# PROTEOMIC BASIS OF DROUGHT TOLERANCE IN CHICKPEA

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**by**
**Hatice ŞELALE**

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**İZMİR**

We approve the thesis of **Hatice ŞELALE**

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**Prof. Dr. Anne FRARY**
Supervisor

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**Assoc. Prof. Dr. Talat YALÇIN**
Committee Member

---

**Assoc. Prof. Dr. Bahattin TANYOLAÇ**
Committee Member

**28 June 2010**

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**Assoc. Prof. Dr. Sami DOĞANLAR**

Head of the Department of
Molecular Biology and Genetics

---

**Assoc. Prof. Dr. Talat YALÇIN**

Dean of the Graduate School of
Engineering and Sciences

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# ABSTRACT


## PROTEOMIC BASIS OF DROUGHT TOLERANCE IN CHICKPEA

In this study our aim was to identify differentially expressed proteins in root and leaf samples of the drought tolerant chickpea cultivar *Gokce* using proteomics approaches.

For this aim we carried out 2D gel electrophoresis from total proteome extracts of root and leaf samples of *Gokce* cultivar from drought treated and control samples. In root 2D gels we obtained approximately 430 proteins; 14 of them were newly formed and 4 of them were disappeared in drought stress. Also we obtained 12 over-expressed protein and 4 down-regulated spot as a result of drought stress. In leaf 2D gels we obtained approximately 450 proteins 4 of them were newly formed spots, and 3 of them were disappeared in drought stress. For these samples we obtained 24 over-expressed proteins and 17 down-regulated proteins in drought stress. We identified differentially expressed proteins in MALDI-TOF/TOF mass spectrometer via peptide mass fingerprinting. Identified proteins are zinc finger (C2H2 type, AN1-like) family protein, pathogenesis-related family protein, STRS2 (STRESS RESPONSE SUPPRESSOR 2), 26S proteasome non-ATPase regulatory subunit 3, pentatricopeptide repeat-containing protein, RABB1C (ARABIDOPSIS RAB GTPASE HOMOLOG B1C); serine hydroxymethyltransferase, fiddlehead protein, aluminum-activated malate transporter, phloem protein 2-A8, ribosomal protein L30 family protein, N-rich protein with known function and we identified 14 hypothetical proteins with unknown function. Identified proteins are WRKY DNA-binding protein 6, myb family transcription factor, porin family protein, pentatricopeptide repeat (PPR) protein and transmembrane protein and 2 hypothetical proteins with unknown function.

# ÖZET

## NOHUT'TA KURAKLIĞA TOLERANSIN PROTEOMİK ESASLARI

Bu çalışma ile kuraklığa dirençli olduğu bilinen nohut çeşidi *Gökçe*'nin kök ve yapraklarında kuraklığa cevaben ifadelenen proteinlerin proteomiks yaklaşımı ile tanımlanmıştır.

Bu amaçla *Gökçe* çeşidinin kök ve yaprak örneklerinden kuraklık ve kontrol koşullarında izole edilen total proteinleri kullanılarak iki boyutlu jel elektroforezi yapılmış. Kök örnekleri için elde edilen iki boyutlu jellerde yaklaşık 430 protein bulunmuştur; bunlardan 14'ünün kuraklığa cevaben ortaya çıktığı 4'ünün ise kuraklık koşullarında kaybolduğu tespit edilmiştir. Ayrıca 12 kök proteinin ifadelenmesi kuraklığa cevaben artmış 4'ünün ise ifadelenmesi azalmıştır. Yaprak örnekleri için elde edilen iki boyutlu jellerde yaklaşık 450 protein bulunmuştur; bunlardan 7'sinin kuraklığa cevaben yeni oluştuğu, 3'ünün ise kuraklık koşullarında kaybolduğu tespit edilmiştir. Ayrıca 24 yaprak proteinin ifadelenmesi kuraklığa cevaben artmış 17'sinin ise ifadelenmesi azalmıştır. Farklı ifadelenen proteinler MALDI-TOF/TOF kütle spektosu ile PMF analizleri yapıldı. Analizler sonucunda farklı ifadelenen yaprak proteinleri çinko parmak C2H2 type, AN1-like) ailesi proteinleri, patojen-ilgili aile proteinleri, STRS2 (STRESS RESPONSE SUPPRESSOR 2), 26S proteasome non-ATPase düzenleyici albirim 3, pentatricopeptide tekrar içeren protein, RABB1C (ARABIDOPSIS RAB GTPASE HOMOLOG B1C); GTP binding/GTPase, serine hydroxymethyltransferase, fiddlehead protein, aluminum-activated malate transporter, phloem protein 2-A8, ribosomal protein L30 ailesi proteini, N-zengin protein gibi fonksiyonu bilinenler ile birlikte 14 adet fonksiyonu bilinmeyen yaprak proteini belirlendi. Farklı ifadelenen kök proteinleri WRKY DNA-binding protein 6, myb ailesi transkripsiyon faktörü, porin ailesi proteinleri, pentatricopeptide tekrar (PPR) proteinleri ve transmembrane proteini gibi fonksiyon bilinenler ile birlikte 14 adet fonksiyonu bilinmeyen kök proteini tespit edildi.

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# CHAPTER 1

# INTRODUCTION

## 1.1. Importance of Chickpea

Chickpea (*Cicer arietinum L.*) is a self-pollinating and diploid annual grain legume with 16 chromosomes. The genome size of chickpea is estimated as 740 Mb (Arumuganathan and Earle 1991). Chickpea is an ancient legume crop and it most probably originated in a region of southeastern Turkey and neighboring areas of Syria (Singh 1997). Chickpea is the second most produced legume crop in the world after dry bean with 9, 7 million tons produced in 2007 (FAO 2007). Turkey is one of the major producers of chickpea and ranks third in world production with 0, 55 million tons production after India and Pakistan with 6,3 million and 0,83 million tons, respectively (FAO 2007).

Chickpea is of great importance in terms of its usage as food for humans and for livestock. Moreover its seed coat is used as fodder. It is consumed as fresh immature green seed, whole seed, as flour and the top shoot from each chickpea plant is consumed as greens in Bangladesh.

Chickpea is also a very nutritive legume crop with high content of protein and carbohydrate which together make-up 80% of the total dry seed weight. The crude protein content of chickpea seed varies from 17% to 24% containing essential amino acids like tryptophan, methionine and cysteine (Williams and Singh 1987). Thus chickpea is a very important dietary component especially in regions with low income. Supplementation of diet with high protein legumes is potentially one of the best solutions to protein-calorie malnutrition, particularly in developing countries.

Chickpea is a dry-land crop with deep roots, which can grow to full maturity in environmental conditions which are fatal for most crops. It is grown in arid and semi-arid regions and rarely receives fertilizers or protection from diseases and pests (Singh and Reddy 1991). Nearly 90% of the crop is grown under rain fed conditions mostly on decreasing soil moisture. The nitrogen-fixing root nodules produced by chickpea can enrich the soil with at least 50 kg of nitrogen per hectare every growing season.

Furthermore chickpea can mobilize phosphorous in the soil that is not available to other crops. Although, chickpea has a number of bacterial and fungal pathogens, it has relatively few insect pest as compared to other legumes in consequence of malic acid and oxalic acid secretion (Khanna-Chopra and Sinha 1987).

When we consider sowing time and photoperiod these factors vary among the chickpea producing regions where most of the precipitation is received before or during the early crop season. Therefore, the crop matures under progressively declining soil moisture and increasing temperature. In most of the areas where chickpea is grown, drought is the most important factor limiting crop yield and even causing total crop lost. In many areas chickpea is subjected to terminal drought in the seed filling period, which limits seed yield (Turner et al. 2001).

Although chickpea is badly affected by drought it is much more resistant to drought than other crop plants. Its wide production in dry-lands worldwide makes the crop significant in term of research in order to develop more resistant varieties and moreover it can be considered as model organism to understand drought resistance mechanisms.

## 1.2. Drought as a Stress

Stress is altered physiological condition caused by factors that tend to alter equilibrium of metabolism. Plant reactions exist to circumvent the potentially harmful effects caused by a wide range of both abiotic and biotic stresses, including light, drought, salinity, and high temperatures. Drought is the major economically significant abiotic stress affecting crop production in the world (Araus et al. 2002; Boyer 1982). Drought is known to cause yield losses up to 50%. The definition of drought is: reduction in normal precipitation which limits plant production (Kramer and Boyer 1995). Drought can be classified as terminal and intermittent drought. During terminal drought, the availability of soil water decreases progressively and this leads to severe drought stress at the later period of crop growth and development. Intermittent drought is the result of finite periods of inadequate rain or irrigation occurring at one or more intervals during the growing seasons and is not necessarily lethal.

Plants have both physiological and molecular defense mechanisms against drought. These plant responses to drought include both physiological and biochemical

responses. And many physiological processes regarding crop growth and development covering photosynthetic CO2 assimilation, transpiration and stomatal regulation, cell growth, hormonal regulation and enzyme concentration and activities and many other processes are influenced by water deficits (Hsiao 1973, Boyer and McPherson 1975, Begg and Turner 1976). A summary of plant drought responses are shown in Figure 1.1.

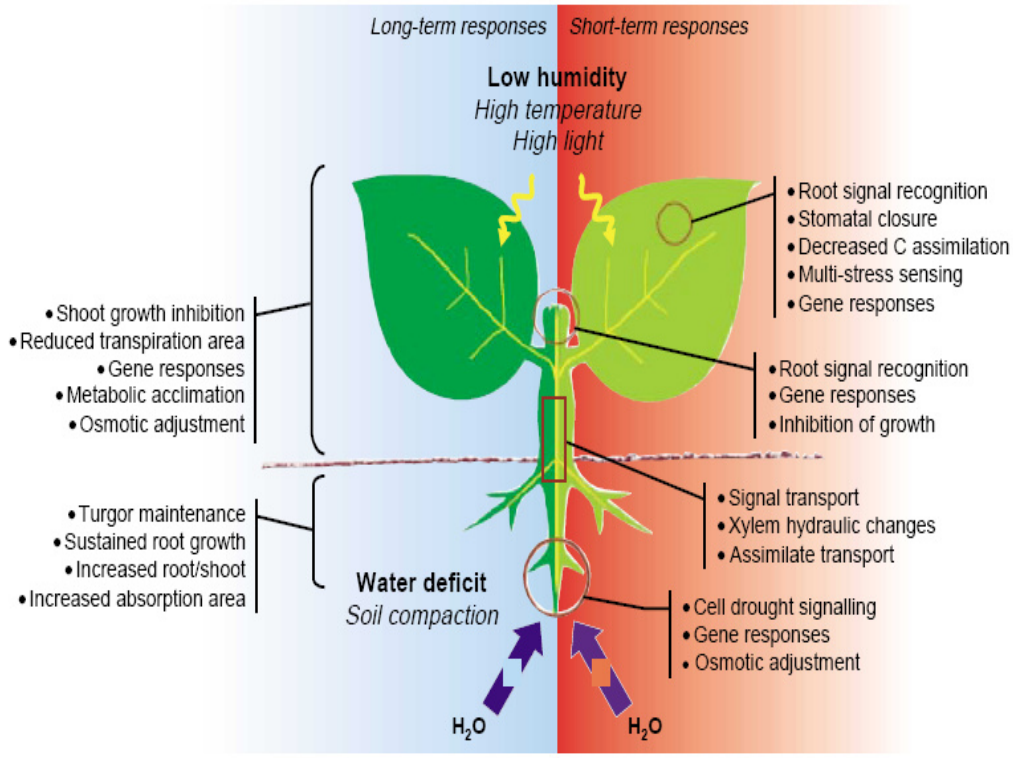



Figure 1.1. Whole plant response to drought stress
(Source: Chaves et al. 2003)

Until now there have been many attempts to classify and identify drought tolerance mechanisms in plants. May and Milthorpe (1962) classified drought resistance into three categories: drought escape, the ability of a plant to complete its life cycle before serious soil and plant water deficits develop; drought tolerance with high tissue water potential; and drought tolerance with low tissue water potential. From Levitt's (1972, 1980) point of view plants adapt to drought by drought escape and drought resistance. Quisenberry (1982) described drought resistance as the ability of a plant variety to produce a higher yield than another at a given limiting level of water availability. Plants try to sustain their homeostasis in both drought escape and avoidance mechanisms by balancing water uptake and avoiding water loss to prevent the hazardous effects of drought stress on tissue water potential. Both in drought escape and

avoidance mechanisms stress is dealt with outside the plant tissue. If this cannot be achieved and the plant tissue does experience low water potential, then dehydration tolerance mechanisms must respond to ensure plant tissue growth and survival by preventing excessive water loss from the plant. Most plants combine these three strategies to achieve drought tolerance.

### 1.2.1. Physiological and Molecular Responses to Drought

Many different physiological and biochemical changes occur in response to drought stress. These changes include turgor loss, reduction in photosynthesis rate, increase in leaf senescence, increased oxidative stress, increased stomatal closure, ABA (absisic acid) accumulation, changes in membrane fluidity and composition, changes in solute concentration, and protein–protein and protein–lipid interactions (Chaves et al. 2003).

In plant tissues, turgor is maintained during drought by avoiding dehydration, tolerating dehydration or both (Kramer and Boyer 1995). An important mechanism to maintain turgor is increased water uptake by the plant. Maintenance of turgor by dehydration avoidance and tolerance are controlled by developmental and morphological traits including root thickness, root depth and mass and the ability of roots to penetrate compacted soil layers, which increase water uptake by plants. (Pathan et al. 2004). The phenotypic traits mentioned above (e.g. root thickness) are present even in the absence of stress conditions in drought resistant plants. By contrast, there are adaptive traits, such as osmotic adjustment and dehydration tolerance which arise in response to water deficit (Serraj and Sinclair 2002).

Another important mechanism to maintaining turgor is achieved by decreasing water loss. This is accomplished by stomatal closure and synthesis of osmoprotectants, osmolytes or compatible solutes. These molecules, which act as osmotic balancing agents, are accumulated in plant cells in response to drought stress and are subsequently degraded after stress relief (Tabaeizadeh 1998). Osmoprotectants also stabilize proteins and cell membranes against the denaturing effect of stress conditions on cellular functions. Osmoprotectants include amino acids (proline), polyols (mannitol, xylitol) , and quaternary ammonium (glycinebetain) and tertiary sulfonium compounds (DMSP) (Rontein et al. 2002).

Changes in carbohydrate metabolism are also reported in response to drought. Early studies on changes in the carbohydrate metabolism of plants exposed to drought stress suggested that, under dry conditions, the hydroxyl group of polyhydroxy compounds can form a hydrogen bond with the polar heads of membrane phospholipids, and that these hydrophobic interactions are important for membrane stability (Chaves et al. 2003, Crowe et al. 1998, Villadsen et al. 2005). Several types of protective proteins including dehydrins and late-embryogenesis abundant (LEA) proteins are known to accumulate in response to decreases in tissue water content (Close 1997). These proteins act as chaperones that protect protein and membrane structure (Bravo et al. 2003, Hara et al. 2001).

Reduction of photosynthetic activity is one of the most yield-limiting responses caused by drought and is due to several coordinated events, such as stomatal closure and the reduced activity of photosynthetic enzymes, which are commonly dependent on decreased $CO_2$ uptake and increased light absorption. C4 and CAM plants have evolved resistance mechanisms that increase $CO_2$ uptake under drought conditions. In addition, some plants undergo some architectural and morphological changes for ridding themselves of excessive light and some changes in photochemical activity is reported (Chaves et al. 2003).

ROS (reactive oxygen species) are known to form in plant tissues as a result of water deficiency and excess light. As a resistance mechanism, some antioxidant enzymes such as catalase, SOD (superoxide dismutase) and antioxidant molecules such as ascorbate- glutatione and α-tocopherol are over-expressed. (Simirnoff 1998, Dat et al. 2000) For example, a high level of α-tocopherol was found in the chloroplast membrane of tolerant plants as compared to susceptible plants (Mune-Bosch and Alegre 2000). Some successful applications for genetic modulation by production of ROS scavengers in plants have also been reported to increase drought stress (Imai et al.1996).

It has been demonstrated that ABA accumulates in plants when they are exposes to drought stress. ABA is a plant hormone responsible for regulation of gene expression in stress conditions. In drought resistance mechanisms, both ABA-dependent and ABA-independent pathways are known to be activated. Activation of ABA-dependent pathways is known to be responsible for stomatal closure and expression of some osmoprotectants and protective proteins like LEA (late embryogenesis abandoned protein) and chaperonins. An important point for discussion at this stage is that different drought mechanisms do not necessarily occur in a linear progression in time after the

stress begins or from mild stress to severe stress. For example, some decrease in water content and turgor is required to trigger accumulation of ABA (Pierce and Raschke 1980, Creelman and Zeevaart 1985) which then causes stomatal closure to prevent further decrease in water content. Another important point to consider is that stressful environments are often characterized by the simultaneous or sequential occurrence of more than one stress. There is also growing evidence of multiple tolerances to stresses in plants, with plants showing tolerance to more than one stress. In addition, there is evidence of cross-adaptation, where tolerance from one stress enhances the tolerance against other stress. For example, ABA increases tolerance against cold/drought and also enhances tolerance against diseases through increasing the thickness of the cell wall.

## 1.3. What is Proteomics ?

Proteomics is the study of the proteome, the protein complement of the genome. The terms “proteomics” and “proteome” were coined by Marc Wilkins and colleagues in the early 1990s and mirror the terms “genomics” and “genome,” which describe the entire collection of genes in an organism (Wilkins et al. 1995). Biochemical basis of genomics and proteomics is shown in Figure 1.2.

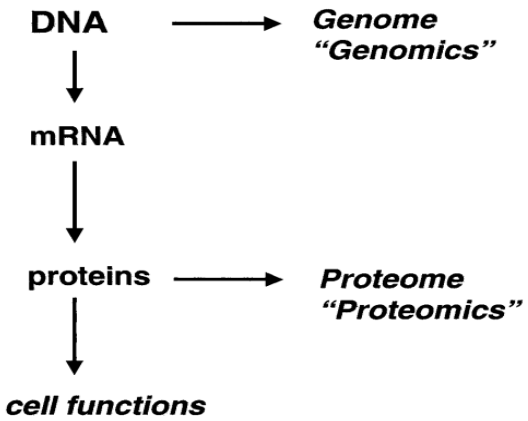



Figure 1.2. Biochemical context of genomics and proteomics
(Source: Liebler 2002)

Until the mid-1990s, biochemists, molecular biologists, and cell biologists studied individual genes and proteins or small clusters of related components of specific biochemical pathways. Three developments changed biology and formed the foundation

of a new discipline. The first was the growth of gene, expressed sequence tag (EST) and protein sequence databases during the 1990s. These resources became more useful as partial catalogs of expressed genes in many organisms. Genome-sequencing projects revealed the complete genomic sequences of many organisms. These genome sequences are organized in databases from which the information regarding living systems will be extracted. The second key development was the introduction of browser-based bioinformatics tools which make it possible to search entire genomes for specific nucleic acid or protein sequences in seconds. Such database search tools are combined with other tools and databases to predict the functions of the protein products based on specific functional domains or motifs. The third key development was the oligonucleotide microarray. By applying a mixture of fluorescently labeled DNAs from a sample of interest to the array, one can probe the expression of thousands of genes at once.

Although the development of the abovementioned techniques helped in the understanding of molecular aspects of biological systems, there were still some missing points for analysis of functional components. Gene microarrays offer a glimpse of the expression of many or all genes in a cell. Unfortunately, the levels of mRNAs do not necessarily predict the levels of the corresponding proteins in a cell. Differing stability of mRNAs and different efficiencies in translation and posttranslational modifications can affect the generation of new proteins. Once formed, proteins differ significantly in stability and turnover rates according to their function and localization (Futcher et al. 1999, Gygi et al. 1999). Many proteins involved in signal transduction, transcription-factor regulation, and cell-cycle control are rapidly turned over as a means of regulating their activities. Finally, mRNA levels tell us nothing about the regulatory status of the corresponding proteins, whose activities and functions are subject to many endogenous posttranslational modifications and other modifications by the environment. (Figure 1.3). These features of proteins make genomics and transcriptomics insufficient to understand the functional component of cells (Pandey and Mann 2000). Therefore studying proteomics is a way to understand the functional components of the cell and their rapidly changing responses.

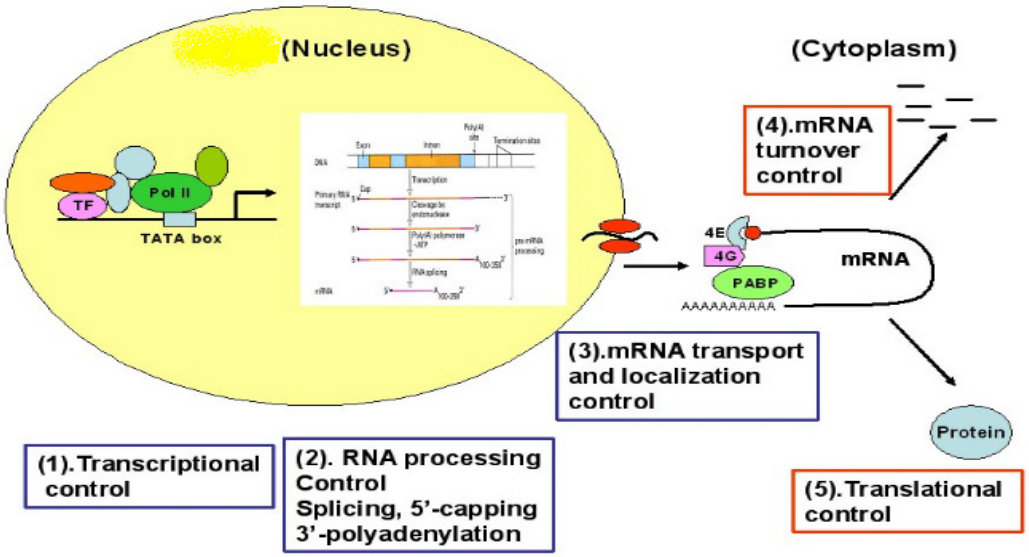



Figure 1.2. Regulation in gene expression
(Source: Lee et al. 2007)

Proteomic analysis includes identification of the amino acid sequence of proteins, determination of their relative amounts, their modification patterns, determination of protein function and their relation with other proteins. Thus, according to the aim of an analysis, we can classify proteomics into three categories: expression, structural, and functional proteomics. Expression proteomics includes the identification of protein expression levels of biological systems in terms of their response to physical and biochemical stresses. Therefore, it deals with changes in the entire proteome in a cell, tissue, or organism at a certain time. The aim of structural proteomics is to identify proteins' three dimensional structures and their locations within the cell or organelle. Functional proteomics involves the functions, activities, and interactions of all the proteins in a proteome (Bradshaw 2004)

Proteomics has different experimental strategies and steps. The most basic approach for proteomics study is as described below. First, the proteins of interest should be extracted from the organ, tissue or cell of interest. Secondly to reduce the complexity of the protein extract, proteins have to be separated. There are several different approaches for separation of proteins including 2D gel electrophoresis, non-gel based electrophoresis and chromatographic approaches. After separation of proteins, they have to be identified. For gel based proteomics approach steps of experimental procedure can be seen in Figure 1.4. There are different strategies for identifying proteins including mass fingerprinting and determination of amino acid sequence of a

protein. One of the important criteria for identification of proteins is amino acid sequence. For amino acid sequencing adman degradation or mass spectrometry can be used. At the present time 2D gel electrophoresis, column chromatograpy and mass spectrometry are the major experimental tools for studying proteomics.

