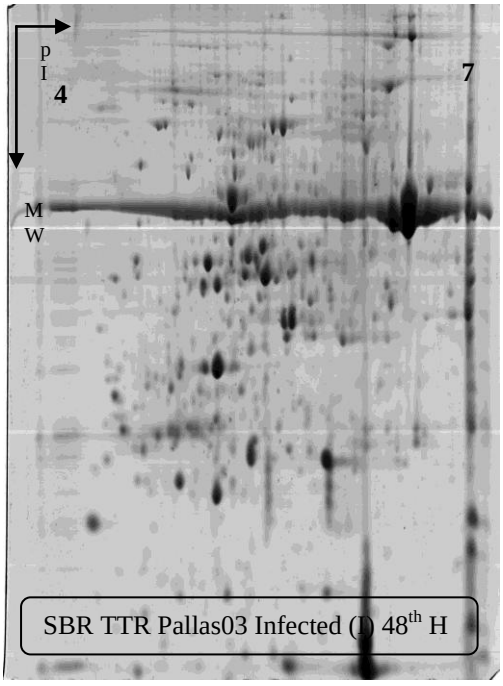
SBR TTR Pallas03 Control (C) 48th H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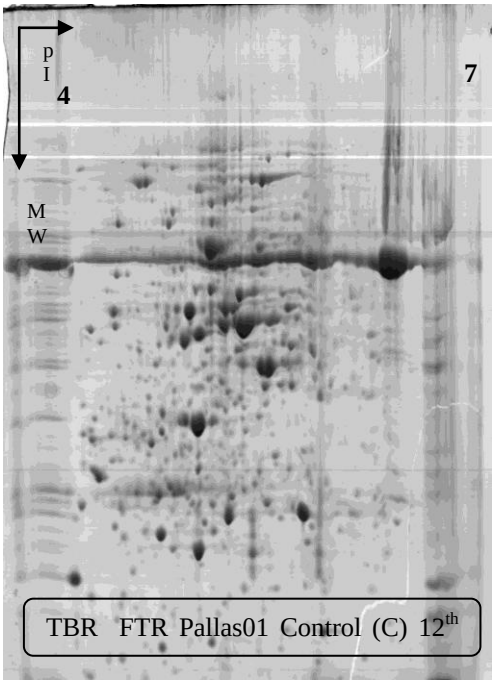
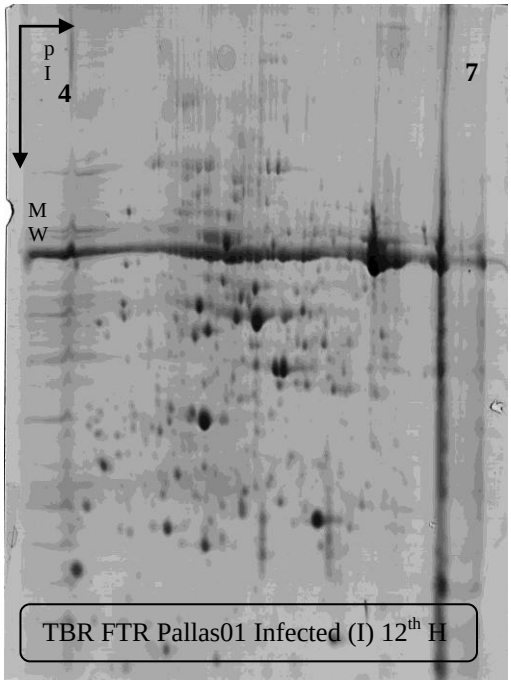
SBR TTR Pallas03 Infected (I) 48th H

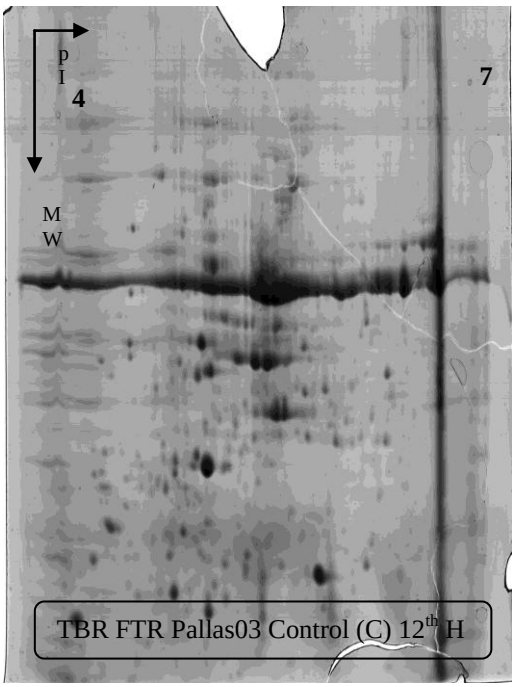
Third Biologic Replicate (TBR) First Technical Replicate (FTR) Gel Pictures



TBR FTR Pallas01 Control (C) 12th



TBR FTR Pallas01 Infected (I) 12th H



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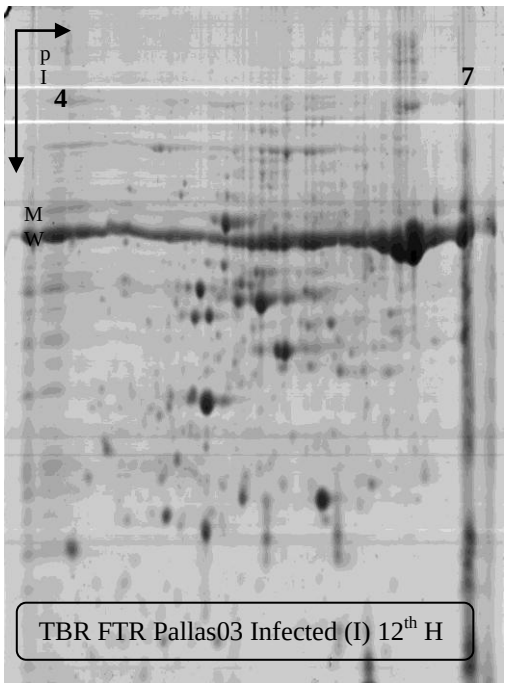

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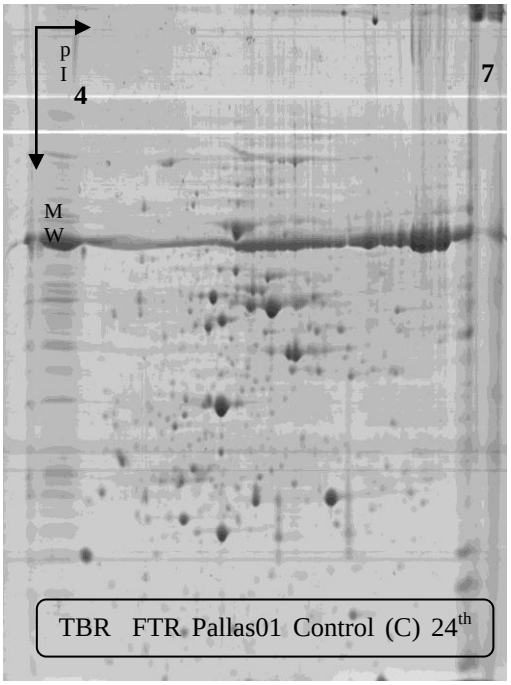
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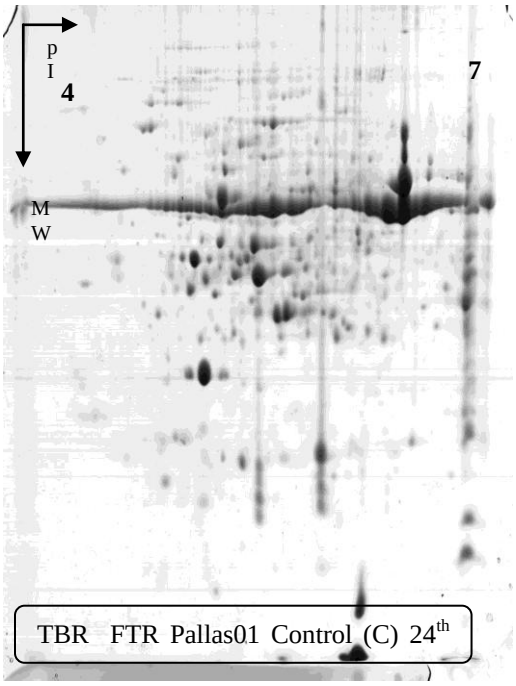
TBR FTR Pallas01 Control (C) 24th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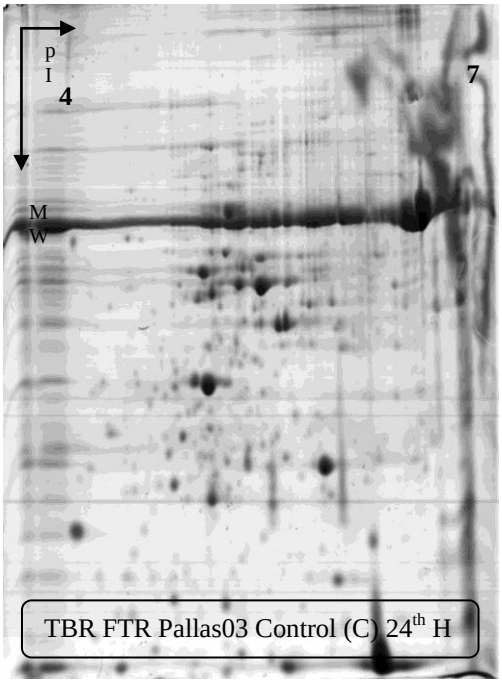
TBR FTR Pallas01 Control (C) 24th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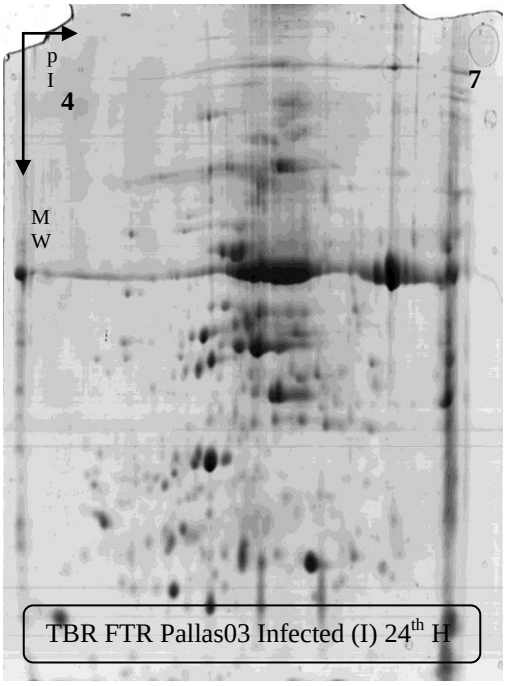
TBR FTR Pallas03 Control (C) 24th H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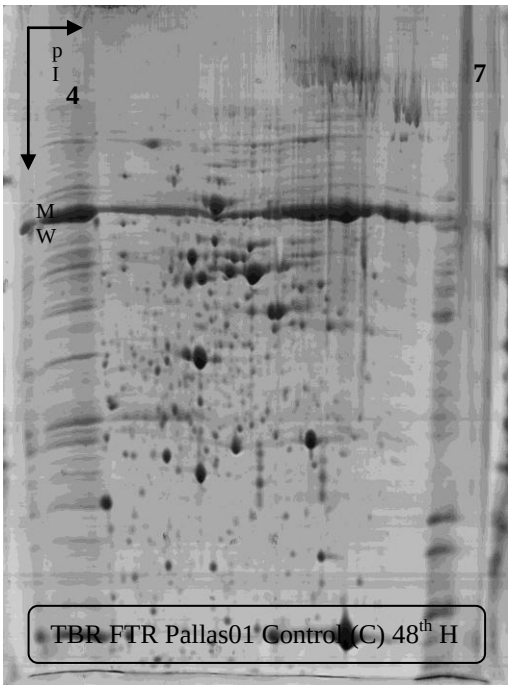
TBR FTR Pallas03 Infected (I) 24th H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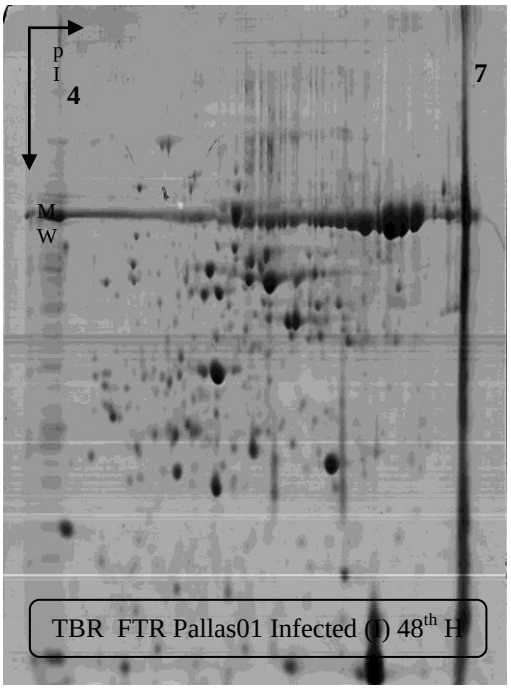
TBR FTR Pallas01 Control (C) 48th H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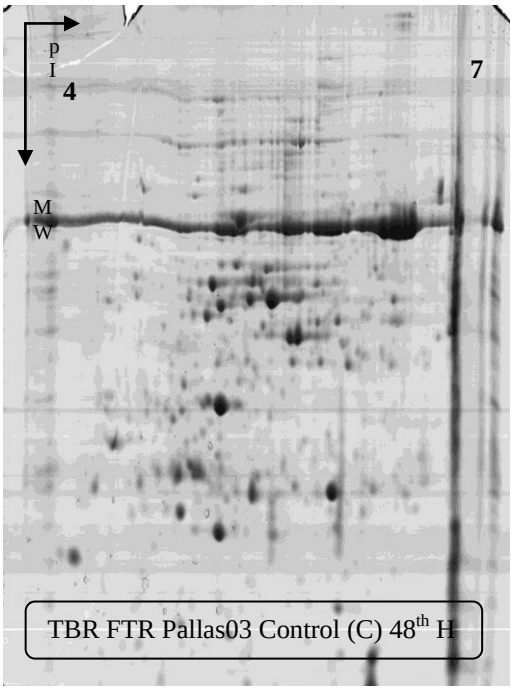
TBR FTR Pallas01 Infected (I) 48th H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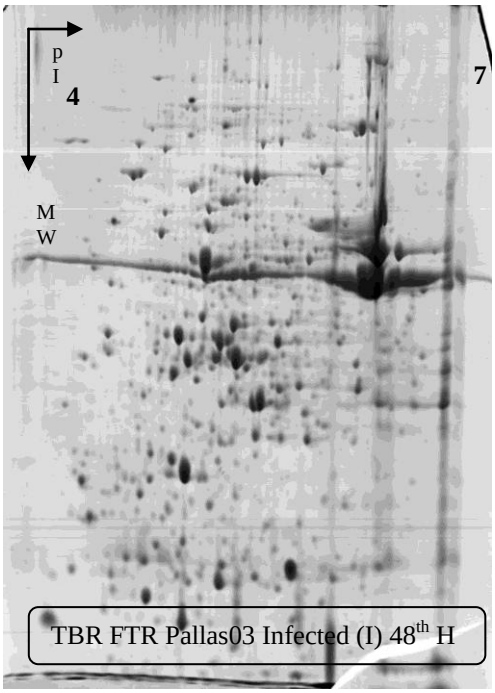
TBR FTR Pallas03 Control (C) 48th H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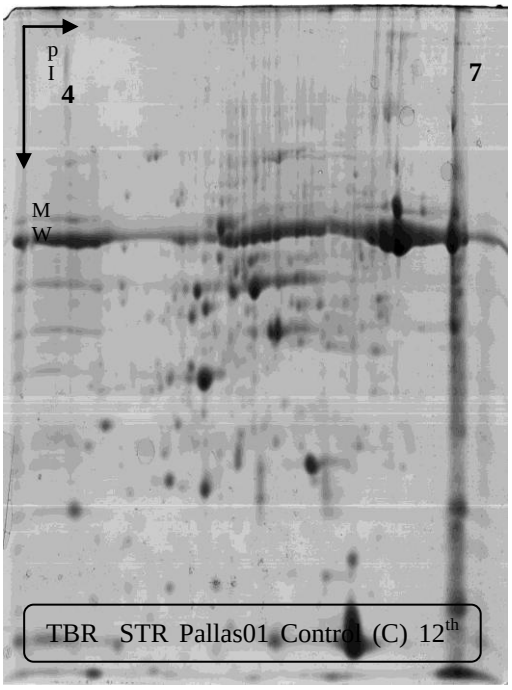
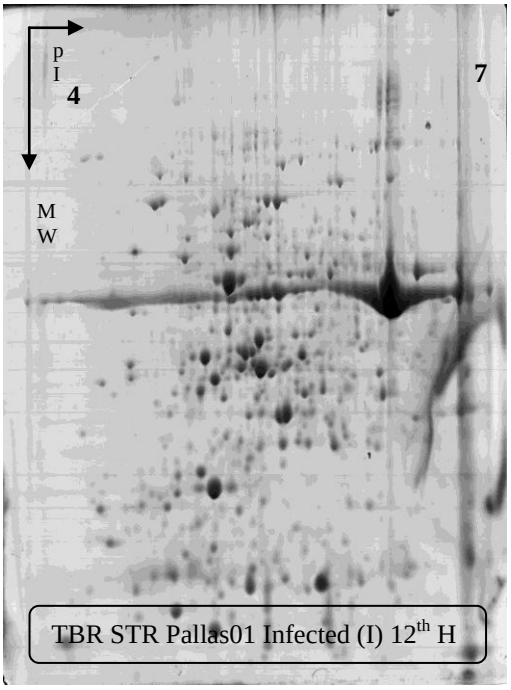
TBR FTR Pallas03 Infected (I) 48th H

