Proteomic analysis of *Vigna radiata* (L.) R. Wilczek leaves under salinity stress

Mahendran VS[1,2], M Prasanna Rajesh Kumar[1,3] , V Balakrishnan[4]
[1] *Research Scholar, R & D Centre, Bharathiar University, Coimbatore-641046, Tamil Nadu, India*
[2]*Assistant Professor, Sri Ramakrishna Engineering College, Coimbatore,*
[3]*Assistant Professor, KSR College of Arts of Science, Tiruchengode*
[4]*Associate Professor, School of Biotechnology,KSR College of Technology, Tiruchengode*

Abstract

Proteomic analyses of stress response and tolerance helps in understanding of physiological mechanisms behind and importantly identification of candidate genes for transgenics that put up with stress and increased crop productivity. In the current investigation, the effect of salinity on *Vigna radiata*(L) R. Wilczek in its vegetative stage was studied. The seeds were sown in red, clayey soil in pots and watered with different concentrations of NaCl viz., 0(Control), 50, 100,150, 200mM. Proteome analysis was done with the leaf tissues of 45 DAS plants that included protein extraction, assay of total proteins, 2 Dimensional gel electrophoresis, spotting of proteins common to control, treated, and differentially expressed proteins. Selected spots were subjected to Peptide Mass Finger printing and MASCOT search. The analysis revealed the presence of proteins, protein subunits similar to 50S ribosomal protein L17, Aspartate carbamyl transferase.

1. Introduction

Cultivated crop plants are often subjected to abiotic stress such as heat,drought, salinity, cold and freeze, water logging. They undergo dramatic metabolic changes and induce defense mechanisms to respond and tolerate the stress. Stress responsive proteins are expressed in response to wide range of stress conditions, either as responsive proteins or stress tolerating proteins, that regulate, coordinate various cellular and metabolic activities during stress and stress response. (Zhang et al., 2012)

Alkalinity is a major abiotic stress and the problems are low osmotic potential of the soil solution, nutritional imbalance, generation of reactive oxygen species and plants tolerate by adaptive mechanisms operating at all levels of organization embracing subcellular, cellular, tissue and systemic level. The mechanisms, for example, include (i) accumulation of intracellular osmolytes and reducing water loss (ii)i ncreasing antiporter and co-porter ion channel activity across the plasma and vacuolar membranes (iii) Development of enzymic and non enzymic antioxidant systems that scavenge free radicals (iv) complex signaling networks and metabolic networks such as protein kinase pathways.(Chattopadhyay et al., 2011 and Munns et al., 2008)

Development of improved levels of tolerance to these stresses is an urgent priority for crop breeding programmes. Generation of stress tolerant crops requires identification of proteins and genes involved in regulatory pathways such that they can be used as candidates for transgenesis.

The current investigation focuses on identification of proteins that may be involved in stress response or tolerance through conventional proteomic technologies.

2. Materials and Methods

Seeds and Salinity treatment

Seeds of Vigna radiata were obtained from Tamil Nadu Agricultural University. Seeds were sterilized by 0.01 M HgCl2 solution for 3minutes, washed thoroughly with distilled water. The pots were filled with red soil and divided into five groups of three pots each. The pots of first group were used as control, irrigated with tap water. The remaining groups were irrigated with aqueous solutions of 50mM, 100mM, 150mM and 200mM NaCl respectively. All the pots were irrigated once in three days to maintain soil moisture. After 7 days of sowing the seeds, thinning was done to leave five uniform seedlings in each pot for experimentation.

Leaves of 45 DAS plants were used for experimentation.

