IDENTIFICATION OF SALT STRESS RESPONSIVE PROTEINS IN WILD SUGAR BEET (*BETA MARITIMA*) USING 2D-PAGE WITH MALDI-TOF/TOF SYSTEM

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ABSTRACT

IDENTIFICATION OF SALT STRESS RESPONSIVE PROTEINS IN WILD SUGAR BEET (*BETA MARITIMA*) USING 2D-PAGE WITH MALDI-TOF/TOF SYSTEM

High salinity is one of the abiotic stresses, which affects the homeostasis, growth and productivity of plants. In plants, uptake of the non-essential salt ions negatively affects the anatomy, physiology and metabolism, changes the osmotic balance in cells and causes abundant dehydration. In this case, higher plants develop salt tolerance mechanisms such as induction of related signaling pathways, effluxion of salt ions, accumulation of these toxic ions in their vacuoles, activation of their detoxification mechanisms and production of osmoprotectans.

In this study, identification of salt responsive proteins in moderately halophyte wild type sugar beet *Beta vulgaris ssp. maritima* was aimed. In order to investigate the protein-based natural stress tolerating mechanisms, plants were exposed to 150 mM NaCl and total proteins were extracted. Differentially expressed proteins were identified by proteomic approaches including MALDI-TOF/TOF mass spectrometry combined two dimensional polyacrylamide gel electrophoresis. The results revealed that enzymatic antioxidants and secondary members of antioxidative pathways are responsive in salt stress. In conclusion, these detected proteins demonstrate that antioxidative system may be the major defense mechanism in halophytic plants.

ÖZET

2D-PAGE İLE MALDI-TOF/TOF SİSTEMİ KULLANILARAK YABANİ ŞEKER PANCARINDA (*BETA MARİTİMA*) TUZ STRESİNE DUYARLI PROTEİNLERİN BELİRLENMESİ

Yüksek tuzluluk bitkilerde homeostaziyi, büyümeyi ve üretkenliği etkileyen abiyotik streslerden biridir. Bitkilerde esansiyel olmayan tuz iyonlarının alımı, hücrelerin osmotik dengesini değiştirerek ve aşırı susuzluğa neden olarak, bitkilerin anatomisini, fizyolojisini ve metabolizmasını olumsuz yönde etkiler. Bu durumda yüksek bitkiler ilgili sinyal yolaklarını uyarmak, tuz iyonlarını dışarı atmak, bu toksik iyonları vakuollerinde biriktirmek, detoksifikasyon mekanizmalarını aktive etmek ya da osmoprotektan üretmek gibi tuz tolerans mekanizmaları geliştirirler.

Bu çalışmada kısmi halofit yabani tür şeker pancarı *Beta vulgaris ssp. maritima*'da tuza duyarlı proteinlerin belirlenmesi amaçlanmıştır. Protein esaslı doğal stres tolerans mekanizmalarını keşfetmek için bitkiler 150 mM NaCl'e maruz bırakılmış ve total proteinleri elde edilmiştir. Farklı olarak ifadelenen proteinler MALDI-TOF/TOF ile kombine edilmiş iki boyutlu poliakrilamid jel elektroforezini kapsayan proteomik uygulamalarıyla belirlenmiştir. Sonuçlar ortaya çıkarıyor ki enzimatik antioksidanlar ve antioksidatif yolakların ikincil üyeleri tuz stresine duyarlılık göstermektedir. Sonuç olarak, tespit edilen proteinler gösteriyor ki halofitik bitkilerde antioksidatif sistem majör savunma mekanizması olabilir.

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$\bullet O_2^-$	Superoxide Radical
^oC	Centigrade degree
1,5-DAN	1,5-diaminonaphtalene
$^1O_2$	Singlet Oxygen
2D-PAGE	Two Dimensional-Polyacrylamide Gel Electrophoresis
ABA	Abscisic Acid
ABC	ATP Binding Cassette
ABF3/4	Abscisic Acid Responsive Elements-Binding Factor 3/4
ABI1/2	ABA Insensitive-1/2
ACTH	Adrenocorticotropic hormone
AGP	Arabinogalactan Protein
Al^{3+}	Aluminum ion
ALMT	Aluminum Activated Malate Channels
APX	Ascorbate Peroxidase
ASC	Ascorbic acid
ATP	Adenosine Triphosphate
AVP1/2	Arabidopsis Vacuolar H^+-Pyrophosphatase 1/2
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
cAMP	cyclic Adenosine Monophosphate
CAT	Catalase
CBB G-250	Coomassie Brilliant Blue G-250
CCC	Cation-Coupled Cl^-
cGMP	cyclic Guanidine Monophosphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
CID	Collision Induced Dissociation
Cl^-	Chloride ion
CLC	Chloride Channel
CNGC	Cyclic Nucleotide Gated Channel

CO_2	Carbon Dioxide
Cu	Copper
Da	Dalton
DHA	Dehydroascorbate
DHAR	DHA Reductase
DHB	2,5-dihyroxybenzoic acid
DMSP	Dimethyl Sulfonium Propironate
dS/m	deciSiemens per meter
DTT	Dithiothreitol
EC number	Enzyme Commission number
EC	Electrical Conductivity
ECD	Electron Capture Dissociation
EF-1α	Elongation Factor-1 alpha
ESI	Electrospray Ionization
ETD	Electron Transfer Dissociation
Fe^{2+}	Iron (II) ion
Fe^{3+}	Iron (III) ion
g	gram
g	gravity
GB	Glycinebetaine
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Oxidized GSH
ha	hectare
H_2O_2	Hydrogen Peroxide
HKT1	High Affinity K^+ Transporter 1
HSP	Heat Shock Protein
IEF	Isoelectric Focusing
IPG	Immobilized pH Gradient
K^+	Potassium ion
KEA	K^+ Efflux Antiporter
KIRC	K^+ Inward Rectifying Channel

KORC	K^+ Outward Rectifying Channel
KUP/HAK/KT	K^+ Uptake Permease / High Affinity K^+ Transporters
LC	Liquid Chromatography
LCT1	Low Affinity Cation Transporter1
Li^+	Lithium ion
m/z	mass to charge ratio
MALDI	Matrix-Assisted Laser Desorption / Ionization
MAP	Mitogen-Activated Protein
MDA	Monodehydroascorbate
MDAR	MDA Reductase
Mg^{2+}	Magnesium ion
MKK2	MAP Kinase Kinase 2
Mn^{2+}	Manganese ion
MS	Mass Spectrometry
MscS	Mechanosensitive Channels of Small Conductance
MSL	MscS-like
N_2	Nitrogen
Na^+	Sodium ion
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NHX1/5	Na^+/H^+ Antiporter 1/5
NL	Non-linear
NO_3^-	Nitrate ion
NRT	(NOD)s Nitrate and Peptide Transporter
O_2	Oxygen
$OH^\bullet$	Hydroxyl Radical
PA	Picolinic Acid
P-ATPase	Plasma Membrane-ATPase
PCIS	Precursor Ion Selector
pI	Isoelectric point
PLP	Pyridoxal-5-phosphate
PMSF	Phenylmethylsulfonyl fluoride

PN/PL/PM	Pyridoxine / Pyridoxal / Pyridoxamine
POX	Peroxidase
PP	Pyrophosphate
PPI	Protein Phosphatase Interaction
ppm	pounds per million
RH	Relative Humidity
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SA	Sinapinic Acid
SCX	Strong Cationic Resin
SDS	Sodium Dodecyl Sulfate
SID	Surface Induced Dissociation
SLAC1	Slow Anion Channel Associated Protein1
SOD	Superoxide Dismutase
SOS	Salt Overly Sensitive
SSP	Salt Shock Protein
ssp	*subspecies*
TEMED	*N*, *N*, *N*', *N*'-Tetramethyl-ethane-1,2-diamine
TFA	Trifluoro Acetic acid
THAP	2,4,6-trihydroxyacetophenone
TOF	Time of Flight
Trx	Thioredoxin
UP	Ultrapure
UV	Ultraviolet
v/v	volume per volume
V-ATPase	Vacuolar-ATPase
VDAC	Voltage Dependent Anion Channel
Vh	Voltage hour
VIC	Voltage Independent Cation Channel
V-PPase	Vacuolar Pyrophosphatase
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1. A Potential Halophyte: *Beta maritima*

Beta maritima, also known as sea beet, is classified as subspecies of *Beta vulgaris* in taxonomy (Lange, Brandenburg, and De Bock 1999). *Beta maritima* is the wild type of sugar beet and grown in coastal areas (Srivastava et al. 2000). Its ecologic situation indicates that sea beet is resistant to several abiotic stresses such as high salinity.

In horticultural and agricultural sciences, plants are divided into several categories as a result of their salt stress responses: sensitive, moderately sensitive, moderately tolerant and tolerant, respectively. According to these categories, salt-sensitive plants are renamed as glycophytes and salt-tolerant ones are halophytes. In this case, sugar beet (*Beta vulgaris ssp. maritima and ssp. vulgaris*) is a halophytic plant related to its moderately tolerant characteristic which refers to tolerating 7 dS / m electrical conductivity (EC) of soil salinity (Blaylock 1994; Glenn, Brown, and Blumwald 1999; Chinnusamy, Zhu, and Zhu 2006).

1.2. Soil Salinity and Effects on Plants

Salt affects over 800 million ha soil on earth (Teakle and Tyerman 2010). All soils have differential salt contents. Calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{3+}), aluminum (Al^{3+}), phosphorus (P) and sodium (Na^{+}) are the cations found in all soil types (Bronick and Lal 2005). In several soil regions on earth, these cations are accumulated excessively with their soluble salt forms such as sodium chloride (NaCl), the most abundant form (Koca et al. 2007). Optimal electrical conductivity unit (EC) of NaCl in soil is 4 dS/m which refers to 40mM approximately. Soils containing NaCl in higher concentrations are accepted as saline soils (Munns and Tester 2008).

Soil salinity, excessive accumulation of salt in soil, is one of the agricultural problems that affect the crop yield through damaging germination, growth and fruit production. High concentrations of salt ions have three different effects on plants basically: Water stress, salt stress and ionic imbalance stress (Mudgal, Madaan, and Mudgal 2010).

First, in water stress; salt ions prevent the efficient uptake of water via negative osmotic potential. In general, plants use most of their energy for growth, flowering and fruiting. However, in salty environment, they consume their energy to take sufficient amount of water instead of growing processes. In addition, high uptake of salt ions increases the osmotic potential in plants. This increment affects the tension of xylem in a parallel way and plant takes more water in order to balance the osmotic potential. Large amounts of water in plant cells increase the turgor pressure in long-term. Thus, high salinity causes osmotic stress in addition to water stress (Parida and Das 2005).

Second, in salt stress; some salt ions such as sodium, chloride or boron have toxic effects on plant metabolism. NaCl, a phytotoxic salt, causes oxidative damage on plant metabolism (i.e. inhibition of transpiration) via overproduction of reactive oxygen species (ROS) such as superoxide radical ($\bullet O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$). These radicals cause peroxidation of membrane lipids, destruction of skeleton structure and dysfunction of cell (Hu et al. 2011). On the other hand, boron forms strong complexes with metabolites such as ATP, NADH and NADPH, which have a high number of hydroxyl groups and affect the energy production negatively (Reid 2010).

Third, in ionic imbalance stress; non-essential ions (i.e. Na^+ monovalent cations), compete with essential ions (i.e. K^+ monovalent cations) and interfere to uptake or usage of them in biochemical reactions and cause nutritional imbalance (Blaylock 1994; Chinnusamy, Zhu, and Zhu 2006). This competition between Na^+ and K^+ ions in plant metabolism results from their physicochemical similarity (Maathuis and Amtmann 1999).

1.3. Effects of Salinity on Plant Anatomy, Physiology and Metabolism

1.3.1. Effects on Plant Anatomy

Salt ions are highly effective on plant growth. Previous researches have demonstrated that plants exposed to high concentrations of salt, have a loss in their biomass. Dry weights of leaves, root, shoot, tubers and nodules decrease when the salt concentration increases. Excess salt reduces the shoot growth 50%, approximately. Flowering is delayed, number of flowers and pods, nodulation ratio and fixation efficiency of nodules decreases (Mudgal, Madaan, and Mudgal 2010). Moreover, since leaves cannot expand, their surface areas remain smaller than non-exposed ones (Marcelis and Van Hooijdonk 1999; Meloni et al. 2001). Despite of deceleration in expansion of leaf surface, there is a significant increment in both epidermal and mesophyll thickness according to palisade and spongy layers of leaf structure (Longstreth and Nobel 1979).

Increment in thickness is the result of chloroplast, mitochondria and endoplasmic reticulum swelling, formation of more Golgi bodies and larger vacuoles in plant cell (Mitsuya, Takeoka Y., and H. 2000).

In addition to intracellular organelles, plasma membrane is also reacted against salt ions. In order to control the ion fluxes, both stability/permeability and enzyme activities are regulated by differentiating the lipid composition. Excess concentrations of salt ions decrease the amount of sterols, phospholipids in contrast to glycolipids. Ratio of unsaturated fatty acids to saturated fatty acids is also slightly decreased (Wu, Seliskar, and Gallagher 1998).

1.3.2. Effects on Plant Physiology and Photosynthesis

Physiology of a plant, including homeostasis and molecular contents, can be differentially affected by salt ions depending on plant age, types of ions, intensity and period of exposure (Chaves, Flexas, and Pinheiro 2009). Major physiological changes in plants, as a result of salt stress, are osmotic imbalances, differentiations in cellular

rigidity, variations in ion levels, production of alternative metabolites and defensive molecules such as antioxidants (Parida and Das 2005).

It is found that when mangrove plants were exposed to different concentrations of NaCl, Na^+ and Cl^- levels increased, Ca^{2+}, Mg^{2+}, Cu^{2+} and Mn^{2+} levels decreased and Fe^{2+} and K^+ levels stabilized (Parida, Das, and Mittra 2004).

Oxidative effect of salinity is another physiological concept which induces the expression of antioxidative enzymes and generation of antioxidant molecules. Superoxide dismutases containing metals such as Cu, Fe, Zn etc., ascorbate peroxidases, monodehydroascorbate reductases and glutathione reductases are some of the enzymes which play role in antioxidative mechanism in the presence of salt stress (Hernandez et al. 1999).

High salinity also affects the efficiency of photosynthesis which is an essential physiological process for plants enabling them to produce their own nutrition. In saline environment, highly uptake of salt ions decreases the water potential in plants. This dehydration limits the stomal opening process which is regulated by root- and shoot-generated hormones. Therefore CO_2 permeabilization of cell membranes and CO_2 conductance on mesophyll are reduced. Intercellular absence of CO_2 lowers the activity of basic photosynthetic enzyme RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase, EC 4.1.1.39) and other related enzymes (Chaves, Flexas, and Pinheiro 2009).

Besides, salt ions inhibit several photosynthesis responsible enzymes, decrease the production efficiency of photosynthetic pigments such as protochlorophyll, chlorophyll and carotenoid and cause chlorosis in further phases (Agastian, Kingsley, and Vivekanandan 2000; Parida and Das 2005). Consequently, photosynthesis is affected significantly as a physiological process by high concentrations of salt ions.

1.3.3. Effects on Plant Metabolism

Carbon taking place in all nutritional productions and nitrogen playing role in nodule formation through fixation, are the cornerstones of plant metabolism.