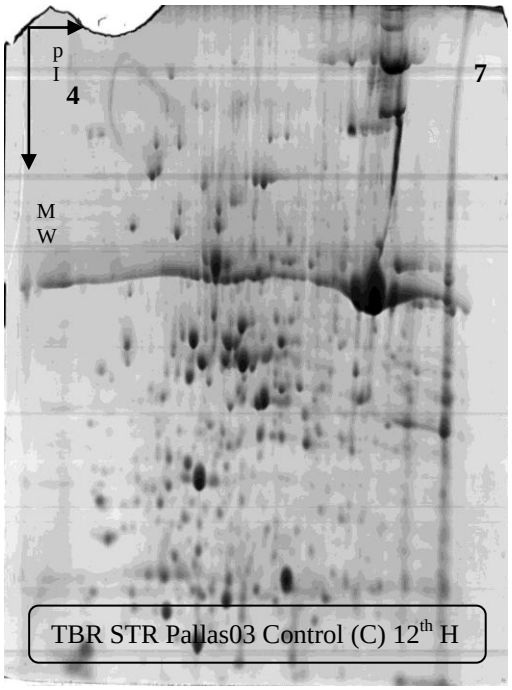
Third Biologic Replicate (TBR) Second Technical Replicate (STR) Gel Pictures



TBR STR Pallas01 Control (C) 12th



TBR STR Pallas01 Infected (I) 12th H



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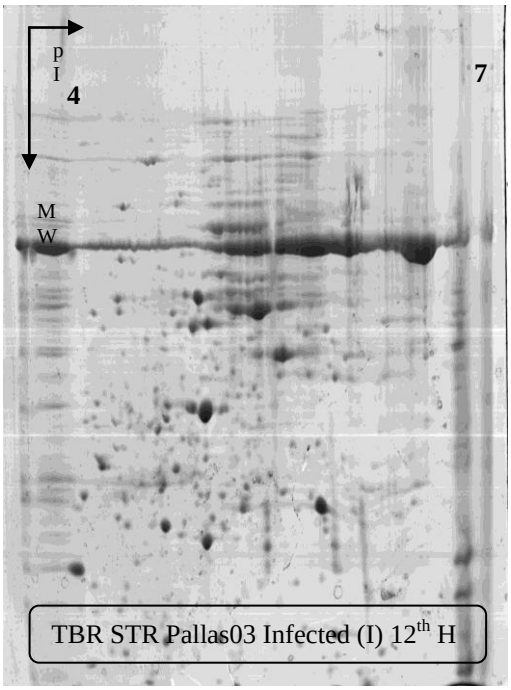
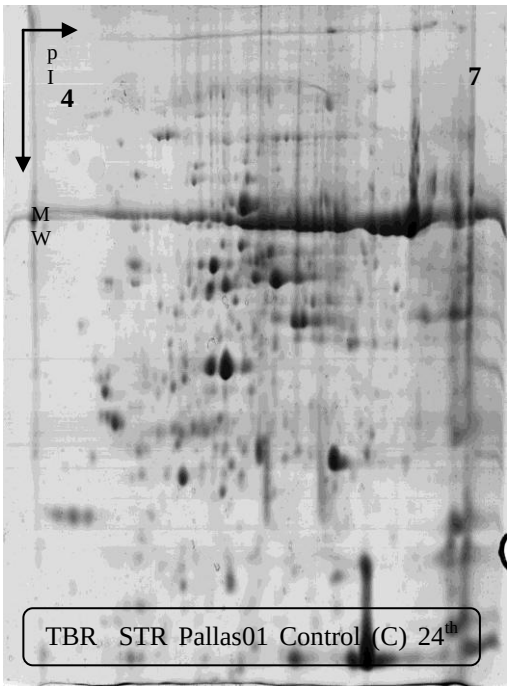


TBR STR Pallas03 Infected (I) 12th H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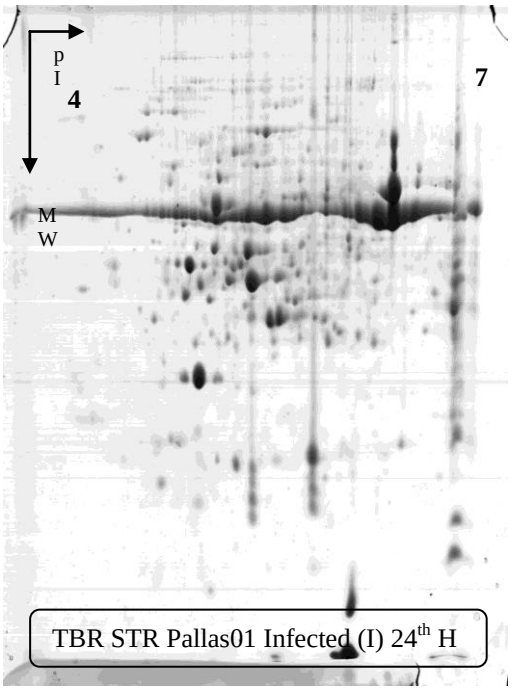
TBR STR Pallas01 Control (C) 24th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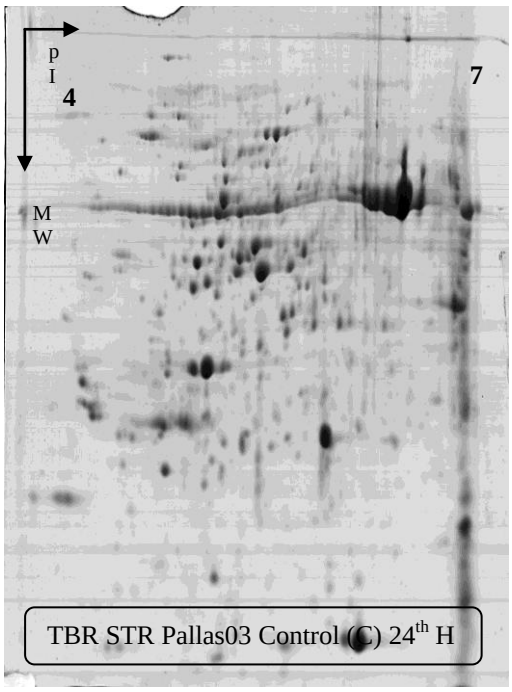
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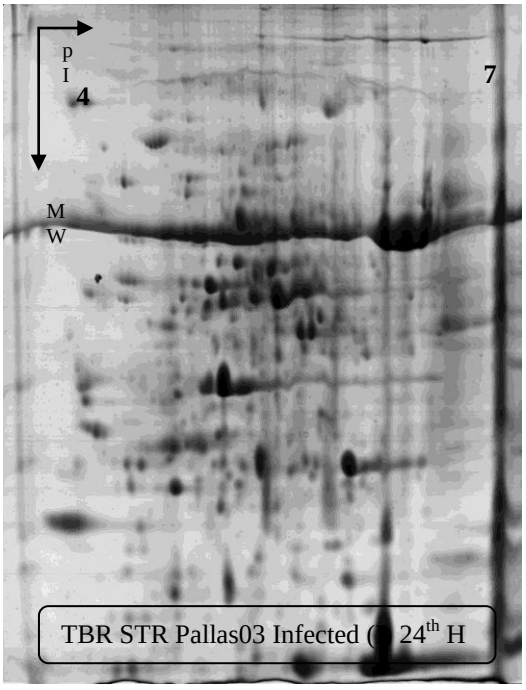
TBR STR Pallas03 Control (C) 24th H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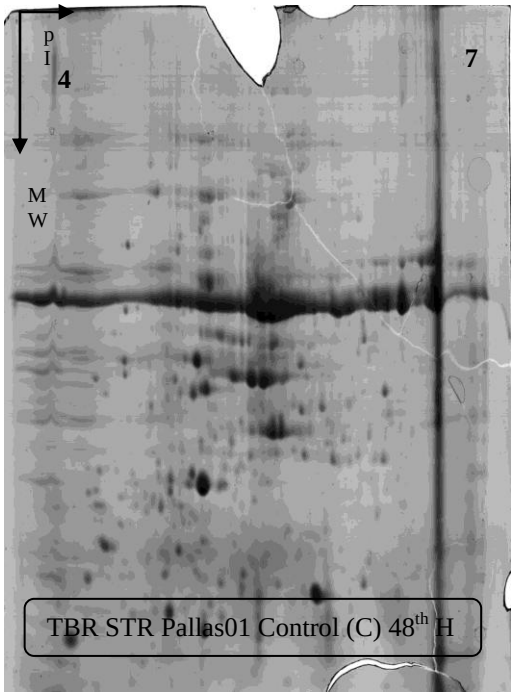
TBR STR Pallas03 Infected (I) 24th H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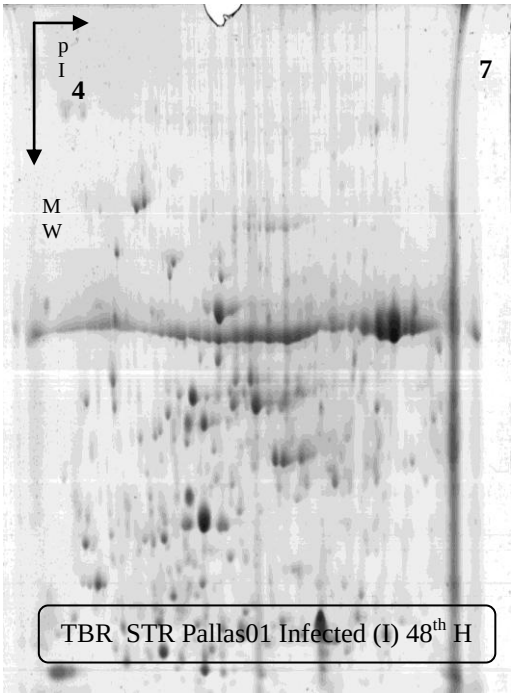
TBR STR Pallas01 Control (C) 48th H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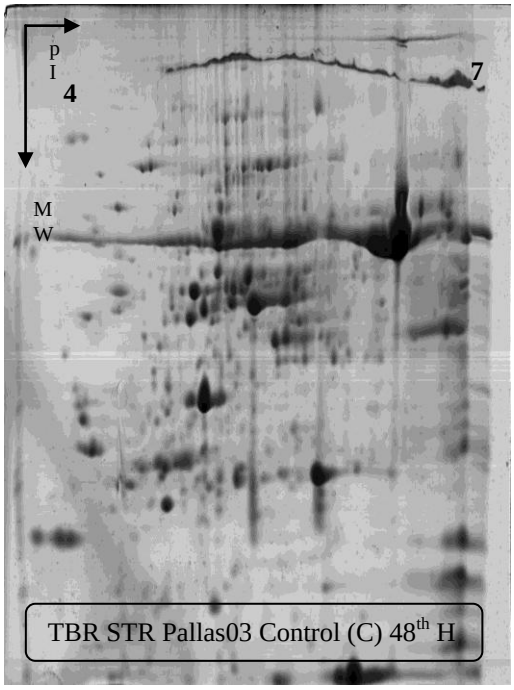
TBR STR Pallas01 Infected (I) 48th H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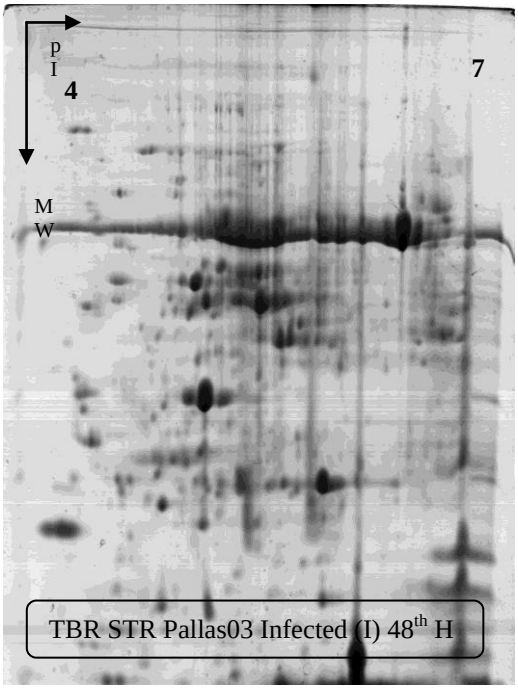
TBR STR Pallas03 Control (C) 48th H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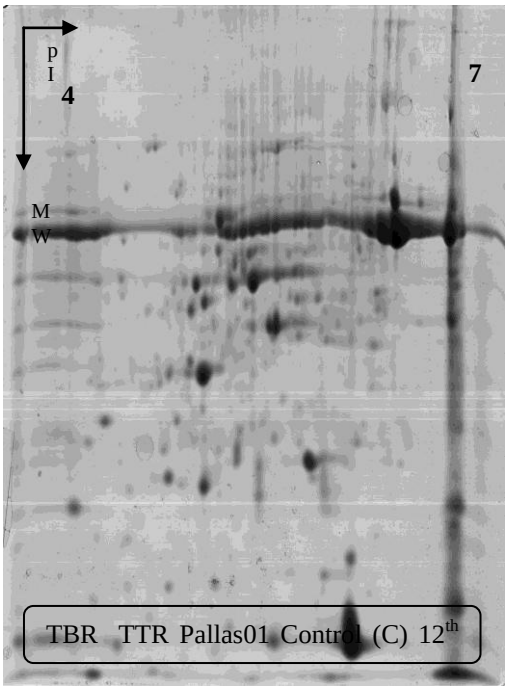
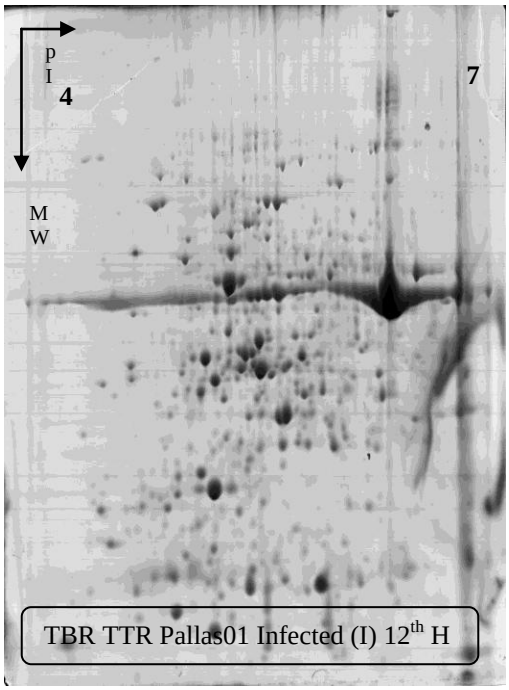
TBR STR Pallas03 Infected (I) 48th H

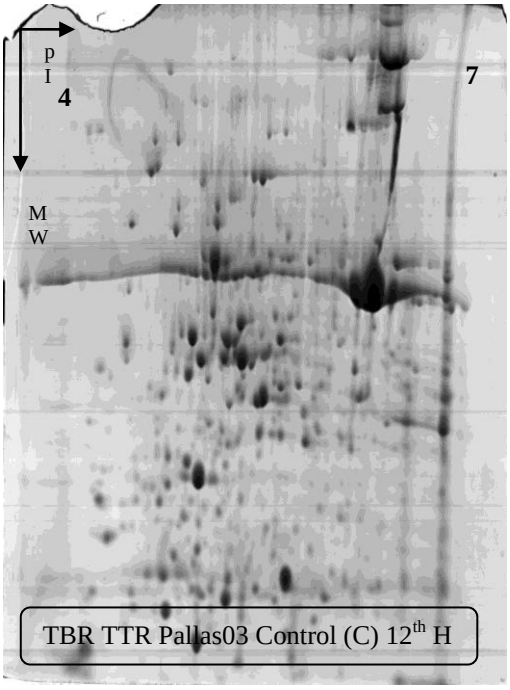
Third Biologic Replicate (TBR) Third Technical Replicate (TTR) Gel Pictures