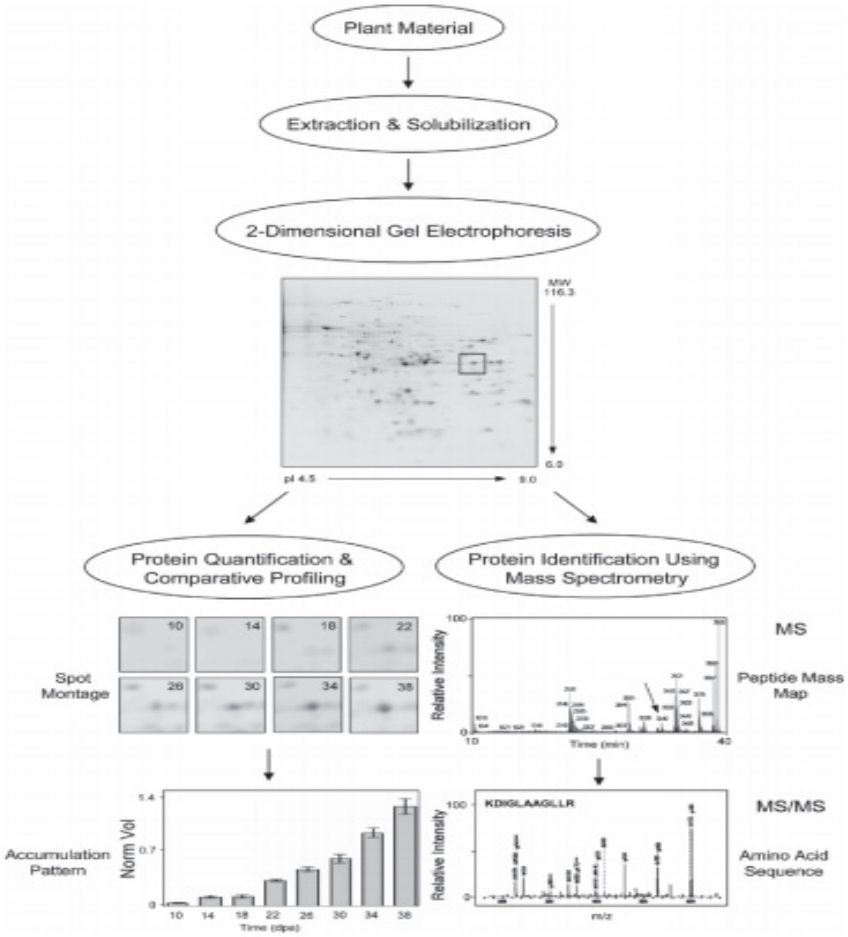



Figure 1.3. Steps of proteomics analysis
(Source: Šamaj and Thelen 2007)

## 1.4. Gel-Based Proteomics

Two dimensional gels were introduced by O'Farrel (O'Farrel et al. 1975 ) and Klose (Klose et al.1975) in 1975, and this approach fascinated many scientists owing to its separation power. The combination of two techniques including isoelectric focusing and SDS (sodium dodecyl sulphate) gel electrophoresis, provides space for resolving more than 10,000 different proteins in a single 2D gel. In 2D gels isoelectric focusing separates proteins according to their pI in the first dimension. In the second dimension,

SDS gel electrophoresis separates proteins according to their molecular mass. Consequently, 2D gels were the method of choice when dealing with very complex protein mixtures like proteomes. Unfortunately, gel-based proteomics has inherent limitations in reproducibility and dynamic range. Standard operating procedures had to be carefully followed to get almost reproducible results even within one lab. A significant improvement was the introduction of the DIGE technique, a multiplexed fluorescent Cy-Dye staining of different proteome states, which largely eliminated technical irreproducibility. Despite the limitations in load capacity, difficulties in handling and interfacing problems in mass spectrometry resolving power of makes 2D gel electrophoresis mostly used method for studying proteomics.

## 1.5. Two- Dimensional Gel Electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) separatse proteins according to two independent parameters, i.e., isoelectric point (pI) in the first dimension and molecular mass (Mr) in the second dimension. This is done by coupling isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension  With high-resolution 2-DE up to 5,000 different proteins (~2,000 proteins routinely), can be resolved simultaneously and detect at a sensitivity of <1 ng of protein per spot (Görg et al. 2000). Because proteomes are highly complex and proteins have extremely diverse physico-chemical properties, 2-DE will not enable comprehensive characterization of all protein. In addition it is technically challenging. Notwithstanding the above mentioned disadvantages of 2-DE, it is still the most widespread method for the majority of ongoing proteome projects and will probably remain so in the foreseeable future. Although alternative and complementary technologies have been developed, there is no technology that matches 2-DE in its ability for routine parallel expression profiling of large sets of complex protein mixturesand preparing a map of intact proteins which reflects changes in protein expression level, isoforms, or post-translational modifications. In spite of its wide usage throughout the 1980s, carrier-ampholyte-generated pH gradients, which are used in IEF suffer from several limitations with respect to reproducibility, resolution, separation of very acidic and/or very basic proteins, and sample loading capacity. The disadvantages at issue have been largely overcome with the introduction of immobilized pH gradients

(IPGs) for IEF. In IPGs, bifunctional Immobiline reagents are used. These reagents are a series of chemically well-defined acrylamide derivatives with the general structure $CH_2$=CH–CO–NH–R, where R contains either a carboxyl or an amino group (Bjellqvist et al. 1982). With these chemicals, a series of buffers with different pK values ranging between 1 and 13 are used. Since the reactive end is co-polymerized with the acrylamide matrix, extremely stable pH gradients are generated, allowing true steady-state IEF with increased reproducibility. Although alkaline proteins with isoelectric points up to pH 12 and hydrophobic proteins are really difficult to separate (Nilsson C. L. et al. 2000), they have been separated under truly steady-state conditions using IPG technology. Current research to further advance 2-DE technology has focused on improved solubilization and separation of hydrophobic proteins and display of low abundance proteins. With the development of difference gel electrophoresis (DIGE) technology, analysis of mixed samples differentially labeled by fluorescent dye molecules on a single 2-DE gel become possible.

The major steps of a proteomics experiment with 2-DE-MS include: sample preparation/prefractionation and protein solubilization; protein separation by 2-DE; protein detection and quantitation; computer-assisted analysis of 2-DE patterns; protein identification and characterization by MS; and database construction (Pasinko et al. 2002).

### 1.5.1. Isoelectric Focusing (IEF)

Initially according to your interest, you have to select an IEF strip with suitable pH range. Commonly used strips are 18- to 24-cm long and available for broad (3 to 10) and narrow (e.g., 5.5 to 6.5) pH ranges. After selecting proper IPG strips, rehydration is applied overnight with or without voltage applied to the strips. For the rehydration solution, 25ml 8-M urea, 2% (w/v) CHAPS, 10 mM DTT, and 2% (v/v) are used. Rehydrated strips are transferred into the IEF chamber and covered with mineral oil to prevent water from evaporating during the focusing. Loading capacity of analytic gels is nearly 50mg. The devices that are commonly available (Pharmacia, Bio-Rad) are used for IEF. Generally, focusing is started slowly (e.g., voltage is linearly increased over 3 h from 300 to 3,500 V, followed by 3 h at 3,500 V and, in the end, 5,000 V overnight). As far as the duration of the focusing is concerned, represented in volt hours

(Vh), the literature offers all values between 40,000 and 400,000 Vh. After focusing, the proteins in the IEF gel have to be saturated with SDS. For this, you incubate the focused gel strip for 10 to 12 minutes in 50 mM Tris-Cl pH 6.8, 6 M urea, 30% (v/v) glycerine, 2% (w/v) SDS, and 2% (w/v) DTT. Afterward, the free SH groups are blocked with 2.5% (w/v) iodoacetamide in 50 mM Tris-Cl pH 6.8, 6 M urea, 30% (v/v) glycerine, and 2% (w/v) SDS for five minutes. Divergence principle of proteins according to their pI is represented in Figure 1.5.

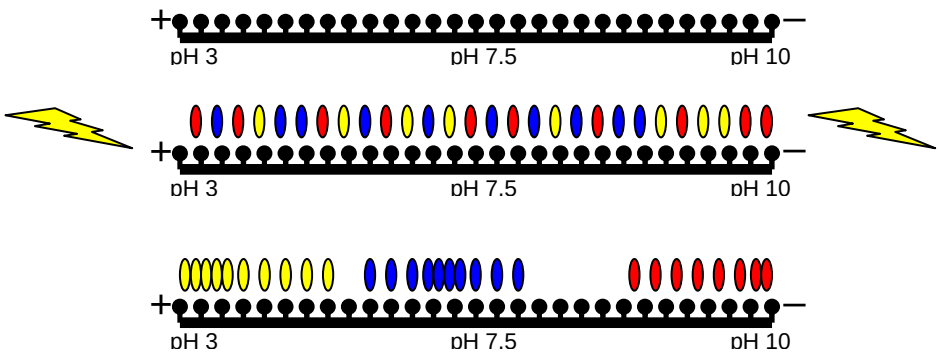



Figure 1.4. Diagrammatic representation of IEF

### 1.5.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After equilibration of focused IPG strips, proteins are ready for separation in the second dimension, the SDS gel. The IEF gel strip equilibrated with SDS is laid on an SDS plate gel. For SDS-PAGE gels according to the sample and the range of the proteins that are used, gels with different concentration and gradient are used. Nine to 16% gradient gels are popular at the moment (they capture proteins of 200–8 kd) and 12% gels (they capture proteins of 150–14 kd). For polymerization of acrylamide, bisacrylamid is used as a cross-linker and TEMED is used as polymerizing agent. The addition of 5mM Na-thiosulfate to the running buffer is also done for better staining. Many experimenters do not use a stacker gel. The IEF gel (in Tris-Cl pH 6.8) together with the agarose used for fixing is considered to be sufficient as a stacker. The SDS separation gel finally separates the focused proteins according to size. Depending on the sample, the experimenter gets up to 5,000 spots (Lopez 2007)

After running the SDS-PAGE, gels have to be stained to visualize the separated protein spots. Some different dyes used for staining the SDS-PAGE gels include

coomassie brilliant blue, silver staining, negative staining with metal cations and staining or labeling with organic or fluorescent dyes. Coomassie brilliant blue (CBB) staining is widely used because of its low cost and ease of use. CBB staining has working detection range of approximately 50 ng to 1000 ng whereas colloidal Coomassie blue staining has working detection range 20 ng to 100 ng (Lopez 2007). Separation principle with SDS-PAGE can be seen in Figure 1.6.

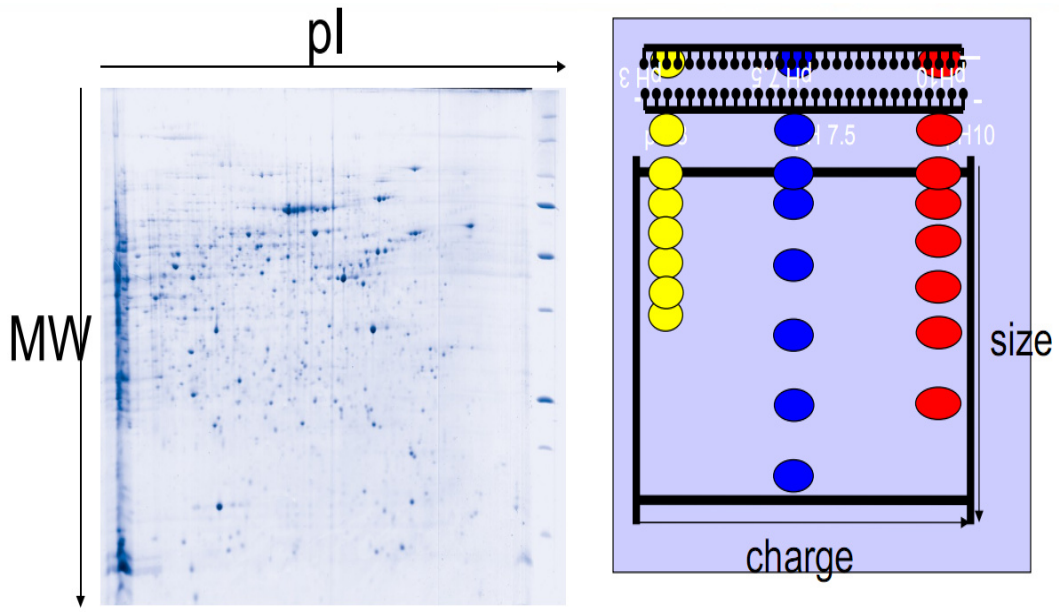



Figure 1.5. Separation principle of SDS-PAGE

## 1.6. Mass Spectrometry

The principle of mass spectrometry (MS) was established more than 100 years ago by J.J.Thomson after he noticed that the movement of ions is proportional to their mass-to-charge (m/z) ratio. Up to now, MS has developed further and is one of the most powerful tools for analyzing biomolecules.

Analysis with MS is based on measurement of the m/z ratio of molecular ions which are characteristic for a given compound. It can be used for many purposes such as for discovering the composition of a sample, identifying unknown compounds, and for determining the structure of a compound by its fragmentation pattern. With modern MS techniques, more detailed and challenging analysis such as the identification of

posttranslational modifications (PTMs) including, phosphorylation, methylation, acetylation, and ubiquitination can be done.

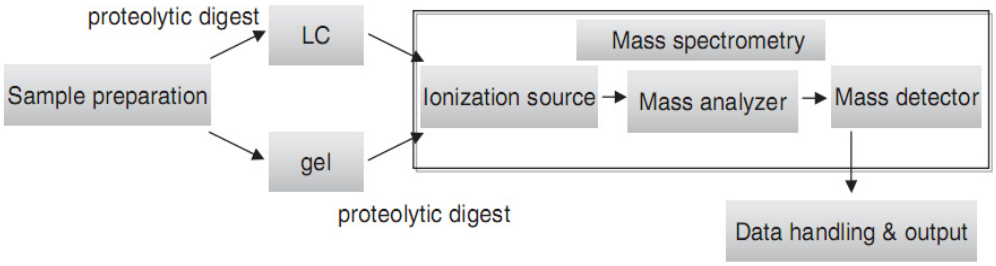



Figure 1.6. Schematic work flow of MS
(Source: Agrawal and Rakwal 2008)

## 1.6.1. Instrumentation

A mass spectrometer measures the m/z ratio of ions. For measuring the m/z ratio of ions, molecules should be ionized, different masses should be separated, and intensities of the ions should be recorded. Mass spectrometers consist of the ion source that ionizes the molecule; the mass analyzer, which separates the gas-phase ions according to their m/z values; and the mass detector that records the signals (Fig.2.6.).

With the MS the data outcome of the analysis is the mass of a molecule or ions which are derived from the parent ion after fragmentation when tandem MS (MS/MS) is applied. These fragmentation patterns are especially important for identification of the amino acid sequence of a peptide and sites for PTMs.

## 1.6.2. Ionization Source

In the ionization source, molecules are converted and transferred into gas-phase ions, which are then transported to the mass analyzer by a magnetic or an electric field. This is achieved by the loss/gain of an electron or the loss/gain of charge by protonation. Electron ionization (EI) and chemical ionization (CI) were the most commonly used methods before the development of the soft-ionization techniques MALDI and ESI (Domon and Aebersold 2005). Nevertheless, these techniques result in intensive thermal decomposition so they were only appropriate for small molecules and

they are not suitable for proteins and peptides analysis. With the development of MALDI and ESI in 1988 and 1989, characterization of proteins by MS become possible because no excessive fragmentation of large compounds occurred (Bradshaw and Burlingame 2005, Palcy and Chevet 2006).

### 1.6.2.1. MALDI

MALDI is one of the soft ionization techniques which is capable of analyzing proteins and other large molecules. Co-crystallization of the analyte and the matrix with an excess of the matrix is the basis of the principal of MALDI. With the bombardment of the matrix/ analyte with N2-laser, the matrix and analyte are protonated because of the energy transfer from matrix to analyte. Ions are then detached from the crystal in a non-fragmentation manner that makes it possible to acquire the molecular mass of the compounds, and they are subsequently transferred into the gas phase. Ions are accelerated toward the mass analyzer by the help of a high electric field (10–30kV). Aromatic molecules are usually used as matrix, which absorbs and transfers energy at a specific laser wavelength (337nm). Most commonly used matrix compound can be seen in Figure 1.8.

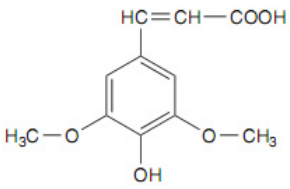
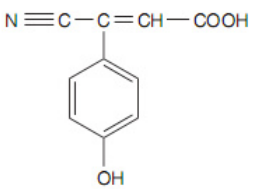
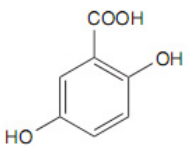
| Matrix | Structure |
|---|---|
| 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid) |  |
| α-Cyano-4-hydroxycinnamic acid |  |
| 2,5-Dihydroxybenzoic acid |  |

Figure 1.7. Structures of Commonly Used Matrix Compounds for MALDI (Source: Agrawal and Rakwal 2008)

Although the ionization mechanism is not fully understood, initial ionization of the compounds is carried out by matrix molecules. With MALDI instruments, generally singly charged molecules $[M+H]^+$ are formed because of protonation and these ions are separated according their m/z. Different mass analyzers can analyze vaporized ions. MALDI can be coupled to several mass analyzers, including TOF mass analyzer (MALDI–TOF–MS). TOF mass analyzers have a problem of low resolution due to different desorbtion and ionization times of molecules with the same molecular mass (Cho 2007). The abovementioned problem has been solved by the development of delayed ion extraction and reflectron technology. MALDI-TOF–TOF instruments are also capable of MS/MS.

### 1.6.2.2. ESI (Electro Spray Ionization)

John Fenn was awarded the Nobel Prize in 2002 for the development of ESI (Palcy and Chevet 2006). With the modification of ESI, nano-ESI which is capable of carrying out analysis with only a few microliters of sample was developed. When the liquid sample is passed through a small capillary, a high voltage is applied and during this process charged droplets are generated. After that the solvent evaporates at the tip of the column so that the gas-phase ions, which are directed under increasing vacuum to the mass analyzer, are formed. Acetonitrile or 2-propanol is used to generate gas-phase ions as solvents. Depending on the sample composition including the pH of the solvent and the chemical nature of the peptides analyzed, the charged molecules can be singly or multiply charged. Doubly charged and sometimes singly charged ions are formed for peptides smaller than 2000 Da. Multiple charge ions are formed from peptides larger than 2000 Da. Multiple charges produce lower m/z ratio, so that large peptides give values in a range that can be measured (Cho 2007).

## 1.6.3. Mass Analyzers

The mass analyzer separates peptides according their m/z ratio. Different types of mass analyzers exist, all of them having advantages and disadvantages. In most cases, more than one mass analyzer is used for the analysis of peptides and thus results in MS/MS, a technique that provides information about the amino acid sequence of a

peptide (Domon and Aebersold 2005). There are several mass analyzers including quadrupole, ion traps, orbitraps, Fourier transform ion cyclotron resonans and time of flight (TOF) mass analyzers.

### 1.6.3.1. TOF

With the mass analyzers, the flight time of ions until they reach the detector is measured. Ions are accelerated with an electrical field and the time they take to reach the detector is measured. Ions with the same initial translational energy and m/z ratio have the same flight time, however heavier particles have a longer travel time. However, because of the spreading of the initial energy, the mass resolution of a TOF analyzer is reduced. With help of the reflectron which is implemented at the end of the flight tube, the mass resolution of a TOF analyzer is increased (Bradshaw and Burlingame 2005). Ions with the same m/z ratio but different kinetic energies are focused by the reflectron in space and time, so that they arrive at the same time at the detector. TOF analyzers are commonly coupled to MALDI. But also they have now been successfully coupled to ESI. Both ESI and MALDI are often used in combination with Q–TOF analyzers. These instruments have the advantage of high resolution of the TOF analyzer and the efficient ion selection of the quadrupole (Li 2005, O'Farrell 1975).

## 1.6.4. Ion Detectors

Mass analyzers need an ion detector as a component of the mass spectrometer but FTICR and Orbitraps do not contain ion detectors. When an ion hits the surface of an ion detector, it creates a current of voltage and this is measured by the ion detector. The magnitude of current produced at the detector as a function of time is dependent on the m/z of the ion and is used to calculate the m/z and the intensity of the signal. To obtain a significant signal, the signal is multiplied because the number of ions that hit to the detector is low. The intensity of peaks in a mass spectrum can be normalized and given as relative abundance in percentage. The most abundant peak is called the base peak and is normalized to 100. Secondary electron multipliers (SEMs) and

microchannel plate (MCP) detectors are the most common ion detectors (Dubois et al. 1999).

### 1.6.5. Protein Identification with MS

The identification of proteins with MS can be done in a database-dependent or independent manner. With construction of protein databases, peptide mass finger print (PMF) has become one of the fastest methods to identify proteins, and also does not require MS/MS. Peptide fragmentation identification and de novo sequencing both need MS/MS data, while the latter one does not use a database as a reference. Basic principles of PMF and de novo sequencing can be seen in Figure 1.9.

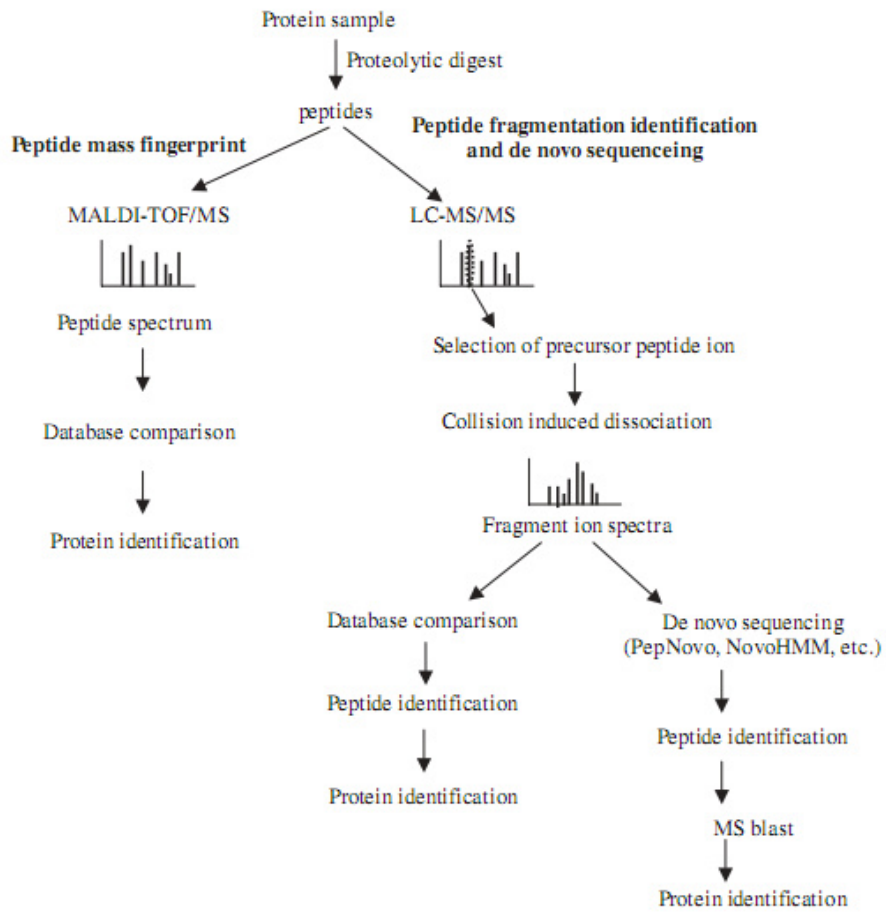



Figure 1.8. Differences between PMF, peptide fragmentation identification, and de novo sequencing. (Source: Agrawal and Rakwal 2008.)

### 1.6.5.1. Peptide Mass Fingerprint (PMF)

If a proteolytic digest of a protein of interest is analyzed by MS, the mass spectrum of the peptide mixture can provide a PMF. PMF is specific for a given protein and thus allows the identification of this protein (Unlu et al. 1997, Tonge et al. 2001). For PMF analysis, generally complex protein samples are separated by 2-Dgels, then protein spots are excised from the gel and digested with proteases (e.g., by trypsin). Then the peptide mixture is analyzed by MS especially with MALDI–TOF and the MS spectrum is compared in-silico with the genome of an organism. Computer programs use protein sequences which are stored in databases and theoretically digests them with the respective enzyme. The obtained virtual digestion data is known as an in-silico digest. The masses of the peptides originated from each protein are calculated and compared to the protein of interest. Statistics are used to calculate the best match, which results in a respective score. PMF analysis is very applicable for high throughput analysis, since it is very fast. But PMF has several limitations. These are caused by the occurrence of more then one protein in one gel spot, the presence of contaminants or PTMs (post translational modifications) may complicate PMF identification.

### 1.6.5.2. Peptide Fragmentation Identification

Peptide fragmentation identification requires MS/MS and allows the unambiguous identification of proteins, even from complex protein mixtures. Thus, this analysis of peptides requires the fragmentation of peptides into different ions by collusion-induced dissociation (CID). With MS/MS, partial sequence of a peptide and fragmentation pattern can be obtained. The partial sequence information, plus the precursor mass and knowledge of the proteolytic cleavage rules are generally sufficient information to query a gene or protein database and find a matching peptide. (Perkins et al.1999, Clauser et al.1999). The correct peptide interpretation is based on MS/MS search algorithms that are provided by search engines such as Mascot (Kislinger et al. 2005.), SEQUEST (Dunkley et al. 2004.) and Phenyx (Turecek 2002). To obtain MS/MS spectra, two mass analyzers and a collision cell need to be coupled. The first mass analyzer measures the parent mass of the ion and the second records the fragment ions after CID. Fragmentation of peptides results in specific internal ions. The most

abundant ions are b-ions, if the N-terminus is included; and y-ions, if the C-terminus is included (Ong et al. 2002, Blagoev et al. 2003). By calculating the mass difference between the ion series, amino acid sequence information is obtained. Besides these ions, other internal fragment ions are also obtained. The fragmentation pattern of a certain peptide is highly dependent on its amino acid sequence.