Chloride (Cl^-) is the most common ion that causes stress in nitrogen metabolism of plants. Recent studies have indicated that Cl^- found in soil decreases the uptake of

nitrate (NO_3^-) ions and inhibits the nitrate reductases. Thus, nitrogen fixation through nodulation is deactivated in plants (Flores et al. 2000).

The linkage of carbon and nitrogen pathways requires NADP-specific isocitrate dehydrogenase (EC 1.1.1.42). This linkage is provided by assimilation of nitrogen atom according to carbon allocation in metabolic compounds. Long-term exposure of salt stress in plants reduces the activity of this crucial enzyme (Popova et al. 2002).

Another metabolic crosslink enzyme, NADP-malate dehydrogenase (EC 1.1.1.82) which reduces the oxaloacetate to malate in chloroplasts is increased by salt stress (Cushman 1993).

1.4. Salt Tolerance Mechanisms in Plants

Hypothetically, halophytes may be evolved from survived glycophytes under salinity stress. Evidence to this idea is that highly glycophytic Arabidopsis has some close relatives which are extremely halophytic (Zhu 2000). Taking Zhu's hypothesis as a theoretical framework, in this section how halophytes can survive under salt stress is discussed in details.

Salt tolerant (halophytic) plants can develop many alternative survival pathways against salinity stress. This tolerance can be explained by three different characteristics of a plant: 1- Plant may export the ions or accumulate in their vacuoles by specific transporters, 2- Their morphological features, biomass distributions, control of transpiration rate by stomal closure may provide adaptation, 3- Metabolic and physiological regulations may stabilize the intracellular ion levels (Winicov 1998).

In tolerance mechanisms, genetic based strategies include chromosomal changes via epigenetic modifications such as DNA methylation and also polyploidization, amplification of specific sequences, DNA elimination or transcriptional regulation via induction of specific transcription factors such as ABF3 and ABF4 (Abscisic acid responsive elements-Binding Factor 3 and 4) (Wang, Vinocur, and Altman 2003; Parida and Das 2005).

Furthermore, halophytes use many biochemical regulation points such as ion accumulations in specific compartments of cells, controlling the activity of plasma membrane transporters and/or water channel proteins, generating of compatible

products, differentiating in photosynthetic pathway including carbon metabolism and energy production, modification of cell wall/membrane composition, induction of osmoprotectans, molecular chaperons or defensive molecules (i.e. antioxidants, detoxifying enzymes, proteases or hormones) which enable plants to deal with salinity stress. (Parida and Das 2005; Winicov 1998).

1.4.1. Genetic Profiles of Plants and Signaling Pathways of Salt Tolerance

Salt induction in many plants may be concluded as transcriptional, translational or post-translational regulation following the receiving of stress signals to related receptors. Plants regulate the expression of metabolic pathway proteins and signaling proteins as well as transcription factors. During salt exposure transcripts of ribosomal proteins, homologous of abscisic acid responsive genes and elongation factor-1α (EF-1α) increase in order to control transcriptional activity (Kawasaki et al. 2001).

Regulation of signaling has also crucial roles in tolerance mechanisms in plants. Most of the abiotic stresses trigger similar regulation pathways, though some of them behave specifically.

One of the signaling mechanisms induced by salt stress is *Salt Overly Sensitive* (*SOS*) mediated pathway (Figure 1.1). SOS1, SOS2 and SOS3 are the three main components of the pathway and have an essential role in salt tolerance mechanism. Hence, all of *sos1*, *sos2* and *sos3* mutant plants are hypersensitive to salt and they accumulate Na^+ or Li^+ ions excessively (Zhu 2000; Chinnusamy, Zhu, and Zhu 2006).

When a plant is exposed to salt stress, firstly Ca^{2+} signals are generated in order to serve as a secondary messenger against stress factor (Knight, Trewavas, and Knight 1997). Then, Ca^{2+} ions are sensed by SOS3 which includes an N-myristoylation motif and three Ca^{2+} binding EF hand which is a helix-loop-helix structural domain. The myristoylation domain of SOS3 leads to SOS2 in order to recruit on plasma membrane (Quintero et al. 2002).

SOS2, which encodes a serine/threonine protein kinase with an N-terminal catalytic domain and a C-terminal regulatory domain, is activated after recognition of Ca^{2+} signals by SOS3. (Chinnusamy, Zhu, and Zhu 2006; Liu et al. 2000). Activation of SOS2 kinase triggers a cascade mechanism via phosphorylation through mitogen-

activated (MAP) kinases, MAP kinase kinase 2 (MKK2) and the two other MAP kinases (MPK4 and 6) (Teige et al. 2004). SOS3 and SOS2 together phosphorylate SOS1 which is a Na^+/H^+ antiporter embedded in plasma membrane in order to regulate the expression level, (Liu et al. 2007; Chinnusamy, Zhu, and Zhu 2006). Moreover, the SOS3-SOS2 couple inhibits the activity of low-affinity Na^+ transporter (HKT1) and activates the ion accumulators on vacuole membranes (NHX1, NHX5, AVP1 and AVP2) under salt stress (Mahajan, Pandey, and Tuteja 2008).

SOS4 and SOS5 are the other SOS pathway members discovered during exposing plants to higher concentrations of salt. *SOS4* encodes a pyridoxine / pyridoxal / pyridoxamine (PN / PL / PM) kinase that catalyzes the biosynthesis reaction of pyridoxal-5-phosphate (PLP), the active form of vitamin B6. It is proposed that PLP may function as a regulator of ion channels or transporters included in salt tolerance mechanisms (Mahajan, Pandey, and Tuteja 2008; Shi et al. 2002).

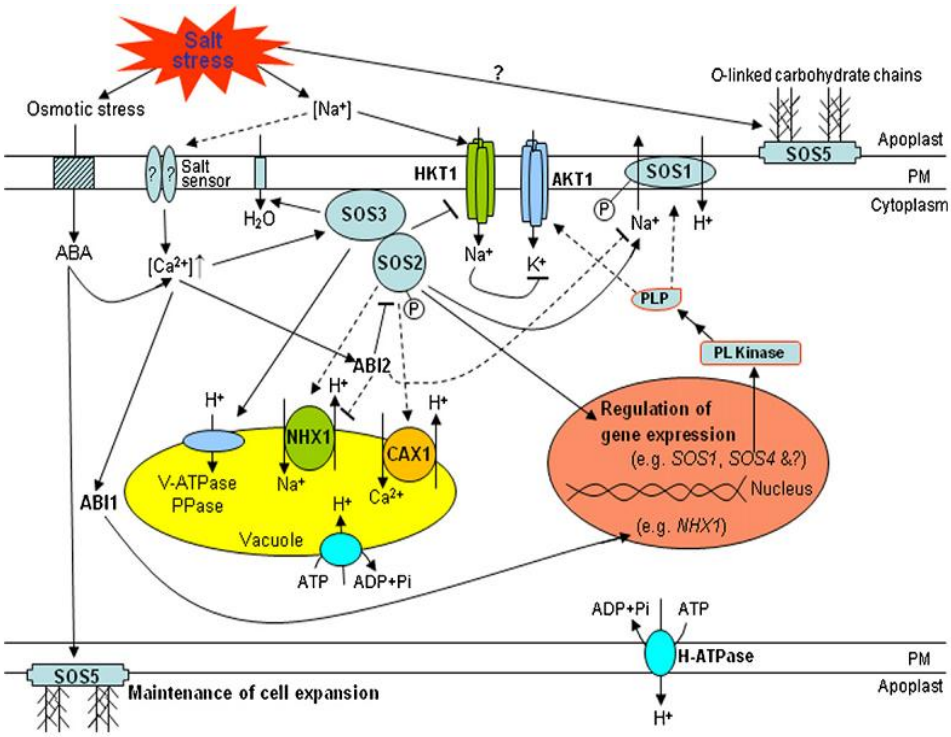

Figure 1.1. Salt Overly Sensitive (SOS) and abscisic acid (ABA) pathways in plants. Salt stress triggers these signaling pathways and initiates the tolerance mechanisms (Source: Türkan and Demiral 2009)

SOS5 encodes a protein which is highly similar to AtAGP8, an arabinogalactan protein (AGP) belonging to *Arabidopsis thaliana*. SOS5 possibly plays role in cell adhesion. It may aggregate on cell walls by their polysaccharide tails and form a unique network (Shi et al. 2003).

Another regulative signaling mechanism is Abscisic acid (ABA) pathway activated by salt stress indirectly. One of the cellular effects of salt stress is osmotic imbalance. This situation causes biosynthesis and accumulation of phytohormone ABA in cytosol as a conclusion of Ca^{2+} signals (Chinnusamy, Jagendorf, and Zhu 2005). ABA is a well known plant hormone and it has different functions such as acting as an osmo-regulator under drought and salt stresses (Barrero et al. 2006), taking place in developmental phases of plants, adjusting the physiological state under stress conditions (Chandler and Robertson 1994), depolarizing the plasma membrane potential and regulating the redistribution of ions and solutes from tonoplast and cytoplasm to apoplast in order to control the osmotic balance (Rock 2000; Chinnusamy, Zhu, and Zhu 2006).

High levels of ABA, activates the ABA insensitive-1 (ABI1). ABI1 regulates the stomata behaviors on leaves and mitotic activity of root meristems. On carboxyl terminus, ABI1 includes a domain related to serine/threonine phosphatase 2C, however in amino terminus, a unique Ca^{2+} binding EF hand domains. This specific structure may provide the aggregation of ABA and Ca^{2+} signals for phosphorylation based response pathways (Leung et al. 1994).

Another activated factor in the presence of ABA, ABA insensitive-2 (ABI2) protein phosphatase 2C, inhibits the SOS pathway through interaction with protein phosphatase interaction (PPI) motif of SOS2. Thus, ABA down-regulates both of the Na^+/H^+ antiporters that localize on plasma membrane (SOS1) and vacuolar membrane (NHX1) (Chinnusamy, Jagendorf, and Zhu 2005).

1.4.2. Transport Mechanisms: Accumulation or Exclusion of Ions by Ion Pumps, Antiporters and Channels

It is generally known that plants are defected by nutrient imbalance or ionic toxicity of salt. Increment in several toxic ions such as Na^+ or Cl^- and/or decrement in essential ions such as K^+ or Ca^{2+} may be the basic reason of this defection. Therefore,

plant develops strategies in order to export the toxic ions and stabilize the levels of essential ions in cells (Mansour, Salama, and Al-Mutawa 2003).

Ion transportation occurs in both plasma membrane and vacuolar membrane. Hence, toxic ions may be either excluded through the plasma membrane or accumulated in vacuoles. Transport systems activated in salt stress include non-selective cation channels, anion channels for Cl^- ions, plasma membrane ATPases (P-ATPases), vacuolar ATPases (V-ATPases), vacuolar pyrophosphatases (V-PPase), plasma membrane and vacuolar Na^+/H^+ antiporters, K^+/H^+ antiporters, $Cl^-/2H^+$ symporters, K^+ channels and water channels (aquaporins) for osmotic adjustment (Mansour, Salama, and Al-Mutawa 2003; Munns and Tester 2008; Maser, Gierth, and Schroeder 2002; Bohnert, Su, and Shen 1999) (Figure 1.2.)

1.4.2.1. Influx Mechanisms of Ions

Sodium and chloride, phytotoxic ions, induce several cellular reactions and accelerate the tolerance mechanisms. When plants absorb Na^+ ions from soil, they cannot discriminate these ion types from K^+ ions due to their chemical similarity and import Na^+ ions into the cells. There are three types of channels that are responsible for K^+ transport and keep the intracellular K^+/Na^+ ratio constant. K^+ inward rectifying channels (KIRC) such as AKT1 localize in plasma membrane and activate K^+ influx via hyperpolarization. In high concentrations of Na^+ ions, they may leak through these channels. K^+ outward rectifying channels (KORC) are the second group which the efflux K^+ ions to outer side of the plasma membrane via depolarization. KORC also import Na^+ ions to balance the intracellular K^+/Na^+ ratio. The last group, voltage-independent cation (VIC) channels also localize in plasma membrane and transport cationic ions selectively, in contrast to voltage-dependent channels, shaker-type K^+ channels such as KIRC and KORC (Blumwald 2000; Yokoi, Bressan, and Hesagawa 2002; Maser, Gierth, and Schroeder 2002).

VIC channels were first identified in wheat with their action nutrition, osmotic adjustment and charge compensation functions (White 1997). VIC channels select the monovalent cations according to their affinity. In addition, these channels are permeable

to divalent cations such as Ca^{2+}. In other words, Ca^{2+} may regulate the intracellular homeostasis through these channels (White and Davenport 2002).

HKT, KUP/HAK/KT, LCT1 and CNGC are some of the other cation transporters that play role in ion accumulation in cytoplasm. HKT and KUP/HAK/KT are the two high affinity K^+ transporter families. HKT family contains four P-loop domains that are effective on ion transportations. HKT1, the first identified member of this family, is a symporter which supports the K^+ uptake via Na^+ coupling. In the presence of excess amount Na^+, K^+ accumulation through HKT1 is repressed and low-affinity Na^+ uptake occurs. Moreover, point mutations on HKT1 increase the Na^+ tolerance of plants. Thus, HKT family may be a defensive factor against salt stress (Maser, Gierth, and Schroeder 2002; Rubio, Gassmann, and Schroeder 1995; Munns and Tester 2008). KUP/HAK/KT, another high-affinity family imports the Na^+ ions as well as K^+. However, transportation may occur competitively and K^+ transportation may be inhibited by high amount of Na^+ ions (Maser, Gierth, and Schroeder 2002).

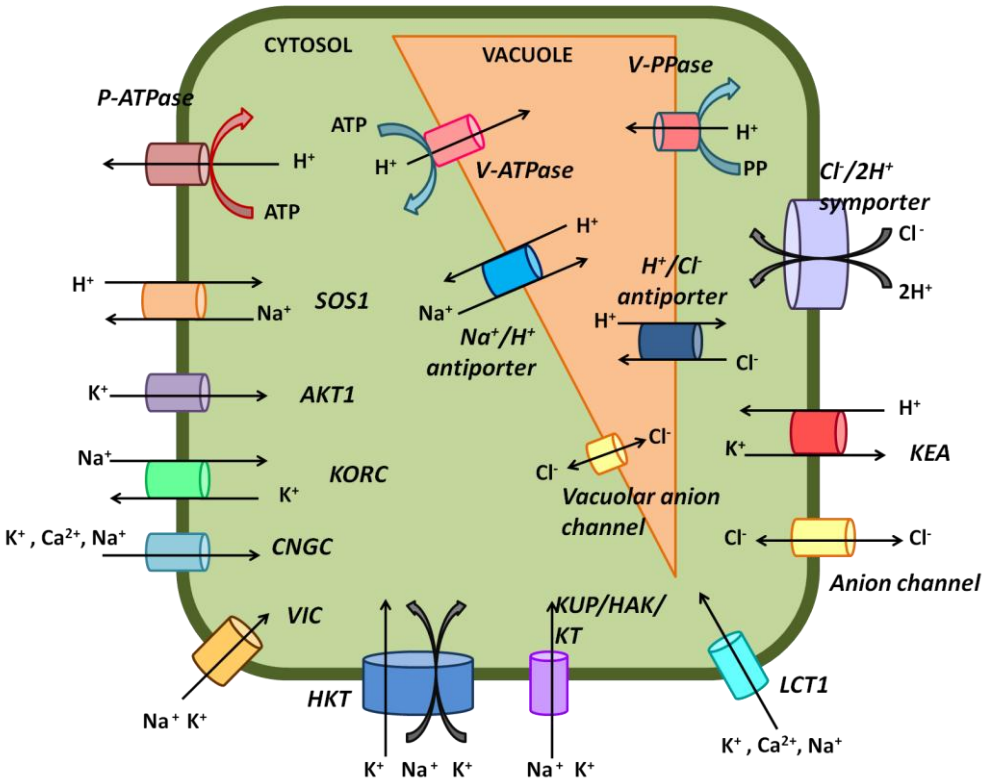

Figure 1.2. Ion transport systems on plasma and vacuolar membrane of plant cells (The figure was modified from Maser, Gierth et al. 2002; Mansour, Salama et al. 2003; Munns and Tester 2008; Teakle and Tyerman 2010).