Protein Extraction

The leaf proteome of control and treated leaves was isolated following the method of phenol ammonium acetate with minor modifications. Briefly, 1 g of leaves was powdered in liquid N2 with mortar and pestle and suspended in 0.5 mL of extraction buffer (700 mM sucrose, 500 mM Tris–HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% w/v β-mercaptoethanol and 1mM PMSF) by vortexing on ice. An equal volume of phenol saturated-500 mM Tris–HCl, pH 7.5 was added and the mixture was stirred for 30 min on ice and then centrifuged at 5000 g for 30 min at 4 °C. The upper phenol phase was removed and extracted twice with the extraction buffer. Proteins were precipitated from the phenol phase by addition of five volumes of 0.1 M ammonium acetate in methanol, overnight at −20 °C. Precipitated proteins were pelleted by centrifugation (20,000 g, 20min, 4 °C) and washed thrice with ice-cold 0.1 M ammonium acetate in methanol and twice with ice-cold acetone. The resultant pellet was air dried under laminar air flow and solubilized in rehydration or sample loading buffer.

Sample Preparation for 1D

Sample were solubilised in sample loading buffer (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.1% bromphenol blue, pH 6.8 and 2%V/V Beta mercaptoethanol)) for 30mins by vortexing and centrifuged at 10000xg 4^0C temperature to remove insoluble debris and supernatant was collected for SDS PAGE and maintained at 4^0C.

SDS PAGE

SDS Poly Acrylamide Gel Electrophoresis was carried out using a model vertical slab gel (7x 8cm) electrophoresis apparatus (Protean Mini-PROTEAN 3 cell Biorad USA). SDS-polyacrylamide denaturing gels (separating gels (12%) and stacking gels (6%) were prepared and extracted protein with 2X sample buffer (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.1% bromphenol blue, pH 6.8 and 2%V/V Beta mercaptoethanol) loaded to the gel after boiling at 95^0C for 3 Mins. Blank wells were loaded with 1X sample buffer. Molecular Weight Marker (BioRad containing MW- Myocin:200kd, Beta Galactosidase:116.3kd,:Phosporylase b:97kd, bovine albumin: 66kd,Ovalbumin:45kd Carbonic anhydrase:31kd Trypsin Inhibitor:21.5kd Beta Lactalbumin 14.4 ,Aprotinin 6kd) was applied to gels in the experiment. Constant voltage (8 V/cm) was applied to stacking gel. After the tracing dye attained the separating gel, the voltage was adjusted to 15 V/cm. After electrophoresis, gels were stained by silver staining protocols.. Stained gels were captured and annoted on Versa Doc 4000 (BioRad).

Protein quantification

Protein concentration in the supernatant was quantitated using the Bio-Rad Quick Start kit (Bio-Rad, Hercules, CA), based on the Bradford technique. A standard curve was generated using the Serum Albumin Standard Set (Bio-Rad). Equal quantities of protein (200 µg) from each lysate were taken for extraction and clean up.

Protein Extraction for 2D electrophoresis

200 µg of in rehydration buffer sample was diluted with µl of 100 %TCA in water to yield final 25 % concentration. The mixture was incubated overnight at -20^0C and centrifuged at 15 000g, 4^0C for 15 min. The supernatant was removed and 1000 µl of 90 % ice-cold acetone were added to wash the pellet. The sample was incubated at -20^0C for 30 min and centrifuged as above. The acetone containing supernatant was removed and the pellet was air dried. For 2D gel electrophoresis, the protein pellet was suspended in 100 µl of rehydration buffer containing 7 M urea , 2M thiourea,60 mM DTT, 4% w/v CHAPS ampholyte (3-10)and traces of bromophenol blue .The cleaned protein sample was stored frozen at -20^0C until analysis.

2D electrophoresis

Cleaned Protein sample containing 200 µg of protein was diluted to 300 µl rehydration buffer(7M urea,2M thiourea,2% CHAPS,65 mM DTT,0.5% IPG buffer(3-10 pH) and traces bromophenol blue) and passively rehydrated on 17cm (3-10pH) strip in rehydration tray and overlayed with 3ml of mineral oil to avoid evaporation and crystallization of urea,rehydartion lasted for 16 hrs. After rehydration strips were transferred to focusing tray with electrode wicks and Isoelectric focusing was performed in a PROTEAN® IEF Cell (Bio-Rad) at optimum,maximum strength of 600 V/cm and a 50mA limit/IPG strip. The strips were focused at 10,000 V for 2 h 30 min with linear voltage amplification and finally to 60,000 Vh with rapid amplification.