## 1.7. Aim of the Project

With this project our aim was to identify the proteins that are differentially expressed in drought exposed and control, non drought-exposed, chickpea plants. For this aim, we first grew our plants for 3 weeks with regular watering. Then we did not water our drought-exposed plants for one week but continued watering the control plants. We extracted total protein from both control and drought-exposed plants. This was followed by 2D gel electrophoresis. 2D gel images were analyzed with image analysis software to define differentially expressed proteins. Then we excised the spots from the gels and carried out in-gel digestion. Finally we identified our proteins with MALDI/TOF-TOF MS.

With this project we will shed light on the mechanisms related to drought resistance by using chickpea as a model organism. “Gökçe” the chickpea cultivar that we used is known to be drought tolerant according to the data of ICARDA. Examination of the proteome of such a resistant plant will help us to identify the proteins that take part in drought tolerance (Kusmenoglu et al. 2006). Identification of these proteins will enlighten us on strategies for the development of new drought resistance chickpea cultivars by molecular breeding. With further studies, the genes corresponding to those proteins can be identified and with recombinant DNA technology it will be possible develop drought resistant crop plants.

# CHAPTER 2

# EXPERIMENTAL

## 2.1. Plant Growth Conditions and Drought Treatment

During our studies we used a drought resistant chickpea cultivar, Gokce. Five chickpea seeds were planted in 18cm, 1,5 lt pots. Plants were grown in growth chamber at 25oC and%50 relative humidity. Each day, each pot was watered with 100ml tap water for three weeks to keep the relative humidity at approximately 30%. After three weeks drought treatment group plants were not watered for a week while control plants were watered. Finally both roots and leaves were harvested and stored in liquid nitrogen until used for total protein isolation.

## 2.2. Protein Extraction from Leaves and Roots Protein Extraction from Leaves and Roots

Phenol extraction method for total protein extraction is reported to be more efficient for plant recalcitrant tissues so we performed phenol extraction and proteins were precipitated with ammonium acetate in methanol during our studies. This method allowed efficient protein recovery and removed non-protein components in the case of plant tissues rich in polysaccharides, lipids, and phenolic compounds.

The same procedure was followed for both leaf and root samples without any modifications. The chemicals that were used were electrophoresis or biological grade and ultra pure water was used during the studies. Experiments were carried out at 4 ˚C unless another temperature is stated. Un-powdered gloves were used throughout to avoid contamination.

Buffers and solutions required for the experiment were as follows;

- Phenol: Tris-HCl saturated, pH 6.6/7.9
- Preparation of extraction buffer: A solution of 500 mM Tris-HCl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl was prepared and adjusted to pH8.0 with HCl. This solution can be stored for a week at 4°C. Just before extraction 2% β-

mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to the buffer.

    - 7,88g Tris Base, 1,86 g EDTA, 11,98g sucrose, 0,746g KCl were dissolved in 90ml ultra pure water then pH was adjusted to 8 then finally 500µl of PMSF (200Mm solution prepared in 2-propanol) and 2 ml of β-mercaptoethanol were added and volume was adjusted to100ml.
- Preparation of 200mM PMSF solution in 2-propanol.
    - A total of 0.348g of PMSF was dissolved in 10ml 2-propanol aliquoted and stored at -20°C.
- Preparation of precipitation solution: 0.1 M ammonium acetate in cold methanol. This solution was stored at –20°C.
    - 1.927g ammonium acetate was dissolved in 250ml cold methanol.
- Cold acetone

After preparation of above mentioned buffers and solutions experiments were carried out as follows;

- 2g of fresh plant tissue was frozen in liquid nitrogen after harvest and ground to a fine powder with mortar and pestle.
- Ground tissue was suspended in 3 mL of extraction buffer in a 15-mL

Falcon tube, vortexed, and incubated by shaking for 10 min on ice

- Afterward, an equal volume of Tris-buffered phenol was added, and the solution was incubated on a shaker for 10 min at room temperature.
- To separate insoluble material (in the pellet), for aqueous and organic phases, the sample was centrifuged for 20 min at 4000rpm and 4°C. The phenolic phase, which was on the top of the tube, was recovered carefully to avoid contact with the interphase and poured into a new tube.
- This phenol phase was then back-extracted with 3 mL of extraction buffer. The sample was shaken for 3 min again and vortexed. Centrifugation for phase separation was repeated for 20 min at 4°C and 4000rpm.
- The phenol phase still on the top of the tube was carefully recovered and poured into a new tube; 4 volume of precipitation solution was added. The tube was shaken by inverting, and the sample was incubated overnight at –20°C.

- Proteins were finally pelleted by centrifugation (10 min, 5500g at 4°C).
- After centrifugation, the pellet was washed three times with cooled precipitation solution and finally with cold acetone. After each washing step, the sample was centrifuged for 5 min at 5500g and 4°C.
- The pellet was recovered to eppendorf tubes and centrifuged for 5 min at 5500g and 4°C and supernatant was removed. Finally protein pellet was dried in SpeedVac (Thermo Electron Corporation) vacuum concentrator for 2 minutes.

## 2.3. Protein Solubilization with Rehydration Buffer

Then the lyophilized pellet was dissolved in rehydration buffer to dissolve all protein species in the protein pellet.

- Preparation of rehydration buffer: 7 M urea, 2M thiourea, %4 CHAPS, 65Mm DTT, %2 ampholyte pH3-10
    - The preparation of rehydration stock solution: 4.2 g of urea, 1.52 g of thiourea, and 0.4 g of CHAPS were weighed and dissolved in ultra pure water to a final solution volume of 10 ml. During dissolving solution should not be heated over 30 ˚C to prevent protein carbamylation. This stock solution was stored at 4 ˚C up to one month.
    - Just prior to use, for each 1 ml of stock solution 0.01 g of DTT and 25 µl of ampholyte was added.

The volume of rehydration buffer added to the protein pellet depended on the amount of the buffer needed to solubilize all of the pellet. Approximately,700 µl of rehydration buffer was used per sample pellet. After addition of enough buffer to pellet, it was thoroughly mixed and vortexed for 30 minutes. Then the eppendorf tubes were centrifuged at 8,000 x g for 5 minutes at 4 ˚C to remove the non-soluble particles and supernatant was transferred to a new eppendorf tube. After quantification of protein concentration, protein solutions were used for IEF.

## 2.4. Bradford Protein Assay for Protein Determination

For protein quantification, we used Bradford protein assay which is one of the spectroscopic analytical methods used to determine total protein concentration (Bradford 1976). The principle of this method is based on the shift in absorbtion of Coomassie brilliant blue G-250 dye when it binds to proteins. After binding of dye to proteins, its color changes to blue and it becomes darker proportional to protein concentration. The Bradford assay has a linear dynamic range, generally from 2µg/ml to 120 µg/ml. To measure protein concentration of an unknown sample, a series of protein standards was needed. Bovine serum albumin (BSA) was used as a standard protein to compare with sample protein concentration.

- Preparation of Coomassie Reagent:
    - 100 mg of CBB G-250 was dissolved in 50 ml of 95 % ethanol then 100 ml of 85 % phosphoric acid was added and the whole was diluted to 1l with ultra pure water. The final solution was filtered through filter paper and was stored in an amber bottle at 4 ºC.
- Preparation of 2µg/µl stock BSA solution
    - 20mg of BSA was weighed and dissolved in water to a final volume of 1.0 ml. Then this solution was diluted 1/10.

Table 2.1. Preparation of BSA Standards from 0.2 mg/ml BSA and Test Sample for the Bradford Protein Assay

| **Test Sample** | **Sample Volume, µl** | **1N HCl Volume, µl** | **Water Valume, µl** | **Rehydration Buffer Valume, µl** | **Coomassie Reagent Volume, ml** |
|---|---|---|---|---|---|
| **Blank** | 0 | 10 | 80 | 10 | 5 |
| **BSA Standard – 5 µg** | 2,5 | 10 | 77,5 | 10 | 5 |
| **BSA Standard – 10 µg** | 5 | 10 | 75 | 10 | 5 |
| **BSA Standard – 20 µg** | 10 | 10 | 70 | 10 | 5 |
| **BSA Standard – 40 µg** | 20 | 10 | 60 | 10 | 5 |

(cont. on next page)

Table 2.1. (cont.)

| | | | | | |
|---|---|---|---|---|---|
| **BSA Standard – 50 µg** | 25 | 10 | 55 | 10 | 5 |
| **BSA Standard – 75 µg** | 37,5 | 10 | 42,5 | 10 | 5 |
| **BSA Standard – 100 µg** | 50 | 10 | 30 | 10 | 5 |
| **Protein Sample** | 10 | 10 | 70 | 10 | 5 |

Blank, BSA standards, and protein samples were prepared according to Table 2.1. Sample mixtures were incubated at room temperature for 10 minutes. Finally, absorbance of each sample was measured at 595 nm using a UV-visible spectrophotometer.

The standard curve was obtained by plotting the absorbance at 595 nm versus µg of protein in BSA standard samples. We obtained a linear graph as is expected and calculated the line's equation via Microsoft Excel software. Using the equation we calculated the concentrations of unknown protein samples. Table 2.2 shows the absorbance values for various BSA standards.

Table 2. 2. Absorbance Values for BSA Standards

| Concentration (µg) | Absorbance at 595 nm |
|---|---|
| 5 | 0,65 |
| 10 | 0,69 |
| 20 | 0,78 |
| 40 | 0,97 |
| 50 | 1,03 |
| 75 | 1,033 |
| 100 | 1,22 |

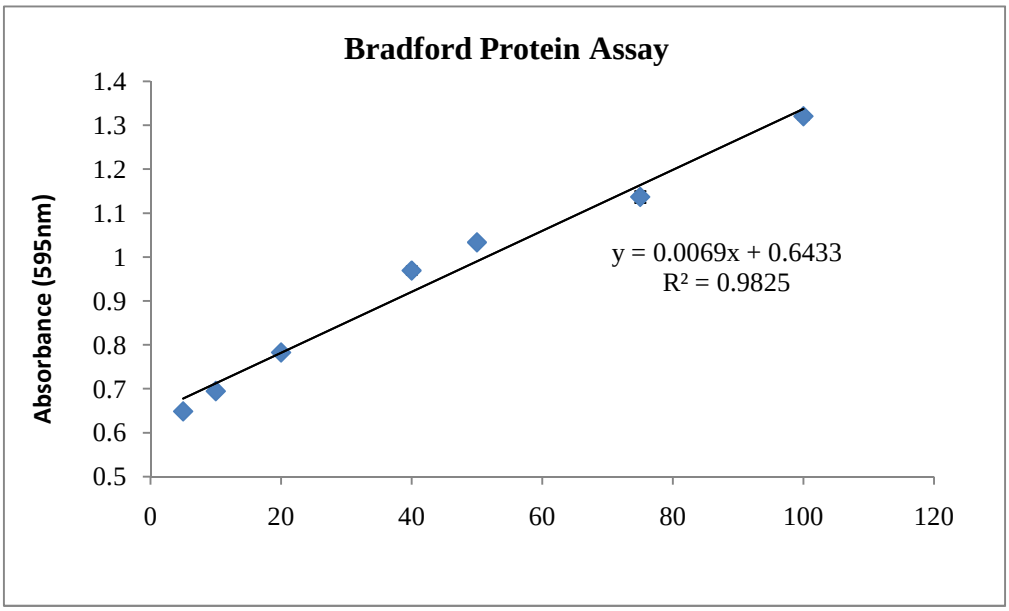



Figure 2. 1. Standard Curve for BSA

## 2.5. 2-DE of Total Proteins from Chickpea Roots and Leaves

### 2.5.1. Isoelectric Focusing

For isoelectric focusing (IEF), the first dimension of 2D-PAGE, we used immobilized pH gradient (IPG) strips at both pH 3-10 and pH 4-7, linear gradient and carried out the experiment by using a Protean IEF Cell (BioRad). In the first step of IEF, we applied our protein samples and the rehydration procedure was carried out in IEF focusing tray. After rehydration, we carried out focusing.

We used a 17cm length focusing tray suitable for our IPG strips which was cleaned before use. First of all we placed paper wicks above the electrodes and soaked them with 8µl ultra-pure water. Than we diluted our protein samples to 500µg with rehydration buffer as mentioned previously. We loaded both root control and drought treatment samples isolated from Gökçe chickpea cultivar. Then we placed 300µl of our protein sample into a certain point in IEF focusing tray and avoided bubble formation. Then IPG strips were taken out of -20 ºC and allowed to thaw for 5 minutes at room temperature. Afterwards the protective layer above the IPG strips was removed and the strips were positioned in the IEF focusing tray gel side down and positioned with the positive end of the strip to the positive electrode and the negative end to the negative electrode. To minimize evaporation and urea crystallization, the strip was then covered

with 2 ml of mineral oil. Finally the focusing tray was covered and allowed to stand for one hour at room temperature.

After one hour, the IEF tray was placed into Protean IEF Cell then IPG strips were actively rehydrated with 50V voltage application per strip for 12 hours. After rehydration, we carried out 6-stepped focusing which was as follows: phase 1, linear gradient up to 250 Volts in 15 minutes; phase 2, linear gradient up to 500 Volts in 30 minutes; phase 3, rapid gradient up to 1000 Volts in 1 hour; phase 4, linear gradient up to 5000 Volts in 3 hours; phase 5, linear gradient up to 10,000 Volts in 3 hours; and phase 6, linear gradient up to 10,000 Volts at 70,000 Volts h-1. Working temperature was set to 20 ºC in IEF. It took approximately 20h for focusing to finish.

After the IEF run was complete, IEF focusing tray and IPG strips were removed and strips were placed at -80 ºC or immediately equilibrated with equilibrium buffers for the second dimension of the experiment.

## 2.5.2. Equilibration of Strips

After IEF, the strips containing the focused proteins were equilibrated. This procedure is applied for formation of SDS-protein complexes, reducing the disulphide bonds and to alkylate the sulfhydryl groups. After we applied this procedure, proteins had completely unfolded structure and carried only negative charges. We used two equilibration buffers both of them containing SDS, Tris-HCl pH 8.8, glycerol, and urea. Equilibration buffer I contained DTT and equilibrium buffer II contain iodoacetamide instead of DTT. DTT is a reducing agent required for cleavage of disulphide bonds between cysteine residues. Iodoacetamide is an alkylation agent used for preventing disulphide bond formation by alkylating free sulfhydryl groups in cysteine residues.

- Preparation of equilibrium buffer I: 6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20 % glycerol, and 2 % DTT.
    - 1.81 g of urea, 1.25 ml of Tris-HCl pH 8.8, 0.1 g of SDS, 1 ml of glycerol, and 0.1 g of DTT. The final volume was adjusted to 5 ml with water for one strip.
- Preparation of equilibrium buffer II: 6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20 % glycerol, and 2.5 % iodoacetamide.

- 1.81 g of urea, 1.25 ml of Tris-HCl pH 8.8, 0.1 g of SDS, 1 ml of glycerol, and 0.125 g of iodoacetamide (AppliChem). The final volume was adjusted to 5 ml with water for one strip.

Equilibration buffers were prepared fresh just before use and focused IPG strips were placed into equilibration trays gel side up. Afterwards strips were treated with equilibration buffer I for 15 minutes with gentle shaking. After 15 minutes, strips were taken to a new dry tray and treated with equilibrium buffer II for 15 minutes with gentle shaking.

### 2.5.3. SDS-PAGE

After the equilibrium step, strips were rinsed with 1X Tris-Glycine-SDS (TGS) running buffer and they were attached to the top of a polyacrylamide gel.

- Preparation of equilibrium buffer II: 6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20 % glycerol, and 2.5 % iodoacetamide.
- Preparation of 5X Tris-Glycine-SDS (TGS) running buffer: 0,125M Tris-base, 1.25M glycine and 0,05% SDS pH is adjusted to 8,3.
    - 15.1 g of Tris-base (AppliChem) and 94 g of glycine (AppliChem) were weighed and dissolved in 900 ml of water. Then, 50 ml of a 10 % (w/v) SDS solution was added and the final volume was adjusted to 1000 ml with water. It wasstored at 4 ºC.
- Preparation of 1X Tris-Glycine-SDS (TGS) running buffer: To prepare 1l of 1X TGS buffer, 200 ml of 5X TGS buffer was diluted to a final volume of 1l with ultra pure water.

SDS-PAGE procedure was carried out in PROTEAN II xi Cell (Bio-Rad). We used 12 % polyacrylamide gel slabs. Glass plate sizes were 16 cm cells with 16 x 20 cm for inner plate and 18.3 x 20 cm for outer plate. Preparation of 12 % polyacrylamide gel and required solutions were as follows;

- Preparation of 12 % polyacrylamide gel mixture: 16.5 ml of water, 20.0 ml of 30 % acrylamide mixture, 12.5 ml of 1.5 M Tris-HCl (pH 8.8), 500 µl of 10 % SDS, 500 µl of 10 % ammonium per sulfate, 20 µl of TEMED were mixed to a final volume of 50 ml.

- Preparation of 30 % acrylamide mixture: 29.0 g of acrylamide and 1.0 g of N, N′-methylenebisacrylamide were dissolved in a total volume of 60 ml of water and the solution was heated to 37 ºC to dissolve the chemicals. The total volume was filled to 100 ml with water. Solution was stored at 4 ºC in dark bottles for not more than 1 month.
- Preparation of 1.5 M Tris-HCl, pH 8.8:1.5 M Tris-base pH8.8buffer.
    - 3.634 g of Tris-base was dissolved in 15 ml of water and pH was adjusted to 8.8 with concentrated (~6 M) HCl. Then the volume was filled to 20 ml
- Preparation of 10 % SDS: 1.0 g of SDS was dissolved in water to a final volume of 1.0 ml.
- Preparation of 10 % ammonium persulfate (APS): 1.0 g of APS was dissolved in water to a final volume of 1.0 ml. This solution should be prepared just before use.

While preparing the 12 % polyacrylamide gel mixture, TEMED and ammonium persulfate were added just before pouring the gel because polymerization was started immediately after adding APS and TEMED. After preparation of 12 % polyacrylamide gel mixture, it was then poured between two glass plates.

To reduce protein modifications caused by free acrylamide or reagents causing polymerization, the casted SDS-polyacrylamide gel was kept for at least one day at 4 ºC prior to electrophoresis.

Equilibrated IPG strip was placed onto the polyacrylamide slabs and sealed with 1 ml overlay agarose. While placing the IPG strips above the polyacrylamide slabs, bubble formation between IPG strip and the resolving gel interface must be avoided. When the overlay gel was solidified, the gel was placed into the central cooling core of the system and finally placed into buffer tank. The buffer tank was half-filled with 1X TGS running buffer and electrophoresis was begun. During electrophoresis, central cooling core was cooled with circulating water to hold the temperature near 10 ºC. Electrophoresis run was carried out at 200V for nearly 7 hrs until the blue dye front reached the bottom of the gel.

## 2.5.4. Staining and Destaining of Gels

After electrophoresis was finished, gels were removed from glass plates and transferred to a large tray. Gels were rinsed with ultra pure water. Afterwards coomassie colloidal blue staining solution was poured into trays containing the gels until the gels were covered with solution. Gels were left for at least 12h staining with gentle shaking.

- Preparation of coomassie colloidal blue staining solution: 40 g of ammonium sulfate was dissolved in 280 ml of ultra pure water and mixed with 8 ml of 85 % phosphoric acid. Then 100 ml of methanol was added and the final volume was adjusted to 500 ml with ultra pure water. Finally, 0.5 g of CBB G-250 was added and mixed thoroughly. This solution was stored at 4 °C.

After 12h the staining solution was poured off and gels were rinsed with ultrapure water at least three times. Then, gel was treated with neutralization buffer for three minutes with gentle shaking. The buffer solution was removed and the gel was rinsed with ultrapure water.

- Preparation of neutralization Buffer: 0.1 M of Tris-phosphate at pH 6.5.
    - 10.96 g of Tris-dihydrogen phosphate dissolved in water to a final volume of 500 ml. The pH was adjusted with NaOH to 6.5. It was stored at 4 ºC.

After neutralization of gels, destaining procedure was applied by adding destaining solution and incubating less than one minute with gentle shaking. Then the destaining solution was removed and the gel was rinsed with ultrapure water.

- Preparation of destaining solution: 25 % methanol solution.
    - 125 ml methanol was mixed with 375 ml water. It was stored at 4 ºC.

After destaining gels were treated with fixation solution for at least one day.

- Preparation of fixation Solution: 20 % of ammonium sulfate solution.
    - 100 g of ammonium sulfate was dissolved in water to a final volume of 500 ml. It was stored at 4 ºC.

## 2.5.5. Image and Data Analysis of Gels

After staining and destaining procedures, gel images were obtained by CCD camera. Ludesi Redfin image analysis soft ware was used for gel scanning and analysis.

Background subtraction and smoothing processes were applied. More importantly, control and drought treated gels were aligned and superposed to find differentially expressed proteins between two groups. Also we obtained the statistical data concerning differentially expressed proteins while comparing sample's gels.

## 2.6. In-Gel Digestion

According to the image analysis results, differentially expressed protein spots were excised from gels as closely as possible with a sharp scalpel, and divided into smaller pieces. After the protein spots were excised from gels, in-gel digestion procedure was applied. In-gel digestion protocol is really significant in terms of proteomics experiments because the efficiency of protein identification in the next step, mass spectrometry, depends on obtaining all possible peptides species formed via in gel digestion.

We used the protocol described by Shevchenko and co-workers which is well-adapted with mass spectrometric experiments (Shevchenko et al. 1996). The in-gel digestion procedure includes removal of stain from gel pieces, reduction and alkylation of protein spots, in-gel digestion and extraction of peptide fragments from gel pieces.

The preparation of in-gel digestion chemicals are described in the following:

- Preparation of wash solution: 50 % (v/v) methanol and 5 % (v/v) acetic acid.
    - 10 ml of methanol (Merck) was added to 5 ml of water followed by addition of 1 ml of acetic acid and the total volume was adjusted to 20 ml with water.
- Preparation of 100 mM ammonium bicarbonate: 0.2 g of ammonium bicarbonate was dissolved in 20 ml of water.
- Preparation of 50 mM ammonium bicarbonate: 2 ml of 100 mM ammonium bicarbonate was mixed with 2 ml of water.
- Preparation of 10 mM DTT: 1.5 mg of dithiothreitol was placed in a 1.5 ml plastic centrifuge tube followed by addition of 1 ml of 100 mM ammonium bicarbonate for complete dissolving of DTT.
- Preparation of 100 mM iodoacetamide: 18 mg of iodoacetamide was placed in a 1.5 ml plastic centrifuge tube followed by addition of 1 ml of 100 mM ammonium bicarbonate for complete dissolving of iodoacetamide.

- Preparation of trypsin solution: 12.5µg/ml trypsin.
    - 1 ml of ice cold 50 mM ammonium bicarbonate was added to 20 µg of proteomics grade modified trypsin and dissolved. The trypsin solution was kept on ice until use. Afterwards 625µl of trypsin solution was mixed with 375µl ultrapure water.
- Preparation of extraction buffer: 50 % (v/v) acetonitrile and 5 % (v/v) formic acid.
    - 10 ml of acetonitrile was added to 5 ml of water followed by addition of 1 ml of formic acid and the final volume was adjusted to 20 ml with water.

**Washing the Gel Pieces**

- After cutting the protein spots from gels, gel pieces were placed in a 1.5 ml plastic microcentrifuge tube. The gel pieces were covered with 200 µL of wash solution and rinsed overnight at room temperature.
- The gel slices were washed with water for 15 minutes.
- The liquid remaining from the previous step was removed. 100 µl of water was added, followed by 100 µl of acetonitrile to the gel pieces and incubated for 15 minutes at room temperature.
- The liquid was removed. 200 µl of acetonitrile was added to cover the gel pieces. The pieces were dehydrated, shrunk and turned white and sticky.
- The acetonitrile was removed. Gel pieces were rehydrated by adding 100 µl of 100 mM $NH_4HCO_3$. After incubation for 5 minutes, 100 µl of acetonitrile was added and incubated for 15 minutes at room temperature.
- Finally the liquid was removed. Gel pieces were dried in a speed vacuum for 15 minutes.

**Reduction, Alkylation, and In-Gel Digestion**

- 100 µL of 10 mM DTT was added and the protein was reduced for 30 minutes at room temperature. Then, DTT was carefully removed from the sample and discarded.
- Next 100 µl of IAA solution was added and incubated for 30 minutes at room

temperature in the dark.

- The liquid was removed. The gel was dehydrated by adding 200 µl of acetonitrile.
- After the gel pieces shrunked and turned white and sticky (~5 minutes), acetonitrile was removed. 200 µl of 100 mM $NH_4HCO_3$ was added and incubated for 10 minutes at room temperature to allow rehydration.
- The liquid was removed. 200 µl of acetonitrile was added. Incubated for 15 minutes.
- The liquid was removed. The gel slices were dried completely in a speed vacuum for at least 30 minutes.
- Samples were rehydrated in 100 µl of In-gel digestion buffer containing 12.5 µg/ml trypsin and incubated overnight at 37°C.