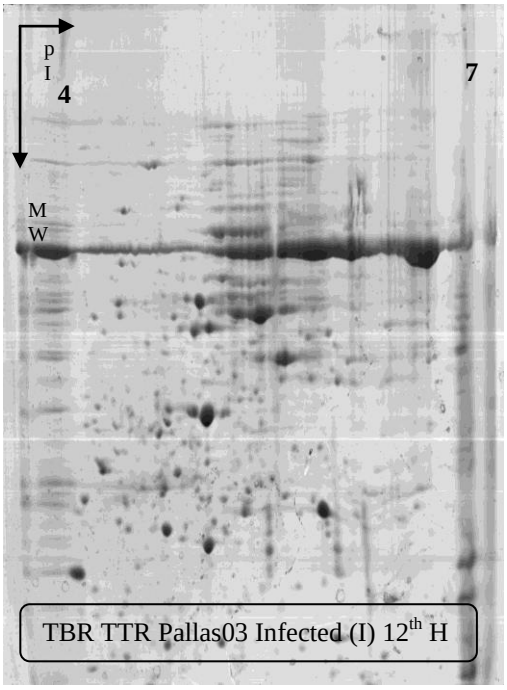
TBR TTR Pallas01 Control (C) 12th



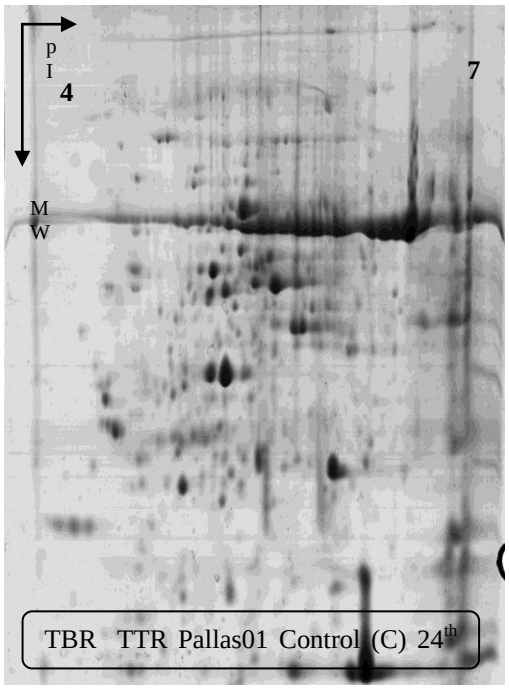
TBR TTR Pallas01 Infected (I) 12th H



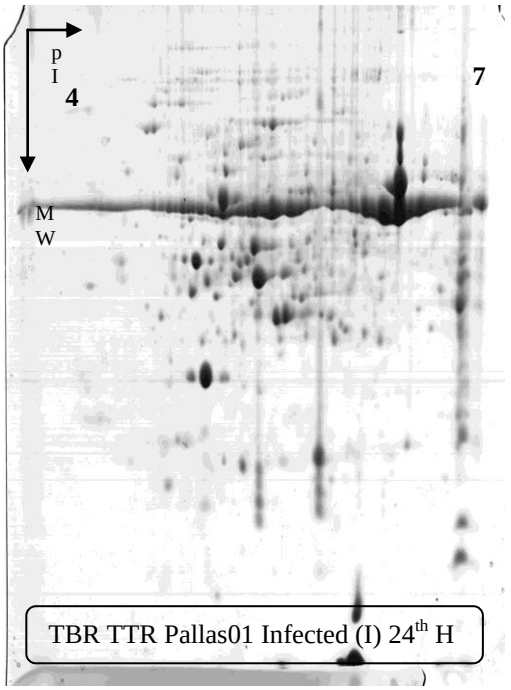
TBR TTR Pallas03 Control (C) 12th H



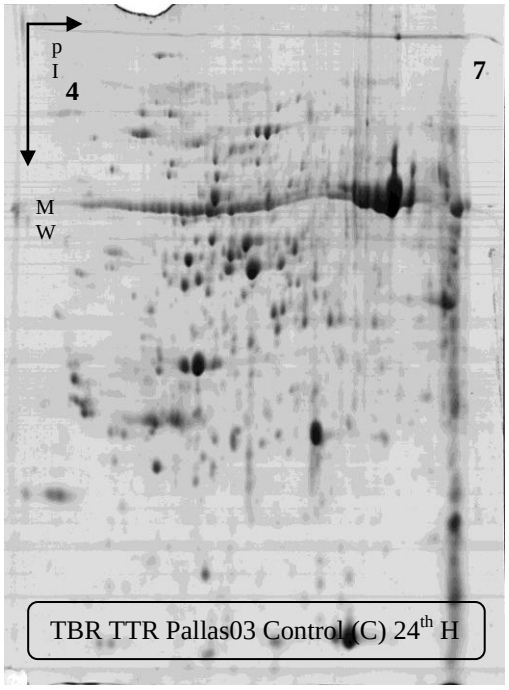
TBR TTR Pallas03 Infected (I) 12th H



TBR TTR Pallas01 Control (C) 24th



TBR TTR Pallas01 Infected (I) 24th H



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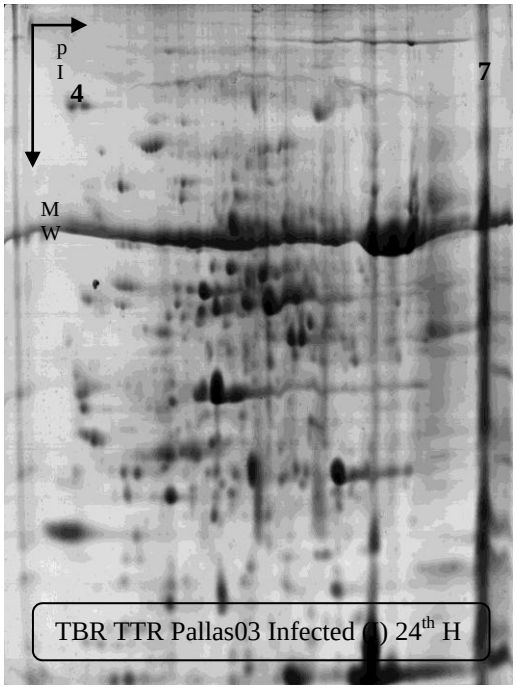

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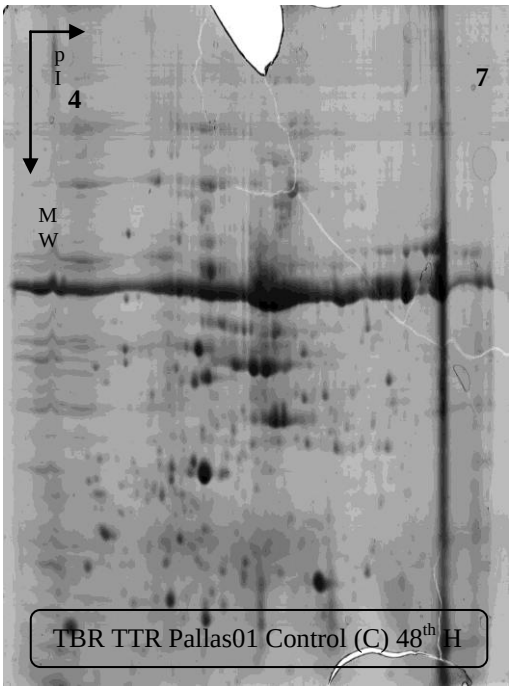
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7
M
W
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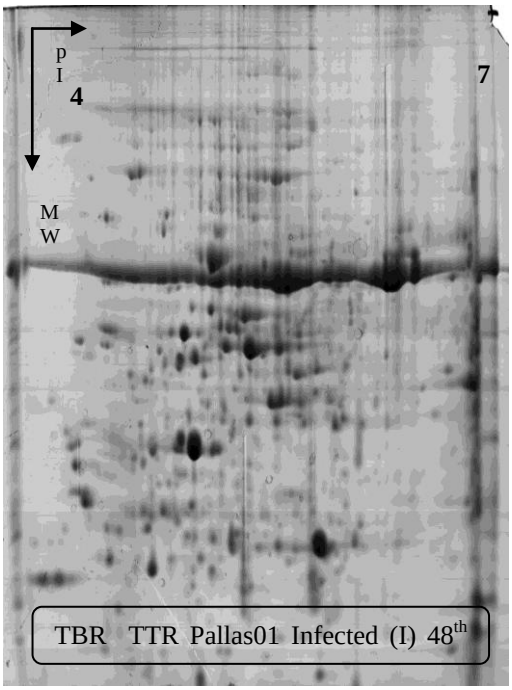
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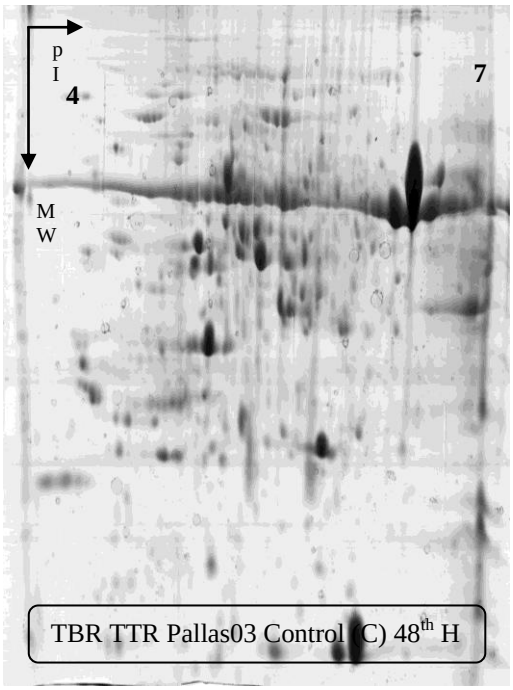
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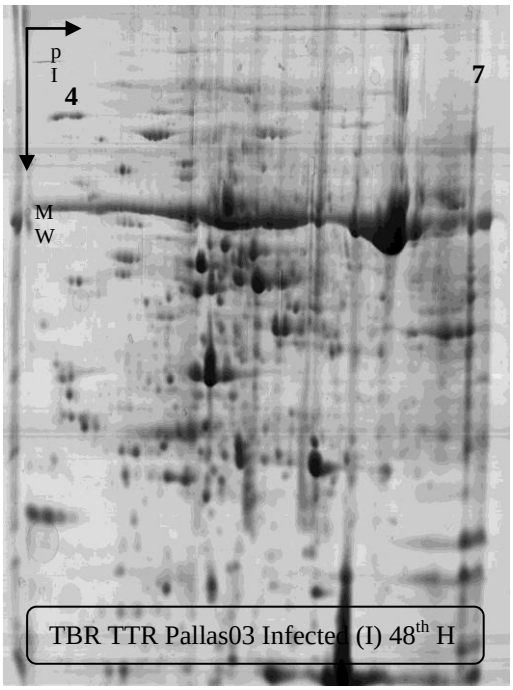
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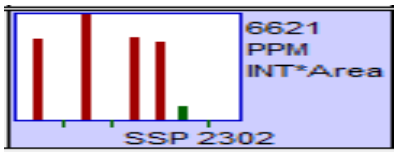
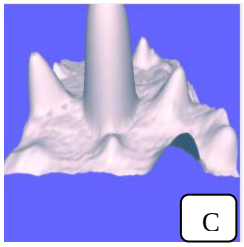
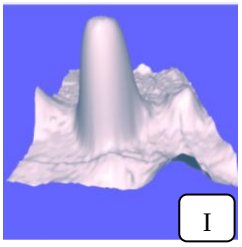
APPENDIX B

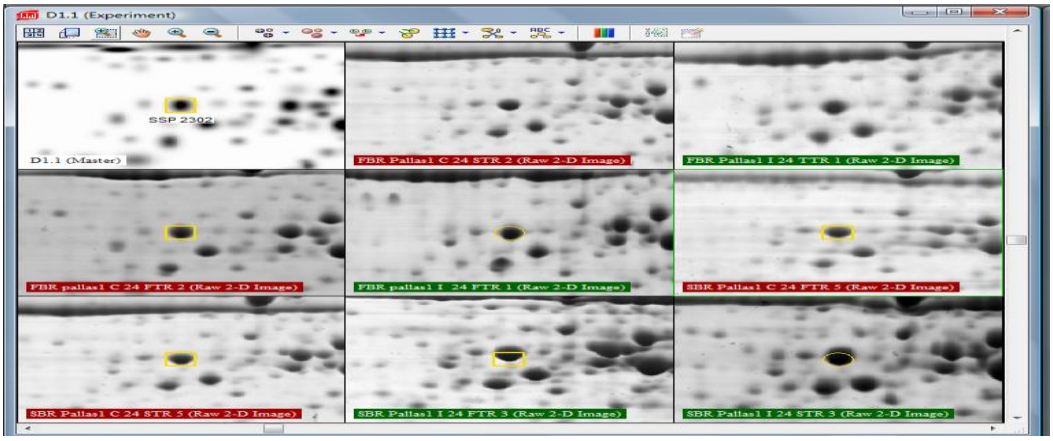
Selected Proteins Accoring to PDQuest

SSP2302









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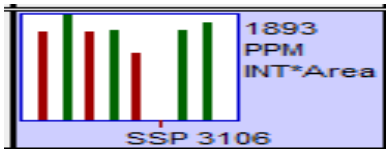

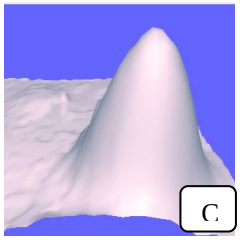

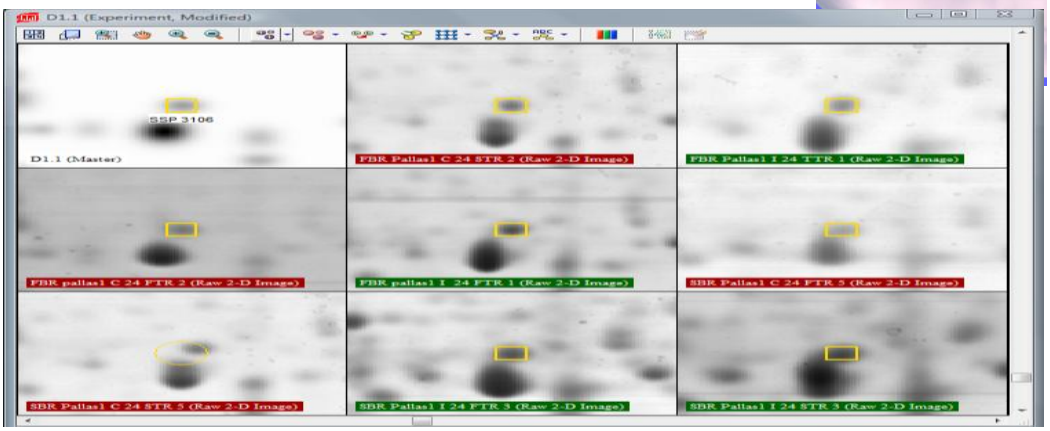

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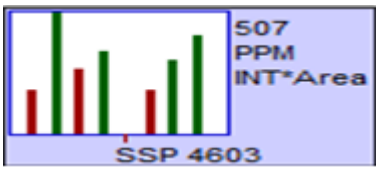

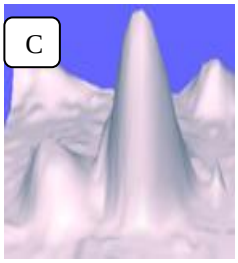

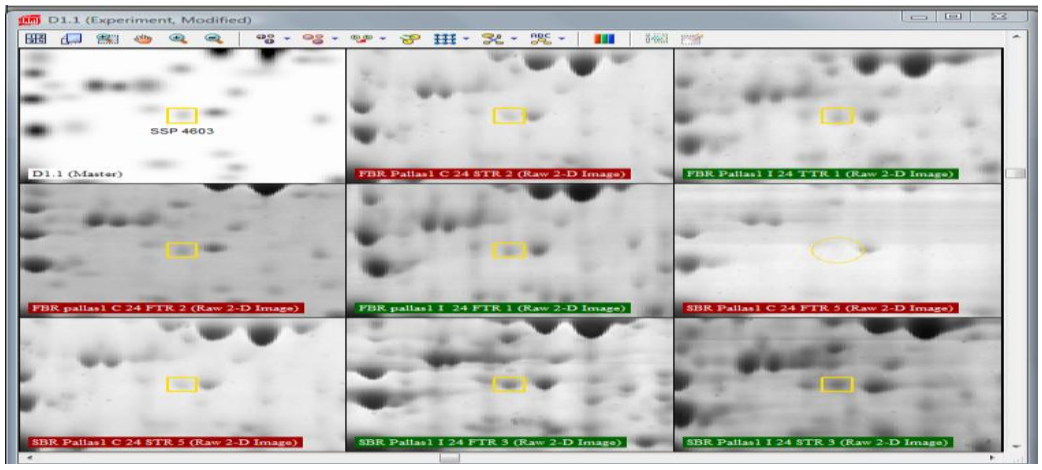

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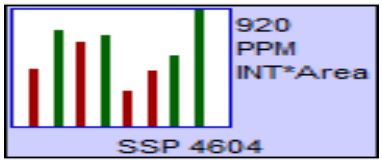

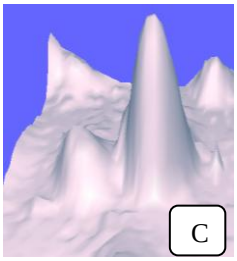

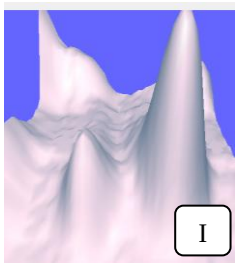

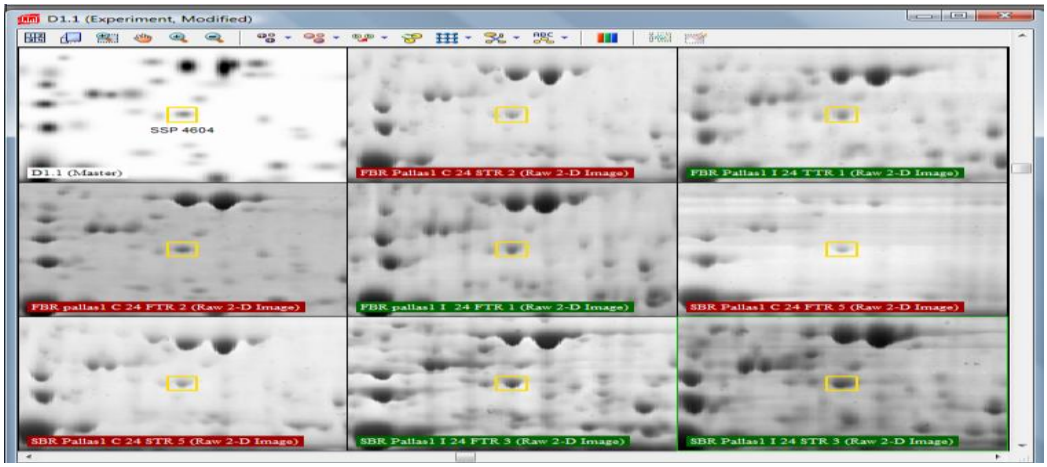

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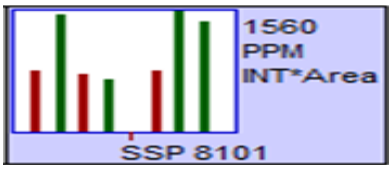