LCT1, low affinity cation transporter, imports Na^+, K^+ besides Ca^{2+} and Cd^{2+} (Clemens et al. 1998). It is also demonstrated that Ca^{2+} ions, at the above of determinant concentrations, inhibit the N^+ uptake of LCT1 (Amtmann et al. 2001).

CNGCs, cyclic nucleotide gated channels, are permeable to several cations. As it is mentioned on its name, these gated channels are controlled by cyclic nucleotides, cAMP and cGMP. Control of the gate is concluded as regulation of Na^+ uptake (Maathuis and Sanders 2001).

In addition to cationic transports, there are also several anionic transportation mechanisms. Chloride is the most common anion belongs to high amount of NaCl in soils. In general, plants need micro levels of Cl^- ions as a regulator of enzyme activity, an essential cofactor in photosynthesis or a stabilizer of membrane potential and turgor pressure of cell (Teakle and Tyerman 2010). In related literature, $Cl^-/2H^+$ symporters and several anion channels which can flux Cl^- ions in cells are the only identified transporters (White and Broadley 2001).

1.4.2.2. Efflux Mechanisms of Ions

In nature, plant cells protect themselves from cytotoxic ions by either effluxing the ions to outer side of the membrane or accumulating in their storage organelles vacuoles. Most of the effluxion processes occur via regulating the proton (H^+) gradient in cytoplasmic and vacuolar systems. Energy dependent H^+ pumps change the membrane potential in addition to electrochemical balance of the cell and facilitate the transportation of phytotoxic ions on reverse directions (Hussain et al. 2010).

Plasma membrane ATPase, vacuolar ATPase and pyrophosphatases are the basic proton pumps. They hydrolyze adenosil triphosphate (ATP) or pyrophosphate (PP), and pump the protons either outer side of the plasma membrane or inner side of the vacuolar membrane tonoplast (Mansour, Salama, and Al-Mutawa 2003). As these pumps remove the protons from the cytoplasm; several antiporters such as SOS1 or KEA are activated against electrochemical gradient. These antiporters are integral membrane proteins which exchange the protons and Na^+/K^+ ions across the plasma membrane or tonoplast in order to accumulate ions in vacuoles (Blumwald 2000; Maser, Gierth, and Schroeder 2002; Mansour, Salama, and Al-Mutawa 2003).

Since the Cl^- transport mechanisms are not identified well, it is predicted that several candidate anion transporter genes may also be permeable for Cl^-. These anion transporters include mechanosensitive channels of small conductance (MscS)-like (MSL), voltage dependent anion channels (VDAC), porins, the CLC (chloride channel) anion channels, anion/H^+ antiporters, the NRT (NOD)s nitrate and peptide transporter family, the ATP-binding cassette (ABC) transporter family, aluminum activated malate channels (ALMT), cation-coupled Cl^- (CCC) and recently identified slow anion channel associated protein (SLAC1) (Teakle and Tyerman 2010). There are at least three types of Cl^- permeable channels on plasma membrane. They are rapidly activated anion channels (R-type), slowly activated anion channels (S-type) or stretch-activated anion channels. These channels facilitate the flux of Cl^- ions outer side of the plasma membrane (White and Broadley 2001).

1.4.3. Antioxidative Response Mechanisms of Plants

Reactive oxygen species (ROS) are the variations of oxygen (O_2) which are produced by changing electron/energy distribution of O_2 molecules during aerobic cellular processes such as chloroplast/mitochondrial electron transport or chlororespiration (Chinnusamy, Zhu, and Zhu 2006; Apel and Hirt 2004). Hence, the main ROS generators in cells are mitochondria, chloroplasts and peroxisomes.

In chloroplasts, electron acceptor of photosystem I reduces oxygen molecules and produce superoxide anion ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2). Also excited chlorophyll molecule transfers its electron to oxygen molecule and produces singlet oxygen ($^1O_2$) during photosynthesis. The main component of the photosynthesis, ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) contributes to ROS production via oxygenase reactions. Chlororespiration, another ROS production process, begins with the reduction of oxygen by NAD(P)H dehydrogenase on respiratory chain and terminates with oxidases that compete with photosynthetic electron transport chain in chloroplasts. Similarly, in mitochondria most of the ROS are generated on electron transport chain (Jithesh et al. 2006; Apel and Hirt 2004).

When a plant is exposed to any of the abiotic stresses such as salinity, oxidative stress occurs related to over-production of ROS (Ashraf and Harris 2004). Oxidative

stress affects membrane integrities, enzyme activities and photosynthesis efficiency via damaging ROS to proteins, lipids and other cellular components (Jithesh et al. 2006; Bohnert, Su, and Shen 1999).

In order to deal with ROS, plants develop enzymatic and non-enzymatic antioxidant defense mechanisms (Koca et al. 2007). These mechanisms include ROS scavengers which are antioxidants and detoxifying enzymes (Figure 1.3.).

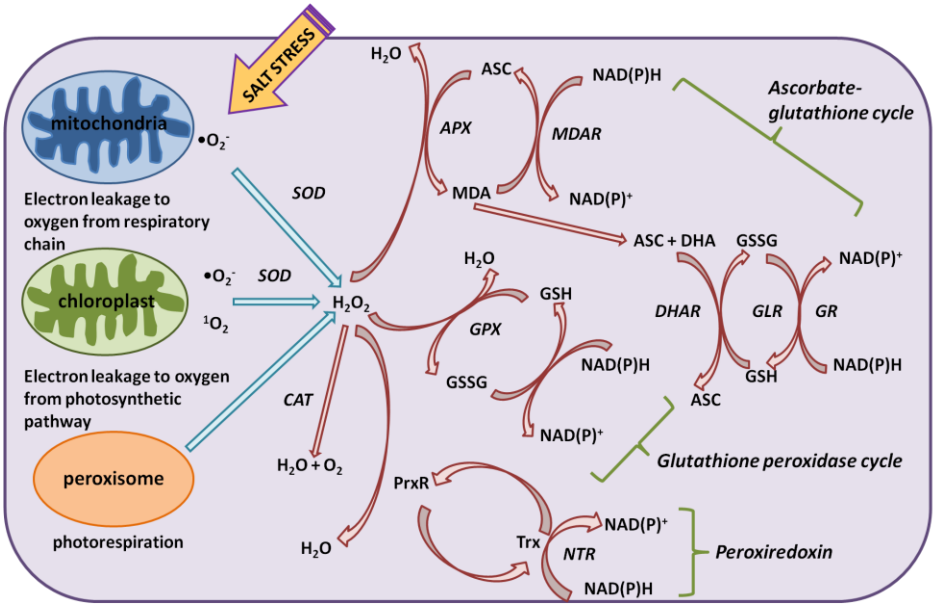

Figure 1.3. ROS production and scavenging mechanisms under salt stress (The figure was modified from Dietz 2003; Apel and Hirt 2004; Jithesh, Prashanth et al. 2006; Abogadallah 2010).

Non-enzymatic antioxidant products are α-tocopherol, flavonoids, alkaloids, carotenoids, ascorbate (ASC) and glutathione (GSH). On the other hand, enzymatic ROS scavengers which are detoxifying enzymes in plants are superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POX; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione peroxidase (GPX; EC 1.11.1.9), catalase (CAT; EC 1.11.1.6), MDA reductase (MDAR; EC 1.6.5.4), DHA reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2) and peroxiredoxin (PrxR; EC 1.11.1.15) (Hsu and Kao 2003; Türkan and Demiral 2009; Dietz 2003; Eltayeb et al. 2006; Hossain and Asada 1985; Mittler et al. 2004).

Superoxide dismutase, initial step of detoxification, converts the superoxide oxygen anion ($\bullet O_2^-$) to hydrogen peroxide (H_2O_2) which is then reduced to H_2O via *ascorbate-glutathione* and *glutathione peroxidase cycles*. Thus, ascorbate and glutathione are the key points of antioxidative mechanism through the NAD(P)H dependent *ascorbate-glutathione cycle*. In the presence H_2O_2, ascorbate is oxidized by APX to form monodehydroascorbate (MDA) and dehydroascorbate (DHA). Then, MDA is recycled to ascorbate via NAD(P)H oxidation by MDAR. However, DHA is reduced to ascorbate by DHAR as glutathione is oxidized to GSSG. Similarly, GSSG reduction depends on NAD(P)H oxidation by GR. In another antioxidative mechanism, *glutathione peroxidase cycle*, H_2O_2 directly reacts with glutathione and oxidize to GSSG by GPX. Conversion of GSSG to GSH again depends on NAD(P)H oxidation and GR. Beside these multistep pathways; H_2O_2 can be reduced to H_2O directly by CAT in cells. (Jithesh et al. 2006; Apel and Hirt 2004).

Peroxiredoxins (PrxR) which are located in distinct cell regions such as mitochondria and chloroplast are the other actors of antioxidant defense mechanisms. PrxR reduces the H_2O_2 to H_2O via *water-water cycle* in photosynthetic electron transport system. This pathway aims to exterminate the excessively absorbed excitation energy and prevents the photo-inhibition as a result of heating. Regeneration of PrxR is provided by thioredoxin (Trx) or glutaredoxin electron donors (Dietz 2003).

For many years, antioxidative enzyme activities have been phenomena related to salinity stress and tolerance of plants. In many independent studies it has been demonstrated that salinity increases the ROS production and, both of ascorbate and glutathione concentrations decrease as a conclusion of ROS increment under salt stress (Hernandez et al. 2000). Besides, ROS increase the activity of detoxifying enzymes such as CAT, GPX or PrxR whereas decreases the SOD (Vaidyanathan et al. 2003; Dionisio-Sese and Tobita 1998; Dietz 2003). Interestingly, overexpression of mitochondrial Mn-SOD or chloroplastic Cu/Zn-SOD has a role in salt tolerance of different plants (Wang, Reyes, et al. 2004; Badawi et al. 2004).

1.4.4. Production of Osmolytes / Osmoprotectans

Plants lose their intracellular water in salty environments as a result of osmotic imbalance (Türkan and Demiral 2009). A general strategy developed by halophytes is overproduction of osmolytes or osmoprotectans in contrast to glycophytes. Some essential ions such as K^+ can also act as an osmolyte in addition to common organic metabolites (Hussain et al. 2010). Accumulation of organic compounds provides the adjustment of cellular homeostasis and osmotic balance.

Some major groups of organic osmoprotectans include simple sugars (majorly fructose, sucrose and glucose), sugar alcohols/polyols (glycerol, mannitol, methylated inositol, sorbitol, cyclic forms (cyclitols), ononitol and pinitol), complex sugars (starch, trehalose, raffinose and fructans), quaternary amino acid derivatives (basically proline and also arginine, glycine, leucine, valine, glutamine, asparagine, ectoine, citrulline and ornithine), quaternary ammonium compounds (glycinebetaine (GB), osmotin, trigonelline, hydroxyprolinebetaine, pipecolatebetaine, β-alaninebetaine and prolinebetaine), tertiary amines (1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrimidine), sulfonim compounds (choline o-sulfate and dimethyl sulfonium propironate (DMSP)) and polyamines (putrescine, spermidine, spermine and diamine, diaminopropane, cadaverine less commonly) (Türkan and Demiral 2009; Ashraf and Harris 2004; Yokoi, Bressan, and Hesagawa 2002; Hussain et al. 2010; Parida and Das 2005). Out of these osmoprotectans, the most common and effective osmolytic regulators are proline and glycinebetaine (GB).

In higher plants, proline derivates from ornithine or glutamate. Salinity stress may induce either one or both of glutamate and ornithine pathways to produce proline. This production depends on plant development and/or aging (Türkan and Demiral 2009). Regulative roles of proline are adjustment of vacuolar and cytoplasmic water balances, accumulation of other amine groups which are also osmotically active, supporting ROS scavenging, buffering redox potential, providing the macromolecular and structural stability of membrane, equilibrating the electronic distribution, pH adjustment, balancing the $NADP/NADPH^+$ ratio and prevention of disruptive salt effects on plasma membrane (Ashraf and Harris 2004; Matysik et al. 2002; Ashraf and Foolad 2007).

Increment in proline content of cells belonging to salinity induces the proline accumulation through activation of glutamate kinase which catalyzes the first step of proline synthesis. On the contrary, accumulated proline inhibits self-biosynthesis. In addition, highly accumulated proline and another form of hydroxyproline in salt exposed plant cells are used for biosynthesis of proline-rich stress proteins. Moreover, proline and hydroxyproline participate in the production of specific molecules that are defensive on salt stress. These defensive molecules are proline-rich glycoproteins, lipoproteins, proteins, phosphoproteins, polypeptides, peptides and hydroxyproline-rich glycoproteins, glycoproteins, phosphoproteins, polypeptides, peptides (Ashrafijou et al. 2010).

Second osmolytic regulator glycinebetaine (GB) is the major quaternary ammonium compound which plays an active role in salt stress is very soluble and mostly abundant in chloroplasts and plastids (Ashraf and Foolad 2007; Chen and Murata 2008). GB is synthesized from serine with the pathway that includes ethanolamine, choline and betaine aldeyhde (Ashraf and Foolad 2007). Other protecting characteristics of GB, as well as adjusting the osmotic balance, are stabilizing proteins (mainly RuBisCO), protecting the photosynthetic apparatus and thylakoid membrane, acting as a ROS scavenger and regulating the activity of ion channels (Heuer 2003; Chen and Murata 2008).

1.5. Proteomic Approaches for Salt Exposed Plants

1.5.1. Proteomics of Salinity Proteins in Plants

As mentioned in previous topics, plants response to salt stress with several mechanisms in which many proteins are activated and/or inhibited. Photosynthesis, photorespiration, signal transduction, metabolic regulation, oxidative stress and ionic transportation include different salt responsive proteins (Joseph and Jini 2010).

In addition, it has been observed that salt exposed plants often express *Salt Shock Proteins (SSP)* and *Heat Shock Proteins (HSP*). Salt shock proteins are accumulated as a survival reflex, which affects the expression or inhibition other responsive proteins (Joseph and Jini 2010). Heat shock proteins/chaperons, on the other

hand, are the most common unspecific responsive proteins in plants including HSP60/chaperonin, HSP70, HSP90, HSP100/Clp and small HSPs are stimulated under salt stress (Wang, Vinocur, et al. 2004).

Identification, characterization and quantification of these stress-related proteins can be obtained by proteomic techniques.

1.5.2. Basic Techniques in Proteomics

Popularity in proteomic studies has been accelerated for last two decades due to the novel developments in mass spectrometry (MS). Understanding the changes in cellular processes or functions at protein levels and also post-translational modifications as a result of several effective factors requires proteomic approaches. Identification of proteins together with the characterization mainly consists of isolation, separation and analysis steps.