**Extraction of Peptides for Analysis**

- The trypsin digests were centrifuged briefly in a microcentrifuge. The peptides (in the supernatant) were transferred to a 0.5-ml microfuge tube.
- 100 µl of 25 mM $NH_4HCO_3$ was added to cover the gel pieces and incubated for 15 minutes.
- 100 µL of extraction buffer was added to the tube containing the gel pieces and incubated for 15 minutes with occasional gentle vortex mixing.
- The liquid was transferred into the 0.5-ml tube containing the peptides.
- 100 µL of the extraction buffer was added to the tube containing the gel pieces, and incubated for 10 minutes with occasional gentle vortex mixing.
- 100 µl of 5% formic acid was added to the gel slices and incubate for 15 minutes.
- 100 µl of acetonitrile was added to the slices and incubate for 15 minutes.
- The supernatant was removed from the slices, and transferred it to the 0.5-ml tube containing the peptides and the previous two steps were repeated.
- The liquid was transferred into the 0.5-ml tube containing the peptides.
- The volume of peptide solution was reduced up to 20µl in vacuum concentrator.
- The volume of the digest was adjusted to ~20 µL with acetic acid. Finally, the sample was ready for mass spectrometric analysis.

## 2.7. Desalting Protein Digests with Zip tip

Desalting procedure of peptide mixtures with Zip tip is as follows:

- We wetted zip tip via aspirating 10 µL 50% acetonitrile solution into tip. Then dispensed to waste. Repeated three times.
- Equilibrated via aspirating 10 µL 0.01% TFA solution into tip. Then dispensed to waste. Repeated three times.
- We aspirated and dispensed 3-7 cycles (simple mixtures), up to 10 cycles (complex). Then dispensed to waste.
- We aspirated 0.01% TFA solution and dispensed to waste. Repeated three times.
- Dispensed 1-4 µL of 50% acetonitrile solution into clean vial (standard pipette tip) (note: if µ-C-18,dispense 0.5-2 µL of elution solution).Aspirated and dispensed eluant through zip tip at least 3 times without introducing air.

## 2.8. MALDI Matrix Preparation

Preparation of two layer a-cyano hydroxy cinnamic acid MALDI matrix is as follows:

- For first layer weighted approximately 5mg α-cyano hydroxy cinnamic acid and dissolve it in 100µl of methanol. Then added 300µl of acetonitrile mix with vortex. Then spoted 1µl to MALDI plate.
- For second layer weighted approximately 5mg α-cyano hydroxy cinnamic dissolved in 300µl methanol than add 100µl 1%TFA (trifluoroacetic acid) than vortexed. Centrifuged for 8 minutes in highest speed. Finally mix protein sample and matrix 1:1 and spot above the first layer.

## 2.9. MALDI-TOF/TOF Analysis

We analyzed our protein in reflectron mode molecular weight between 1-4 kDa and obtained the spectra

# CHAPTER 3

# RESULTS AND DISCUSSION

In this study we examined drought responsive proteins in both leaf and root proteome of drought resistant chickpea cultivar *Gökçe*. For this aim we isolated total leaf and root proteins from drought treated and control plants via phenol extraction method which is suitable for low protein bearing recalcitrant plant tissues and carried 2D-PAGE and obtained differentially expressed protein spots between control and drought treated gels and we identified protein spots with MALDI-TOF/TOF mass spectrometer.

## 3.1. Effects of Drought Stress on Growth

As a result of drought stress exposure plants shows several symptoms including reduced plant mass, reduced shoot and root growth, increased leaf senescence, reduced leaf chlorophyll amount, reduced photosynthesis rate as a result of reduced intake of CO2, reduced chlorophyll and leaf necrosis, reduced yield and depending to the severity of drought even death can be occur. In our experiments after one week of drought treatment without watering we observed most of the symptoms including reduced shoot and root growth rates, increased senescence, leaf necrosis, reduced leaf chlorophyll amount were observed.


Figure 3.1. Control (A) and drought (B) treated chickpea plants before protein extraction.

## 3.2. Drought-Stress Responsive Protein Expression Changes in Chickpea Roots

Gökçe cultivar which was reported to be drought tolerant was examined in terms of protein expression differences in root proteome in response to drought exposure. Drought exposure was applied by without watering plants for 7 days. Total proteins were extracted from control and drought treated plants via phenol extraction method than to see the general protein profile we carried out 2D-PAGE in broad range ph 3-10 IPG(immobilized pH gradient) strips Fig.3.2. As a result of this experiment we saw that vast majority of proteins were distributed between neutral pH region so we decided to use pH 4-7 IPG strips to obtain higher resolution for further experiments.

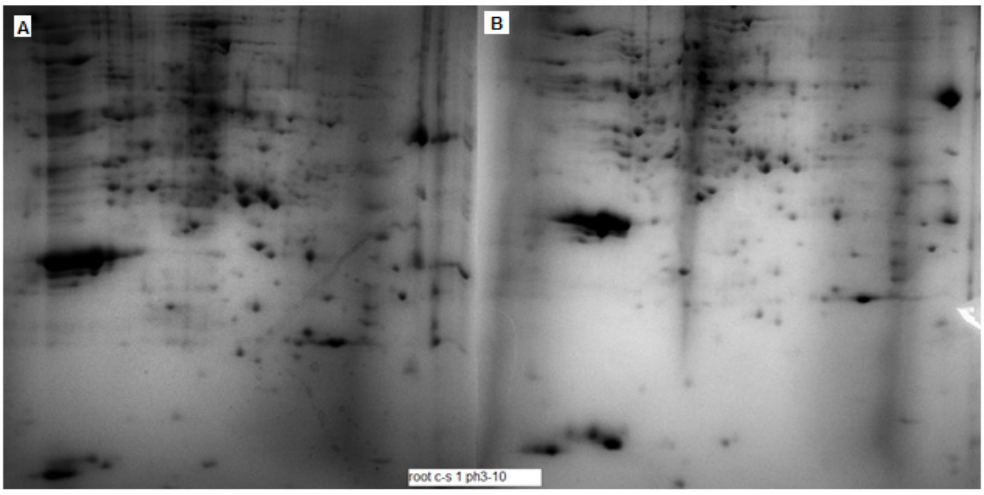



Figure 3.2. Gökçe root proteome map pH 3-10.A control, B stress

With pH 4-7 IPG strips we obtained a high resolution and saturated proteome map for root proteome Fig4.3. Proteins were distributed mostly in middle and low mass regions (proteins less than 50 kDa). Differences were obtained between control and drought treated plant's root and leaf proteomes. We carried out both plant growth, protein extraction and 2D-PAGE experiments three times and we obtained similar proteome maps for root samples.

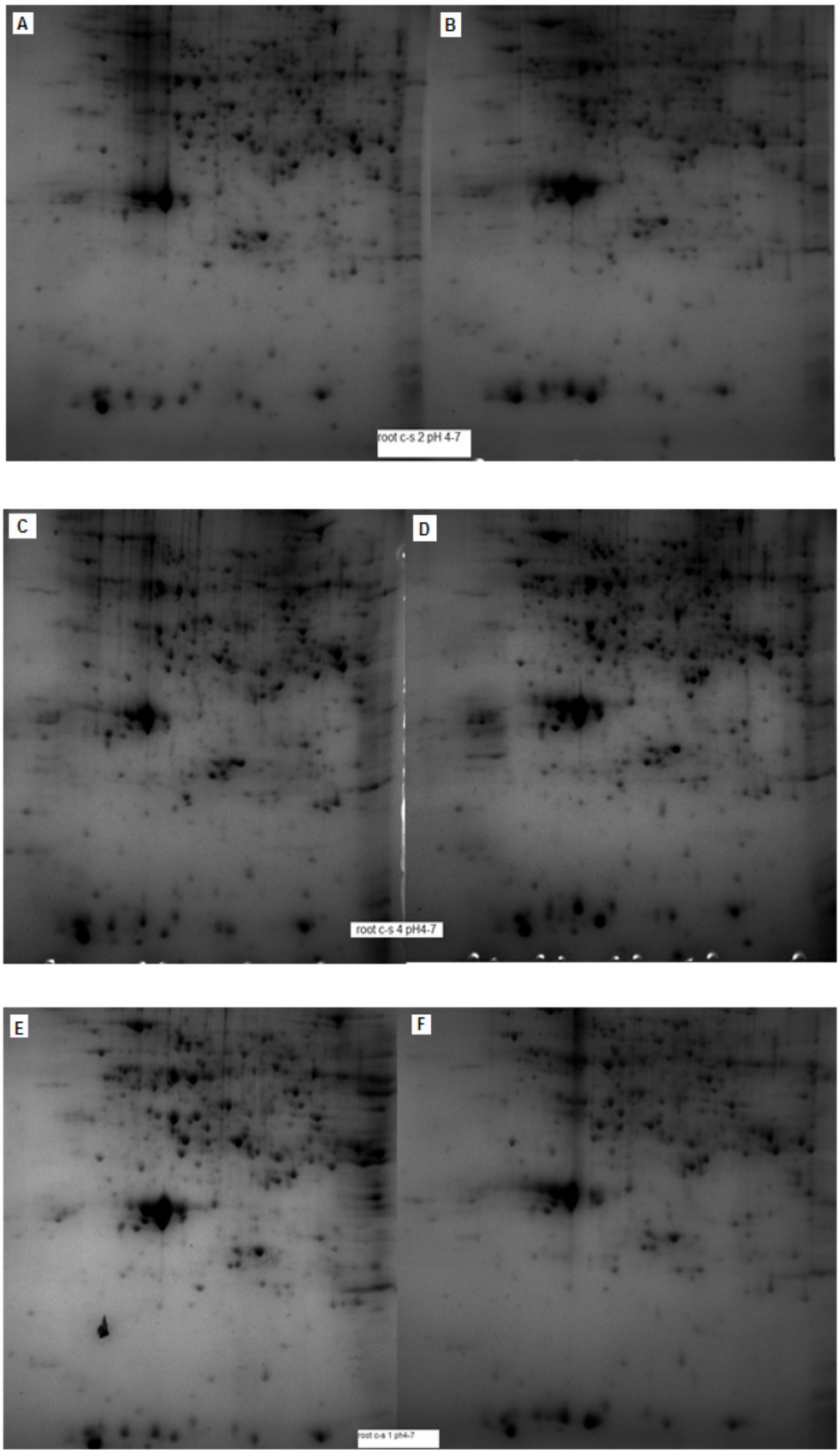



Figure 3.3. Gökçe root proteome map pH 4-7 of three replicaticates. A, C, E control, B, D, F stress

We carried out image analysis via Ludesi REDFIN 3 software to obtain differentially expressed protein spots between control and drought treatment proteomes of both root and leaves. Via the software we analyzed three pairs of both control and drought treatment proteome maps to obtain statistically significant results. Approximately 430 protein spots were obtained both in control and drought treated root proteome maps. Proteins whose expression changed more than 2 fold reproducibly among three replicate gels were selected as differentially expressed proteins between control and drought treatment proteome maps.

As a result of image analysis we obtained 18 newly formed spots: 14 of them formed and 4 of them disappeared in drought treatment proteome map. Also we obtained 12 over-expressed protein spots and 4 spots were down-regulated in drought treatment proteome map. All differentially expressed protein spots can be seen in Appendix A 1.

Table 3.1. Table of differentially expressed spots and their fold changes from root proteom.

| **Spot No** | **Spot Status** | **Fold Change** |
|---|---|---|
| 272 | new spot | - |
| 478 | new spot | - |
| 655 | new spot | - |
| 768 | new spot | - |
| 775 | new spot | - |
| 789 | new spot | - |
| 893 | new spot | - |
| 928 | new spot | - |
| 1008 | new spot | - |
| 1013 | new spot | - |
| 1038 | new spot | - |
| 1079 | new spot | - |
| 1132 | new spot | - |
| 93 | disappeared spot | - |
| 543 | disappeared spot | - |
| 595 | disappeared spot | - |
| 647 | disappeared spot | - |
| 795 | disappeared spot | - |
| 152 | up-regulated spot | 2.39 |
| 155 | up-regulated spot | 3.63 |
| 173 | up-regulated spot | 2.8 |

(cont. on next page)

Table 3.1. (cont.)

| | | |
|---|---|---|
| 174 | up-regulated spot | 2.86 |
| 197 | up-regulated spot | 2.79 |
| 253 | up-regulated spot | 6.01 |
| 271 | up-regulated spot | 2.56 |
| 296 | up-regulated spot | 2.77 |
| 605 | up-regulated spot | 6.62 |
| 635 | up-regulated spot | 2.49 |
| 734 | up-regulated spot | 2.56 |
| 739 | up-regulated spot | 5.36 |
| 39 | down-regulated spot | 11.35 |
| 53 | down-regulated spot | 2.96 |
| 103 | down-regulated spot | 2.92 |
| 761 | down-regulated spot | 2.83 |

We carried out PMF(peptide mass fingerprint analysis) for all differentially expressed leaf proteins via MALDI-TOF/TOF instrument. After obtaining the MS spectra for each protein we carry out database search via Mascot program by searching all entries in NCBIr database. We could carry out MS/MS analysis for just a few proteins. We selected the highest scored protein that matched as a result of the analysis. Also we especially selected plant proteins unless they had the third highest score. We managed to identify 7 proteins from the 34 differentially expressed proteins.

Table.3.2. Drought Root Responsive Proteins, Identified by MALDI-TOF/TOF via PMF

| Spot No | Protein Name | Sequence | AC Number (gi \| NCBI) and Reference Organism | Sequence Covered (%) | heoretical Molecular Mass (kDa) | Theoretical (pI) |
|---|---|---|---|---|---|---|
| **93** | WRKY DNA-binding protein 6 | MDRGWSGLTLDSSSLDLLNPNRITHNNHRR **FSNPLTMSRIDEDDDQK**TK**ISTNGSEFRFP VSLSGIR**DREDNDFSSGVVGDNDREVPGEV DFFSDKKSRVCREDEDGFRVKKEEQDDRTD VNTGLNLRTTGNTKSDESMIDDGESSEMED KRAKNELVKLQDELKKMTMDNQKLRELLT QVSNSYTSLQMHLVSLMQQQQQQNNKVIEA AEKPEETIVPRQFIDLGPTR**AVGEAEDVSNSS SEDR**TRSGGSSAAERRSNGKRLGREESPETE SNKIQKVNSTTPTTFDQSAEATMRKARVSVR **ARSEAPMISDGCQWR**KYGQKMAKGNPCP R**AYYRCTMATGCPVR**KQVQRCAEDRSILIT TYEGNHNHPLPPAAVAMASTTTAAANMLLS GSMSSHDGMMNPTNLLARAVLPCSTSMATI SASAPFPTVTLDLTHSPPPPNGSNPSSSAAAT SNNNNQNSLMQRPQQQMTNLPPGMLPHVIG QALYNQSKFSGLQFSGGSPSTAAFSQSHAVA DTITALTADPNFTAALAAVISSMINGSNHHD GQGNNKSQ | 29782455<br><br>Arabidopsis lyrata subsp. lyrata | 14% | 60.379 | 5.95 |
| **173** | Predicted protein | **MSGWLGGSMAMDFLCCTAALVK**K**GASS GIGTETTRVLALRGVHVIMGSGIWLLEEM SKTQLLR**K**YPLPKLIPWNWTSVHWHISVR** KFASDFSSSGRPLNLLM | 224151730<br><br>Populus trichocarpa | 81% | 11.257 | 9.92 |
| **272** | myb family transcription factor | MVMMIIIYTEPEISLFPLQDRSEELSSNVENGS CNSNEGINPETSSHWIENVVKVRKPYTVTKQ REKWSEEEHDRFLEAIKLYGR**GWRQIQEHI GTK**TAVQIRSHAQKFFSKMAQEADSRSEGS VKAIVIPPPRPKRKPAHPYPRKSPVPYTQSPP PNLSAMEKGTK**SPTSVLSSFGSEDQNNYTT SKQPFKDDSDIGSTPISSITLFGK**IVLVAEES HKPSSYNDDDLKQMTCQENHYSGMLVDTN LSLGVWETFCTGSNAFGSVTEASENLEKSAE PISSSWKRLSSLEK**QGSCNPVNASGFPYKRC LSEREVTSSLTLVASDEK**KSQRARIC | 22330946<br><br>Arabidopsis thaliana | 27% | 37.582 | 7.05 |
| **296** | Os04g0445800 | M**SSWAGPDEIFLSTSLAGFLDK**KLIVLLRD GR**KLLGTLCSFDQFANVVLQGACERVIVG ELYCDVPLGLYVIRGENVVLIGELDR**EKDE LPAHMTCVSEAEIRKAEKAEREARDLKGSM RKRMEFLDFD | 115458610<br><br>Oryza sativa Japonica Group | 57 % | 14.501 | 5.00 |
| **595** | porin family protein | MEGFSPPINTAQVDAKTK**LDEKVDYSNLPC PVLYEELNR**EATMALKPELFEGFRLDYNK**S LNQKFFLSHSILMGPTEVPNPTPSSEIIK**IPT ANYDFGAGFIDPKLYLIGRITTDGRLNRAK**F DLTDNFSVKANALLTDEEDK**SQGHLVIDY KGSDYRTQLQLGNNSVYAANYIQHVTPHLS LGGEAFWLGQQLMSGVGYAARYETDKTVA SGQIASTGVAVMNYVHKVSEK**LSFATDFIY NYLSRDVTSVGYDLITR**QSRLRGKVDSNGV VAAYLEEQLPIGLRFLLSAEVDHVKKDYKFG FGVNAF | 15222963<br><br>Arabidopsis thaliana | 31% | 34.451 | 5.58 |

Table 3.3. Drought Root Responsive Proteins, Identified by MALDI-TOF/TOF via MS/MS

| Spot No | Protein Name | Sequence | AC Number (gi \| NCBI) and Reference Organism | Sequence Covered (%) | Theoretical Molecular Mass (kDa) | Theoretical (pI) |
|---|---|---|---|---|---|---|
| **928** | pentatricopeptide repeat-containing protein | GFCERKVCGNLRLLSKNSPGSSNTMYSASAI SLPTFRLHCSIPTELSSCRPKLSRIQTNPSIGKP VQVPSPNLASFDGGLNEAFQRLDVNGNNSPI EAYAYLLELCGKSRALSQEKVFDEMRDRTA FAWNALIGAYVSNGEPASALFIYRNMRVEG VPLDLYSFPVLLKACGKLRDIRSGTELHCML VKLGFNSTGFIVNALVSMYAKTDHLSAAKR LFDASQEKGDAVLWNSILSSYSTSGKSLETL QLFREMQMTGPASNSYTIVSALTACEGFSYA KLGKEIHAAVLKSTHSFEVYVCNALIAMYA RCGKMLEAGRILRLMNNADVVTWNSLIKGY VQNLMYKEALQFFCDMIAAGHKPDEVSLTS VIAASGRLSNLLAGMELHAYVIKHGWDSNL LVGNTLIDMYSKCNLTCYMGRAFLMMHEK DLISWTTIIAGYALNDCHVEALQLFRDVAKK RMEIDEMMLGSILRACSVLKSMLIVKEIHCHI LRKGLIDTVIQNELVDVYGKCRNMGYASRV FESIKGKDVVSWTSMISSSALNGNENEAVEL FRRMAETGLLADSVALLCILSAAASLSALKK GREIHGYLLRKGFCLEGSIAVAVVDMYACC GDLQSAKAVFDRIERKGLLQYTSMINAYGM HGCGKASVELFNKMRHENVSPDHISFLALLY ACSHAGLLDEGRRFLKIMELEYKLEPWPEH YVCLVDMLGRANCVVEAFEFVKMMKTEPT TEVWCALLAACRSHSEKEIGEIAAQRLLELE PKNPGNLVLVSNVFAEQGRWNDVEKVRAK MKASGMEKHPGCSWIEMDGKVHKFTARDK SHPETKEIYEKLSEVTRKLERESGYLADTKFI LHNVDEGEKVQMLHGHSERLAIAYEGKERD SKGIQNTHFSFLFVIISAFKRTPTKPVDESFID TPLSLCIQTQTTNCGPLDTWSQTKNGPWRKL LHGQDLKSGGGDIGMQGRGSNLFIALLIFCF FNSWCLSPFYPTISLNFQHLIYHAS | 297817672<br><br>Arabidopsis lyrata subsp. lyrata | 2% | 111.866 | 8.15 |
| **93** | transmembrane protein 14, putative | MAVSAPTLSQLSCFSSINRRNLHLHRRPILFP SLPLSRSKLSIVMSVEGGGHGMDTSSSDIKT NTSVESKSYMKGAETAKPHNDNNTDPAKV YGAAKIHDFCFGIPYGGLVLSGGLLGFLFSR NPTILSTGVLYGGALLALSFLSLKIWRQGKSS IPFVLGQAVLSAALSWKHFQAYSLTKKLIPT GFYAVISAAMLCFFSYVMISGGNPPPKKLQS STVGS | 255583841<br><br>Ricinus communis | 8% | 23.706 | 9.90 |

In Table 3.2 and Table 3.3 differentially expressed root proteins which were identified via peptide mass fingerprint analysis (PMF) and MS/MS respectively can be seen. Identified proteins include WRKY DNA-binding protein 6, myb family transcription factor, porin family protein, pentatricopeptide repeat (PPR) protein and transmembrane protein 14. Protein 94 which disappeared with stress was identified as WRKY DNA-binding protein which contains the WRKY DNA binding domain which is found in one or two copies in a superfamily of plant transcription factors. These

transcription factors are involved in the regulation of various physiological programs that are unique to plants, including pathogen defense, senescence and trichome development (Eulgem et al. 2000). Newly formed protein 272 was identified as MYB protein, which are a superfamily of transcription factors that play regulatory roles in developmental processes and defense responses in plants. Myb family transcription factors take part in control of secondary metabolism, regulation of cell morphogenesis and serving in signal transduction pathways responding to plant growth regulators (Yannuhi et al. 2006). Disappeared protein 94 was identified as porin family protein. Porins are voltage-gated diffusion pores found in all eukaryotic kingdoms. They are important for maintenance of osmotic balance and water uptake and ion uptake (Fisher et al. 1994). Newly formed protein 928 was identified as Pentatricopeptide repeat (PPR) protein containing tandem repeats of a degenerate 35 amino acid motif. Some of these proteins have been shown to play a role in post-transcriptional processes within organelles especially in mitochondria and plastids and they are thought to be sequence-specific RNA-binding proteins (Small et al. 2000). Disappeared protein 93 was identified as transmembrane protein 14.

We carried out BLAST search in NCBI database for proteins identified for unknown function. We BLASTed the sequence of protein spot 173 and 296 and they were defined to have similarity to short-chain dehydrogenase Tic32 which is an antioxidative enzyme of *Pisum sativum* and small nuclear ribonucleoprotein LSM1 which is a transcription regulator of *Zea mays*.

## 3.3. Drought-Stress Responsive Protein Expression Changes in Chickpea Leaves

We grew and carried out drought exposure to our plants as we mentioned in root proteome analysis. Also total protein extraction was carried out via phenol extraction method and to obtain high resolution proteome map we used pH 4-7 IPG (immobilized pH gradient) strips. In figure 3.4 three replicates of leaf proteome maps can be seen.

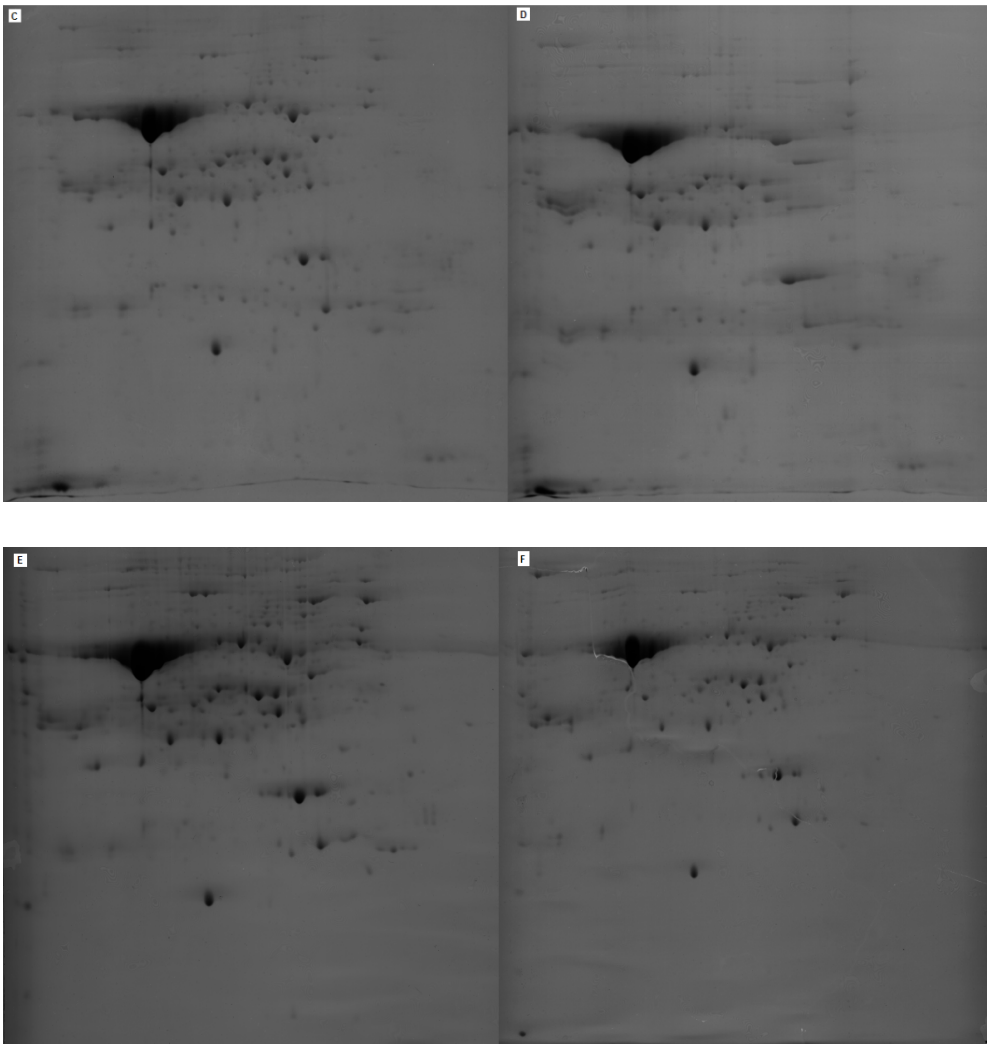


Figure 3.4. Gökçe leaf proteome map pH 4-7 of three replicaticates. A, C, E control, B, D, F stres.