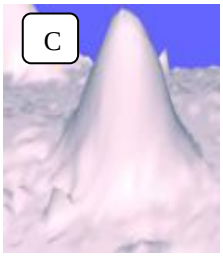

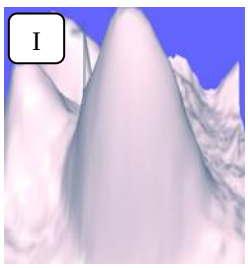

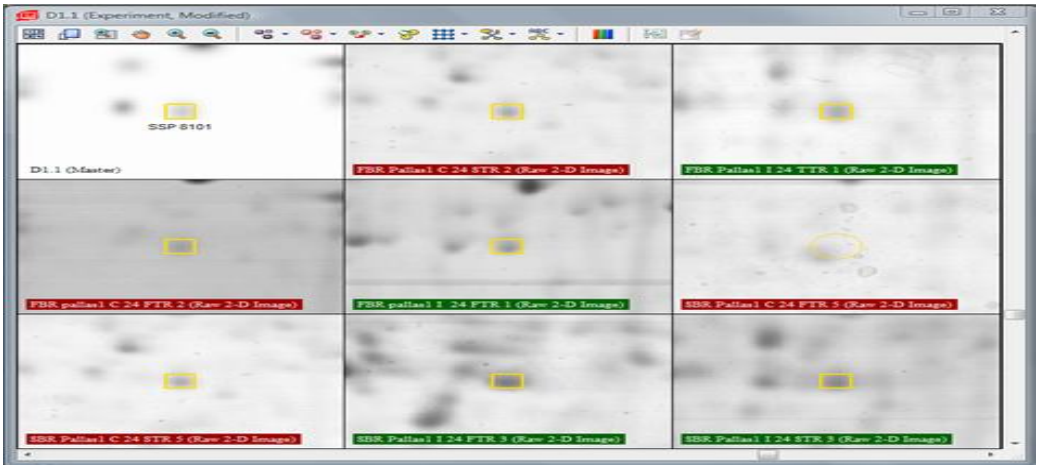

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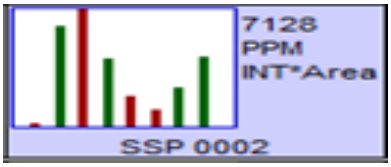

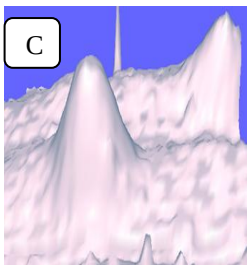

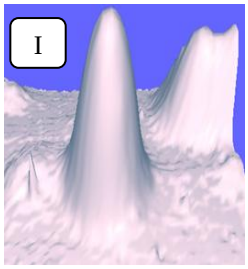

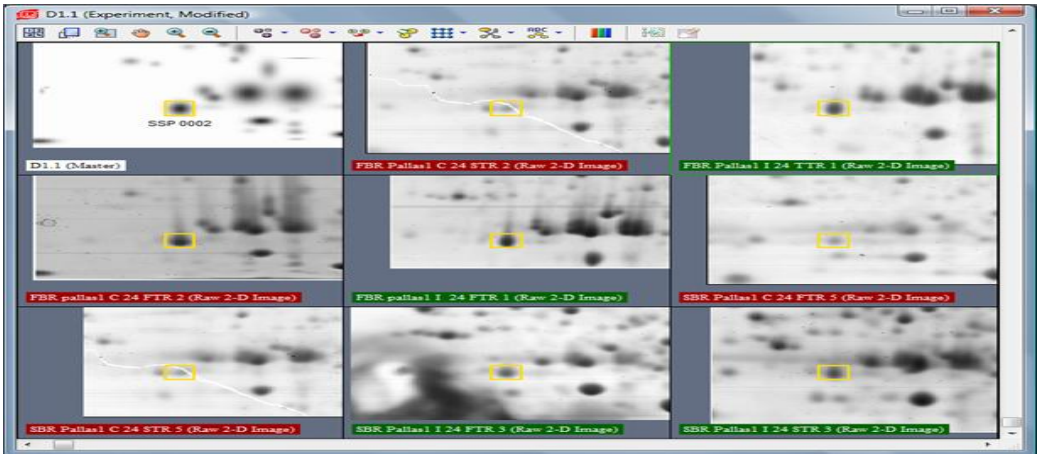

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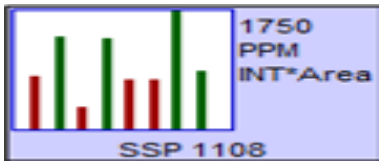

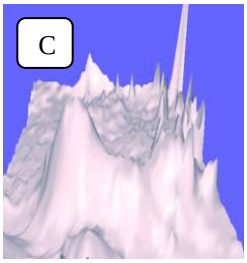

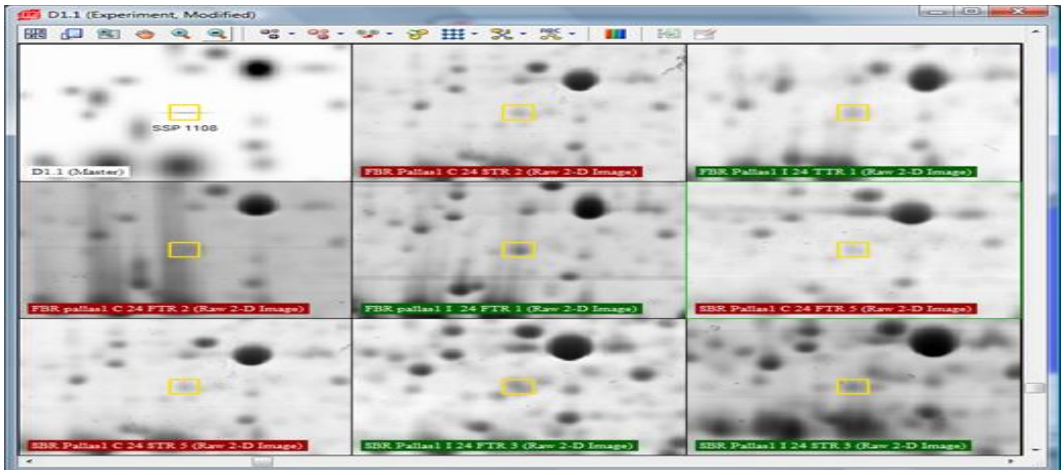

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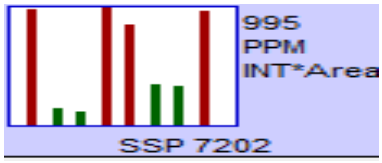

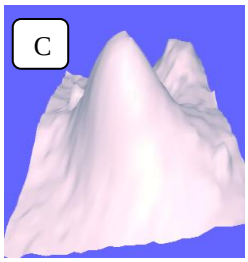

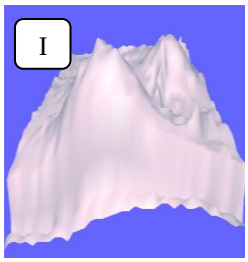

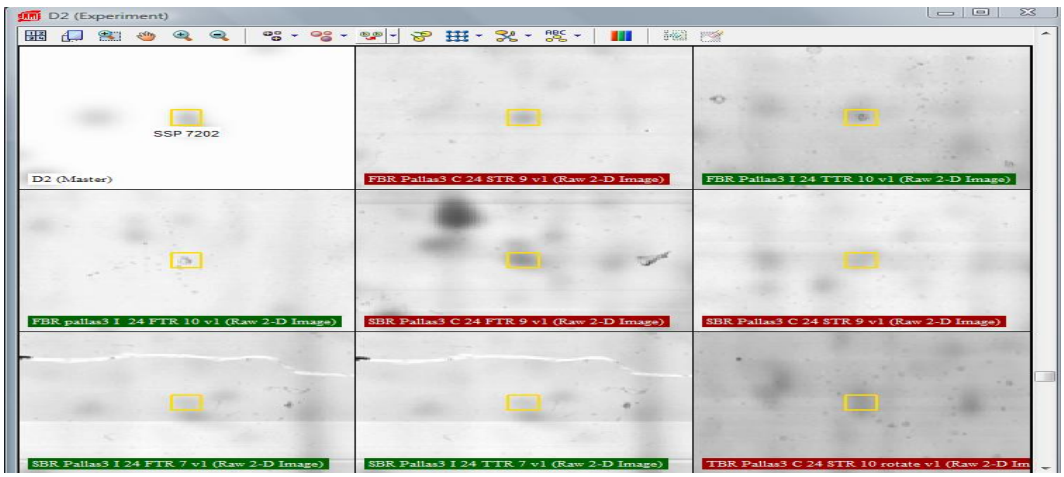

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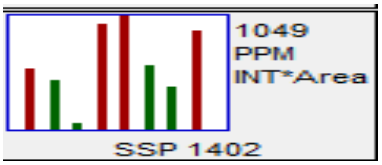

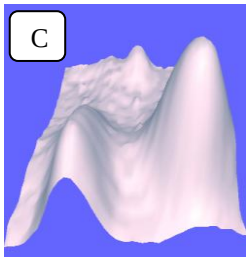

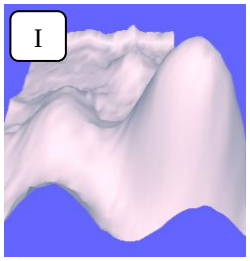

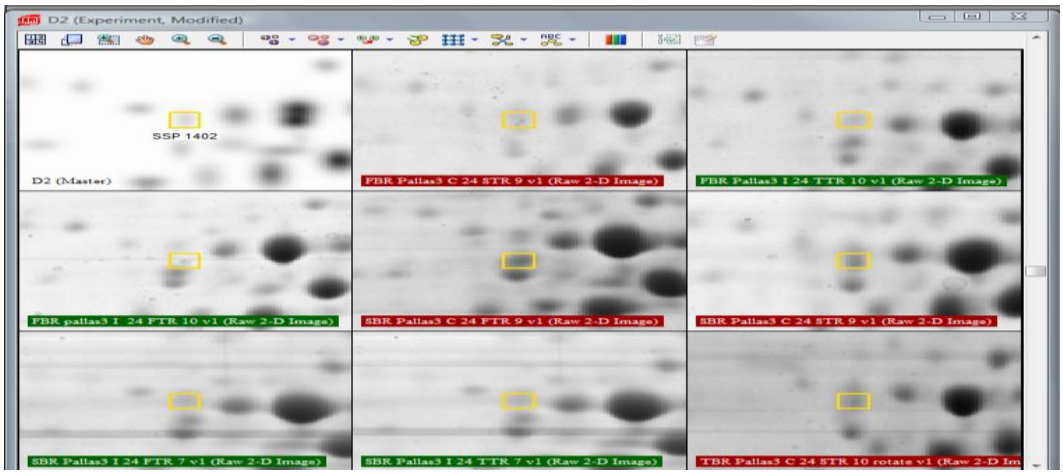

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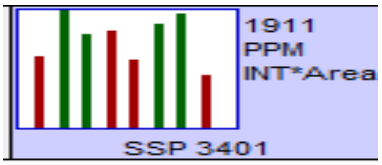

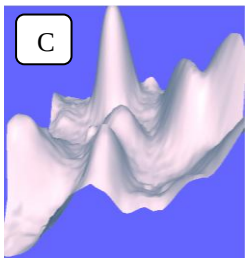

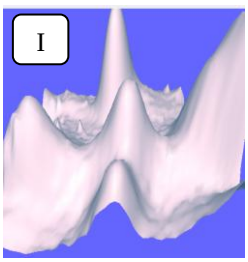

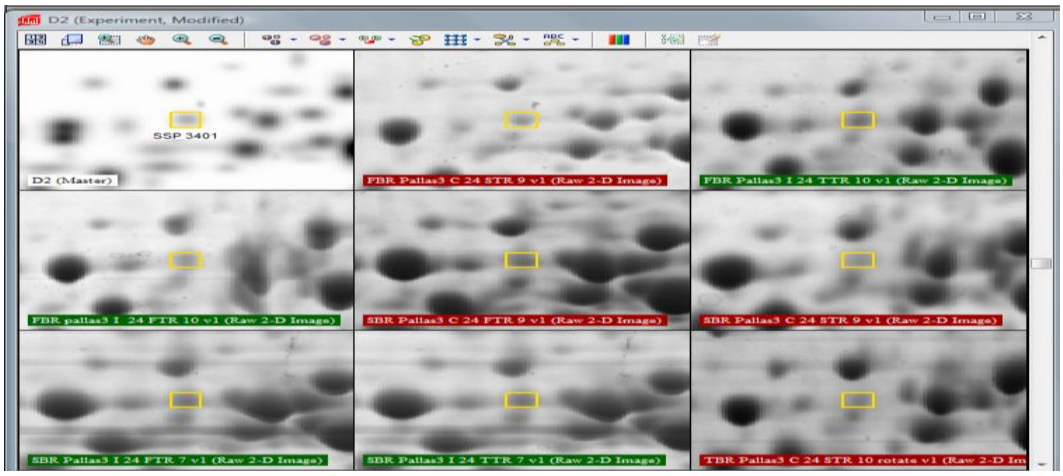

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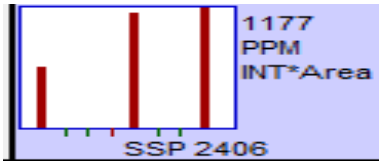

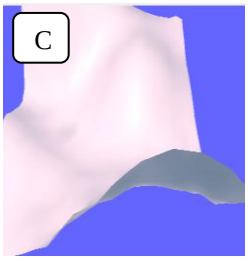

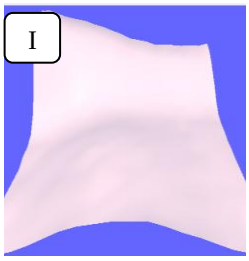

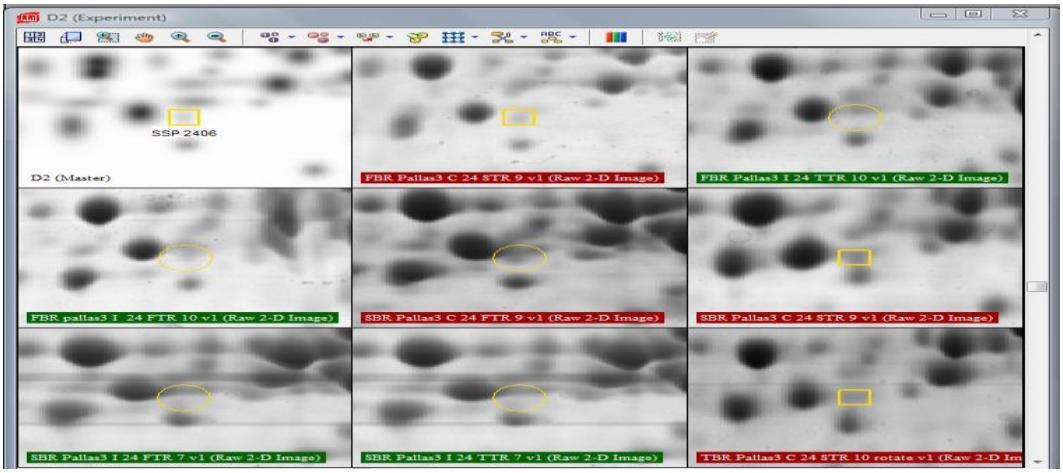

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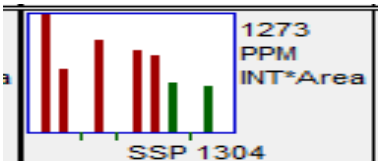
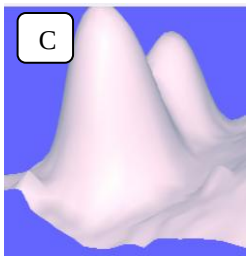
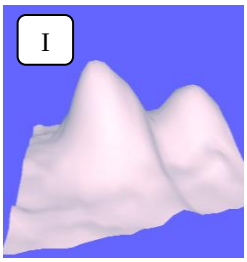
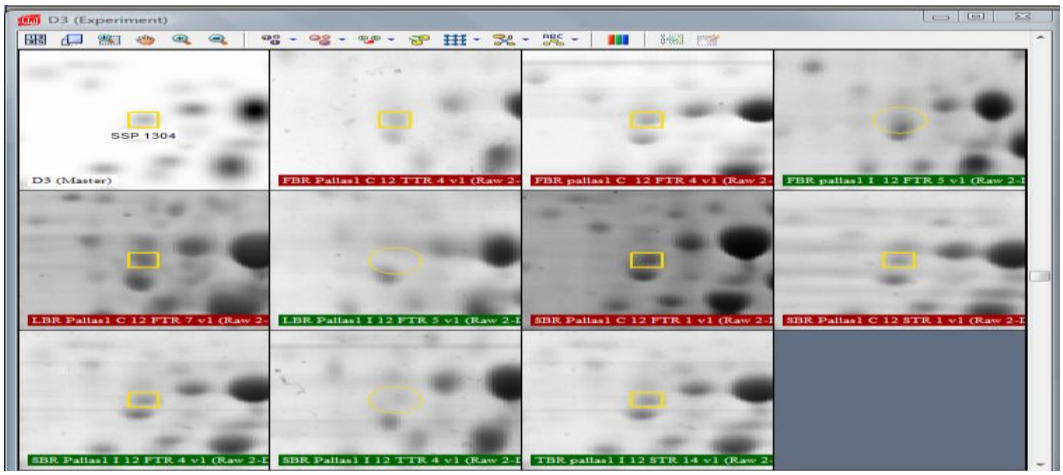
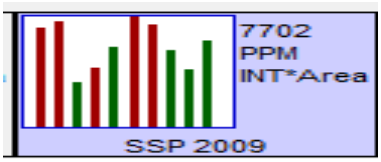