In proteomic approaches, initial step includes preparation of proteins. Hence, proteins are isolated specifically or totally from target cells, tissue, organ or whole organism by performing several techniques. Specific extraction techniques may require recombination technologies in order to insert specific tags and/or antibody production recognizing target proteins. Total protein extractions; however, relies on cell or tissue lysis and purification unless they are isolated from specific organelles or cellular regions such as plasma membrane.

Proteomic technologies offer many different alternatives for protein identification. As an exception, mass-spectrometry based techniques have peaked since last decade. Identification of proteins can be carried out by gel-based separations or gel-free fractionations followed by mass spectrometry (Agrawal et al. 2012).

Gel-based separations are one or multi-dimensional electrophoretic applications that is useful for distinguishing proteins due to their specific characteristics such as molecular weight and pI value. In contrast, gel-free fractionations are one or multi-dimensional chromatographic methods by which protein fractions can be produced according to their specific characteristics again such as size, hydropathy or charge. In gel-free systems, protein fractions can be collected as well as peptide fractions after enzymatic digestion. Though both of multi-dimensional gel-based and gel-free systems

reflect high technology, combination of one-dimensional gel-based and gel-free techniques are more common as a result of easy manipulation (Figure 1.4).

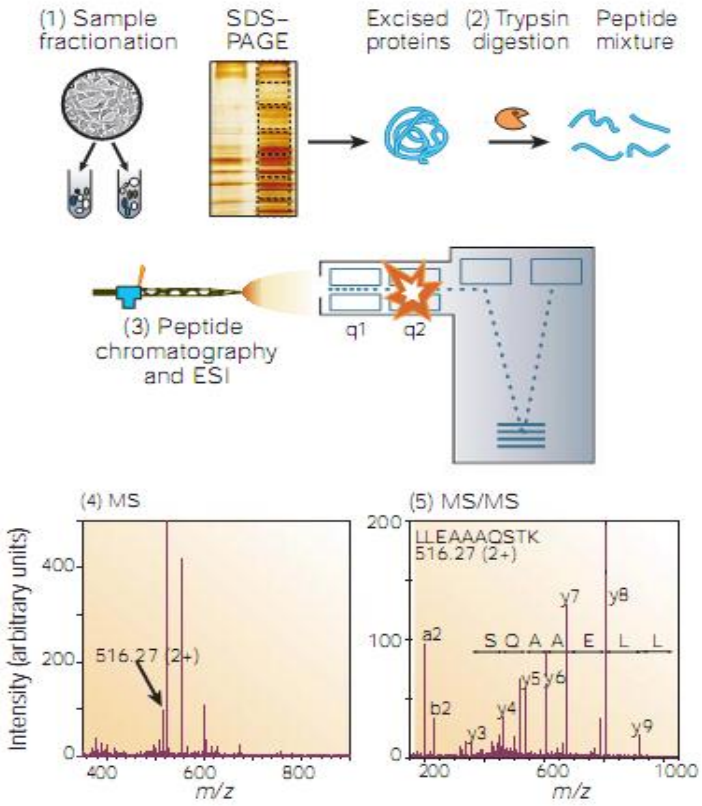

Figure 1.4. Basic steps of proteomic approaches: 1-Sample preparation, 2-Gel-based separation, 3-Enzymatic digestion, 4-Chromatographic fractionation, 5-Mass spectrometry analysis (Source: Aebersold and Mann 2003)

1.5.3. Mass Spectrometry-Based Techniques

In proteomic studies, mass spectrometry has become a crucial analyzing technique in which gas-phase ions of peptides/proteins are generated (Aebersold and Mann 2003). The theory behind the MS is ionization of the sample, subsequent separation and detection of these ions based on their mass to charge *(m/z)* ratios. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are two major ionization methods which are also known as *soft ionization techniques* in mass spectrometry. “Soft ionization” term represents the ionization of large and non-volatile

molecules such as proteins and peptides without causing any degradation during ionization process (Aebersold 2003).

In MALDI, analyte is mixed with a synthetic matrix and irradiated with a pulsed laser beam resulting in the vaporization of the analyte either via protonation or deprotonation. The organic matrix molecules are capable of absorbing the energy of the laser, generally in the UV range. MALDI has become an effective technique with the combination of time-of-flight (TOF) mass analyzer which also enables to analyze the fragment ions derived from parent ion in TOF/TOF systems (Aebersold 2003) (Figure 1.5).

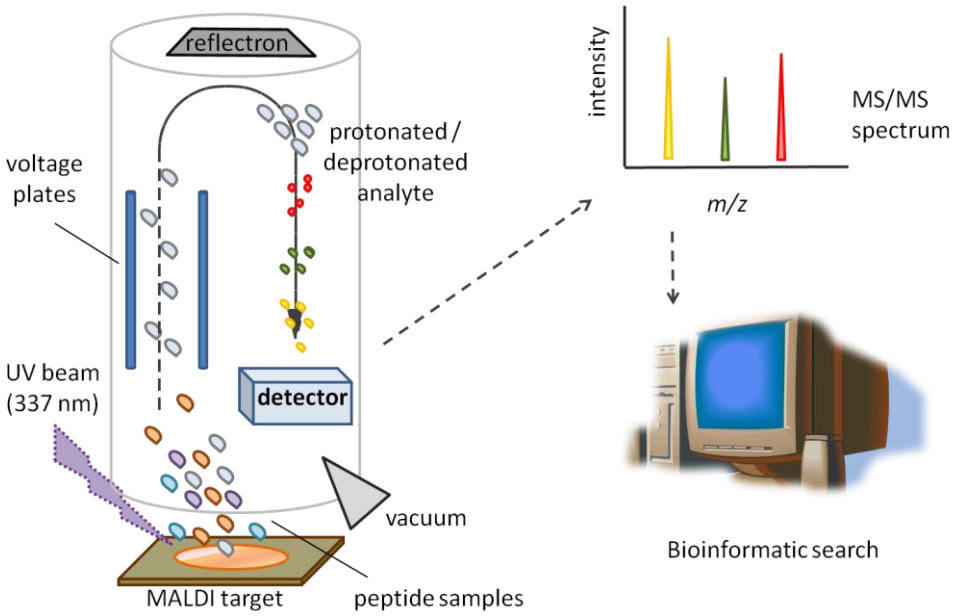

Figure 1.5. Schematic representation of MALDI-TOF/TOF

Common proteomic based matrices used in MALDI are 2,5-dihyroxybenzoic acid (DHB), sinapinic acid (SA), a-cyano-4-hydroxycinnamic acid (CHCA), 1,5-diaminonaphtalene (1,5-DAN), picolinic acid (PA) (Demeure et al. 2007) and 2,4,6-trihydroxyacetophenone (THAP) (Kussmann et al. 1997).

In proteomic studies, two-dimensional polyacrylamide gel (2D-PAGE) and two-dimensional differential electrophoresis (2-DE) separation techniques are analyzed by MALDI-TOF/TOF preferentially (Qureshi, Qadir, and Zolla 2007).

Another ionization technique, ESI, is based on sample ionization via electric field. In ESI, multiply charged molecular ions are measured and detected in ion trap or

quadrupole instruments (Aebersold and Mann 2003; Aebersold 2003). ESI-MS can be combined with one or multi-dimensional liquid chromatography (LC) technique since liquid samples are required. As mentioned in part 1.5.1, sample preparation for LC can be either peptide fractionation after enzymatic digestion or fractionation at protein level.

With the soft ionization techniques tandem mass spectrometry (MS/MS) commonly used for protein identification is based on peptide analysis where this application requires enzymatic digestion initially (Nesvizhskii et al. 2003). Then, ionized peptides are detected individually and each selected peptide is allowed to fragment by collision induced dissociation (CID), electron transfer dissociation (ETD) (Swaney, McAlister, and Coon 2008), electron capture dissociation (ECD) or surface induced dissociation (SID) (Sadygov, Cociorva, and Yates 2004). Each peptide may give a unique result based on *m/z* ratio related to its fragments. In this respect, this unique mass spectrum of a peptide can be identified via mass related bioinformatic tools and protein databases (MASCOT; SEQUEST etc.).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All materials used in experimental procedures are given in Appendix A.

2.2. Methods

2.2.1. Plant Growth and Salt Exposure

Beta maritima seeds were firstly germinated in sterilized sand and then transferred to half strength Hoagland's medium (Appendix B). Conditions of the growth chamber were adjusted as 12 hours dark and 12 hours light photoperiod with a 40 μmol m^{-2} s^{-1} light intensity. The temperature was 25^{o}C and humidity was 50.0 (as % RH).

Plants were divided into two groups. The first group was exposed to 150 mM NaCl in half strength Hoagland's medium for 10 days. This group was renamed as "Stress Group". Second group was growth in the same half strength Hoagland's medium as a "Control Group". During exposure period, all mediums were refreshed once in two days. After exposure, leaves and roots were harvested, frozen by liquid N_2 and stored at −80^{o}C.

2.2.2. Total Protein Extraction with Phenol Extraction Method

In this part Faurobert's phenol extraction protocol was followed (Faurobert, Pelpoir, and Chaib 2007). Protein extraction from "control leaf", "stress leaf", "control root" and "stress root" samples was performed one by one. First, 1 g sample was grinded in the presence of liquid N_2 by mortar and pestle at least three times until the whole sample was powder. 3 ml extraction buffer (Appendix B) was added onto the

powder in 15 ml falcon tube and then shaken on ice for 10 minutes. Sample was centrifuged at 5000 rpm, 4^{o}C for 15 minutes. Supernatant was removed into new tube and Tris buffered phenol (Appendix B) was added with equal volume (1:1). Tube was shaken at room temperature for 10 minutes in dark and centrifuged again at 5000 rpm, 4^{o}C for 15 minutes. After centrifugation three different phases were observed. Top phase (phenol phase) was taken into new tube avoiding to touch the medium phase (white oily phase) and lower phase was disposed. Extraction buffer was added onto phenol phase with equal volume (1:1) and shaken in room temperature for a few minutes. Centrifugation was performed under the same conditions and again the top phase was removed into new tube. Ice cold precipitation solution (Appendix B) was added onto phenol phase with (1:2) volume. That sample was incubated overnight at -20^{o}C.

On the second day, proteins were observed clearly as a whitish mucous-like structure in falcon tube. The sample was precipitated at 5000 rpm, 4^{o}C for 25 minutes. After that, supernatant was removed; pellet was washed with ice cold precipitation solution and taken into 1.5 ml micro centrifuge tubes. Proteins were centrifuged at 5500 g, 4^{o}C for 10 minutes. Supernatant was removed again and protein pellet was washed with 90 % (v/v) ice cold acetone solution. The centrifugation step was repeated and after supernatant was removed, pellet was dried at room temperature for few minutes. The usage of vacuum evaporator was avoided due to the excess dry.

Finally, semi-dried pellet was resolved in 100-200 μl 2D rehydration buffer (Appendix B).

2.2.3. Protein Quantification with Bradford Assay

In Bradford assay, Coomassie Brilliant Blue G-250 (CBB G-250) dye interacts with proteins non-specifically and blue color gives absorbance at 595 nm wavelength. According to Lambert-Beer law, sample concentrations can be measured by comparing with standards.

In this experiment, self-optimized Bradford Assay was performed in order to determine the protein amounts for each sample. 25, 50, 100, 125, 200, 250, 500 μg/ml bovine serum albumin (BSA) solutions were used as standards and ultra pure (UP)

water as blank. Extracted proteins were diluted 20 times with UP water. 20 µl from each standard / sample / water was mixed with 180 µl 1X grayish-green colored Bradford Reagent (Appendix B).

Reactions occurred in Greiner Bio One U-bottom 96 well plate and incubated at room temperature in dark for 10 minutes. Bright blue colors were measured at 595 nm wavelength against blank by spectrophotometer Multiskan Spectrum, Thermo Electron Corporation. Standard graph was created as absorbance vs. BSA concentrations and protein amounts of samples were calculated via equation of trend line considering dilution factor.

2.2.4. 2-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

2.2.4.1. First Dimension: Isoelectric Focusing (IEF)

The total protein separation was performed by 2-dimensional gel electrophoresis. In the first dimension, proteins were focused with BioRad PROTEAN IEF Cell due to their isoelectric points.

400-450 µg protein samples of each group were mixed with 400 µl 2D rehydration buffer including 65mM DTT and 2 % (v/v) pH 3-10 carrier ampholyte. Protein mixture was loaded into a channel of PROTEAN IEF focusing tray and immobilized pH gradient (IPG) strip (ReadyStrip^{TM} IPG Strip, 3-10 non-linear (NL), 17 cm, BioRad) was replaced onto protein solution in corresponding channel by using forceps, avoiding air bubble formation. Gel side of the strip should be at the bottom touching to proteins and pH 3 edge should be on positive, pH 10 edge on negative poles.

Passive rehydration occurred 1-2 hours at room temperature. 2 ml mineral oil was added onto strips to prevent the drying of strips before active rehydration. Focusing steps were set as Voltage-hour (Vh) and after rehydration process, by using forceps pre-wetted electrode wicks were placed between IPG strip and wire electrode to protect the strips from high voltage. Conditions of isoelectric focusing were; 50µA per IPG strip, 20^{o}C, 16 hours active rehydration at 50 V, 300 Vh linear at 200 V, 500 Vh linear at 500 V, 1000 Vh linear at 1000 V, 4000 Vh linear at 4000 V, 24000 Vh rapid at 8000 V and

30000 Vh rapid at 8000 V, respectively. During isoelectric focusing technique two of root protein samples could be focused at the same time whereas leaf proteins were focused one by one.

2.2.4.2. Equilibration of IPG Strips

Equilibration after IEF is an important procedure that provides saturation of IPG strips to sodium dodecyl sulfate (SDS) and reduction of sulphydryl groups in addition to complete denaturation of proteins.

IPG strips were removed into a new disposable tray by the help of forceps. Gel side of the IPG strip should be on top to touch the equilibration buffers. Then, IPG strips were shaken in equilibration buffer I (Appendix B) for 15 minutes in order to break disulfide bridges and then equilibration buffer II (Appendix B) for 15 minutes in dark to alkylate the reduced sulphydryl groups. Following the equilibration, strips were washed at least two times with 1X Running Buffer (Appendix B).

2.2.4.3. Second Dimension: SDS-PAGE

In the second dimension, 12% polyacrylamide gel (pH 8.8) including sodium dodecyl sulfate (SDS) was prepared. Strips were replaced onto the polyacrylamide resolving gel avoiding bubble formation and covered with melted overlay agarose gel (Appendix B). Circuit of the system was completed and gel was run at 16 mA for an hour and then 180 V for 6-6.5 hours at room temperature in the presence of 1X Running Buffer. System was cooled with water flow through the inside of the tank. For gel electrophoresis BioRad PROTEAN II xi Cell system was used.

After the running process gels were separated from glass plates and treated with staining solution (Appendix B) for overnight (~16 hours) in dark at room temperature.

The next day, the staining solution was removed. Gels were washed with deionized water (dH_2O) few times and then gels were treated with neutralization buffer (Appendix B) for 5 minutes in order to neutralize the proteins and reduce the effect of SDS. After the neutralization buffer is removed, gels were destained (Appendix B) for 1 minute. Next, fixation solution (Appendix B) was used to fix the proteins in

polyacrylamide gel. This process was carried out for 5-6 hours at least. Finally, gels were kept in 5 % (v/v) acetic acid solution at 4^{o}C.