With pH 4-7 IPG strips we obtained a high resolution and saturated proteome map for leaf proteome. Proteins were distributed mostly in middle and low mass regions (proteins less than 50 kDa). Differences were obtained between control and drought treated plant's leaf proteomes. We carried out both plant growth, protein extraction and 2D-PAGE experiments three times and we obtained similar proteome maps for leaf samples.

We carried out image analysis via Ludesi REDFIN 3 software to obtain differentially expressed protein spots between control and drought treatment proteomes of Gokce leaves. Via the software we analyzed two pairs of both control and drought treatment proteome maps to obtain statistically significant results. Approximately 450 protein spots were obtained for both control and drought treated leaf proteome maps. Proteins whose expression changed more than 2 fold reproducibly among two replicate

gels were selected as differentially expressed proteins between control and drought treatment proteome maps.

As a result of image analysis we obtained approximately 450 protein spots. From leaf samples we defined 7 newly formed spots, 4 of which were formed as a result of drought stress and 3 of them disappeared in drought stress. For these samples we also obtained 24 over-expressed proteins and 17 down-regulated proteins as a result of drought stress.

Table 3.4. Table of differentially expressed spots and their fold change from leaf proteome

| Spot No | Spot Status | Fold Change |
|---|---|---|
| 215 | new spot | - |
| 289 | new spot | - |
| 515 | new spot | - |
| 555 | new spot | - |
| 76 | disappeared spot | - |
| 346 | disappeared spot | - |
| 435 | disappeared spot | - |
| 16 | up-regulated spot | 2.45 |
| 22 | up-regulated spot | 2.79 |
| 47 | up-regulated spot | 5.04 |
| 57 | up-regulated spot | 2.01 |
| 65 | up-regulated spot | 2.38 |
| 66 | up-regulated spot | 2.09 |
| 77 | up-regulated spot | 2.41 |
| 86 | up-regulated spot | 2.56 |
| 89 | up-regulated spot | 2.65 |
| 98 | up-regulated spot | 2.02 |
| 104 | up-regulated spot | 2.32 |
| 132 | up-regulated spot | 2.29 |
| 160 | up-regulated spot | 2.05 |
| 193 | up-regulated spot | 3.8 |
| 208 | up-regulated spot | 3.14 |
| 210 | up-regulated spot | 2.16 |
| 231 | up-regulated spot | 3.51 |
| 240 | up-regulated spot | 2.31 |
| 286 | up-regulated spot | 2.41 |
| 350 | up-regulated spot | 2.1 |
| 352 | up-regulated spot | 3.34 |
| 523 | up-regulated spot | 4.51 |
| 18 | down-regulated spot | 2.18 |
| 24 | down-regulated spot | 3.23 |
| 30 | down-regulated spot | 2.91 |
| 31 | down-regulated spot | 2.71 |
| 53 | down-regulated spot | 2.34 |
| 56 | down-regulated spot | 2.32 |
| 80 | down-regulated spot | 2.75 |
| 134 | down-regulated spot | 2.35 |

(cont.on next page)

Table 3.4.(cont.)

| | | |
|---|---|---|
| 56 | down-regulated spot | 2.08 |
| 180 | down-regulated spot | 2.51 |
| 219 | down-regulated spot | 2.06 |
| 251 | down-regulated spot | 4.26 |
| 258 | down-regulated spot | 2.94 |
| 273 | down-regulated spot | 2.25 |
| 287 | down-regulated spot | 2.76 |
| 293 | down-regulated spot | 3.27 |
| 471 | down-regulated spot | 2.73 |

We carried out PMF (peptide mass fingerprint analysis) for all differentially expressed leaf proteins via MALDI-TOF/TOF instrument. After obtaining the MS spectra for each protein we carried out database search via Mascot program by searching all entries in NCBIr database. We selected the highest scored protein that matched as a result of the analysis. Also we especially selected plant proteins unless they had the third highest score. We managed to identify 27 proteins from 46 differentially expressed proteins.

Table 3 5. Drought Responsive Leaf Proteins, Identified by MALDI-TOF/TOF via PMF

| **Spot No** | **Protein Name** | **Sequence** | **AC Number (gi \| NCBI) and Reference Organism** | **Sequence Covered (%)** | **Theoretical Molecular Mass (kDa)** | **Theoretical (pI)** |
|---|---|---|---|---|---|---|
| **16** | predicted protein | MYRVLVPSLSSLFANAARDSTLYKLNLSYSL DCSRKLSKSLFFLIECCLSYLCTTNLVIL | 29782455<br><br>Arabidopsis lyrata subsp. lyrata | 93% | 6.792 | 8.82 |
| **18** | Hypothetical protein ARALYDRAFT | MSLFTSFFACFVPKSKSRVNTTDSNLEVLSL KKPKSKTDSPRTPTIVVSYFPVGSNLSRL | 333079<br><br>Arabidopsis lyrata | 55% | 6.652 | 10.13 |
| **22** | unknown protein | MPPSPGFSFGQIWRGGRRVVERRSPGPALRG GGSLKSADGGASVRCGGCHVLPFVCVVVLS WWTAICSQGCRVPSESLVRWFTGPTAATSS GVVISLERCRGLPSPFLGELLWVKTTSF | 52075650<br><br>Oryza sativa Japonica Group | 55% | 12.613 | 10.19 |

(cont. on next page)

Table 3.5. (cont.)

| | | | | | | |
|---|---|---|---|---|---|---|
| **24** | zinc finger (C2H2 type, AN1-like) family protein | MGTPEFPDLGKHCSVDVCKQIDFLPFTCDRC LQVFCLDHRSYMKHSCPKGDREDVTVVICP LCAKGVRLNPNEDPNITWEKHVNTDCDPSN YEKATKKKKCPVPRCKEYLTFSNTIKCRDCN VDHCLKHRFGPDHTCPGPRKLPFMGFLSSST TRKEAKTTRPNKAHPSTSSSSSSSRWSNLLSS AEAGISRLGNDISQKLQFSSSKDNGIVEVCPQ CGAKFSSVTSLVEHVEKTHERNKKQNHGNV TVDVCPRCSRGFRDPVDLVNHIERDHRGTSK A | 30688811<br><br>Arabidopsis thaliana | 29 % | 31.232 | 8.87 |
| **31** | unknown | MVCSGLFSSVKIFRCQLPRNNAFYSAQPPKH DGSDKPLCPGAPVCHWCGTWKGDKICSSCK KARYCYEKHQALHWRTGHKNDCLQIISSVA SNSVLPAVGIALRQIEQGFVWLPNGLLVLK | 194699008<br><br>Zea mays | 44% | 13.434 | 9.28 |
| **53** | unknown | MASSIPPDEQTFLSMLKSERRSVGKQVHVHV EVSGLHSSVYLRNSLIKMYLDAGDVEAEEA MFRCTPTADTVSCNIMLSGYVKGGCGGKAL RFFCGMVSRGIGVDQYSVHGCCSPRLLRAA EEGSSCPPSPPLQLPPPPLAPRGWPKRRGRTE REKRGGGEEEEREEMTGSRARPHVVSKAKV PVRRCGWTSGAQVPKACVPDVAGGGVGLH CGTGDAGALHGIAPVCASVAPA | 50726626<br><br>Oryza sativa Japonica Group | 34 % | 25.303 | 8.79 |
| **57** | pathogenesis-related family protein | MATREERDKYRSVLEDAGQVQWRYDPPDF NSVNQLFEEGQTKVWPEGSLEETVQNAIKS WEMEFSHKIRLQDFKTINPEKFKLFVNGREG LSAEETLRLGSYNALLKNSLPEEFQYYKPEE ESFESSHDAFRSALPRGFAWEILSVYSGPPVI AFKFRHWGYFEGTFKGHAPTGEMVQFLGLG VLKVDESLRAEEIEIYYDPGELFGGLLKGPPI SETKTTDSGDNTAEKQSCPFTH | 18412106<br><br>Arabidopsis thaliana | 14 % | 27.266 | 4.89 |
| **76** | hypothetical protein ARALYDRAFT | MKGIEEEAEASLVGLTIRTSSPASSSSKKGKD LLQTHNNASDSSPSIKNSPFNSPSLVSPPSSAF VSALQSPYISPRATTPITTHKPSPPLSYKGSQS EDVPSSSYTPPSDQYEFSDEQPSDRKLKLSAC TPDPAPPRISFSFPVPRVSLAKVSVSSPATNTK LRSSDVFIGFHGQNPNLVRFCKWLKSELELQ GIACFVADRANYSDTQSHEIADRVICSVTYGI VVVSCSSLLNYLSLEEVRFFAQKKNLIPIFYG TGPSEIMGLLNCNAIDKECKEAIDGLIKSHEF KLEANESNWRSCVGKTATILRAKLGRKSVA DKEIVEGIDELPFPRNRSFLGREKEIIEMEMA LFEEKQLRGRRKKDYPVEEVEVLKLFDEKL GRLSYGLWVVGSLLSELAIPPSALFEAVNKI QIEERSASPFLNVIDEQYCKSNPFVAKVLAFS LAVLEQAEGNRNLLSLKMLLVGAWFAPVPI PVNLLAAAAAKNMPTGGNRFSKWNKCLSHTF AWCGGCGLGRRSEEDAAFLLVRLGLARITN RQPGCWIQFHPITQTFARRRDYILAPKATVQ GVRKIDNPLLNLDHLWASAFLVFGFKSEPPL VQLQAMDMVLYIKRTALPLAITAFTTFSRCN SALELLKVCTNVLEEVEKSFVSQIQDWRQGS LCWKKKTNKKVDEYVWQDVTLLKALLLET RAKLLLRGGHFDSGEELCRTCISIRTVMLGH NHDLTLAAQETLAKLVRMRSKIHCPEEVLFH PFHTWMLNATSAKITLPTEA | 497314<br><br>Arabidopsis lyrata | 14 % | 82.334 | 8.91 |

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Table 3.5. (cont.)

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|---|---|---|---|---|---|---|
| **86** | STRS2 (STRESS RESPONSE SUPPRESSOR 2); ATP-dependent helicase/ RNA binding | MNSDGPKSGKKRREIRAKLVKKLTSDEDGS GKLVKDNNKSLKRGREGKSDVDEPLIKKPA STTPLVTQIAKTSDSYLSKTRFDQFPLSPLTL KGIEDAGFKTMTVVQEATLPLILQGKDI LAKAKTGTGKTVAFLLPSIEAVIAPPASRDN RHPPIIVLVVCPTRELACQAAAEANILLKYHP SIGVQVVIGGTKLPTEQRRLQKSPCQILVATP GRLKDHIDNTSGFATRLMGVKVLVLDEADH LLDMGFRREIERIIAAVPKQRQTFLFSATVSD EVRQICHVALKRDHEFVNCVQEGAGETHQK VSQMYMIASLDRHFSLLYGLLKKHITDNVG YKVIIFCTTAMVTRLVADLLGKLSLNVREIH SRKPQSYRTRVSDEFRKSKSIILVTSDVSARG VDYPDVSLVVQMGLPSDREQYIHRLGRTGR KGKEGEGVLLLAPWEEYFLSSVKDLPITKSS LPPIDHEAVKKVQKGLIQVEMTNKEAAYQA WLGYYKSQKKIARDTTRLVELANEFSRSMG LSIPPAIPVNILGKMGLKNVPGIRVAPGFDKK PAKRNYRSR | 15242323<br><br>Arabidopsis thaliana | 20 % | 62.456 | 9.80 |
| **104** | 26S proteasome non-ATPase regulatory subunit 3 | MTEDVQMNDSEPQPAASAPAVGAPALSTLH HLKEIASVIEAGSLSKEVRRISRAFRLTVALR RRLAARDVSAFLAFALPASSEAYGRLTALVP KEDDTEMDVDAAAPATQISIKHGLPEIEIYCY LLVLIFLIDHKKYDEAKACASVSIARLKNLN RRTVDFLASRLYFYYSYVYELTNSLAEIRGN LLALHRMATLHRDELGQETLLNLLLRNYLH YNLYDQAEKLRSKAPRFEAHSNQQFCRYLF YLGKIRTIQLEYTDAKESLLQAARKAPITAC GFRIQCNKWAIIVRLLLGEIPERTVFMQKGM KKALIPYFELTNAVRVGDLELFRAVADKFAS TFSADRTRNLIVRLRHNVIRTGLRNISISYSRI SLADIAKKLRLDSENPIADAESIVAKAIRDGA IDATIDHANGWMVSKETGDVYSTNEPQIAFN SRIAFLNMHNEAVKAMRFPPNSHKEKESAE KRRERLQQEEELA KHMAEDDDDDF | 226529243<br><br>Zea mays | 22% | 55.621 | 8.54 |
| **134** | predicted protein | MDEGFNEIKQERKECQDYIDSFAVQINSKIA KLDIEREKLQRATDKMAQLEAMVRVLERSN EALGNNNEVIIADNTLFHDKIRCMTKQVEQV TRYAERLHQQATQVGNDVTKY | 224151582<br><br>Populus trichocarpa | 51% | 13.183 | 5.43 |
| **156** | pentatricopeptide repeat-containing protein | MRFVTSSAAGEIFRRDELVVRRLLSQRLCSK LVNTFSETETKLRSLCEDSNPQLKNAVSVFQ QAVDSGGSLSFAGNNLMATLVRSRNHEVAF SFYRKMLETDTFINFVSLSGLLECFVQMRKT GFAHGVLALMLKRGFAFNVYNYNILLKGLC RNLEFGKAVSLLREMRQNSLMPDVVSYNTV IRGFCEGKELEKALQLANEMQGSGCSWSLV TWGILIDAFCKAGKMDEAMGLLKEMKHKG LEADLIVYTSLIRGFCDCGELDRGKALFDEV LERGDSPCAITYNTLIRGFCKLGRLKEASEIF EFMMERGVRPNVVTYTGLIDGLCGVGKTKE ALQLLNLMLQKDEEPNVVTYNIIINKLCKDS LVADALEIVELMKKRRTRPDNITYNSLLGGL CAKDLDEASKLLYLMLKDSSYTDPDVISFNA LIHGLCKGNRLHQALDIYDLLVEKLGAGDIV TTNILLNSTLKSGDVNKAMLWKQISNSKIVP NSDTYTTMIDGFCKTGMLNVAKGLLCKMRL SELPPSVFDYNCLLSSLCKKGTLDQAWRLFE EMQRDDSFPDVISFNIMIDGSLKAGDIKSAEL LVGMSHAGLSPDLFTYSKLINRFLKLGYLDE AISFFDKMIDSGFEPDAHICDSVLKYCISQGE TDKLTEFVKKLVDKDVVLDKELTCTVMDY MCSSSGNMDIAKRLLRVADDKEEGDKWGA | 297803282<br><br>Arabidopsis lyrata subsp. lyrata | 22% | 78.967 | 5.79 |

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Table 3.5. (cont.)

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| **160** | RABB1C (ARABIDOPSIS RAB GTPASE HOMOLOG B1C); GTP binding / GTPase | MSYAYLFKYIIIGDTGVGKSCLLLQFTDKRF QPVHDLTIGVEFGARMITIDNKPIKLQIWDTA GQESFRSITRSYYRGAAGALLVYDITRRETF NHLASWLEDARQHANANMTIMLIGNKCDL AHRRAVSTEEGEQFAKEHGLIFMEASAKTA QNVEEAFIKTAATIYKKIQDGVFDVSNESYGI KVGYGGIPGPSGGRD GSTSQGGGCCG | 15235981<br><br>Arabidopsis thaliana | 35% | 23.150 | 6.96 |
| **165** | predicted protein | MVLSTTDPYFTDTGISRTYDLYYRTDRPYYD DAAYKIVTSGGSVRFGVPFSEIDTVYFGGGV ERSEIKPGTYLPQAYKDYADKYGYSNTGIPL TLGWSRDSRDSALAPNSGLYQRLNTEWSVG GEARYVRANYQIQQYIPLSKKYTLALNGELG YGKGLNGRPFPLFKNFFSGGLGSVRGFEQGS LGPRDSAENIALGGSKKVTLNAEFMVPFPGA GNDRTLRLFTFLDVGNVYGANQNFDLGELR ASTGLGISWISPLGPLRLAFAQPIRKQTGDKI QRLQFQIGTSF | 224157530<br><br>Populus trichocarpa | 31% | 31.882 | 9.15 |
| **180** | serine hydroxymethyltransferase | MDSKLNLGFHRTQIADDSISLQLDCSLRDLP TANPVSPVPLQLLEPLTESHYCNNQNGEKEE DDDDRDVEEFRILGHSLCFKRRRESDSLTSN KREASSSSNGLDVEERRSLVKTWGNQPLSA ADSEIFEIMEKEKERQFKGIELIASENFVCRA VMEALGSHLTNKYSEGMPAARYYGGNQYI DEIELLCCKRALEAFGLDSESWGVNVQPYSC TSANFAVYTGLLLPGDRIMGLDTPSGGNTSH GYYTPHGRKVSGASIFFESLPYKVNPQTGYI DFDKLEERALDFRPKILICGGSSYPREWGYA RLRHIADKCGAVLMCDMAQISGLVAAKECL NPFVYCDIVTST<br>THKSLRGPRGGIIFYRKGTKPRKRGIHLGQG DESDQYDFEEKINFAVFPSLQGGPHNNHIAA LAIAFKQVATPEYKAYMQQVKKNAQYLAA ALLRRKCRL<br>VTGGTDNHLLLWDLRPLGLTGKAYEKVCEL CHITVNKIAIFGENGTITPGGVRIGTPAMTSR GCLESDFETIADFLLKAAHIACMVLREHGKL QKAFMNG<br>LQTKKEILELQKQVENFATQFAMPGFDM | 134142081<br><br>Populus tremuloides | 20% | 64.395 | 6.34 |
| **183** | hypothetical protein OsJ_36716 | MPPAASGSASSPPRCRGSGRPWVSTDQRRRR QRGDEADEKKGVAGAGAALGVETRRRKEE GDVGACGGAMSDTTVFLLLDHFAPS | 25579928 Oryza sativa Japonica Group | 18% | 8.892 | 9.37 |
| **193** | hypothetical protein LOC_Os10g10119 | MAAAGCFEDGIKLTLCSCSYTSILLSKRDAR VREANG | 78707974 Oryza sativa (japonica cultivar-group) | 72% | 3.949 | 7.71 |
| **210** | Hypotheical protein OsI_24876 | MSYRSLLHKRILPAAAAAATAAAGAALRRP AAGSRLLQARLHQATQQQCISDAGFRDSEK DIDREIEQLAQKFEENSKRWKQEREELDNLR RYTSS | 218199083<br><br>Oryza sativa Indica Group | 60% | 10.862 | 9.64 |
| **219** | hypothetical protein | MPSKRSLGEVDYDLCDFGKELSCTLNNEVES GNLRELKGAQAVAAALPPPPPPTSCCRYRFA VDAKKSPPNVSATSRHRRQPPSRTAFAGQST NRTSNVLVFSTSPQPFCPPLSNRHRGQSVDC LRNRH | Oryza sativa Japonica Group | 35% | 14.136 | 9.63 |

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Table 3.5. (cont.)

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| **231** | fiddlehead protein | MGRSNEQDLLSTEIVNRGIEPSGPNAGSPTFS VRVRRRLPDFLQSVNLKYVKLGYHYLINHA VYLATIPVLVLVFSAEVGSLSREEIWKKLWD YDLATVIGFFGVFVLTACVYFMSRPRSVYLI DFACYKPSDEHKVTKEEFIELARKSGKFDEE TLGFKKRILQASGIGDETYVPRSISSSENITTM KEGREEASTVIFGALDELFEKTRVKPKDVGV LVVNCSIFNPTPSLSAMVINHYKMRGNILSY NLGGMGCSAGIIAIDLARDMLQSNPNSYAV VVSTEMVGYNWYVGSDKSMVIPNCFFRMG CSAVMLSNRRRDFRHAKYRLEHIVRTHKAA DDRSFRSVYQEEDEQGFKGLKISRDLMEVG GEALKTNITTLGPLVLPFSE | 8177715<br><br>Arabidopsis thaliana | 93% | 43.765 | 8.38 |
| **258** | Hypothetical protein | MEDAEAPRRLLNDTDLDLVGDRERQAYYM LSDREYANTREYSPELLKKIGMDVEFCAIWK AVGWQKFVVVDEPSSRLLTLQFLCTLKEIED GISFRFFHKEYTLTWKGLSTLLGFHKPRTNDI HNPTLRLIHKWIAMTWFPRGDLRPIREDELII MFAMVRKIKIVPMKYMIRQWLESIKFSAPVE CTSLITRIAKGLGVVSDQIAFISATRPCIDETY LVQGHIVKHGINGSLIYFFPGCTNEIPLPNAG CMRQVGSQLGMHPDGSKLHATASESQAGR VLVRTGGVRLMTYIRGTTNLPDQVTEEIQYN LMEHIAQTQEWQQSVNAQFASINNMMQQQ HDDLQAYFRFQGFNPYQGP | 28209461<br><br>Oryza sativa Japonica Group | 32% | 41.315 | 6.80 |
| **286** | hypothetical protein LOC100274788 | MAAYAGTLVPPFLTVRLAVSHLRSITPGSYS LSHCLPRLAVARAASRGNGDGDGGPPAEGE KERRRSSLPALSEIRWGELLSPDPTNAVAVV LTGALVWAGASLLLQLALISAAFAAAVKYS FVAALLLFVLIALL | 226507448<br><br>Zea mays | 56% | 14.203 | 9.50 |
| **289** | aluminum-activated malate transporter | HGQFRFRRPWSQYQKLGTLCHQCASSMEAL ASCVITTTKTQYPAAANPESFKVRKTCREMS THSAKVLRGLEMAIRTMTVPYLANNTVVVA MKVAERLRSELEENAALLQVMHMAVTAML LADLVDRVKEITECVDVLARLAHFKNPED AKYAIVGALTRGIDDPLPDVVIL | 77166850<br><br>Triticum aestivum | 49% | 19.272 | 8.64 |
| **335** | hypothetical protein ARALYDRAFT_891439 | MAGEVIPYRCYLDELLYVAKEFKVEAMPTF VFMKEGEILDHIIGAEREKIQEKLLKHGGFVL STEYVFSYCLTMNVIQSRD | 297852254<br><br>Arabidopsis lyrata subsp. lyrata | 59 % | 9.420 | 5.11 |
| **350** | phloem protein 2-A8 | MAASSSVRPTTTGPQVFINFRGKDVRNGFLS FLEPAMREANINVFIDKHEVVGTDLVNLFVR IQESRVVVVIFSKDYTSSEWCLDELAQIKDCI DQGGLNVIPIFYKLAPSSVEELKGGFGDSFRV LKCKYKDEPERTQKWEEALKSIPKIKGLTLS EKSDRNEREFMNETIFEIQRSLSQIAVKGNPK LESNSLGGFMVPARRLVITHENPEKWTWSAI YDRPHKADIEIATMINTHSLIKINGDFHTRKLI PGKKYEVVFLVRLHDTSLGWKNDVTLTLKL VMGDKTGNEKEKKLCLDEYIGENWVDILVG EFEAPPKKDDAKIFFSMSQYVDTDKKSGLVV KGFAIRPA | 29779482<br><br>Arabidopsis lyrata subsp. lyrata | 25 % | 40.043 | 7.63 |
| **352** | ribosomal protein L30 family protein | MSGFRAFKAQVPIEWSQSLYITLVRGLPGTR KLHRRTLEAMGLRRCHRTVLHSNNSSIRGMI NQVKRMVVVETEEMYNARKEAEANHKALR PPLVISHSPATDSSNMS | 297792989<br><br>Arabidopsis lyrata | 48% | 12.364 | 10.88 |

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Table 3.5. (cont.)