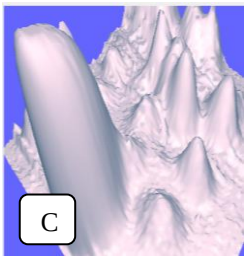


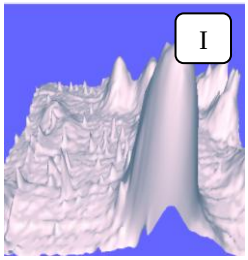


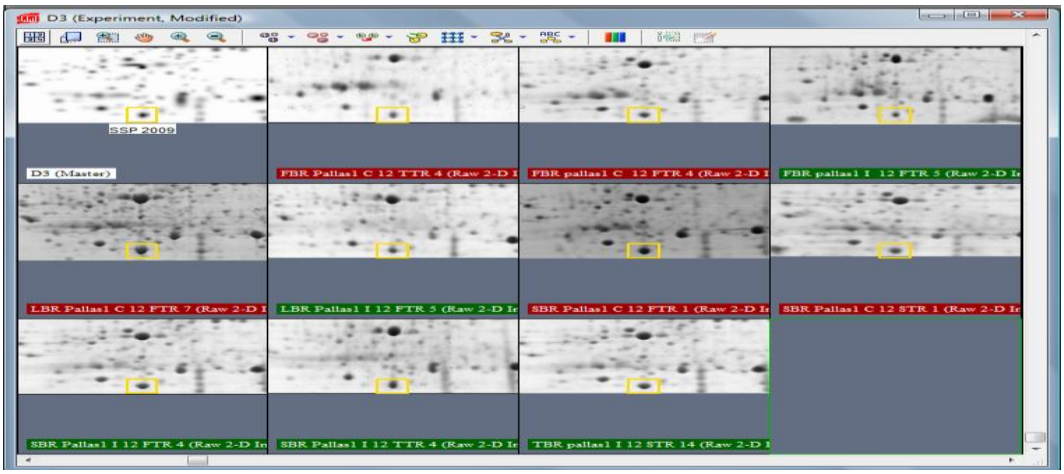


SSP 2009

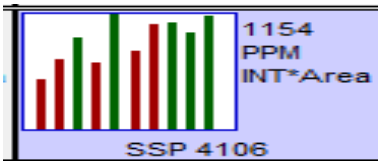


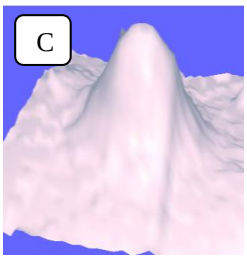


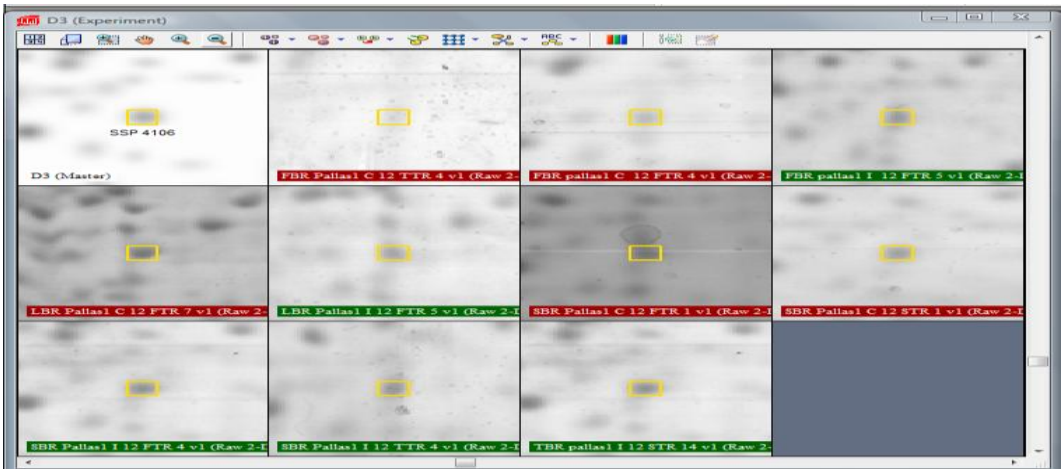




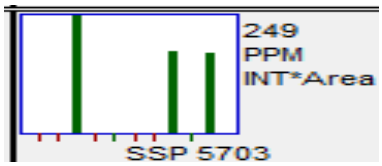
SSP 4106

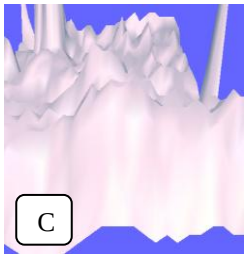


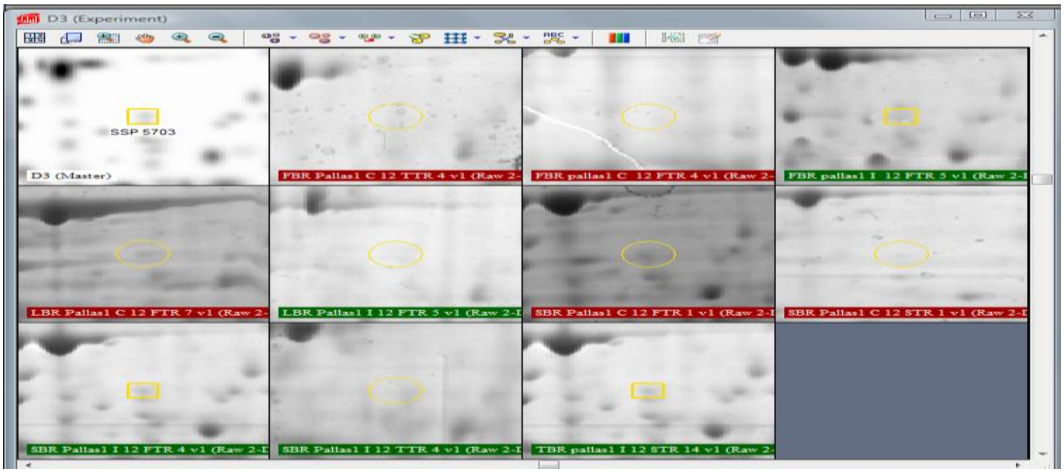




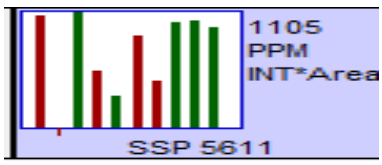
SSP 5703

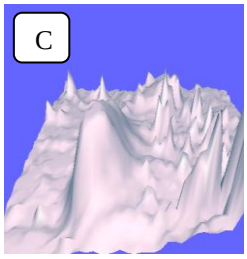


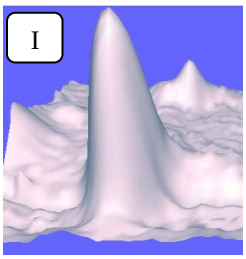


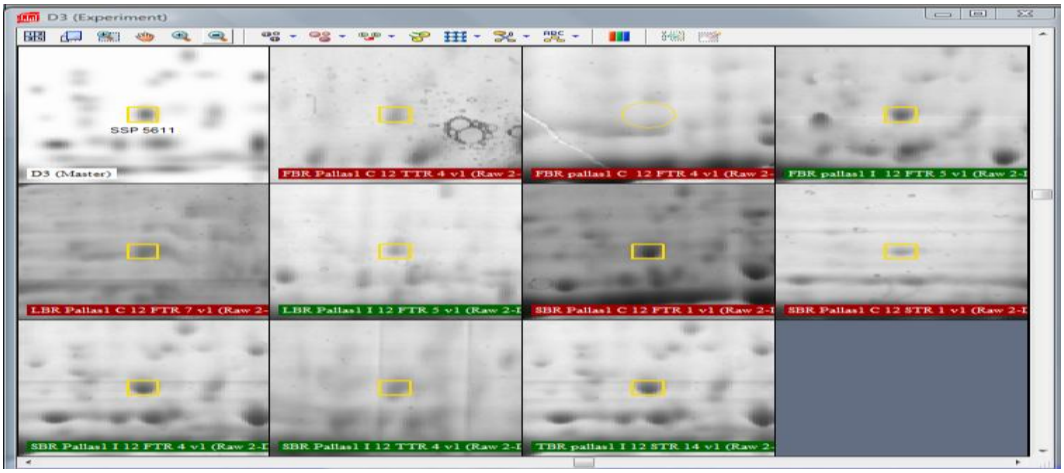


SSP 5611

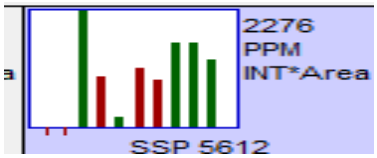


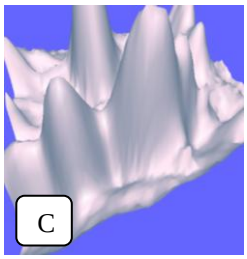


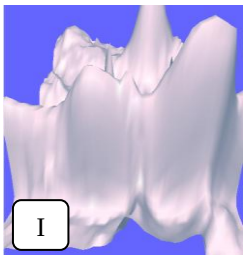


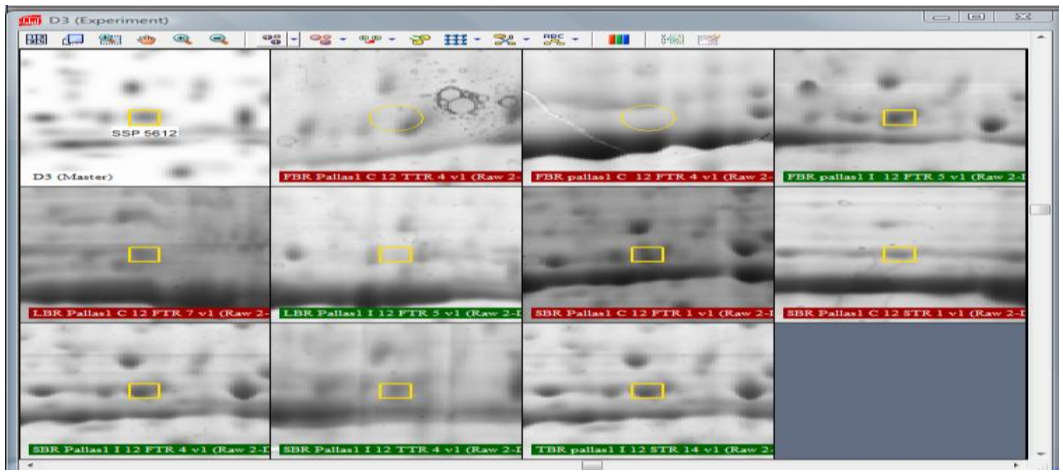


SSP 5612

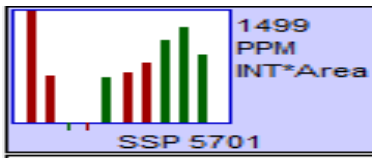


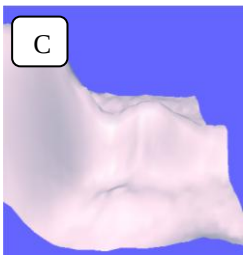


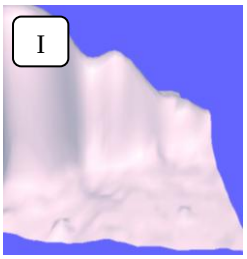


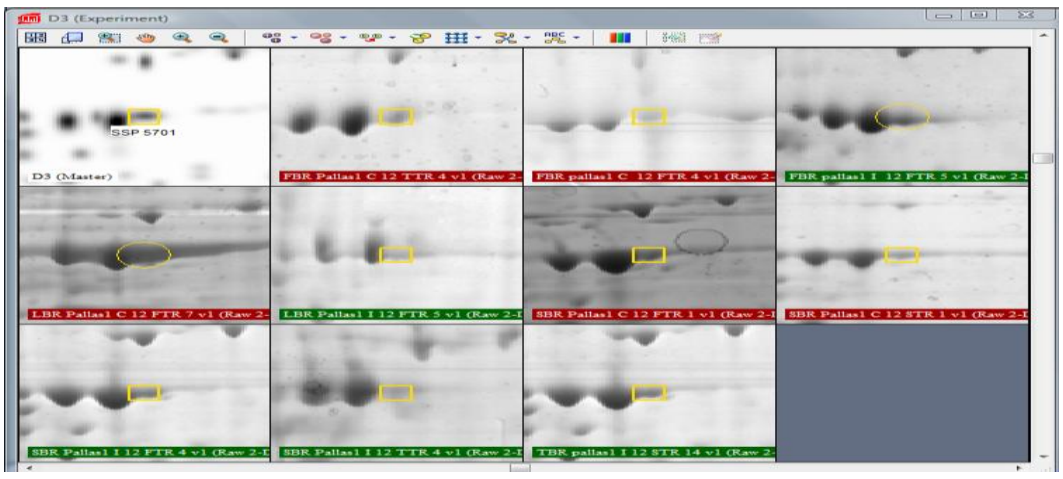


SSP5701

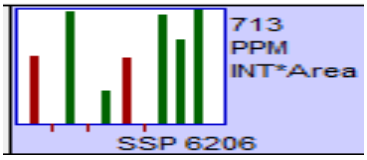


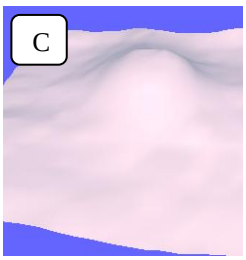


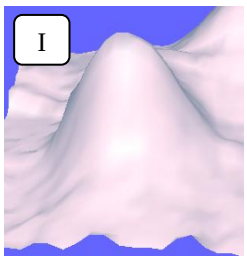


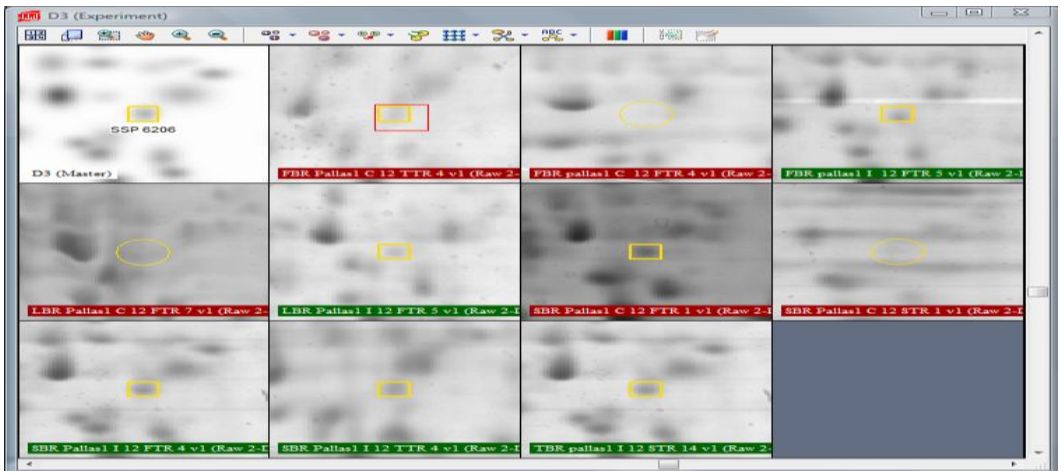


SSP6206

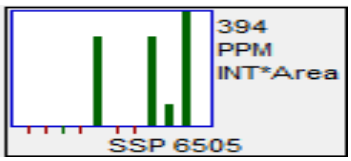


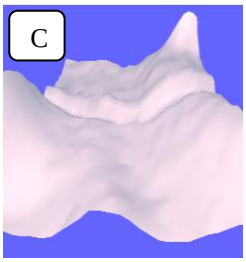


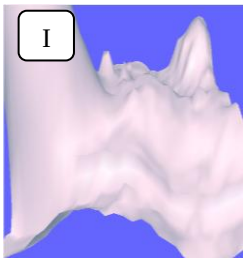


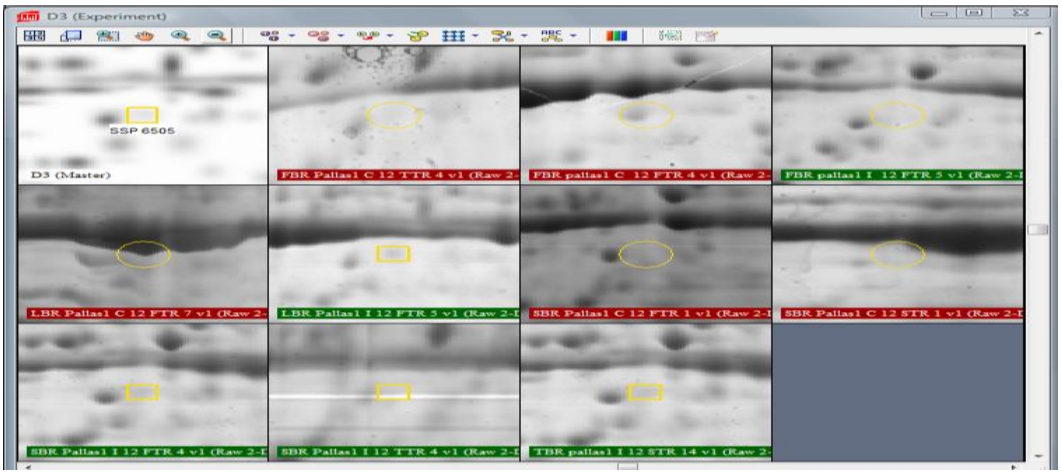


SSP6505

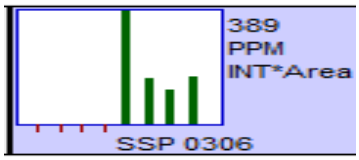


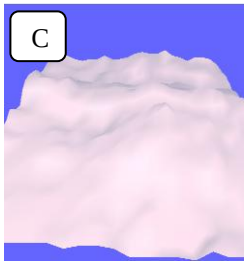


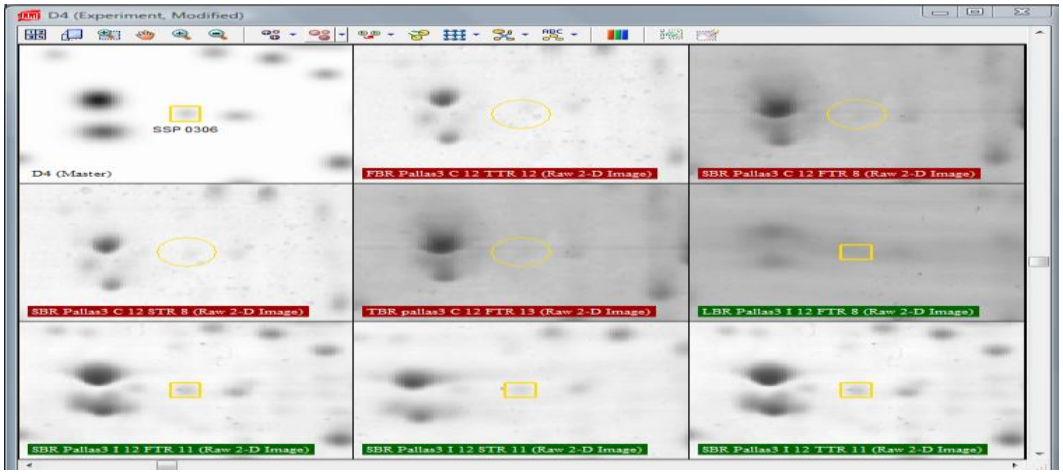




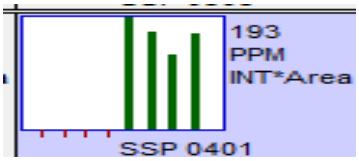
SSP 0306

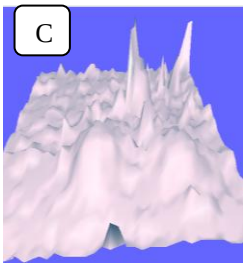


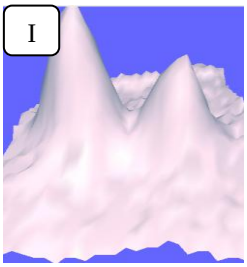


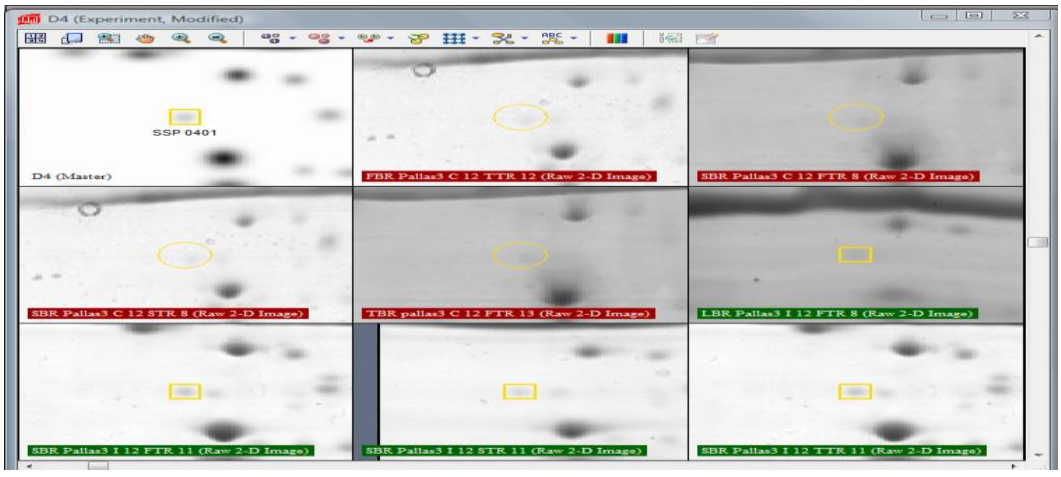


SSP 0401

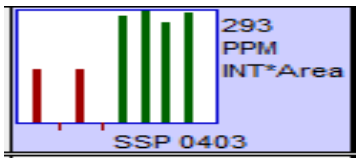


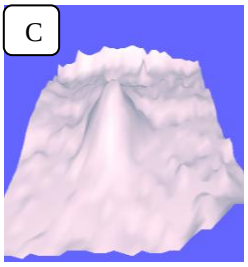


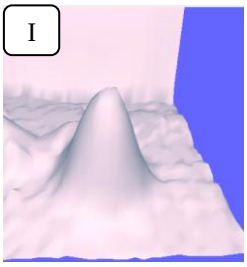


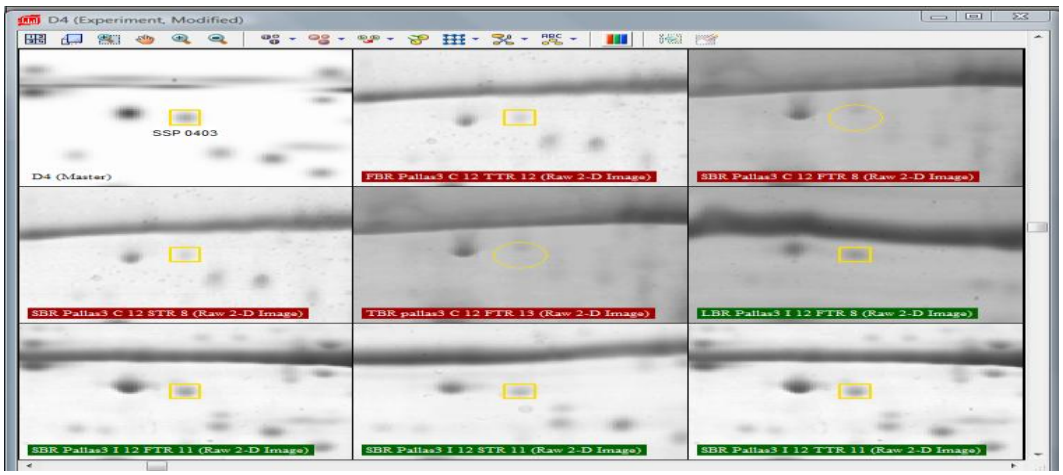


SSP 0403

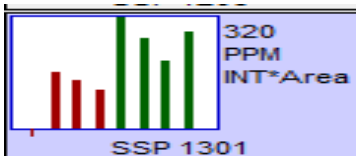


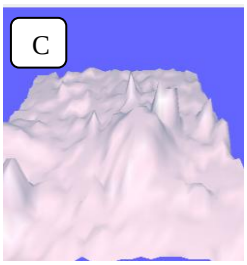


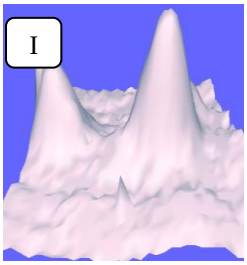


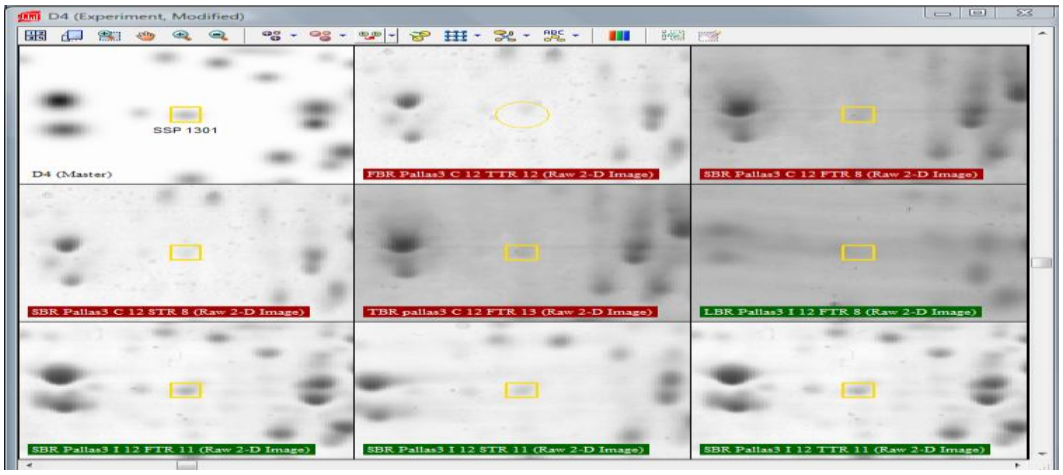


SSP 1301

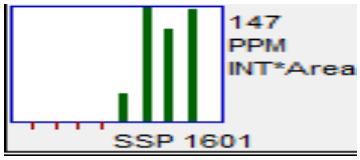


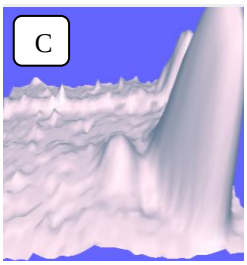


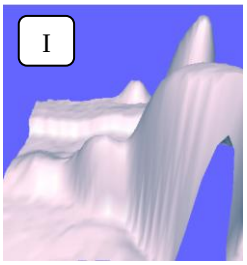


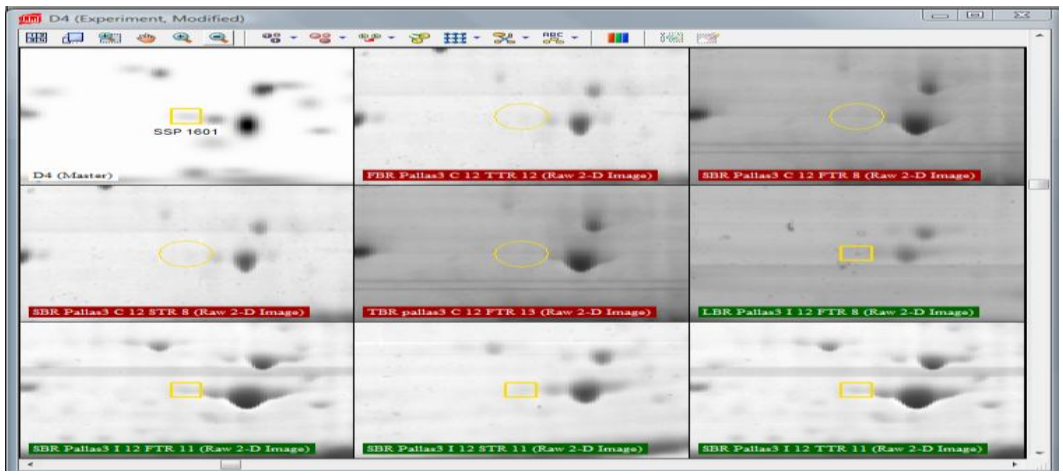


SSP 1601

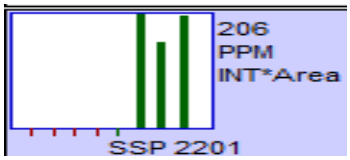


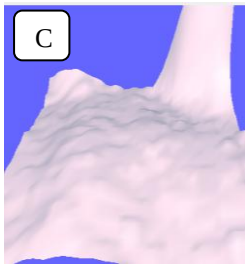


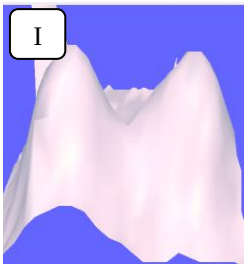


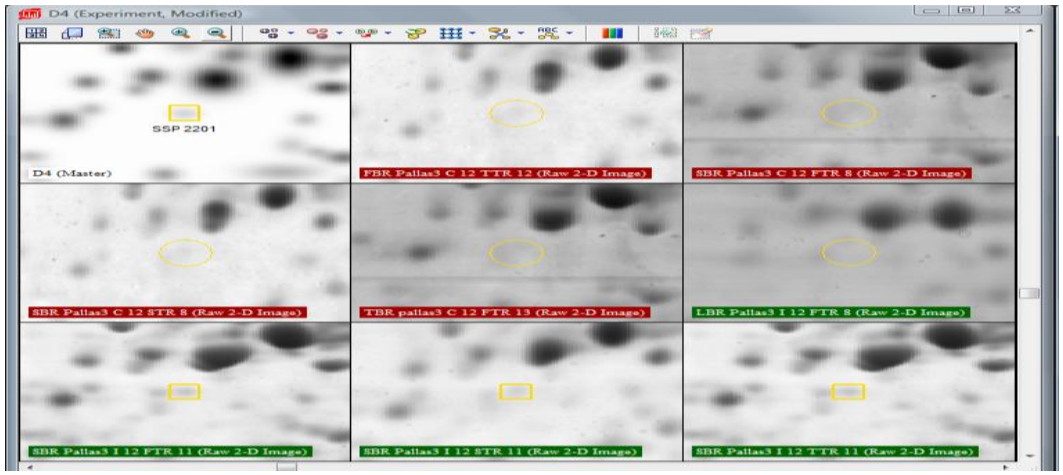


SSP 2201

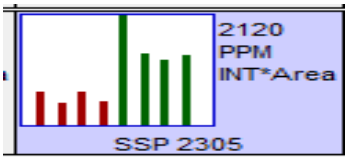


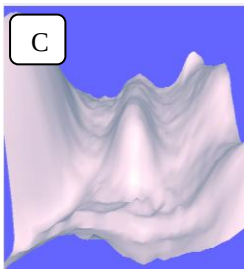


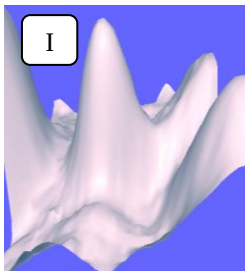


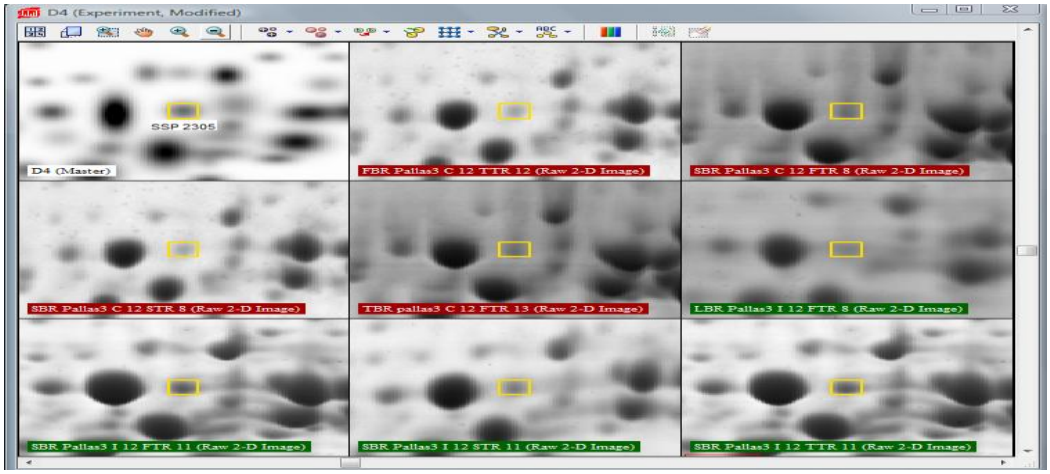


SSP 2305

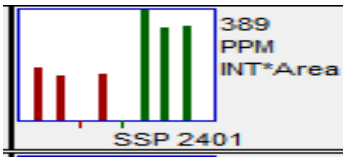


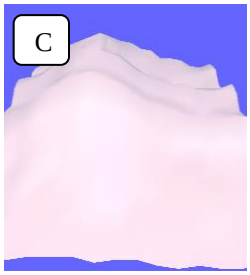


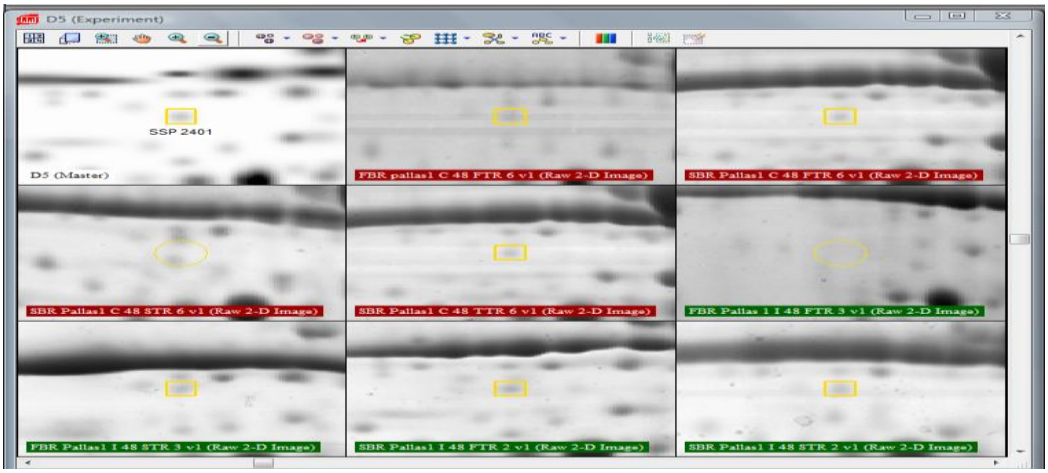




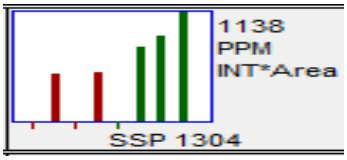
SSP 2401

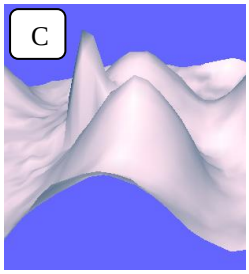


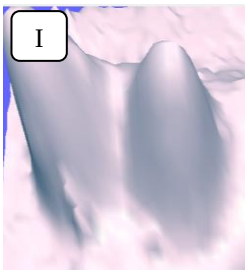


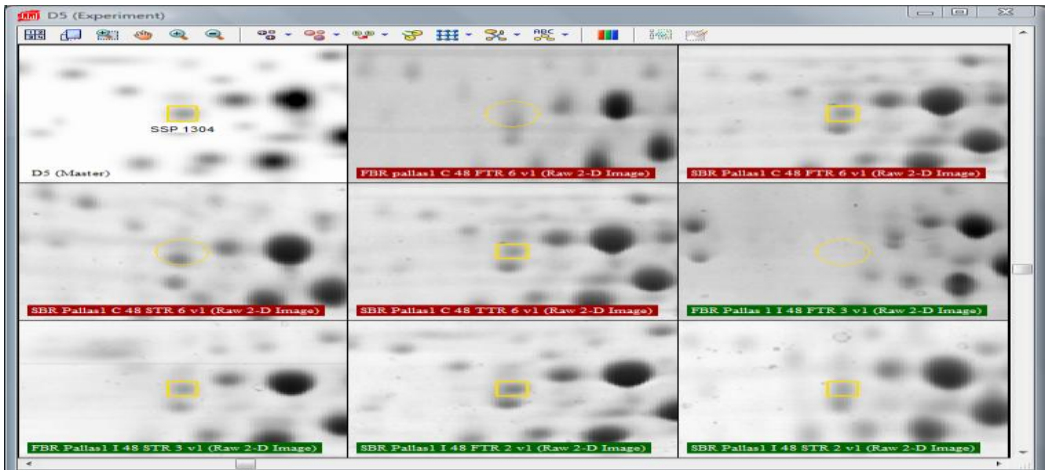


SSP 1304

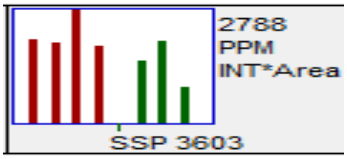


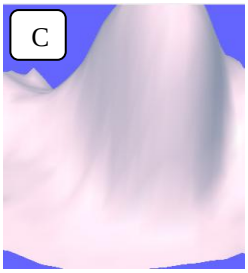


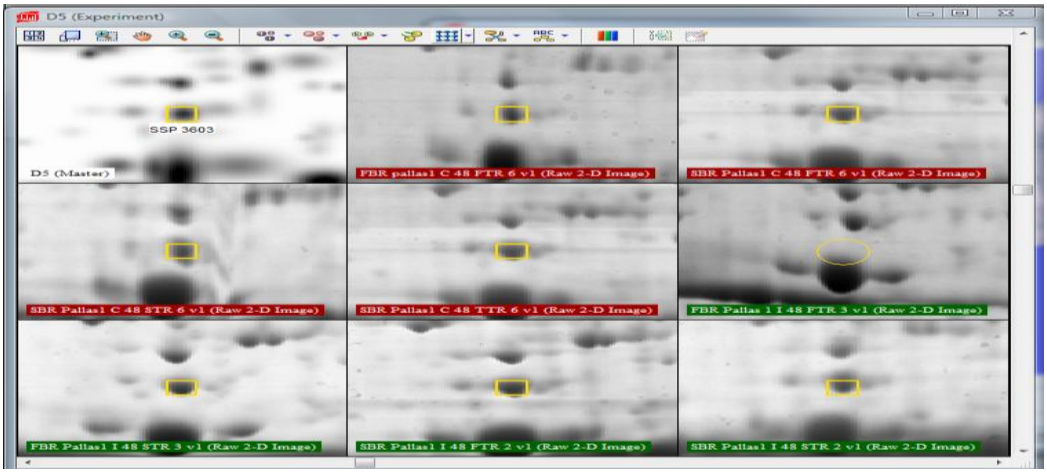




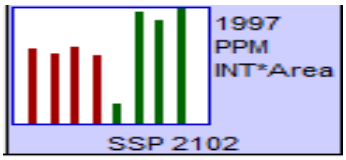
SSP 3603

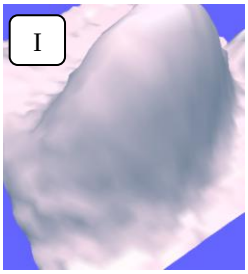


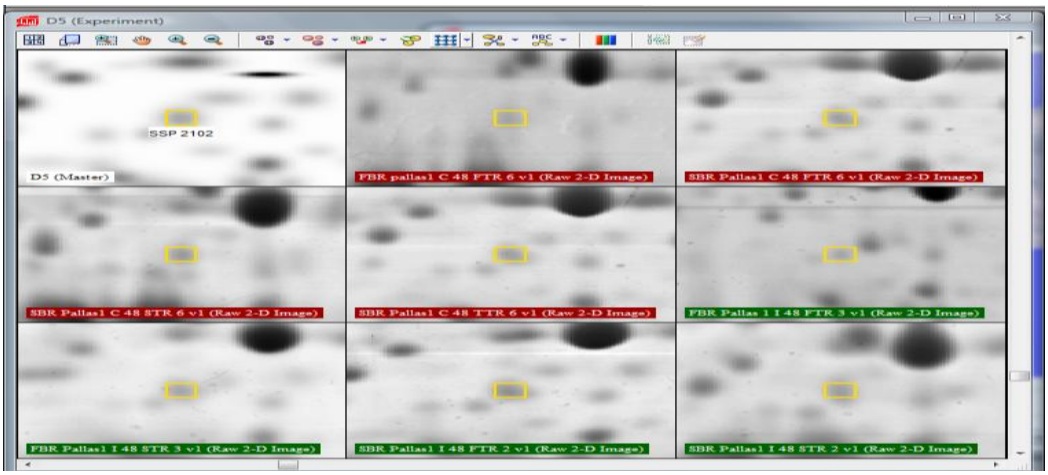




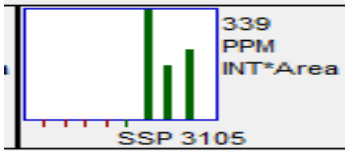
SSP 2102

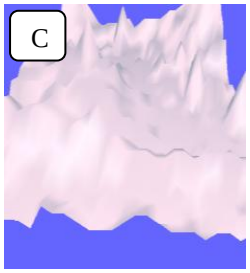


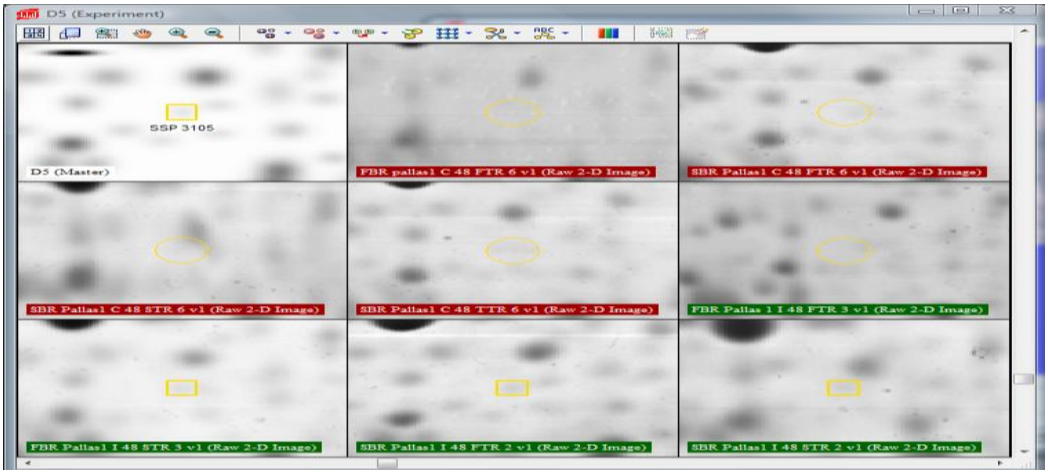




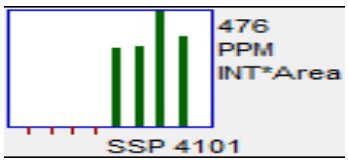
SSP 3105

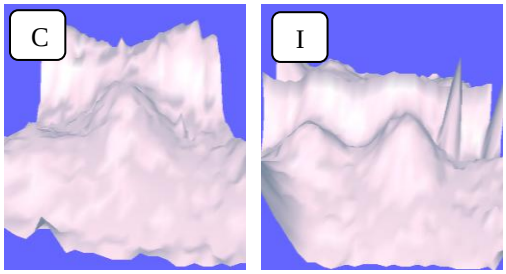


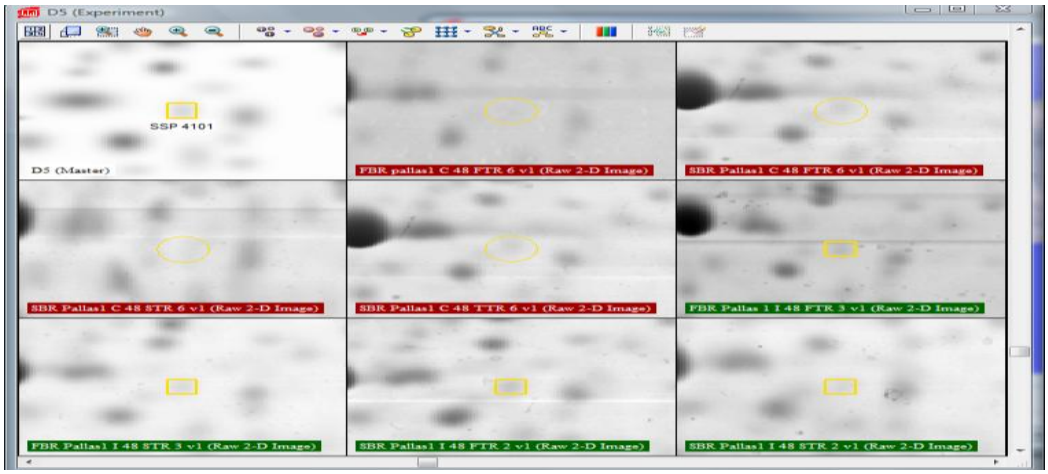




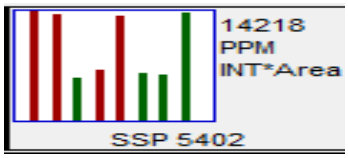
SSP 4101

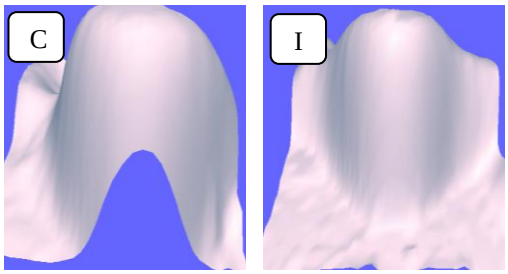


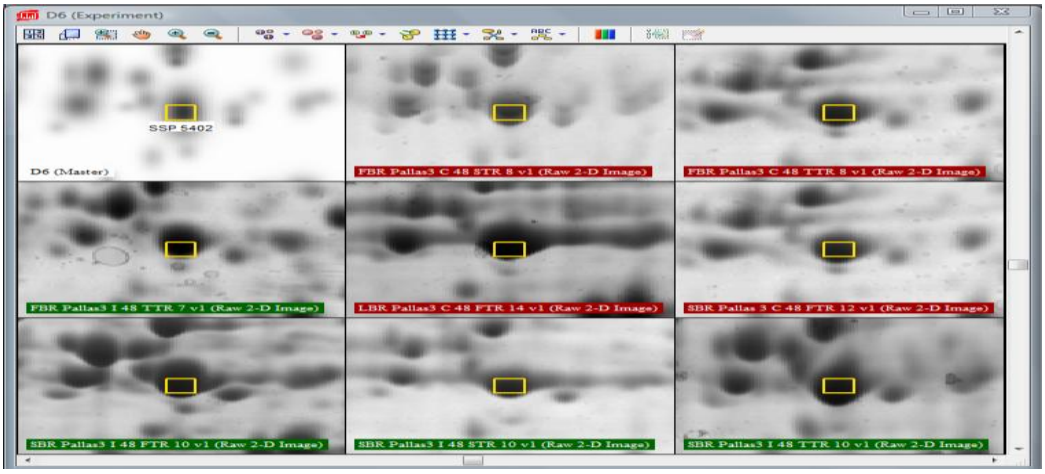




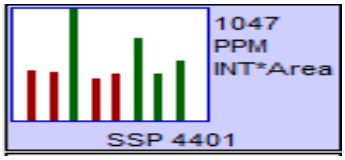
SSP 5402

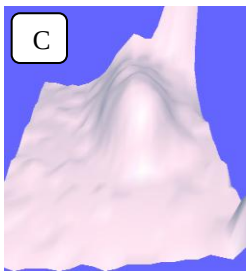


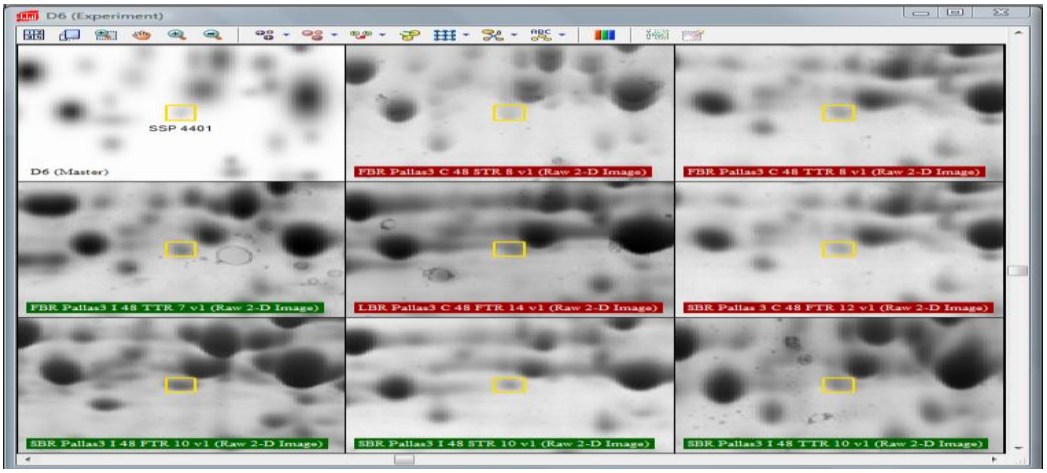




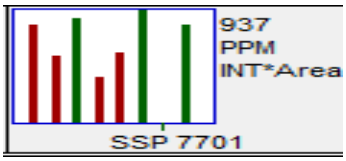
SSP 4101

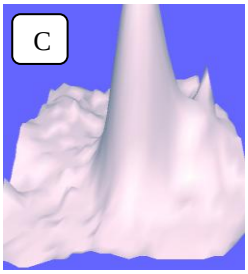


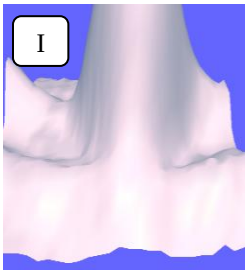


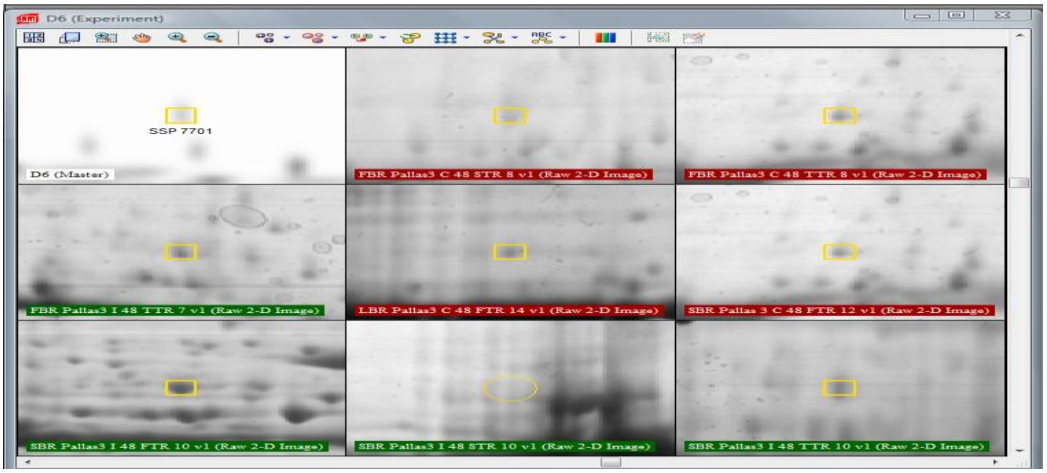


SSP 7701

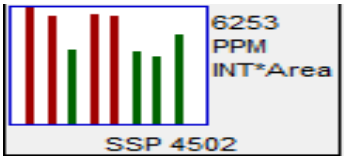


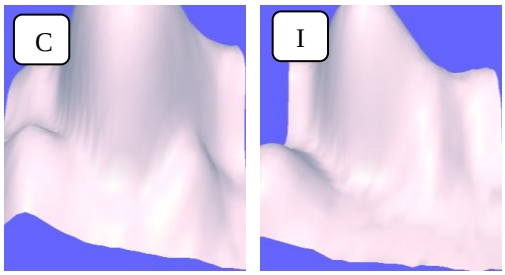


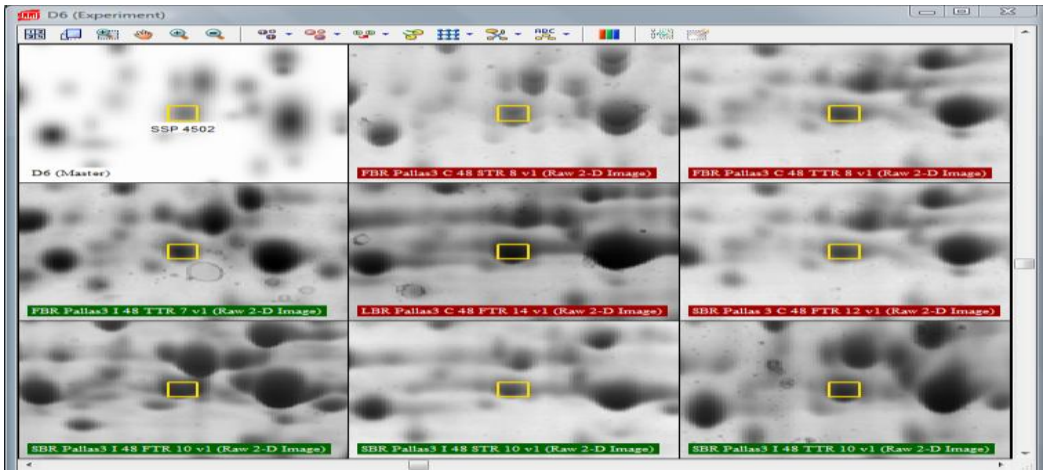




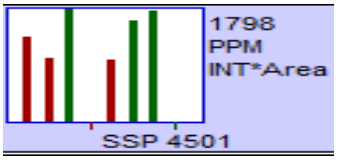
SSP 4502

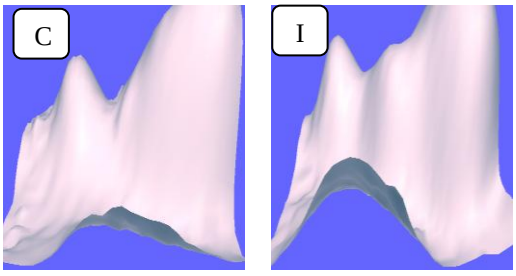


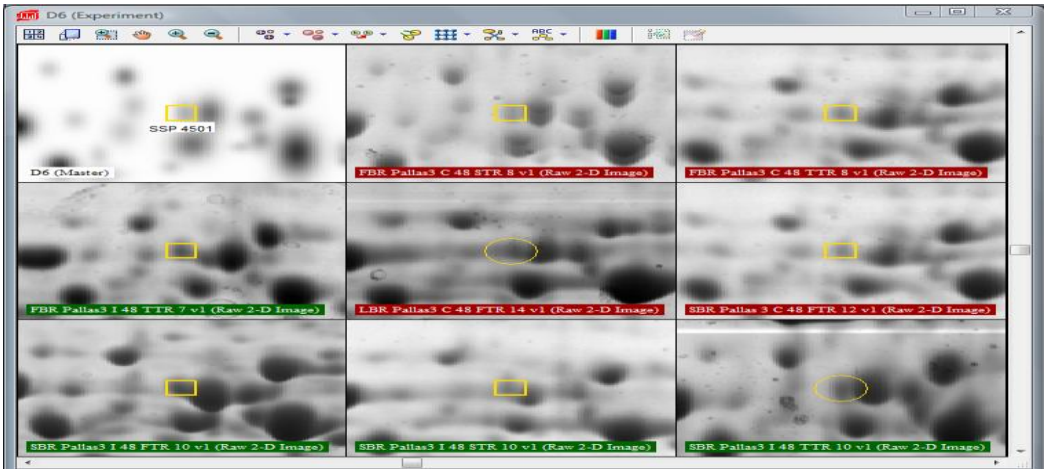




SSP 4501







APPENDIX C

Students' T-test results