2.2.5. Gel Comparison and Spot Selection

Stress and control gel images belonging to root and leaf samples were obtained with CCD camera of BioRad Universal Hood II Gel Imager on epi-white light. Increasing the image quality was provided by background subtraction and smoothing application.

Intensity changes, appearance and/or disappearance of protein spots were selected with naked eye. Each selected spot was excised with the back part of 100 µl micropipette tips and stored in 5 % (v/v) acetic acid solution in 1.5 ml micro centrifuge tubes at 4 ^{o}C. Non-protein part of a gel was also excised and the same protocol was followed in order to eliminate background peaks in mass spectrometry.

Confirmations of gel comparisons were obtained by gel analyzing tool Delta2D (DECODON). For each gel comparison process, gel images were uploaded to the program. Stress and control gels were warped by the program and the matched spots were accepted as reference points. Then, the spot volumes were calculated mathematically. Finally, scattering plots and expression profiles of each selected spots were detected.

2.2.6. In-Gel Digestion with Trypsin

Tryptic in-gel digestion procedure is a common technique mainly used for protein cleavage from excised SDS or 2D gels. Shevchenko and co-workers developed this technique as a sample preparation for analyzers (Shevchenko et al. 2006). In this study, the experiment was self-optimized and performed as a three-day procedure. In the second and third days, all chemical applications were carried out in a flow cabinet to provide sterile conditions and minimize the keratin contamination. In addition, silicon micro centrifuge tubes were used to prevent the adhesion of peptides to tube walls.

On the first day, the storage solution was removed and the pre-excised spots were treated with wash solution (Appendix B) overnight at room temperature which provides destaining of proteins that are found in gel.

On the second day, the wash solution was discarded and the spots were divided into small pieces in micro centrifuge tube with a micro pipette tip. Then, the steps stated below were followed:

- 100 μl acetonitrile was added onto gel pieces for dehydration and incubated at room temperature until the pieces had white opaque color.
- Acetonitrile was removed, 30 μl DTT solution (Appendix B) was added for reduction and gel pieces were incubated at room temperature for 30 minutes after swelling.
- DTT solution was removed and gel pieces were dehydrated with acetonitrile at room temperature to take out the excess DTT from samples.
- Acetonitrile was removed, iodoacetamide solution (Appendix B) was added and gel pieces were incubated at room temperature for 30 minutes in dark place.
- Iodoacetamide solution was removed and then acetonitrile was added to take out the excess iodoacetamide from gel pieces until the gel pieces become opaque.
- Gel pieces were rehydrated with 100 μl of 100 mM ammonium bicarbonate solution.
- The pieces were dehydrated with acetonitrile one more time and dried at ambient temperature in vacuum centrifuge few minutes.
- Finally, they were allowed to swell with 30 μl Trypsin solution (Appendix B) on ice for 10 minutes. Gel pieces were covered with 50 mM ammonium bicarbonate to keep the enzyme in aqueous environment. Tube caps were covered with Parafilm and stored in 37^{o}C incubator. Heater block was not preferred since the solutions vaporized as a result of temperature differences between top and bottom of the tube. Thus gel pieces were dried and enzymatic reaction was inhibited.

On the third day, applications were carried out on ice. 20 μl of 50 mM ammonium bicarbonate was added onto samples and mixed with vortex for 2-3 minutes. Then, they were kept on ice for a few minutes and the same procedure was applied. Tubes were centrifuged at maximum speed for 30 seconds and supernatant was

removed into new 0.5 ml micro centrifuge tube. 30 µl of extraction buffer (Appendix B) was added. In this protocol, extraction buffer takes out the peptides by the dehydrating character of acetonitrile.

In the presence of extraction buffer, vortex and centrifuge processes were repeated. Supernatant was collected in the tube containing previous supernatant. Extraction step was also repeated and all supernatants were collected in same tube. Extract including peptides was concentrated in DNA 120 SpeedVac® System, Thermo Electron Corporation via evaporation of supernatant under vacuum at ambient temperature until the total volume of each sample was 20 µl. Finally, 0.5 µl acetic acid was added to the each sample for acidification of peptides.

2.2.7. ZipTip Assay

ZipTip is a specific micro-column which may be C_4, C_{18} or strong cationic resin (SCX) replaced in the edge of 10 µl micro-pipette tips. This micro-column system is a useful technique that provides purification, desalting and concentration of protein / peptide samples.

In this study, ZipTip 0.6 µl C_{18} resin of Millipore were used with 10 µl micro-pipettes. Initially, ZipTip micro-columns were treated with 10 µl of wetting solution (Appendix B) by aspirating and dispensing for a few times. Same procedure was repeated with the equilibration solution (Appendix B). Then, 10 µl of peptide sample was aspirated and dispensed in sample tube at least 10 times. Removing salt ions and impurities were provided via aspirating washing solution (Appendix B) and dispensing to waste. This step was repeated for a few times. Peptide molecules were eluted from micro-column with 5-10 µl of elution solution (Appendix B) into 0.1 ml micro centrifuge tubes.

2.2.8. Sample Preparation for MALDI-TOF/TOF Mass Spectrometry

α-cyano-4-hydroxycinnamic acid (CHCA) matrix was applied as a two layer system. Initially first layer of matrix was prepared freshly (Appendix B) and spotted as

1 μl on gold plated aluminum target (MTP 384 massive target gold plated T: one-piece aluminum target with transponder technology, BRUKER, DALTONICS). 1st layer was spotted twice if the intensity of first layer was inadequate and spots were transparent. Then, the second layer was prepared freshly (Appendix B), mixed with equal volume of the sample and spotted as 1 μl onto the 1st layer spots. In case the matrix amount was inefficient, the samples were mixed with 2nd layer of matrix as (1:3), (1:4) or (1:5). With the same procedure, peptide mixture for MS calibration including leucine enkephalin, angiotensin I, angiotensin II, bradykinin, adrenocorticotropic hormone (ACTH) and insulin; also ACTH (18-39) peptide for MS/MS calibration were prepared. Spots on target were dried at room temperature for 15-20 minutes.

2.2.9. Mass Spectrometric Analyzes via MALDI-TOF/TOF

MALDI-TOF/TOF analyzes were performed by BRUKER autoflex III smartbeam with flexControl Version 3.0 program. Several parameters were set and fixed for all analyzes as in the following; for MS, mass range: 700-3500 Da, reflector voltage: 1725 V, laser frequency: 50.0, maximum shots: 2000 and laser power: 70-80 %. For MS/MS, CID mode: on, collision gas: Argon, mass range: 40-2020 Da, reflector voltage: 1544 V, laser frequency: 50.0, maximum shots: 5000, laser power: 70-80 % and PCIS window range was optimized based on parent ion. External calibrations were applied.

Peaks were analyzed with flexAnalysis Version 3.0 and ion types were selected as low energy CID ions (a,b,y). Database connection was provided by Biotools Version 3.1 and Mascot MS/MS Ion Search bioinformatic programs. Additionally, optimized parameters were set as default to identify the proteins by Mascot MS/MS Ion Search tool. These parameters were in the following as taxonomy: Viridiplantae (Green Plants), enzyme: Trypsin, variable modifications: Carbamidomethyl (C), mass tolerance: 50 ppm, MS/MS tolerance: 0.9 Da, charge state: +1, monoisotopic.

All peptide peaks were searched in both of NCBInr and SwissProt protein databases. Peptide recoveries, molecular weight of protein and pI values were considered instead of ion scores.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Effects of NaCl on Plant Growth

Freshly growth *Beta maritima* plants were exposed to toxic levels of NaCl (150 mM) for 10 days. During this salt stress period several phenotypic profile changes were observed as a conclusion of physiologic response.

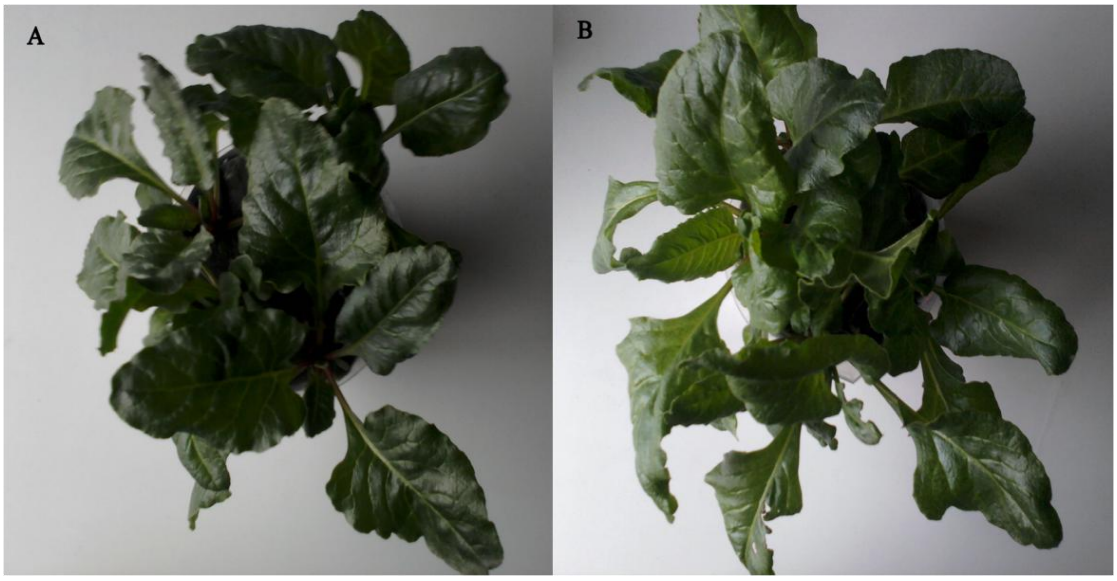

Figure 3.1. Comparisons of plant profiles after salt exposure. (A) Salt exposed "Stress group". (B) Non-exposed "Control group"

NaCl stress, reduced plant growth significantly. High concentrations of salt prevented the uptake of water and essential minerals from media efficiently. This situation is concluded as suppression of leaf and root growth. In stress group, leaves were less and smaller; roots were shorter and weaker than in control group. In addition, as it is shown in Figure 3.1 metabolic affects of salinity increased the thickness and fragility of leaves, decreased their elasticity. Pigments were over-produced; plants lost their bright green color and got darker colors.

3.2. Measurements of Protein Concentrations by Bradford Assay

Extracted protein samples from both leaf and root tissues were measured by a common spectrophotometric technique, Bradford assay. In the presence of Bradford reagent, absorbances of BSA standards were obtained in 595 nm and standard curve was plotted as absorbance vs. concentrations (Figure 3.2). Concentration of each extracted 1 g sample was calculated based on this standard graph (Table 3.1).

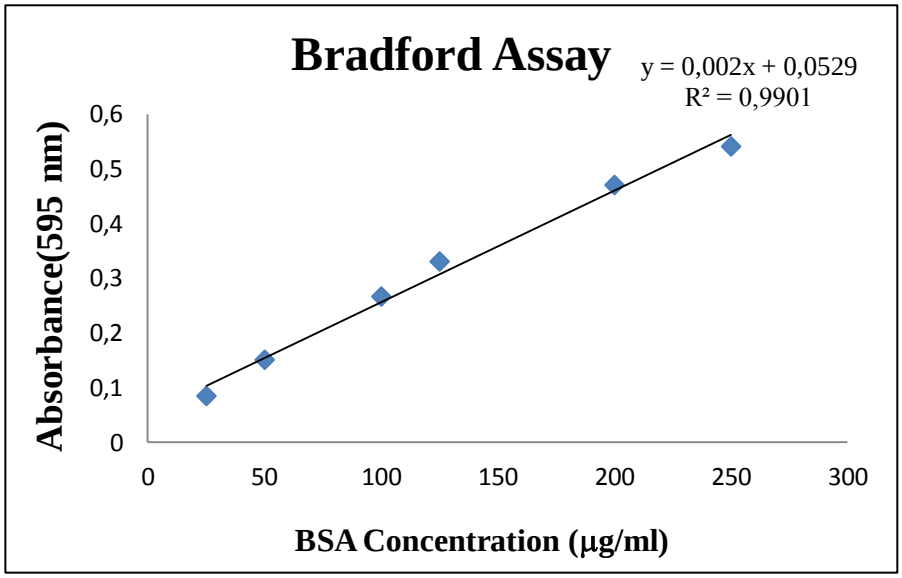

Figure 3.2. Standard Curve for BSA

Table 3.1. Average concentrations of extracted proteins from plant samples

Sample Name	Protein Concentration (mg/ml)	
	Stress Group	Control Group
Leaf (1 g)	14.7 ± 1	13.3 ± 1
Root (1 g)	6.9 ± 1	8.2 ± 1

3.3. Determination of Salt Stress Responsive Proteins

3.3.1. Separation of Proteins via 2D-PAGE

Identification of salt stress responsive proteins was the main strategy to make sense about the tolerance mechanism of a halophyte. After extraction of total proteins from leaf and root samples, 2D-PAGE was performed in order to separate these proteins specifically due to their pI values and molecular weights. This experimental step was independently repeated three times for each sample group.

In the first dimension of 2D-PAGE, isoelectric focusing (IEF) was performed. In each set, 400-450 μg protein was mixed with 2D-rehydration buffer, loaded to channels of isoelectric tray and focused for ~ 2 days including rehydration step. For IEF; non-linear (NL), 17 cm, pH 3-10 IPG strips were used. Leaf protein samples were focused one by one, whereas two groups of root protein samples could be focused at once. In this procedure, proteins were separated according to their pI values.

In the second dimension which consists of SDS-PAGE, pre-equilibrated proteins were run in 12 % resolving gel and separated according to their molecular weights. During this process, protein ladder could not be used. Therefore, some known proteins were accepted as molecular reference such as larger subunit of RuBisCO.

Spot detection was carried out for overnight by staining of gels with colloidal Coomassie Brilliant Blue G-250. Excess dye was destained with methanol after neutralization of proteins with slightly acidic buffer. Proteins were fixed in polyacrylamide gel for ~6 hours and gels were stored at 4 ^{o}C for 2-3 days until the color of background became brighter and spots obtained clearly. Gel images were obtained via epi-white illumination. Several spots were accepted as reference and differentially expressed (completely lost, completely appeared, increased or decreased) proteins were detected and excised from gel. Selected spots, as a result of control-stress comparisons, are demonstrated in Figure 3.3 and 3.4. Second and third sets of gel images are given in Appendix C.