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|---|---|---|---|---|---|---|
| **435** | N-rich protein | MEGYDREFYQFSDQLRLQTAAFSGLSLGDSI WSPAGRRNNSNDVLFAASASPADAAAKTNA VVGLKLNDGGPGLIGSGKLAFGGGGTKADR YNNNNLSNTDNKTVYNSSSSFMGNGSSYAK NNNNAGMLAFDEMGSYGYNSNNGGGNTN NNSNGVEVKSYFNKSAGRAASNNNNNNNS HAGGGNKKGGAGEYGRKKHAKSEGAAAA AATDKQRFKTLPASEALPRGQAIGGYIFVCN NDTMDENLRRELFGLPSRYRDSVRAIRPGLP LFLYNYSTHQLHGIFEAASFGGTNIDPTAWE DKKCPGESRFPAQVRVATRKIYDPLEEDAFR PILHHYDGPKFRLELSVTEALALLDIFADKD DA | 226529894<br><br>Zea mays | 20 % | 38.914 | 8.33 |
| **Rubisco** | ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit 3 | QLTYYTPEYETKDTDILAAFRVTPQPGVPPE EAGAAVAAESSTGTWTTVWTDGLTSLDRY KGRCYHIEPVAGEENQYICYVAYPLDLFEEG SVTNMFTSIVGNVFGFKALRALRLEDLRVPP AYSKTFQGPPHGIQVERDKLNKYGRPLLGCT IKPKLGLSAKNYGRAVYECLRGGLDFTKDD ENVNSQPFMRWRDRFVFCAEALYKAQAET GEIKGHYLNATAGTCEEMIKRAVFARELGVP IVMHDYLTGGFTANTSLAHYCRDNGLLLHI HRAMHAVIDRQKNHGMHFRVLAKALRMSG GDHVHSGTVVGKLEGEREITLGFVDLLRDDF VEKDRSRGIYFTQDWVSMPGVLPVASGGIH VWHMPALTEIFG | 37959650<br><br>Piper nigrum | 30% | 41.876 | 6.42 |

In table 3.5 differentially expressed leaf proteins which were identified via peptide mass fingerprint analysis (PMF) can be seen. Identified proteins include zinc finger (C2H2 type, AN1-like) family protein, pathogenesis-related family protein, STRS2 (STRESS RESPONSE SUPPRESSOR 2); ATP-dependent helicase/ RNA binding, 26S proteasome non-ATPase regulatory subunit 3, pentatricopeptide repeat-containing protein, RABB1C (ARABIDOPSIS RAB GTPASE HOMOLOG B1C); GTP binding/GTPase, serine hydroxymethyltransferase, fiddlehead protein, aluminum-activated malate transporter, phloem protein 2-A8, ribosomal protein L30 family protein, N-rich protein with known function and we identified 14 hypothetical proteins with unknown function. As a positive control we cut the RUBISCO large subunit spot and analyzed it in the same way as our unknown samples. It matched to ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit 3 after database search. Down regulated protein 24 was identified as C2H2 zinc fingers protein which (ZF) have both from DNA or RNA binding activities and take part in protein-protein interactions. Especially they take part in transcriptional regulation, but also involved directly or through site-specific modification and/or regulation of chromatin. **(**Englbrecht et al**.** 2004). Up-regulated protein 57 was identified as pathogenesis related protein family which are a group of plant-coded proteins induced by different stress stimuli, named

"pathogenesis-related proteins" (PRs) is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment (Van Loon et al. 1999). Up-regulated protein 86 was identified as STRS2 (STRESS RESPONSE SUPPRESSOR 2) which is a transcriptional regulator playing a negative regulatory role in stress response. It is demonstrated that The *strs* mutants are more tolerant to salt, osmotic, and heat stresses due to an enhanced expression of *DREB/CBF* and heat shock transcription factor genes (Kant et al. 2007). Up-regulated protein 104 was identified as 26S proteasome non-ATPase regulatory subunit 3 which acts as a regulatory subunit of the 26 proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins. Protein degradation by the 26S proteasome (26SP) is an essential step of many fundamental processes in eukaryotes also important for plant growth (Smalle et al.2002). Down regulated protein 24 was identified as Pentatricopeptide repeat (PPR) protein which contain tandem repeats of a degenerate 35 amino acid motif. Some of these proteins have been shown to play a role in post-transcriptional processes within organelles especially in mitochondria and plastids and they are thought to be sequence-specific RNA-binding proteins (Small et al. 2000). Up-regulated protein 160 was identified as RABB1C (ARABIDOPSIS RAB GTPASE HOMOLOG B1C); GTP binding / GTPase. Rab is a signal transduction protein localized on cis-Golgi membranes and interacts with Golgi matrix proteins. In plants, Rab2 regulates vesicle trafficking between the ER and the Golgi bodies and is important to pollen tube growth (Moore et al. 1997). Down regulated protein 180 was identified as serine hydroxymethyl transferase which is a enzyme involved in photorespiratory pathway and they are known to be down-regulated in stress conditions. SHMT1 functions in the photorespiratory pathway and plays a critical role in controlling the cell damage provoked by abiotic stresses such as high light and salt and in restricting pathogen induced cell death (Moreno et al. 2005). Up-regulated protein 231 was identified as fiddlehead protein which involves in the synthesis of long-chain lipids found in the cuticle and shows similarity to a large class of genes encoding proteins related to β-ketoacyl-CoA synthases and chalcone synthases. It is known that cuticule layer is involved in reduction water loss via this protein synthesis of lipid components that are thought to localize extracellularly and probably modify the properties of the cuticle (Pruitt et al. 2000). Newly formed spot 289 was identified as aluminum-activated malate transporter which is localized to the plasma membrane, and confers resistance to aluminum (Sasaki et al. 2004). It is also demonstrated that ALMT-

type anion channels have multiple functions in anion homeostasis, contributing to the regulation of growth and response to the environment and they are key regulators of stomatal closure. (Sasaki et al. 2010). Up-regulated protein 350 was identified as Phloem protein 2 (PP2) which is one of the most abundant proteins in the phloem sap. Although thought to be associated with structure PP2 also functions in cell-to-cell trafficking, long-distance transport, and in some cases, are reversibly exchanged between the conducting cells, or sieve elements, and the intimately associated companion cells (Thompson and Schulz, 1999). Up-regulated protein 350 was identified as ribosomal protein L30 family protein which take part in protein translation. Disappeared protein spot 435 was identified as N-rich protein. They are activated during programmed cell death. The *NRP*-gene appears to be a new marker in soybean activated early in plant disease resistance (Ludwig and Tenhaken 2001).

We carried out BLAST search in NCBI database for proteins identified for unknown function. We BLASTed sequence of protein spot 53, 76, 165,173 196, 210, 219, 231,296, 335 and they are defined to have similarity to pentatricopeptide repeat-containing protein which is a transcriptional regulator; nucleotide binding protein of *Arabidopsis lyrata* which is a signal transduction protein;; outer membrane protein assembly complex, YaeT protein which is a beta barrel membrane protein of *Delftia acidovorans;* short-chain dehydrogenase which is an antioxidant enzyme of *Ricinus communis;* ClpA/B-type chaperone of *Acinetobacter sp.;* CoA-disulfide reductase of *Geobacillus sp.;* 3-ketoacyl-coa synthase 10 acyltransferase/catalytic/transferase, transferring acyl groups other than amino-acyl groups of *Arabidopsis thaliana;* small nuclear ribonucleoprotein LSM1 which is a transcription regulator of *Zea mays;* oxidoreductase, acting on sulfur group of donors, disulfide as acceptor of *Arabidopsis thaliana* respectively. We also carried out BLAST search for rest of unknown functioned proteins but we could not find similarity between them and proteins with known function.

# CHAPTER 4

# CONCLUSION

In our study we identified drought stress responsive proteins in chickpea. We carried out 2D-gel electrophoresis to resolve differentially expressed proteins from both root and leaf tissue of chickpea. We observed 34 differentially expressed proteins from root tissue and 46 differentially expressed proteins from root tissue. We managed to identify 27 leaf proteins and 7 root proteins via MALDI-TOF/TOF mass spectrometry. In this study we identified candidate genes which will take part in tolerance mechanisms to drought stress.

We identified C2H2 zinc fingers protein which is a transcriptional regulator which will take part in the drought resistance mechanism. Another protein we identified is pathogenesis related family protein which is important in terms of defense to pathogens and take part in general adaptation to stressful environment. We also identified STRS2 (STRESS RESPONSE SUPPRESSOR 2) which is a transcriptional regulator and demonstrated to play a role in tolerance to salt, osmotic, and heat stresses tolerances Another protein we identified is 26S proteasome non-ATPase regulatory subunit 3 which take part in protein degradation and it is demonstrated to be essential step of many fundamental processes in eukaryotes also important for plant growth. We also identified Pentatricopeptide repeat (PPR) protein which are shown to play a role in post-transcriptional processes within organelles and they will potentially play role in expression of drought tolerance genes. Another protein we identified is RABB1C (ARABIDOPSIS RAB GTPASE HOMOLOG B1C); GTP binding / GTPase which is signal transduction protein localized on cis-Golgi membranes and interacts with Golgi matrix proteins will potentially take part in drought tolerance related pathways. Another protein we identified is serine hydroxymethyl transferase which is a enzyme involved in photorespiratory pathway and play a critical role in controlling the cell damage provoked by abiotic stresses such as high light and salt and in restricting pathogen induced cell death. Another protein we identified is fiddlehead protein which involved in the synthesis of long-chain lipids found in the cuticle via up-regulation of this protein the cuticle layer will be thickened and will prevent water loss in drought

stress conditions. Another protein we identified is aluminum-activated malate transporter which involved in anion homeostasis and they are key regulators of stomatal closure which is especially very important for tolerance to drought. Another protein we identified is phloem protein 2 (PP2) which is abandoned in phloem. Another protein we identified is ribosomal protein L30 family protein and involve in protein translation which is known to be activated during programmed cell death.

We identified root protein WRKY DNA-binding protein which functions as transcription factor involved in pathogen defense and senescence. Another protein identified is MYB proteins, a superfamily of transcription factors that play regulatory roles in developmental processes and defense responses in plants. We also identified a porin family protein which are important for maintenance of osmotic balance and water uptake and ion uptake.

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# APPENDIX A

## ROOT PROTEOM DIFFERENTIALLY EXPRESSED SPOTS

Table A. 1. Over-expressed Protein Spots

93


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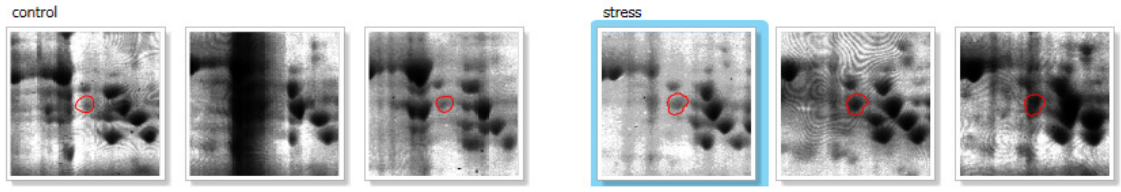


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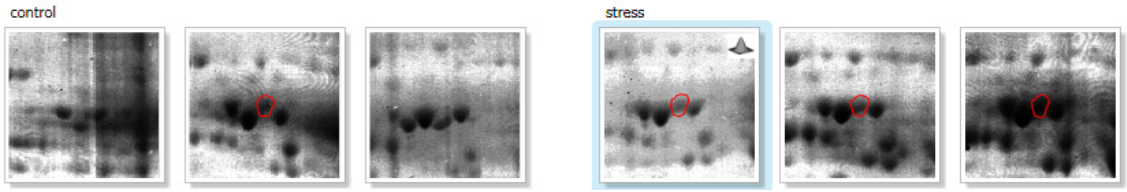


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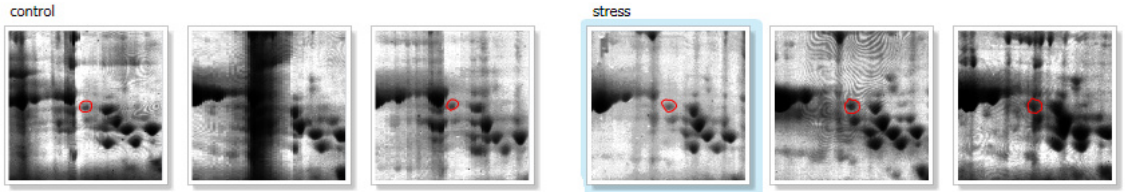


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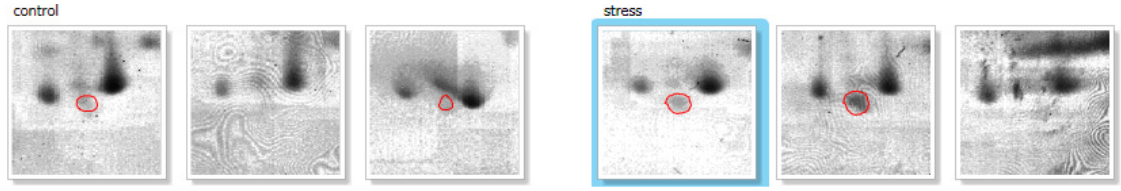


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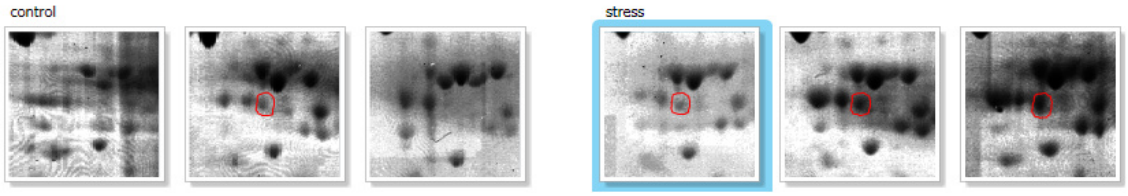


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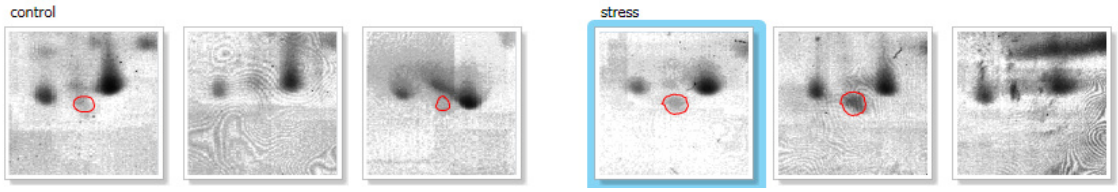


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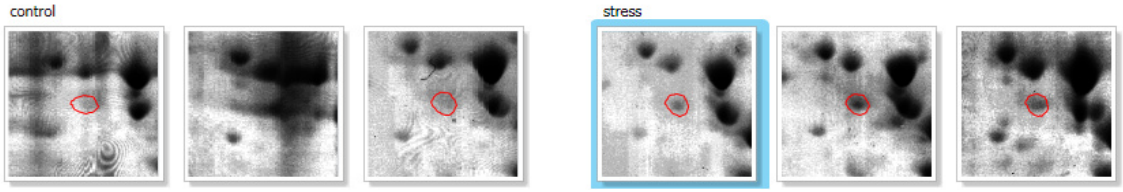


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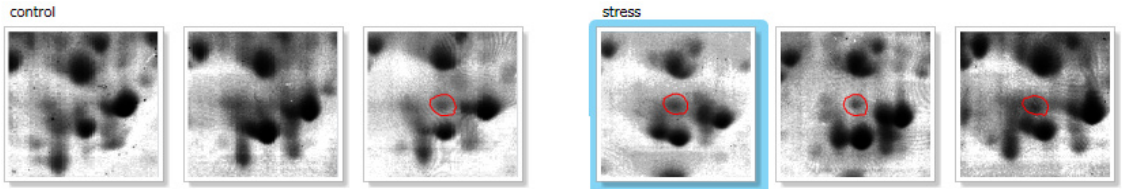


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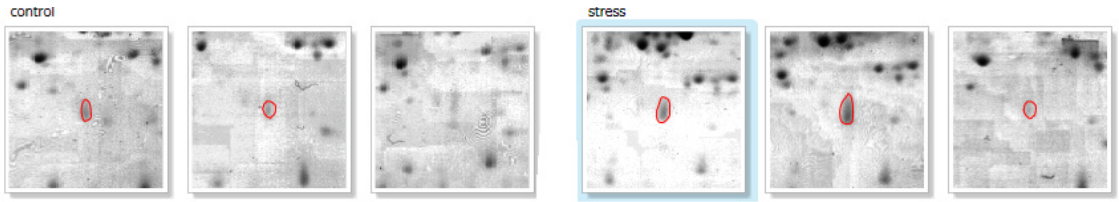


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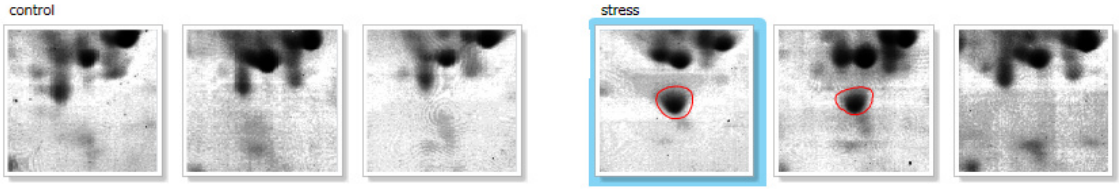


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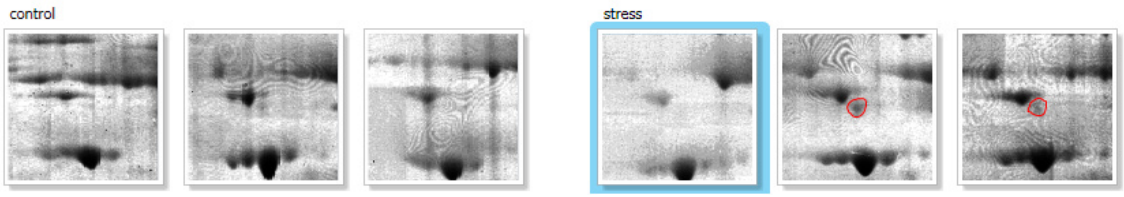


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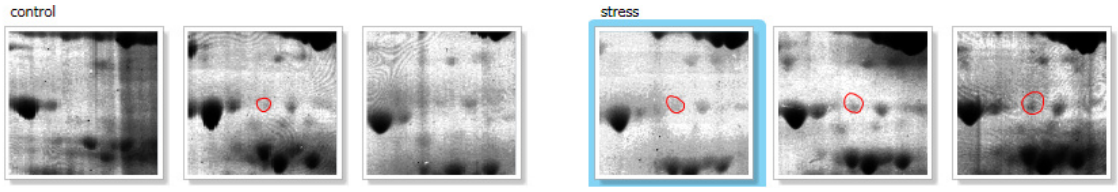


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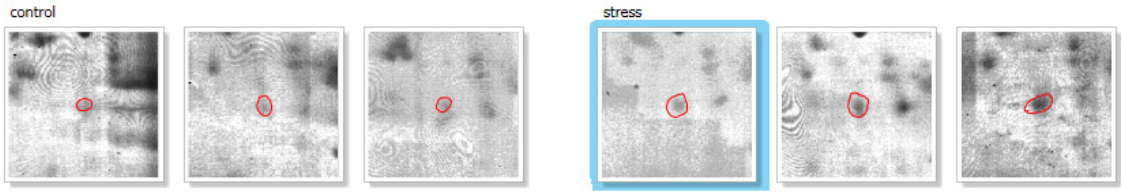


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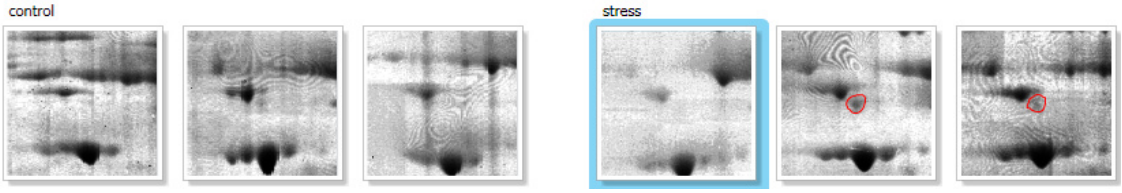


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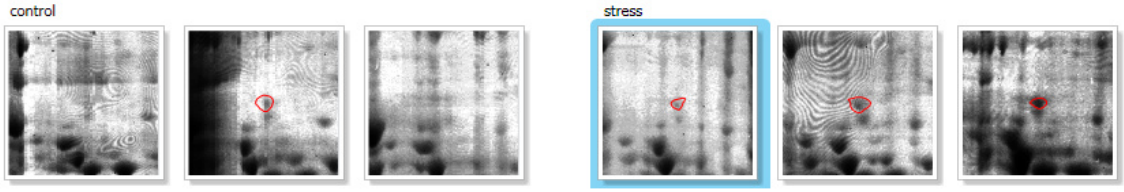


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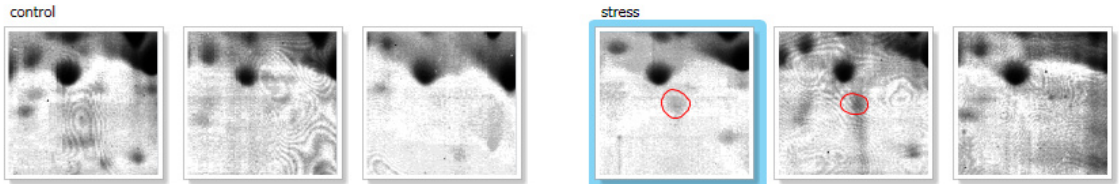


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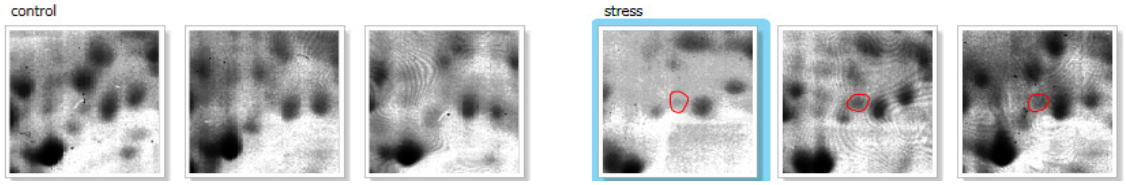


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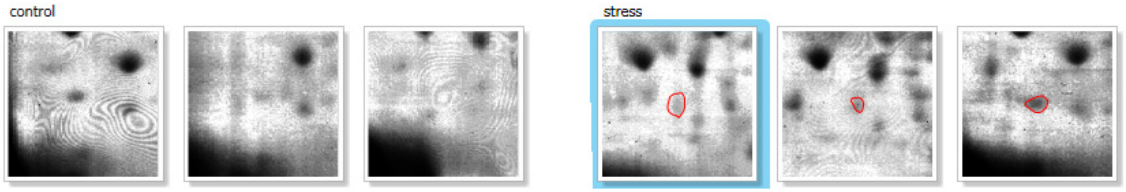


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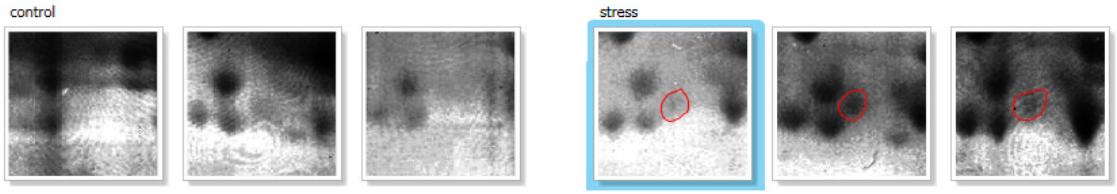


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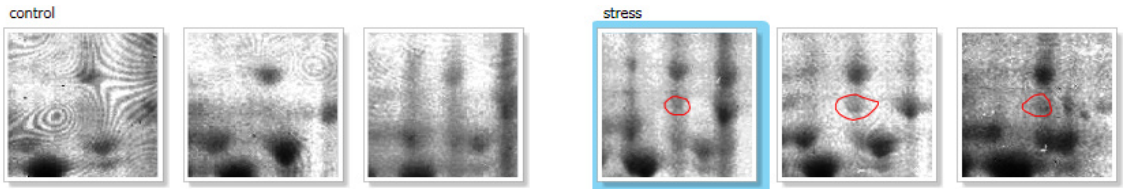