SPOT 1 E:1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	5471,5500	774,11263	387,05631
	2,00	4	203,0750	406,15000	203,07500

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	1,462	,272	12,053	6	,000	5268,4750	437,09501	4198,942	6338,008
	Equal variances not assumed			12,053	4,535	,000	5268,4750	437,09501	4109,360	6427,590

SPOT 2 E:1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	1100,7750	756,92473	378,46237
	2,00	4	1504,2000	531,32653	265,66327

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,413	,544	-,872	6	,416	-403,4250	462,39673	-1534,87	728,01903
	Equal variances not assumed			-,872	5,379	,420	-403,4250	462,39673	-1567,31	760,45780

SPOT 3 E:1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	293,8500	212,29498	106,14749
	2,00	4	741,2500	149,03066	74,51533

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,566	,480	-3,450	6	,014	-447,4000	129,69126	-764,743	-130,057
	Equal variances not assumed			-3,450	5,379	,016	-447,4000	129,69126	-773,838	-120,962

SPOT 4 E1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	157,4000	113,27427	56,63713
	2,00	4	394,3250	86,72510	43,36255

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,093	,771	-3,321	6	,016	-236,9250	71,33075	-411,465	-62,38495
	Equal variances not assumed			-3,321	5,618	,018	-236,9250	71,33075	-414,386	-59,46428

SPOT 5 E:1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	584,5750	390,63704	195,31852
	2,00	4	1298,7500	424,14504	212,07252

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,026	,876	-2,477	6	,048	-714,1750	288,31247	-1419,65	-8,69981
	Equal variances not assumed			-2,477	5,960	,048	-714,1750	288,31247	-1420,80	-7,54521

SPOT 6 E:1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	1016,0667	811,95124	468,78027
	2,00	4	4205,3000	1512,38407	756,19203

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,407	,552	-3,265	5	,022	-3189,2333	976,92651	-5700,50	-677,964
	Equal variances not assumed			-3,585	4,706	,018	-3189,2333	889,70857	-5519,91	-858,560

SPOT 7 E : 1

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	649,3500	217,69757	108,84878
	2,00	4	1334,7750	368,60549	184,30275

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,356	,573	-3,202	6	,019	-685,4250	214,04570	-1209,18	-161,674
	Equal variances not assumed			-3,202	4,866	,025	-685,4250	214,04570	-1240,24	-130,608

SPOT 8 E : 2

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	952,7000	67,98122	33,99061
	2,00	4	231,0500	121,12309	60,56155

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	8,496	,027	10,391	6	,000	721,6500	69,44827	551,71620	891,58380
	Equal variances not assumed			10,391	4,719	,000	721,6500	69,44827	539,88770	903,41230

SPOT 9 E : 2

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	875,1000	214,85735	107,42867
	2,00	4	376,3000	226,52021	113,26011

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,002	,968	3,195	6	,019	498,8000	156,10500	116,82482	880,77518
	Equal variances not assumed			3,195	5,983	,019	498,8000	156,10500	116,56644	881,03356

SPOT 10 E : 2

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	1185,6250	304,45407	152,22704
	2,00	4	1753,3250	174,28634	87,14317

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,414	,544	-3,237	6	,018	-567,7000	175,40525	-996,901	-138,499
	Equal variances not assumed			-3,237	4,776	,025	-567,7000	175,40525	-1025,04	-110,357

SPOT 29 E : 2

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	726,1500	553,46394	276,73197
	2,00	5	,0000	,00000	,00000

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	16,586	,005	2,988	7	,020	726,1500	243,05648	151,41276	1300,887
	Equal variances not assumed			2,624	3,000	,079	726,1500	276,73197	-154,535	1606,835

SPOT 12 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	5	934,7000	221,07516	98,86782
	2,00	5	206,5200	283,36343	126,72398

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	1,886	,207	4,530	8	,002	728,1800	160,72900	357,53826	1098,822
	Equal variances not assumed			4,530	7,553	,002	728,1800	160,72900	353,68588	1102,674

SPOT 13 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	7320,2250	327,22543	163,61272
	2,00	5	4846,7400	1234,99277	552,30556

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	9,940	,016	3,850	7	,006	2473,4850	642,53139	954,13970	3992,830