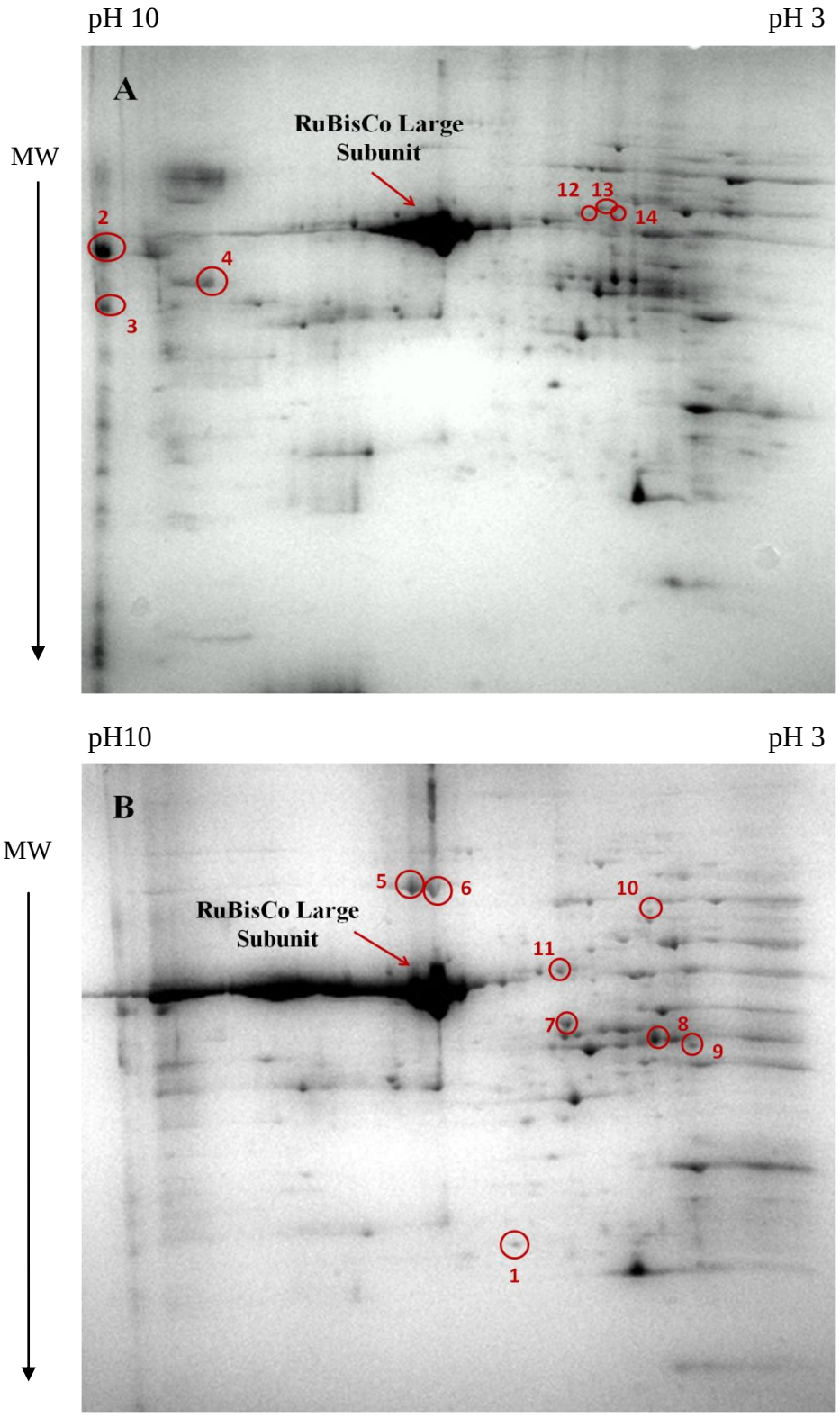

Figure 3.3. 2D gel images of leaf proteins and selected spots which are differentially expressed. (A) Control group (B) Salt stress group

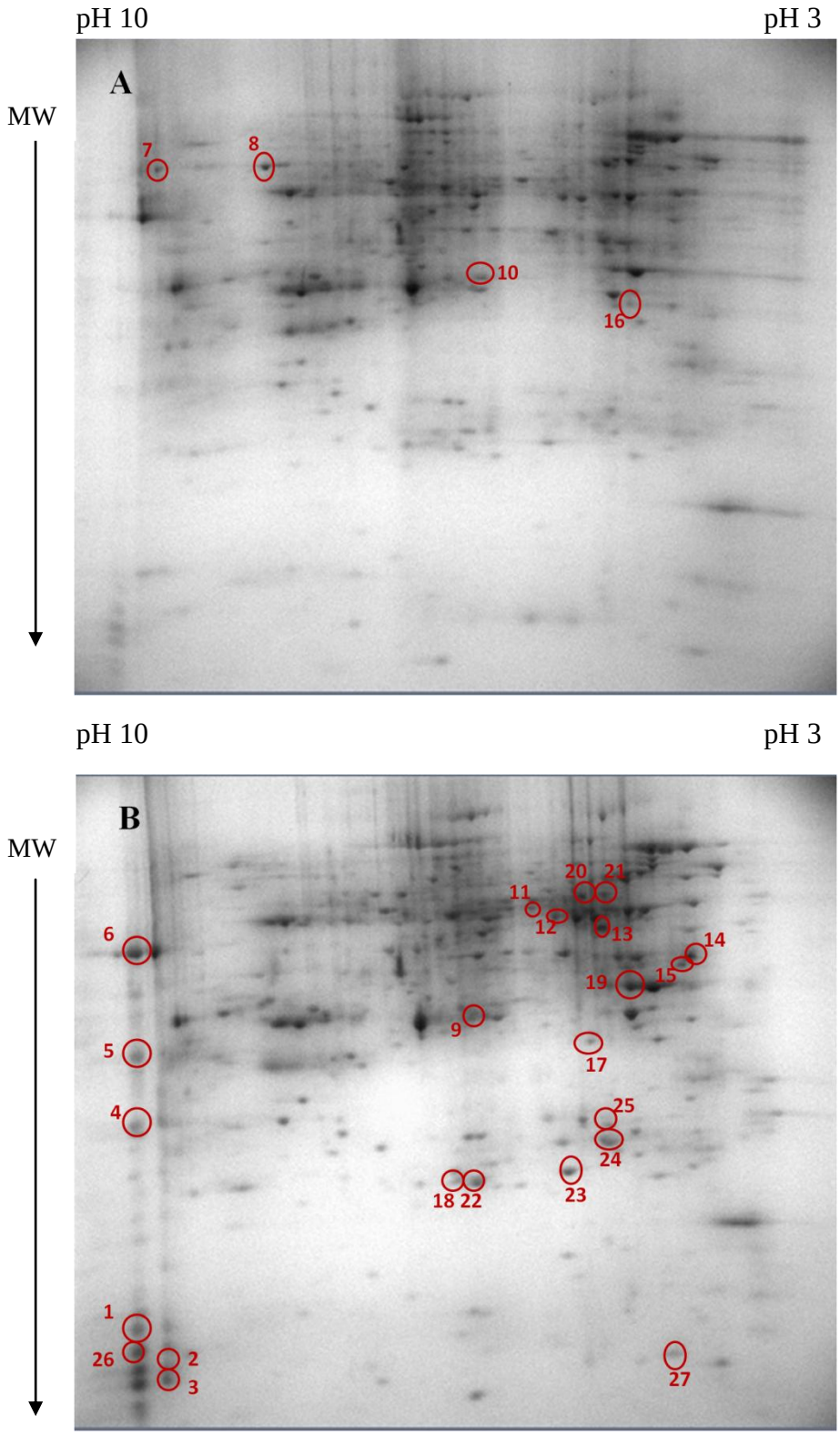

Figure 3.4. 2D gel images of root proteins and selected spots which are differentially expressed. (A) Control group (B) Salt stress group

3.3.2. Bioinformatic Confirmation of Spot Selections

In this study, gel comparisons and spot selections were performed without using a bioinformatic tool in order to eliminate background interference. Instead, protein spots were determined with naked eye and results were approved by a proteomic gel analysis program, Delta2D (DECODON). Gel images were uploaded to program independently. Each set including control and stress gel images were combined together and examined in the same frame. Significantly matching spots were selected by program automatically and gels were warped based on these reference points. Warping feature of this bioinformatic program provide to discriminate the unmatched spots via indicating each gel with different color. Thus, each gel images and matched spots had individual color that facilitates the selection of lost or appeared spots mostly, referring to down-regulated or up-regulated proteins. In this case, pre-selected spots were compared with results of the program and incorrect selections were eliminated. Expression levels of selected proteins were found out via calculation of spot volumes mathematically on warped images. Figure 3.5 demonstrates the scattering plots for leaf and root samples indicating the relative volumes belong to control and stress groups.

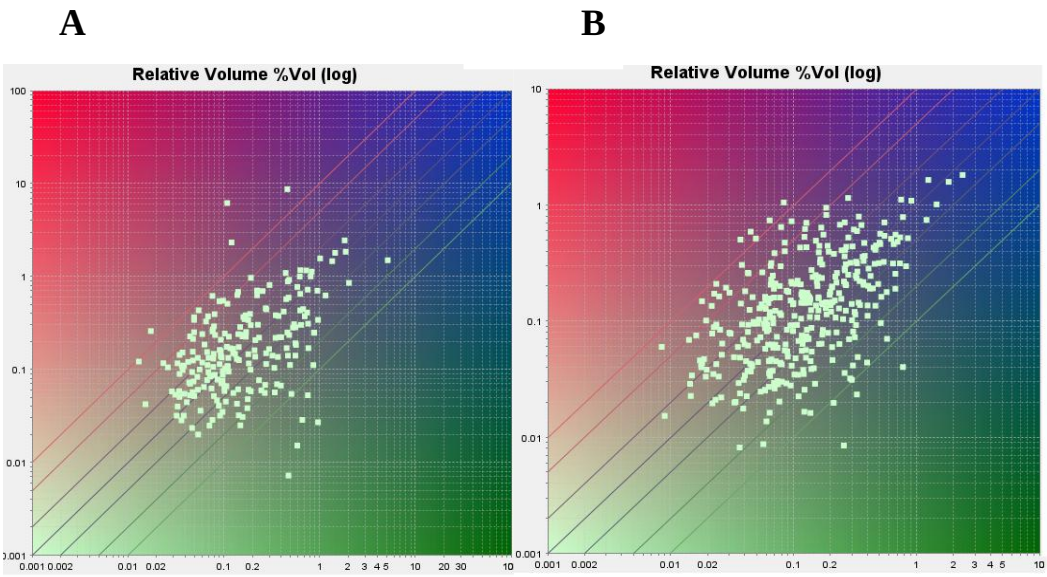

Figure 3.5. Scattering plots of leaf (A) and root (B) protein spots on 2D gel images. Pink area (y axis) refers to stress group proteins; green area (x axis) refers to control group proteins and blue area refers to matching proteins.

Expression profiles due to the changes in relative volumes of spots were also calculated by the program. In this case, statistical evaluations of these changes confirmed our results directly (Figure 3.6). Other data sets are given in Appendix C.

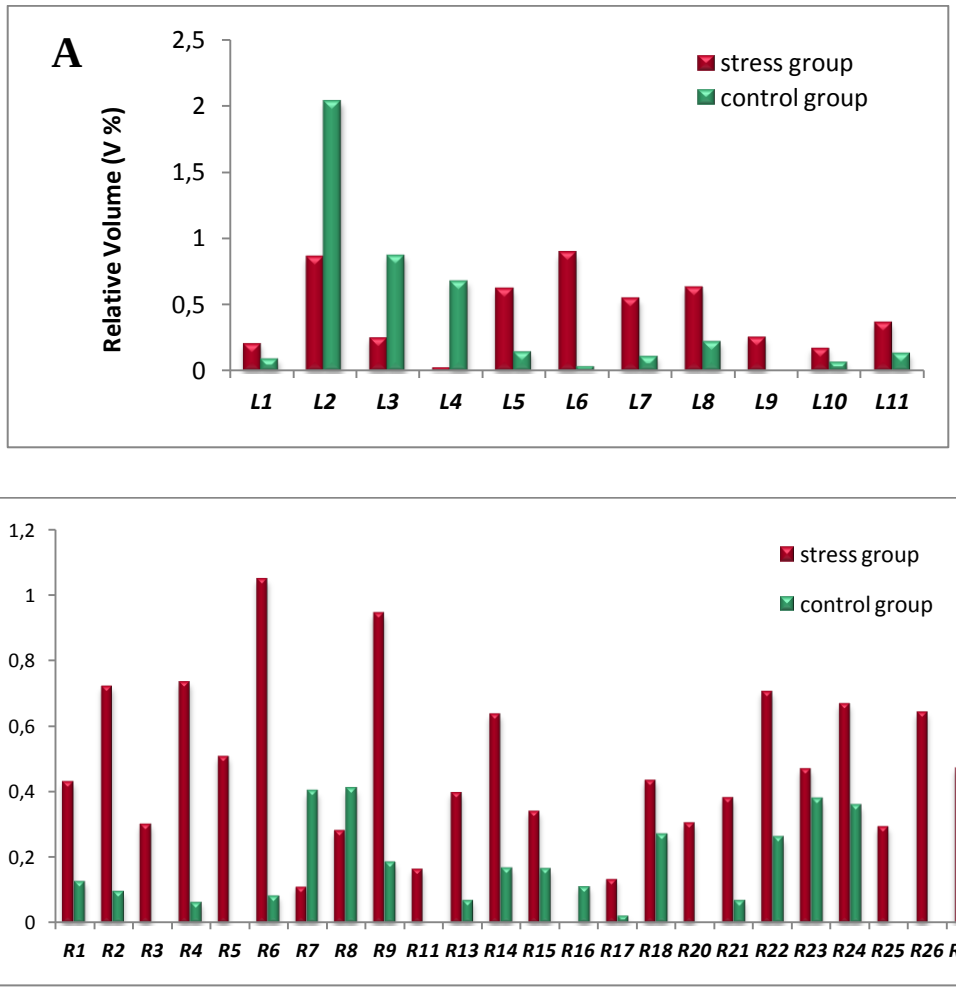

Figure 3.6. Graphical demonstration of calculated relative spot volumes indicating expression profiles. (A) Leaf samples. (B) Root samples.

3.4. Identification of Salt Stress Response Proteins by MALDI-TOF/TOF Mass Spectrometry

Preparation of samples for mass spectrometric analysis was followed with in gel digestion procedure after selection and excision of spots. Protein samples were reduced, alkylated and degraded into peptides by treating with trypsin in gel for an overnight. Extracted peptides were analyzed in MALDI-TOF/TOF and therefore, proteins were identified by searching the related peptides from proteomic databases, NCBInr and

SwissProt. In each analysis, molecular weights and pI values of candidates were considered as well as peptide recovery. All of the leaf and root samples were analyzed and only six leaf proteins could be identified.

The summary of the identified proteins were given in Table 3.2.

Table 3.2. Proteins identified by MALDI-TOF/TOF mass spectrometry

Spot No	**Protein Name**	**Nominal Mass (Da)**	**pI value**	**Protein Sequence Coverage (%)**	**Expression Profile**
L1	Thioredoxin H-type	11836	5.84	16	upregulated
MGGSVIVIDSKAAWDAQLAKGK**EEHKPIVVDFTATWCGPCK**MIAPLF ETLSNDYAGKVIFLKVDVDAVAAVAEAAGITAMPTFHVYKDGVKADD LVGASQDKLKALVAKHAAA					
L2	Elongation factor 1-alpha 1	49471	9.19	5	downregulated
MGKEKFHINIVVIGHVDSGKSTTTGHLIYKLGGIDKRVIERFEKEAAEM NKRSFKYAWVLDKLKAERERGITIDIALWKFETTKYYCTVIDAPGHRDF IKNMITGTSQADCAVLIIDSTTGGFEAGISKDGQTR**EHALLAFTLGVK**Q MICCCNKMDATTPKYSKARYDEIIKEVSSYLKKVGYNPDKIPFVPISGFE GDNMIERSTNLDWYKGPTLLEALDQINEPKRPSDKPLRLPLQDVYK**IGG IGTVPVGR**VETGMIKPGMVVTFAPTGLTTEVKSVEMHHESLLEALPGD NVGFNVKNVAVKDLKRGYVASNSKDDPAKGAANFTSQVIIMNHPGQI GNGYAPVLDCHTSHIAVKFSEILTKIDRRSGKEIEKEPKFLKNGDAGMV KMTPTKPMVVETFSEYPPLGRFAVRDMRQTVAVGVIKSVDKKDPTGA KVTKAAVKKGAK					

(cont. on next page)

Table 3.2. (cont.)