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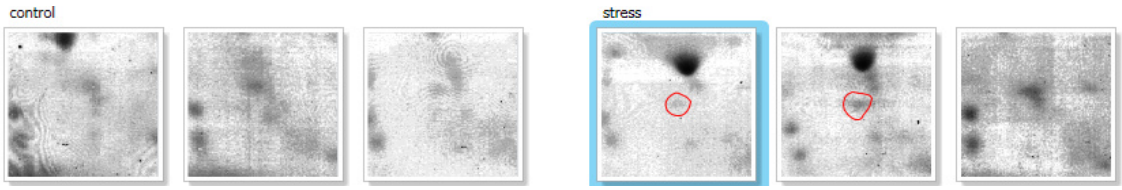


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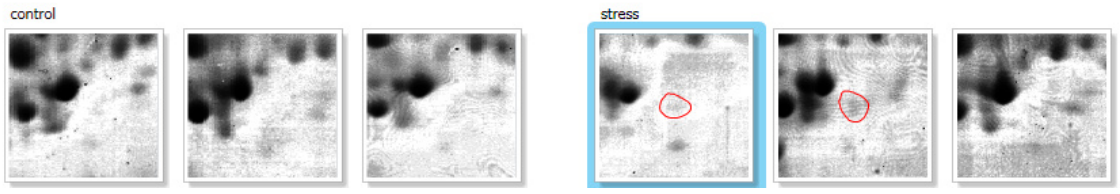


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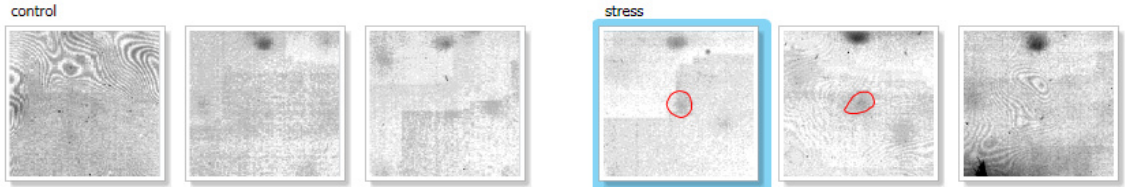


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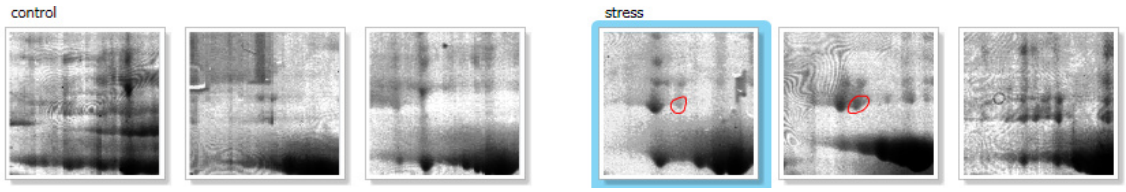


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Table A. 2. Down-regulated Protein Spots

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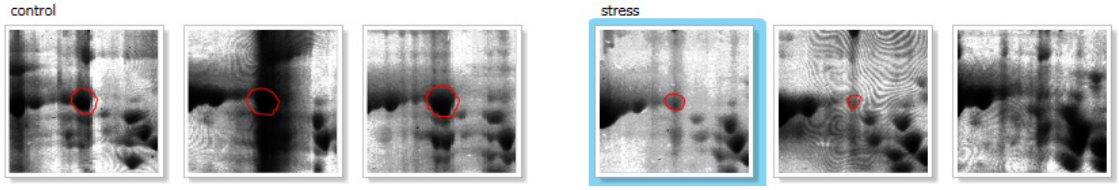


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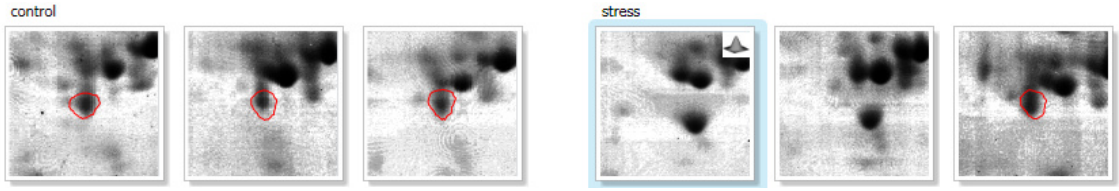


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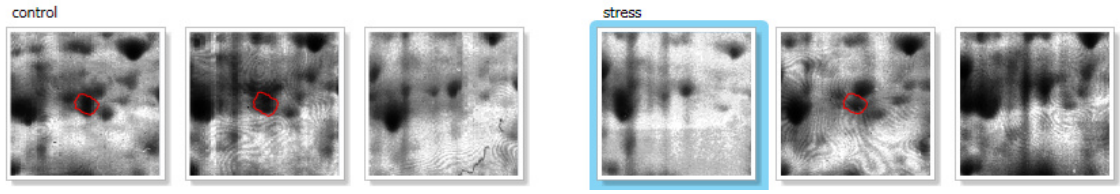


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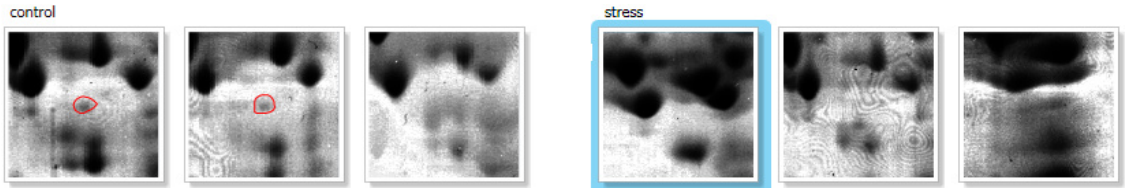
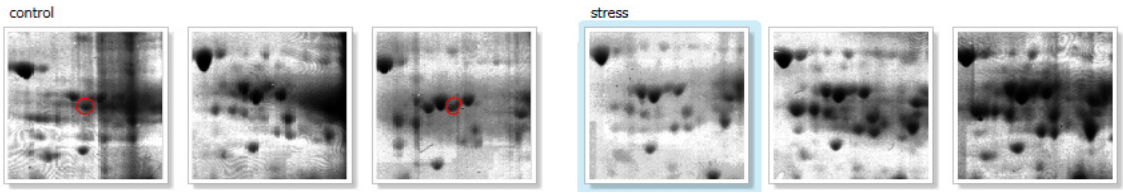
Table A.2. (cont.)

543




595

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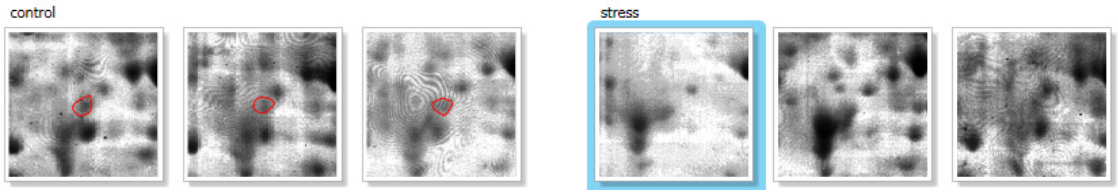


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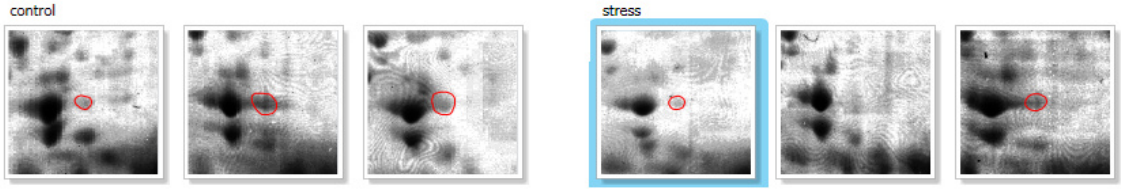


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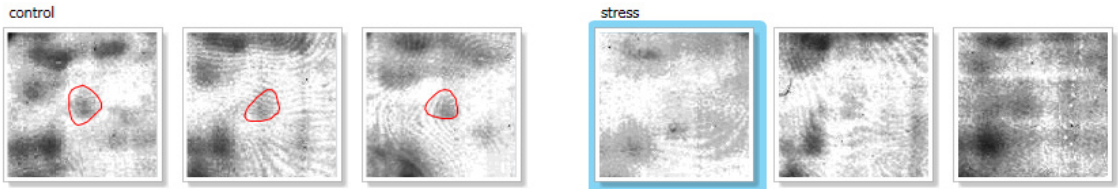
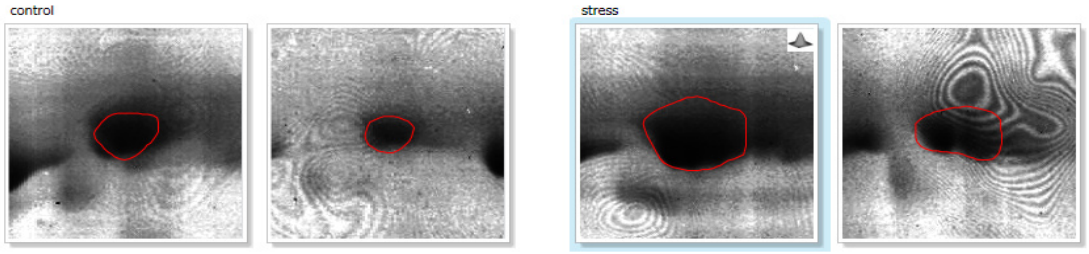


# APPENDIX B

## LEAF PROTEOM DIFFERENTIALLY EXPRESSED SPOTS

Table B. 1. Over-expressed Protein Spots

16

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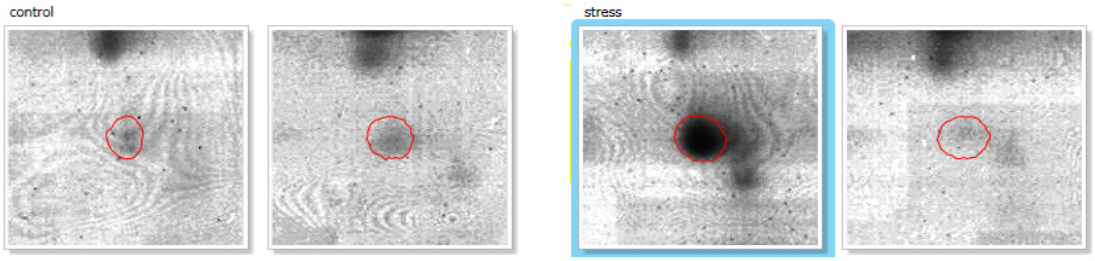
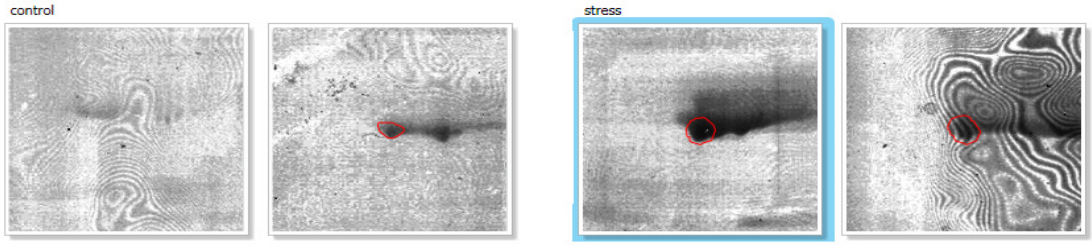



47

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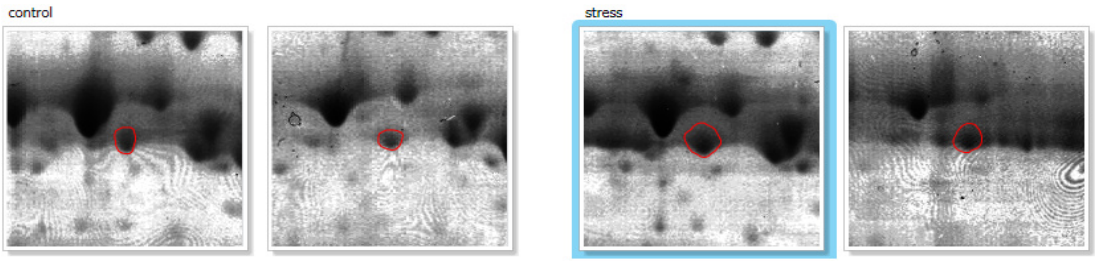


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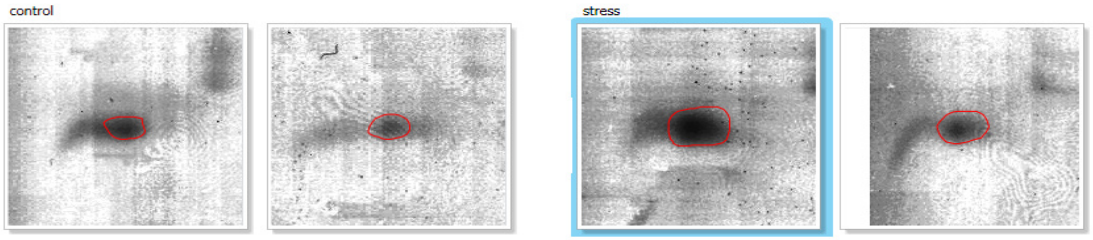
Table B.1. (cont.)

65

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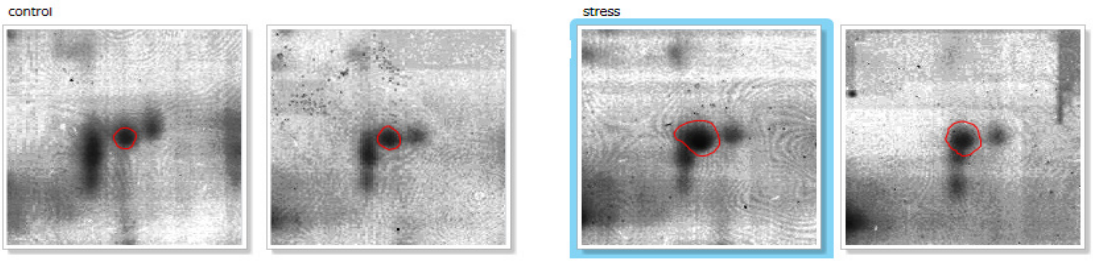
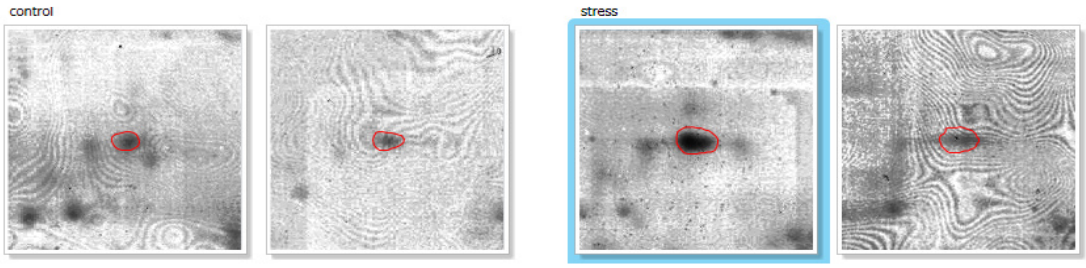



77

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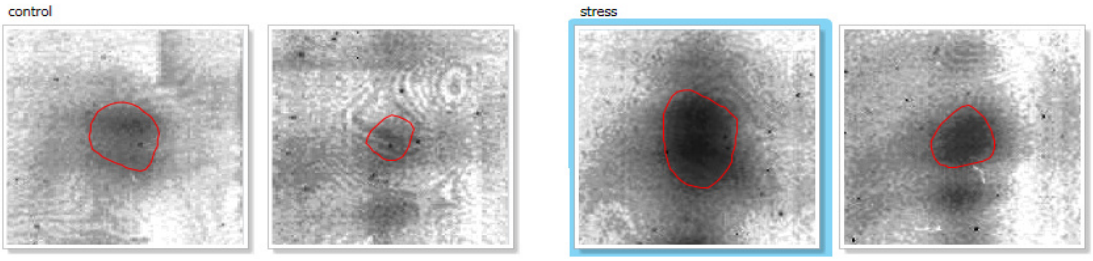


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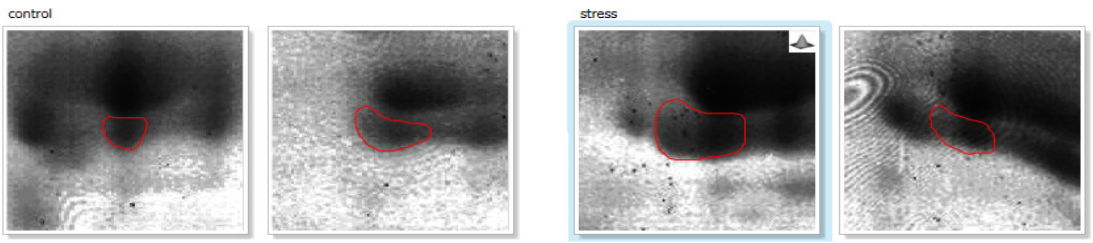


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Table B.1. (cont.)

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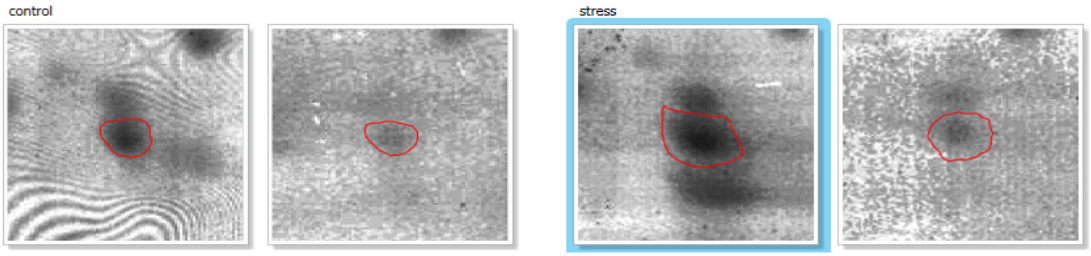
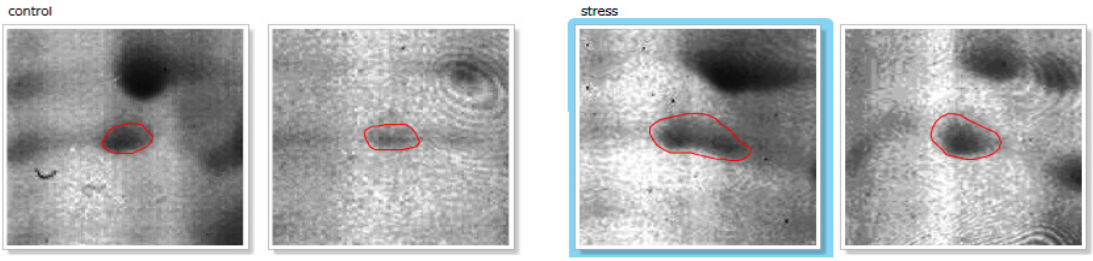



132

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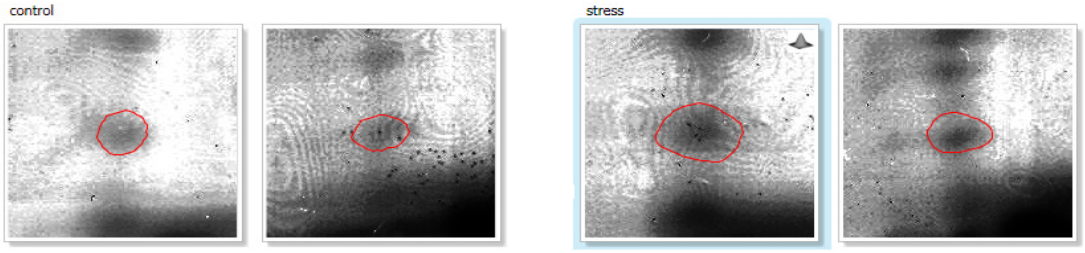


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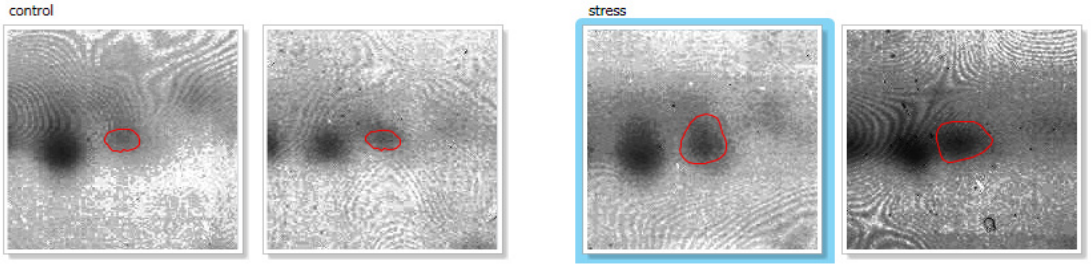


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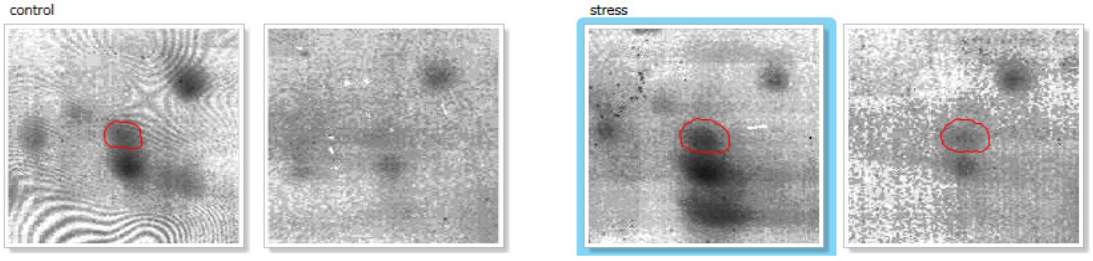


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Table B.1. (cont.)