	Equal variances not assumed			4,294	4,685	,009	2473,4850	576,02999	962,27428	3984,696

SPOT 14 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	5	747,9800	205,30771	91,81640
	2,00	5	1052,8000	105,62448	47,23670

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,858	,381	-2,952	8	,018	-304,8200	103,25482	-542,926	-66,71396
	Equal variances not assumed			-2,952	5,979	,026	-304,8200	103,25482	-557,693	-51,94727

SPOT 15 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	5	,0000	,00000	,00000
	2,00	5	118,7200	112,75501	50,42557

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	31,361	,001	-2,354	8	,046	-118,7200	50,42557	-235,002	-2,43842
	Equal variances not assumed			-2,354	4,000	,078	-118,7200	50,42557	-258,724	21,28383

SPOT 16 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	737,1000	293,96642	146,98321
	2,00	5	885,7000	330,07259	147,61295

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,009	,927	-,703	7	,505	-148,6000	211,37921	-648,432	351,23240
	Equal variances not assumed			-,713	6,865	,499	-148,6000	208,31142	-643,143	345,94266

SPOT 17 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	5	619,2200	571,99263	255,80288
	2,00	4	1724,2250	394,73174	197,36587

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	3,063	,124	-3,270	7	,014	-1105,0050	337,90611	-1904,03	-305,984
	Equal variances not assumed			-3,420	6,913	,011	-1105,0050	323,09194	-1870,94	-339,068

SPOT 18 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	529,6250	361,55269	180,77635
	2,00	5	785,4200	508,29726	227,31745

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,568	,476	-,845	7	,426	-255,7950	302,73314	-971,645	460,05512
	Equal variances not assumed			-,881	6,952	,408	-255,7950	290,43641	-943,532	431,94189

SPOT 19 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	5	167,8600	229,88773	102,80892
	2,00	5	568,9800	219,48620	98,15721

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,432	,530	-2,822	8	,022	-401,1200	142,14258	-728,901	-73,33863
	Equal variances not assumed			-2,822	7,983	,022	-401,1200	142,14258	-729,024	-73,21645

SPOT 39 E : 3

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	5	,0000	,00000	,00000
	2,00	4	272,0000	137,93308	68,96654

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	8,558	,022	-4,490	7	,003	-272,0000	60,57400	-415,235	-128,765
	Equal variances not assumed			-3,944	3,000	,029	-272,0000	68,96654	-491,482	-52,51770

SPOT 20 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	,0000	,00000	,00000
	2,00	4	205,7500	123,50667	61,75333

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	8,103	,029	-3,332	6	,016	-205,7500	61,75333	-356,855	-54,64504
	Equal variances not assumed			-3,332	3,000	,045	-205,7500	61,75333	-402,277	-9,22333

SPOT 21 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	,0000	,00000	,00000
	2,00	4	164,4500	25,82318	12,91159

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	4,135	,088	-12,737	6	,000	-164,4500	12,91159	-196,044	-132,856
	Equal variances not assumed			-12,737	3,000	,001	-164,4500	12,91159	-205,540	-123,360

SPOT 22 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	70,0000	80,82908	40,41454
	2,00	4	283,1000	12,54379	6,27189

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	371,508	,000	-5,210	6	,002	-213,1000	40,89831	-313,175	-113,025
	Equal variances not assumed			-5,210	3,144	,012	-213,1000	40,89831	-339,939	-86,26065

SPOT 23 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	136,5000	26,70225	15,41655
	2,00	4	264,8000	51,69217	25,84608

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,740	,429	-3,866	5	,012	-128,3000	33,19031	-213,618	-42,98158
	Equal variances not assumed			-4,263	4,634	,009	-128,3000	30,09468	-207,533	-49,06747

SPOT 24 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	,0000	,00000	,00000
	2,00	3	138,3000	15,32873	8,85005

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	22,855	,005	-18,678	5	,000	-138,3000	7,40448	-157,334	-119,266
	Equal variances not assumed			-15,627	2,000	,004	-138,3000	8,85005	-176,379	-100,221

SPOT 25 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	,0000	,00000	,00000
	2,00	3	188,9667	28,81412	16,63584

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	22,795	,005	-13,577	5	,000	-188,9667	13,91854	-224,745	-153,188