L3	Peroxisomal (S)-2-hydroxy-acid oxidase	40260	9.16	8	downregulated
MEITNVNEYEAIAKQKLPKMVYDYYASGAEDQWTLAENRNAFSRILFR PRILIDVTNIDMTTTILGFKISMPIMIAPTAMQKMAHPEGEYATARAASA AGTIMTLSSWATSSVEEVASTGPGIRFFQLYVYKDRNVVAQLVRRAER AGFKAIALTVDTPRLGRREADIKNRFVLPPFLTLKNFEGIDLGKMDKAN DSGLSSYVAGQIDRSLSWKDVAWLQTITSLPILVKGVITAEDAR**LAVQ HGAAGIIVSNHGAR**QLDYVPATIMALEEVVKAAQGR**IPVFLDGGVR**R GTDVFKALALGAAGVFIGRPVVFSLAAEGEAGVKKVLQMMRDEFELT MALSGCRSLKEISRSHIAADWDGPSSRAVARL					
L4	Serine--glyoxylate amino transferase	44061	7.63	10	downregulated
MDYMYGPGRHHLFVPGPVNIPEPVIRAMNR**NNEDYRSPAIPALTKTLL EDVKK**IFKTTSGTPFLFPTTGTGAWESALTNTLSPGDRIVSFLIGQFSLL WIDQQKRLNFNVDVVESDWGQGANLQVLASKLSQDENHTIKAICIVHN ETATGVTNDISAVRTLLDHYKHPALLLVDGVSSICALDFRMDEWGVDV ALTGSQKALSLPTGLGIVCASPKALEATKTSKSLKVFFDWNDYLKFYK LGTYWPYTPSIQLLYGLR**AALDLIFEEGLENIIAR**HARLGKATRLAVE AWGLKNCTQKEEWISNTVTAVMVPPHIDGSEIVRRAWQRYNLSLGLG LNKVAGKVFRIGHLGNVNELQLLGCLAGVEMILKDVGYPVVMGSGVA AASTYLQHHIPLIPSRI					

(cont. on next page)

Table 3.2. (cont.)

L5	5-methyl tetrahydro Pteroyltriglutamat —homocysteine methyltransferase	84304	6.09	5	upregulated

MASHIVGYPRMGPKRELKFALESFWDGKSTAEDLKKVSADLRSSIWK
QMADAGIKYIPSNTFSYYDQVLDTTAMLGAVPPRYGWTGGEIEFDVY
FSMARGNASVPAMEMTKWFDTNYHFIVPELGPEVNFSYASHKAVLEY
KEAKALGVDTVPVLVGPVSYLLLSKQAKGVDKSFDLLSLLPKILPIYKE
VVAELKEAGASWIQFDEPLLVMDLESHKLQAFSAAYADLESTLSGLN
VVVETYFADVTAEAYKTLISLKGVTGYGFDLVRGTKTLDLVKAEFPSG
K**YLFAGVVDGR**NIWANDLAASLATLEALEGVVGKDKLVVSTSCSFLH
TAVDLINETKLDDEIKSWLAFAAQKVLEVNALAKALSGQKDEAFFSA
NAAALASRKSSPRVTNEAVQKAATALKGSDHRRATTVSSRLDAQQKK
LNLPILPTTTIGSFPQTVELRRVRREYKAKKISEEEYVKAIKEEISKVVKL
QEELDIDVLVHGEPERNDMVEYFGEQLSGFAFSANGWVQSYGSRCVK
PPIIYGDVSRPNPMTVFWSSMAQSMTARPMKGMLTGPVTILNWSFVR
NDQPRHETCYQIALAIKNEVEDLEK**AGINVIQIDEAALR**EGLPLRKSEH
DFYLKWAVHSFRITNVGVQDTTQIHTHMCYSNFNDIIHSIIDMDADVIT
IENSRSDEKLLSVFREGVK**YGAGIGPGVYDIHSPR**IPPTEELADRIRKM
LAVLESNVLWVNPDCGLKTRKYGEVNPALSNMVAAAKQLRQELASA
K

(cont. on next page)

Table 3.2. (cont.)

L8	glutamine synthetase	47401	5.73	9	upregulated

MAQILAPNMQCQMKLSKSLTNSMIPNSWTSILLKGSQKGSIKCSTKFK
VCAALKTEHGTVNRMEQLLNLDVTPFTDK**IIAEYIWIGGSGIDLR**SKS
R**TLSRPVEDPSELPK**WNYDGSSTGQAPGEDSEVILYPQAIFKDPFRGG
NNILVICDAYTPAGEPIPTNKRHKAAEIFSNPKVASEVPWFGIEQEYTLL
QPNVQWPLGWPVGAYPGPQGPYYCGVGADKSFGRDISDAHYKACLY
AGINISGTNGEVMPGQWEFQVGPSVGIEAGDHIWCARYLLERITEQAG
VVLTLDPKPIEGDWNGAGCHTNYSTKTMREDGGFEVIKKAILNLSLRH
KEHISAYGEGNERRLTGK**HETADIDTFSWGVANR**GCSIRVGRDTEKE
GKGYMEDRRPASNMDPYVVTGLLAESTLLWEPTLEAEALAAQRLSLN
V

CHAPTER 4

CONCLUSION

In this study comprehension of the natural salt tolerance mechanism of the moderately halophytic plant *Beta maritima* (sea beet) on proteomic level was aimed. In this respect, up-regulated and down-regulated proteins were determined on two-dimensional polyacrylamide gel and analyzed by MALDI-TOF/TOF mass spectrometry after in gel tryptic digestion.

Results of the mass spectrometric analyses demonstrated that most of the protein samples were digested by trypsin well and peptides were collected efficiently. Hence the peptide peaks were quite intense referring to high signal to noise ratio in the mass spectrum. For the MS/MS analysis each peptide peak was recorded, selected as a parent ion and dissociated by collision induction in the presence of the inert gas argon. According to MS/MS results it was observed that the fragmentation was homolytic and peaks were intense. However the bioinformatic research was not succeeded as well as mass spectrometric analyses, since *Beta maritima* has not a specific proteomic database. Only some metabolic pathway enzymes, some redox proteins and a transcription regulator, elongation factor-1-α were identified from leaf samples which are similar to leaf proteins of the other plant species. On the other hand, *Beta maritima* has a characteristic root structure including specific proteins which are not similar to the ones found in other species. Therefore solution of the puzzle and identification of the differentially expressed root proteins may require *de novo* sequencing. By this way, the unknown part of the salt tolerance mechanism may be found out.

Finally, this study may lead to produce novel agriculturally important salt tolerant plants in the future. Moreover, if the tolerance mechanism includes vacuolar accumulation, this mechanism may be used for the remediation of the environment.

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APPENDIX A

LIST OF CHEMICALS

- $Ca(NO_3)_2.4H_2O$, $MgSO_4.7H_2O$, H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $NaMoO_4$, EDTA.2Na, $FeSO_4.7H_2O$, KOH, Tris, sucrose, KCl, PMSF, phenol, ammonium acetate , urea, thiourea , CHAPS , DTT, CBB-G250, 85 % phosphoric acid , ethanol, SDS, glycerol anhydrous, acrylamide, bisacrylamide, ammonium sulfate, glycine, agarose, Tris-dihydrogen phosphate, acetic acid and ammonium bicarbonate (**AppliChem)**
- KNO_3, methanol, BSA, iodoacetamide, ammonium persulfate, TEMED and trypsin proteomics grade **(Sigma Aldrich)**
- KH_2PO_4, HCl, acetone, Bromophenol blue, acetonitrile and TFA (**Merck)**
- Acetic acid and formic acid **(Riedel-de Haën)**
- DTT **(Fluka)**
- pH 3-10 Carrier Ampholyte **(Biochemika)**
- CHCA **(Bruker Daltonics)**

APPENDIX B

CHEMICAL CONTENTS OF USED BUFFERS / SOLUTIONS

Half-strength Hoagland: 3.5 mM $Ca(NO_3)_2.4H_2O$, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 22 µM H_3BO_3, 4.5 µM $MnCl_2.4H_2O$, 0.35 µM $ZnSO_4.7H_2O$, 0.2 µM $CuSO_4.5H_2O$, 0.07 µM $NaMoO_4$, 15 µM EDTA.2Na, 14 µM $FeSO_4.7H_2O$ and 0.5 mM KOH are prepared in dH_2O.

Protein Extraction Buffer: 500 mM Tris/HCl pH 8, 50 mM EDTA, 700 mM sucrose, 100 mM KCl are prepared in water; just before usage 2 % (v/v) β-mercaptoethanol reducing agent and 1mM PMSF protease inhibitor are added.

Tris buffered phenol: Phenol is mixed with equal volume of 10X TE and after the phases are separated well upper phase (TE) is discarded. This process is repeated one more time with 10X TE and then two times with equal volume of 1X TE. Finally, pH of the last discarded 1X TE should be ~8.

10X TE Buffer: 50 ml from 1M Tris (pH 8), 10 ml 0.5 M EDTA is mixed and the total volume is completed to 500 ml with dH_2O.

Protein Precipitation Reagent: 100 mM ammonium acetate is dissolved in ice cold methanol.

2D-Rehydration Buffer: 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS zwitterionic detergent mixture is used for dissolving of proteins. IEF requires 65 mM DTT and 8 % (v/v) pH 3-10 carrier ampholyte prepared with this buffer.

5X Red Colored Bradford Stock Reagent: 0.01 g CBB G-250 is dissolved in 5 ml ethanol, 10 ml 85 % phosphoric acid is added and final volume is completed to 25 ml with UP water. Reagent is filtered with Whatman paper and stored at 4^oC in dark.

Equilibration Buffer I: 6 M urea, 375 mM Tris/HCl pH 8.8, 2 % (w/v) SDS, 20 % (v/v) glycerol and 2 % (w/v) DTT

Equilibration Buffer II: 6 M urea, 375 mM Tris/HCl pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol and 2.5 % (w/v) iodoacetamide

12 % Polyacrylamide Gel: 13.5 ml of dH_2O, 16 ml of 30 % (w/v) acrylamide mix, 10 ml of 1.5 M Tris (pH 8.8), 0.4 ml of 10 % (w/v) SDS, 0.4 ml of 10 % (w/v)

ammonium persulfate, 0.016 ml of TEMED are mixed respectively. Total volume is 40 ml.

30 % (w/v) acrylamide mix: 29 % (w/v) acrylamide and 1 % (w/v) bisacrylamide are dissolved in dH_2O.

1X Running Buffer: 250 mM glycine, 25 mM Tris, 0.1 % (w/v) SDS

Overlay Agarose Gel: 90 mM Tris, 190 mM glycine, 0.1 % (w/v) SDS, 0.5 % (w/v) agarose and 0.002 % (w/v) Bromophenol blue indicator pH 3.0-4.6 are prepared in water. Mixture is heated until the agarose is dissolved completely. Then, solution is aliquoted into 1.5 ml micro centrifuge tubes and stored in -20^{o}C.

Polyacrylamide Gel Staining Solution: 8 % (w/v) ammonium sulfate, 1.6 % (v/v) concentrated phosphoric acid, 0.1 % (w/v) colloidal Coomassie Brilliant Blue G-250, 20 % (v/v) methanol. CBB G-250 is pre-resolved in ~10 ml water and mixed with the staining solution. Methanol is added finally.

Neutralization Buffer: 0.1 M Tris-Phosphate pH 6.5

Destaining Solution: 25 % methanol solution

Fixation Solution: 20 % Ammonium sulfate solution

Wash Solution for in Gel Digestion: 50 % (v/v) methanol and 5 % (v/v) acetic acid in UP water.

10 mM DTT for in Gel Digestion: 1.5 mg DTT is dissolved in 1 ml of 100 mM ammonium bicarbonate solution.

100 mM Iodoacetamide for in Gel Digestion: 18 mg iodoacetamide is dissolved in 1 ml of 100 mM ammonium bicarbonate solution and stored in dark.

Trypsin solution: 100 µl of ice cold 50 mM ammonium bicarbonate is added onto 20 µg of sequencing-grade modified trypsin. Final concentration is 200 ng/ µl. Then, 5 µl of trypsin aliquots are stored in -80^{o}C. Before used, ~70 µl of freshly prepared ice cold 50 mM ammonium bicarbonate solution is added and volume is adjusted due to the spot size. Trypsin solution should cover the spot completely.

Extraction Buffer for in Gel Digestion: 50 % (v/v) acetonitrile and 5 % (v/v) formic acid are prepared in UP water.

ZipTip Wetting Solution: Acetonitrile

ZipTip Equilibration Solution: 0.1 % (v/v) Trifluoro Acetic acid (TFA)

ZipTip Washing Solution: 0.1 % (v/v) TFA

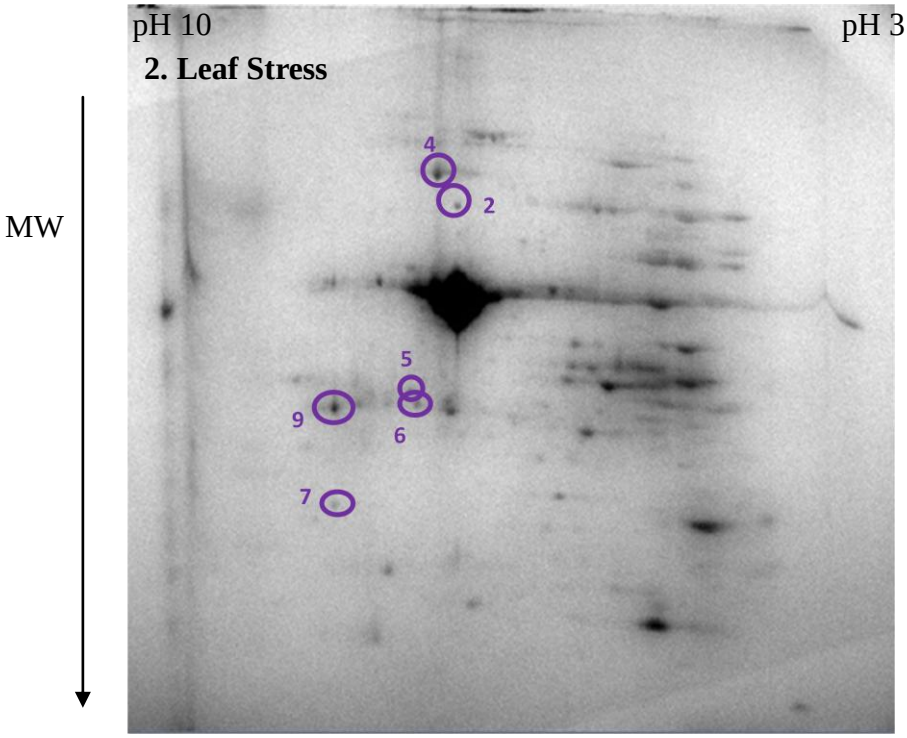
ZipTip Elution Solution: 50 % (v/v) acetonitrile is prepared in 0.1 % (v/v) TFA.