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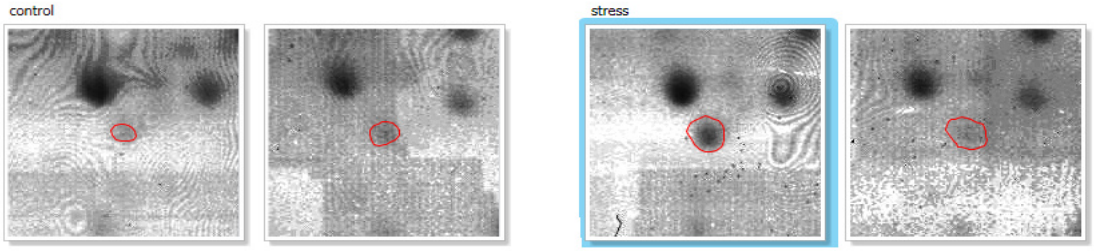


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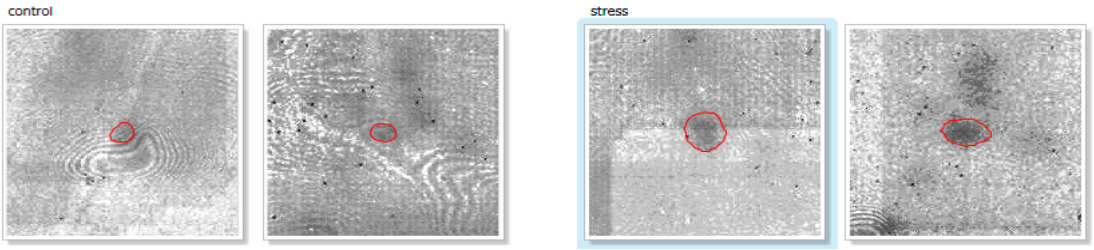


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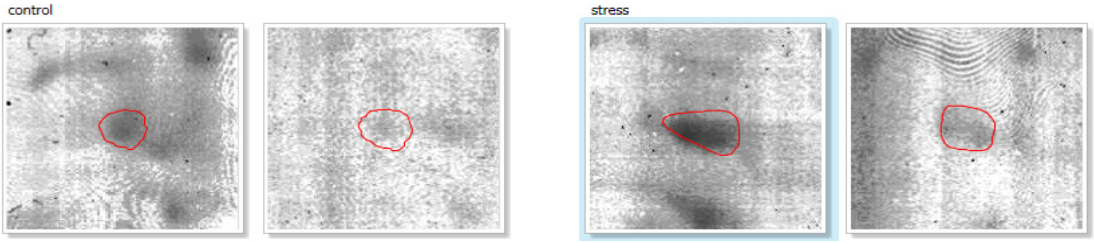


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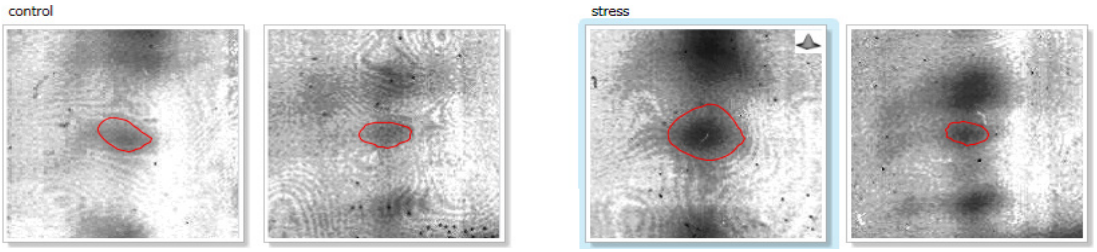


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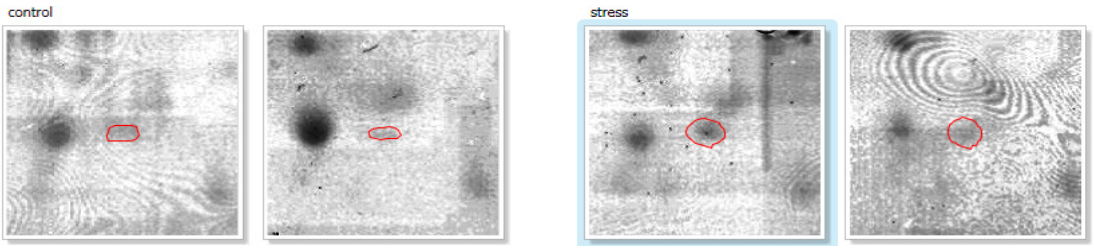


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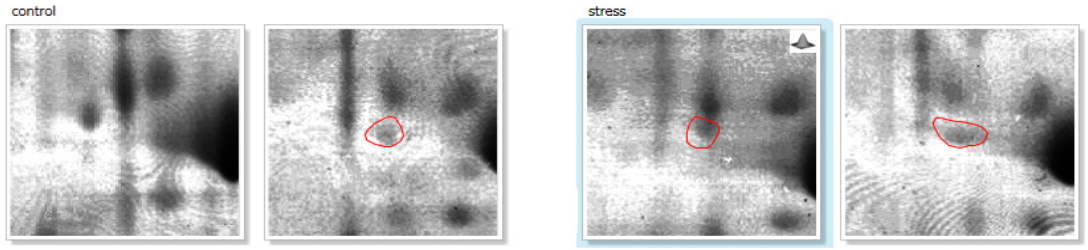


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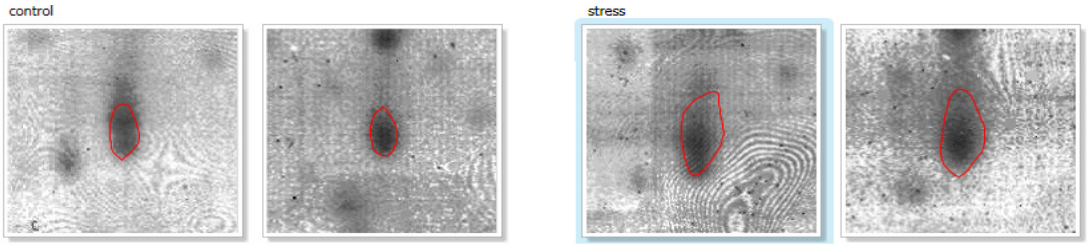


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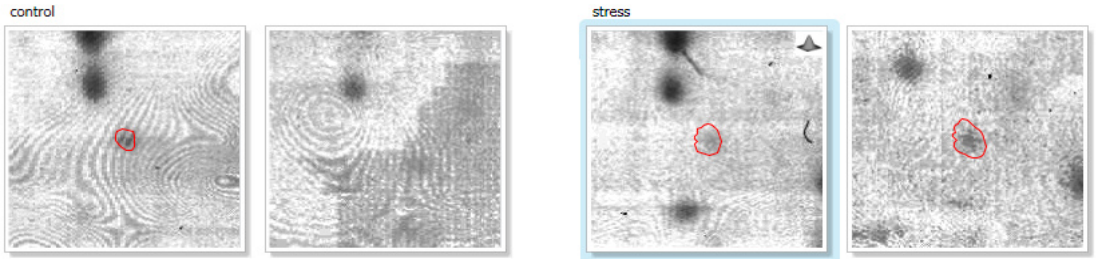


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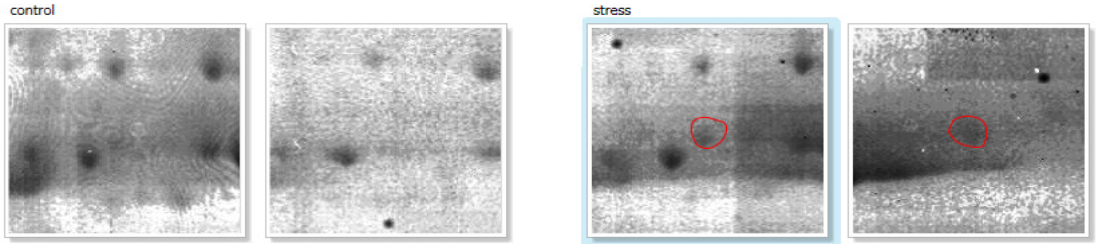


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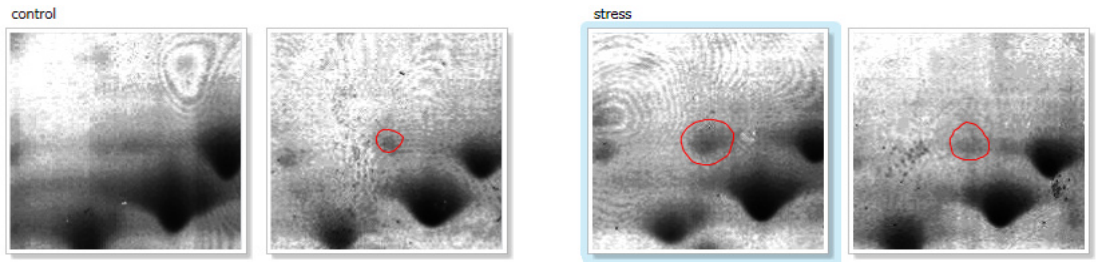


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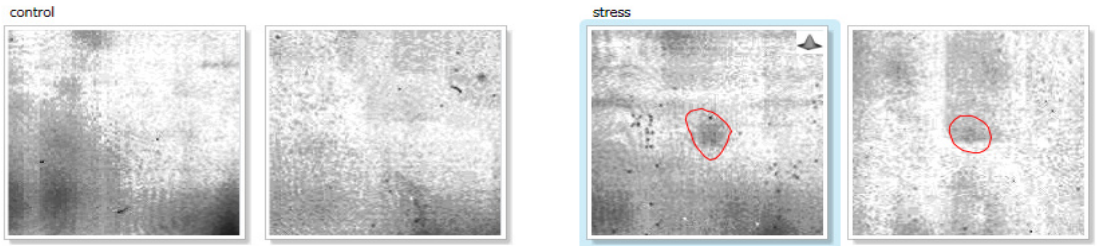
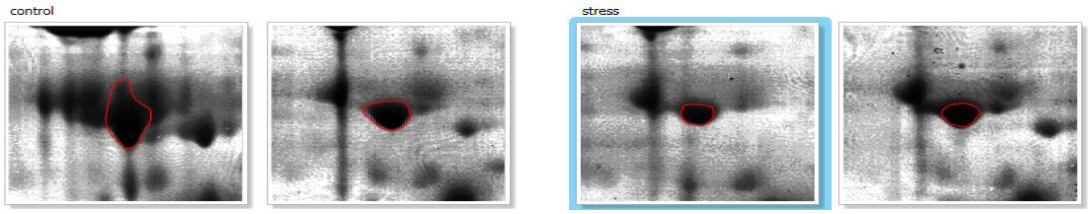
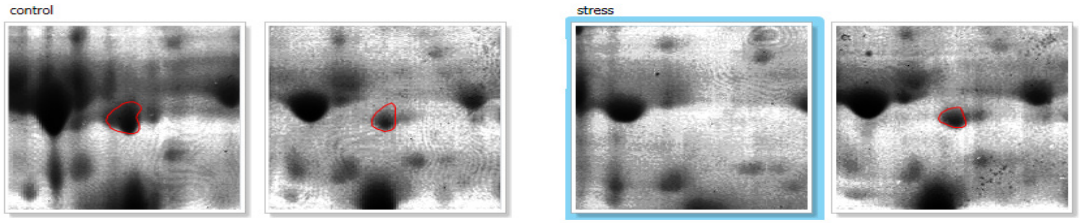



Table B. 2. Down-regulated Protein Spots

18




24

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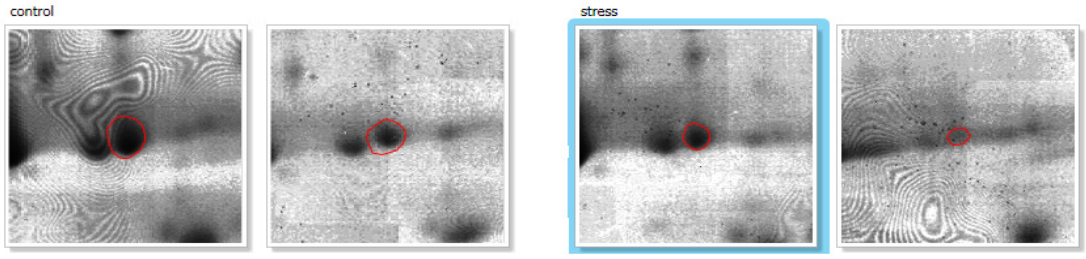


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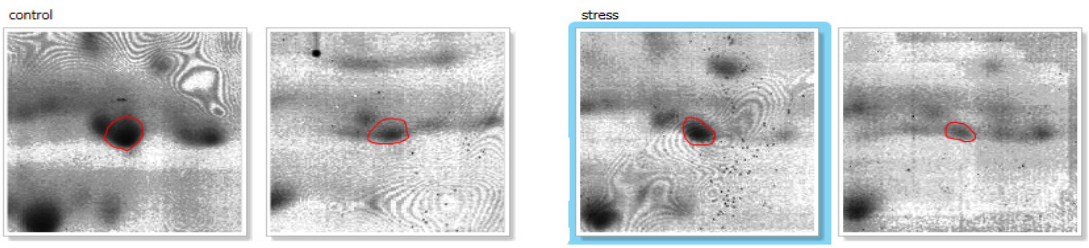


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Table B.2. (cont.)

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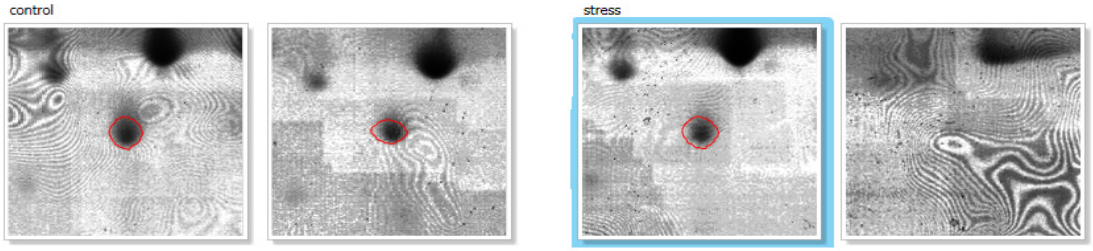
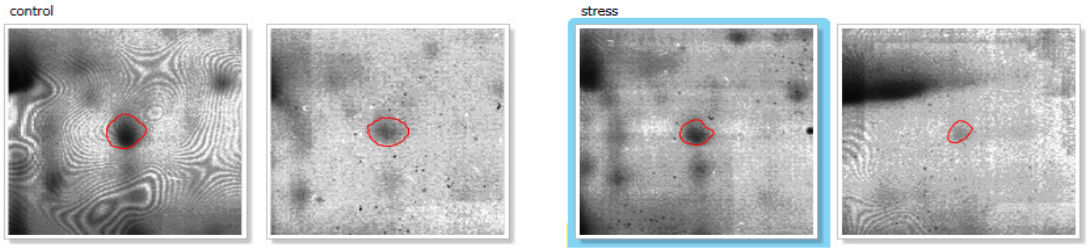
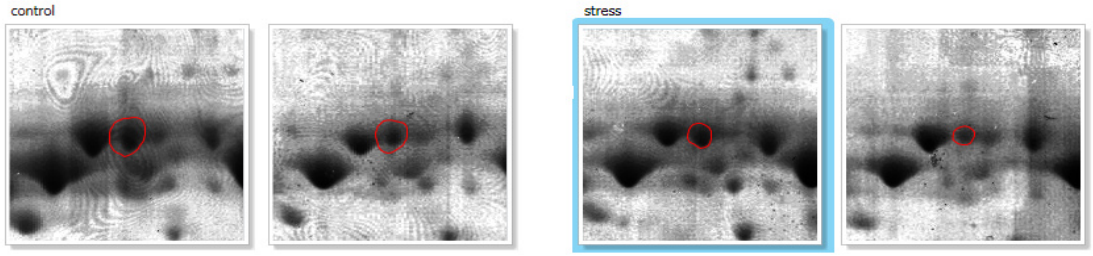


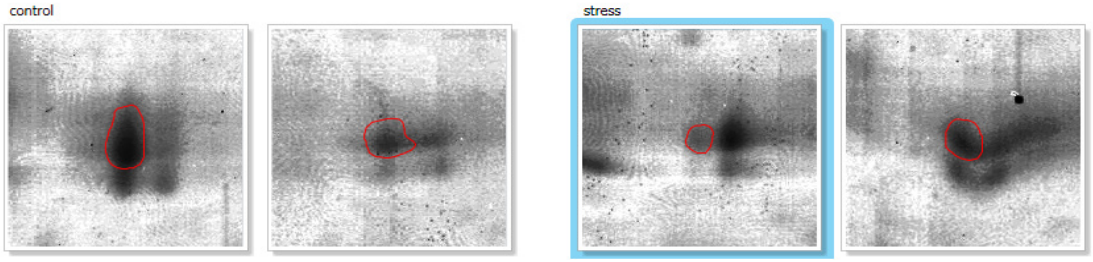

53




56




63

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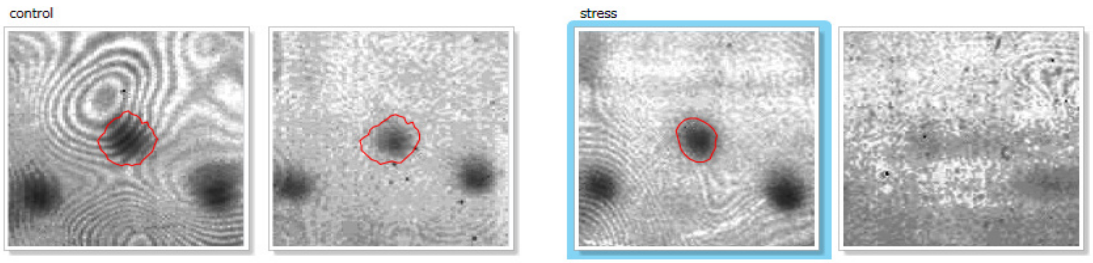


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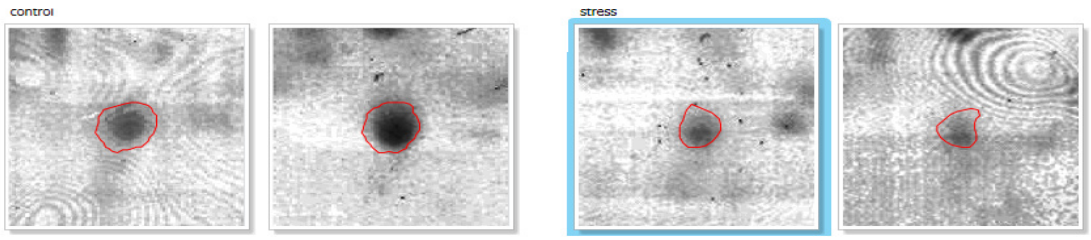
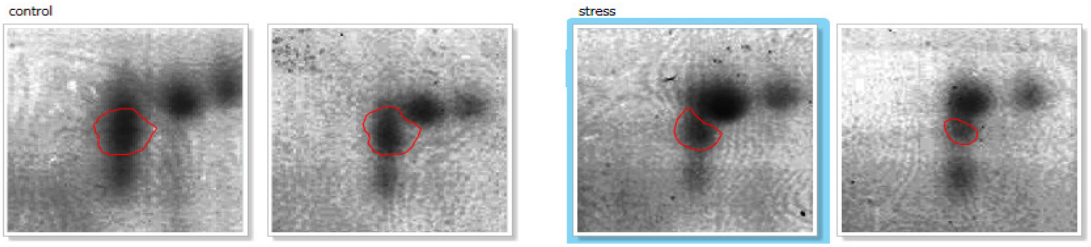
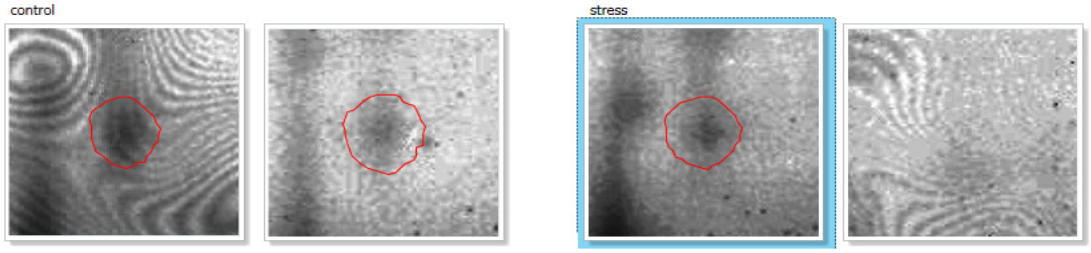



134




156

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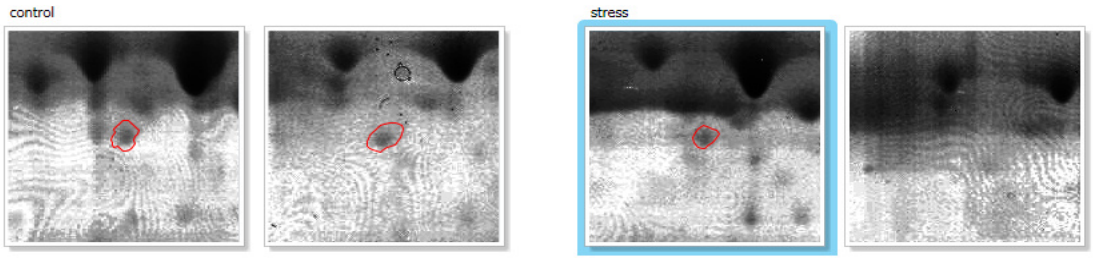


219

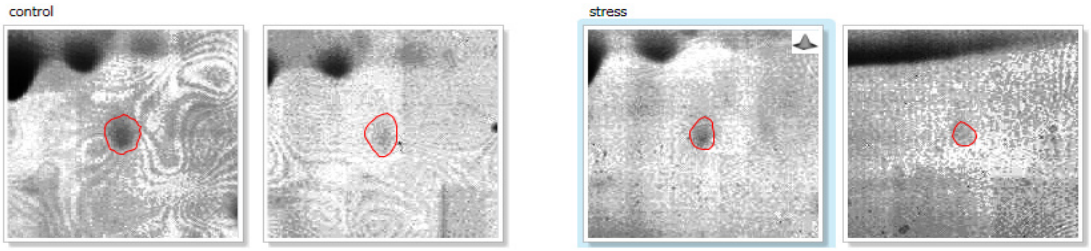


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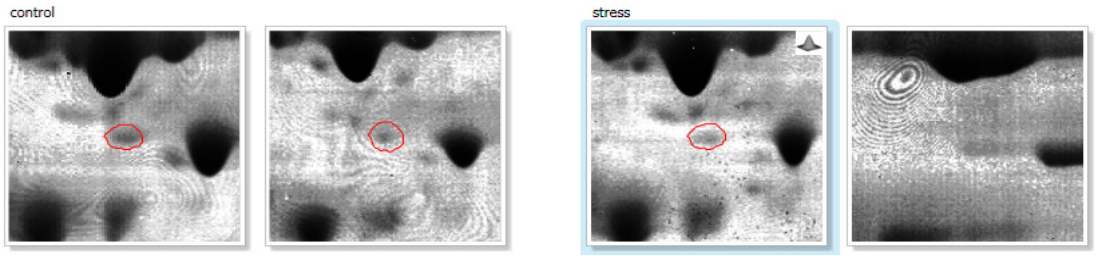
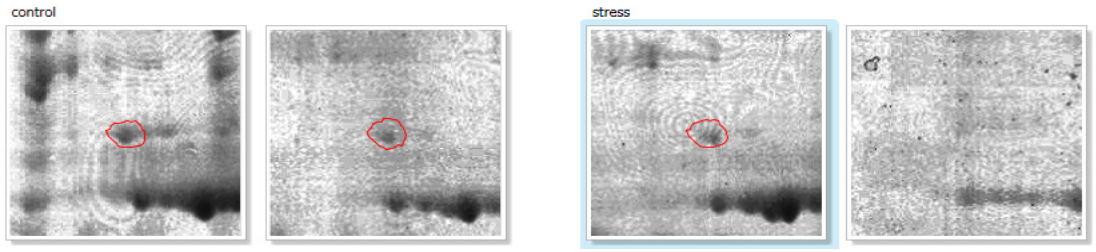
Table B.2. (cont.)

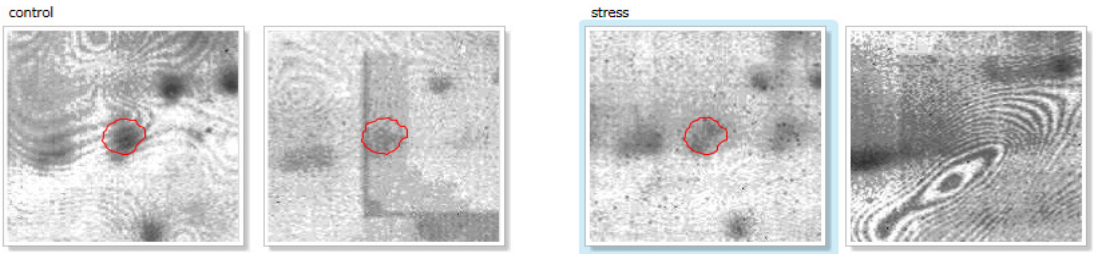
251



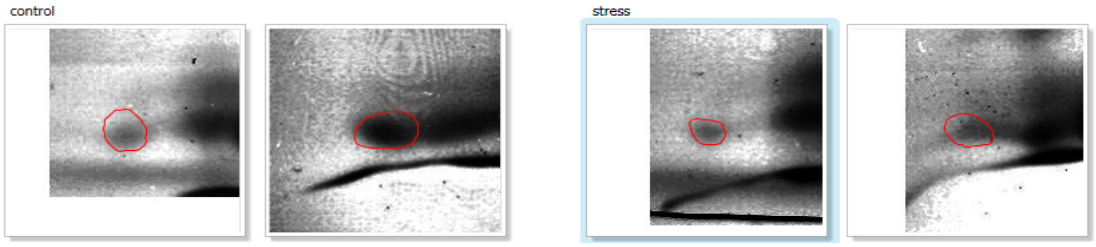

258



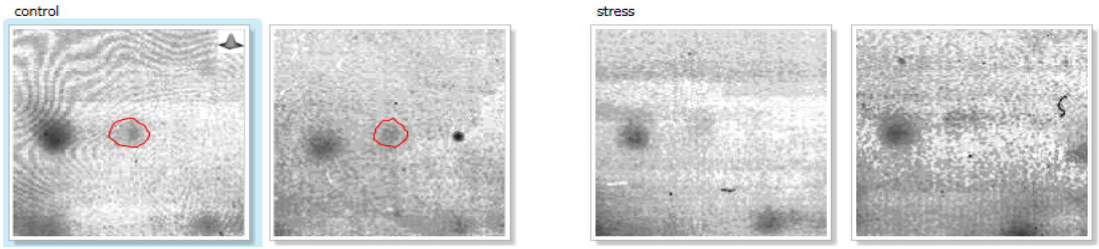

273




287




346

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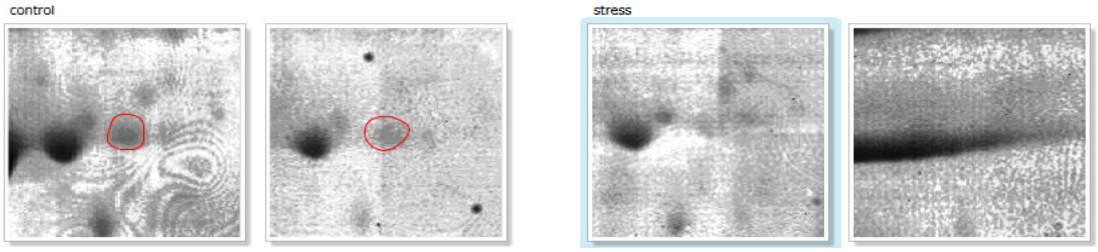


471