	Equal variances not assumed			-11,359	2,000	,008	-188,9667	16,63584	-260,545	-117,388

SPOT 26 E : 4

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	542,8000	115,95582	57,97791
	2,00	4	1526,5750	398,21985	199,10993

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	3,735	,101	-4,744	6	,003	-983,7750	207,37936	-1491,21	-476,336
	Equal variances not assumed			-4,744	3,505	,012	-983,7750	207,37936	-1593,08	-374,474

SPOT 27 E : 5

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	126,4500	85,04275	42,52138
	2,00	3	351,1000	32,98924	19,04635

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	2,065	,210	-4,257	5	,008	-224,6500	52,77527	-360,313	-88,98686
	Equal variances not assumed			-4,822	4,078	,008	-224,6500	46,59218	-353,037	-96,26328

SPOT 28 E : 5

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	249,5500	288,19179	144,09590
	2,00	3	935,8333	183,68368	106,04982

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	8,528	,033	-3,571	5	,016	-686,2833	192,20217	-1180,35	-192,212
	Equal variances not assumed			-3,836	4,951	,012	-686,2833	178,91392	-1147,56	-225,003

SPOT 30 E : 5

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	2282,4667	440,18825	254,14280
	2,00	3	1442,4000	504,90649	291,50790

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,024	,885	2,172	4	,096	840,0667	386,73688	-233,687	1913,820
	Equal variances not assumed			2,172	3,927	,097	840,0667	386,73688	-241,602	1921,735

SPOT 31 E : 5

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	1242,4000	51,04243	29,46936
	2,00	3	1924,6333	97,46960	56,27410

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	2,197	,212	-10,740	4	,000	-682,2333	63,52336	-858,602	-505,864
	Equal variances not assumed			-10,740	3,020	,002	-682,2333	63,52336	-883,630	-480,837

SPOT 32 E : 5

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	,0000	,00000	,00000
	2,00	3	241,2000	89,00135	51,38495

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	12,046	,018	-5,610	5	,002	-241,2000	42,99174	-351,714	-130,686
	Equal variances not assumed			-4,694	2,000	,043	-241,2000	51,38495	-462,292	-20,10839

SPOT 33 E : 5

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	,0000	,00000	,00000
	2,00	4	375,9250	69,83597	34,91798

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	6,427	,044	-10,766	6	,000	-375,9250	34,91798	-461,366	-290,484
	Equal variances not assumed			-10,766	3,000	,002	-375,9250	34,91798	-487,050	-264,800

SPOT 34 E : 6

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	13969,60	224,78425	129,77925
	2,00	3	5905,4000	242,54754	140,03489

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	,040	,852	42,237	4	,000	8064,2000	190,92518	7534,107	8594,293
	Equal variances not assumed			42,237	3,977	,000	8064,2000	190,92518	7532,900	8595,500

SPOT 35 E : 6

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	435,1250	34,88575	17,44288
	2,00	3	798,1000	241,29818	139,31357

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	4,897	,078	-3,066	5	,028	-362,9750	118,37122	-667,258	-58,69210
	Equal variances not assumed			-2,585	2,063	,119	-362,9750	140,40130	-949,771	223,82108

SPOT 36 E : 6

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	511,8333	111,87459	64,59082
	2,00	3	874,2333	61,04034	35,24166

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	2,175	,214	-4,925	4	,008	-362,4000	73,57954	-566,690	-158,110
	Equal variances not assumed			-4,925	3,094	,015	-362,4000	73,57954	-592,596	-132,204

SPOT 37 E : 6

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	4	5962,5000	200,17106	100,08553
	2,00	4	4091,2000	516,18637	258,09318

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	1,841	,224	6,760	6	,001	1871,3000	276,81981	1193,946	2548,654
	Equal variances not assumed			6,760	3,882	,003	1871,3000	276,81981	1093,450	2649,150

SPOT 38 E : 6

Group Statistics

	VAR00002	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	1,00	3	1119,2000	204,37351	117,99510
	2,00	3	1741,0333	96,25177	55,57099

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VAR00001	Equal variances assumed	3,641	,129	-4,768	4	,009	-621,8333	130,42614	-983,954	-259,712
	Equal variances not assumed			-4,768	2,846	,020	-621,8333	130,42614	-1049,94	-193,724