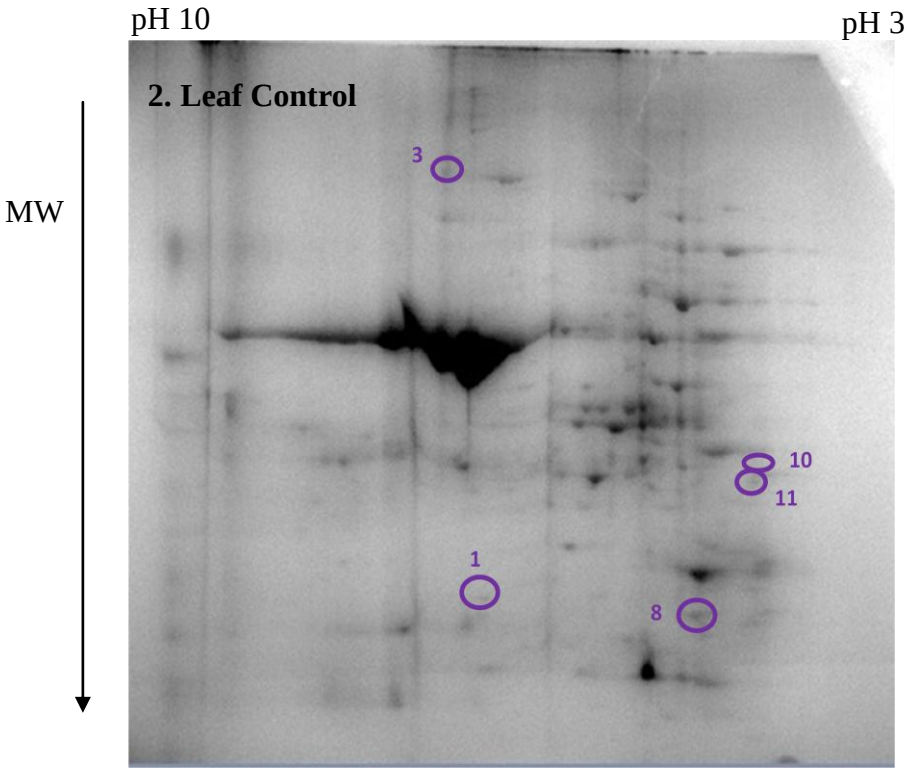
1st Layer of Matrix in MALDI: 3-6 mg of CHCA is dissolved in 100 µl of methanol and 400 µl of acetone is added.

2nd Layer of Matrix in MALDI: 5 mg CHCA is dissolved in 200 µl methanol. 300 µl of 0.1 % (v/v) trifluoro acetic acid (TFA) is added. As a result of aqueous media, CHCA crystal formation is observed. Matrix solution is centrifuged at maximum speed for 10 minutes. Supernatant is used as 2nd layer.

APPENDIX C

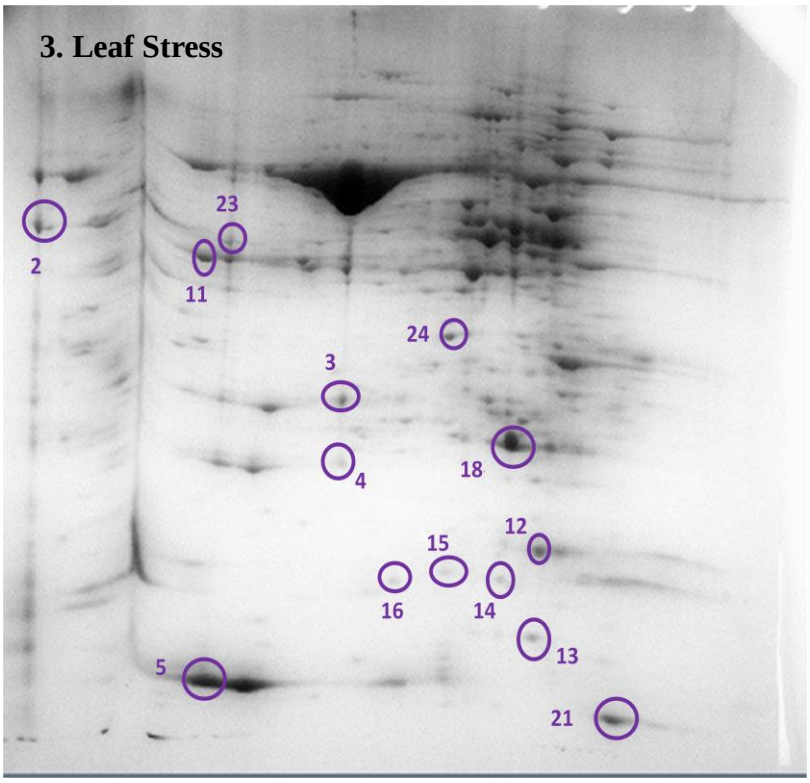
SECOND AND THIRD SETS OF 2D-PAGE AND BIOINFORMATIC RESULTS





pH 10 pH 3

MW



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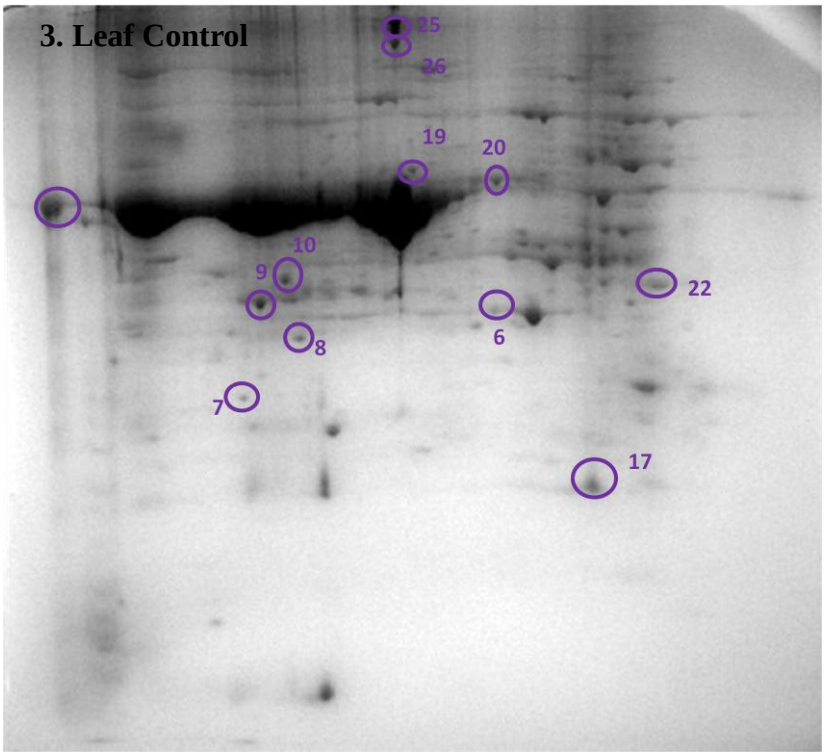
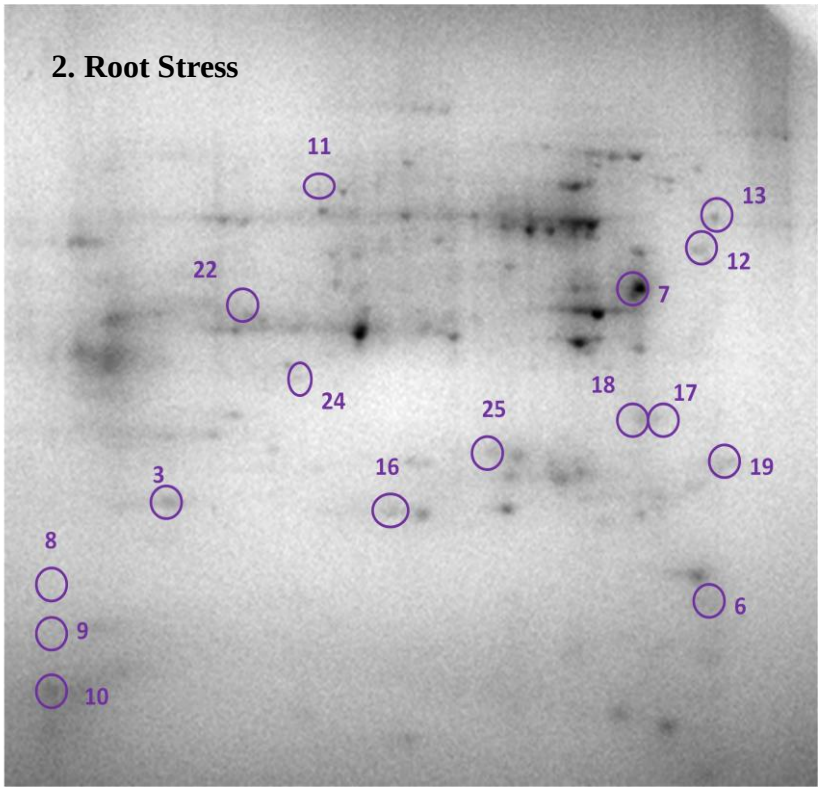
MW



pH 10 pH 3

MW



pH 10 pH 3

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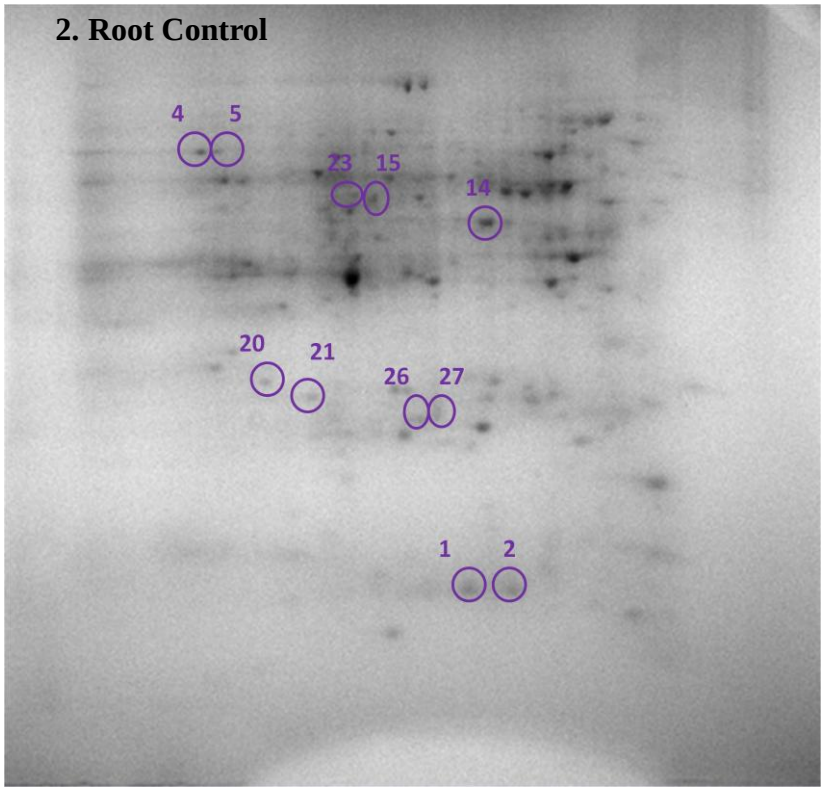

pH 10 pH 3

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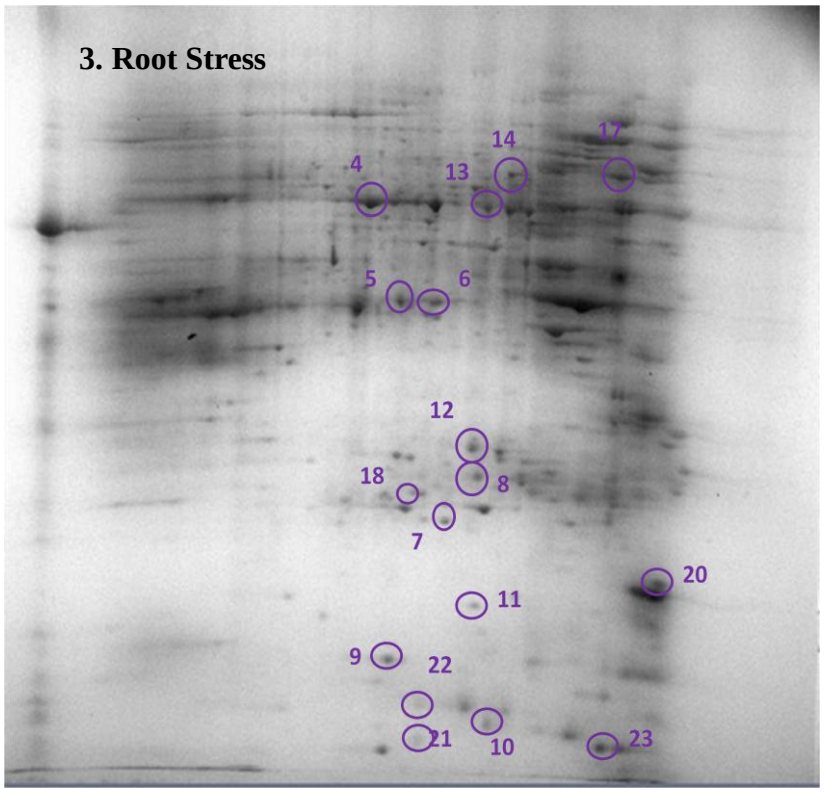
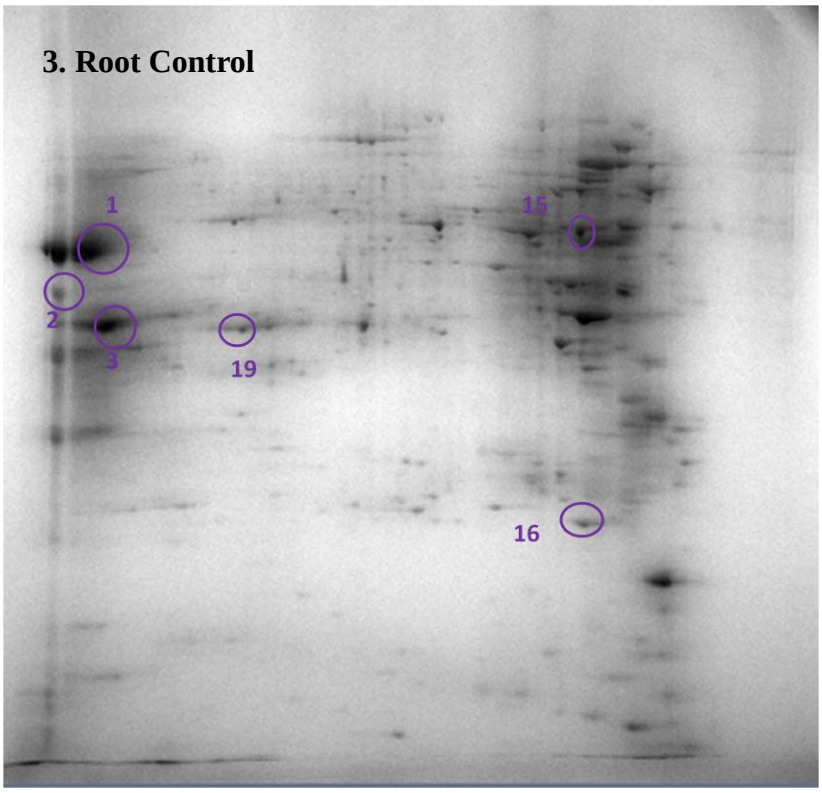


pH 10 pH 3

MW



2nd and 3rd Data Sets of Expression Profiles