For second dimension SDS-PAGE, the focused IPG strips were equilibrated in two steps, first in equilibration buffer I containing 20% v/v glycerol, 0.375 MTris–HCl, pH 8.8, 6 MUrea,2% (w/v) SDS, 130 mM DTT followed by a second equilibration step in buffer containing 20% (v/v) glycerol, 0.375 M Tris–HCl, pH 8.8, 6 M Urea, 2% (w/v) SDS, 135 mM

iodoacetamide. IPG strips were placed at he top of a 12% SDS-PAGE gel and overlaid with 0.5% agarose in running buffer (50 mM Tris-HCl, pH 6.8, 1.44% lycine and 0.1% SDS). The second dimension was performed using 12% polyacrylamide gels (7 cm×8 cm, 1.5mm thickness) in a Mini Protean 3 (Bio-Rad) at constant 200 V for 4 h 30 min. Thereafter, the gels were visualized by silver staining.

Protein Visualization and Imaging

Silver staining: MALDI compatible silver staining was performed by method described by Yan et al .Briefly the gels were immersed in fixative solution (methanol/distilled water/acetic acid, 40/50/10) for one hour. The gels were sensitized by exposure to thiosulfate reagent (0.02% Sodium thiosulfate), followed by impregnation with silver nitrate reagent (0.2% silver nitrate and 0.02% of 37 % formaldehyde) for 30 minutes and developed in developing solution (3% sodium carbonate, 0.05% formaldehyde (37%), 0.0005% sodium thiosulfate). The staining reaction was stopped by using 12% acetic acid solution (for 5 minutes) and gels were preserved in same solution.

Silver Stained gels were digitalized using VersaDoc™ (Model 4000) ImagingSystem (Bio-Rad) and analyzed with PDQuest Advanced™ 2-DAnalysis software (version 8.0.1, Bio-Rad).

Mass spectrometry. MS was performed on selected protein spots using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and MALDI-TOF/TOF.

A) In-gel digestion and protein digest preparation: Protein spots were excised manually with precaution for avoidance for Keratin contamination.Gel spots were diced into small pieces. Gel pieces were destained dehydrated with acetonitrile and then rehydrated with 50 mM ammonium bicarbonate. Proteins in the gel pieces were reduced and alkylated with 25 mM dithiothreitol (30 min at 56 °C) and 55 mM iodoacetamide (45 min in the dark at room temperature), respectively. Digestion was performed with 500 ng of trypsin in 50 mM ammonium bicarbonate buffer overnight. Extraction of peptides was performed using 0.1 % trifloro acetic acid in water (rehydration), followed by dehydration with 100% acetonotrile. For each extraction step, the solution was aspirated, collected, and collated. Three extraction cycles (dehydration and rehydration) were performed per sample. The recovered peptides were stored, in -20^0C.

B) MALDI target spotting: The sample was spotted directly onto a MALDI target that was pre-spotted with 0.5 µL MALDI matrix (CHCA) using 0.5 µL of an protein digest solution (0.1% trifluoroacetic acid/50% acetonitrile).

C) MALDI mass spectrometer parameters: Instrument: ABI 4800 MALDI TOF/TOF analyzer; data acquisition and processing program: 4000 Series Explorer software; MS acquisition in reflector mode positive ion mode; mass range: m/z = 600 – 4000; 400 laser shots per spectrum; minimum S/N = 10 for MS acquisition; 10 strongest precursors chosen for MS/MS; minimum S/N = 30 for MS/MS precursors.

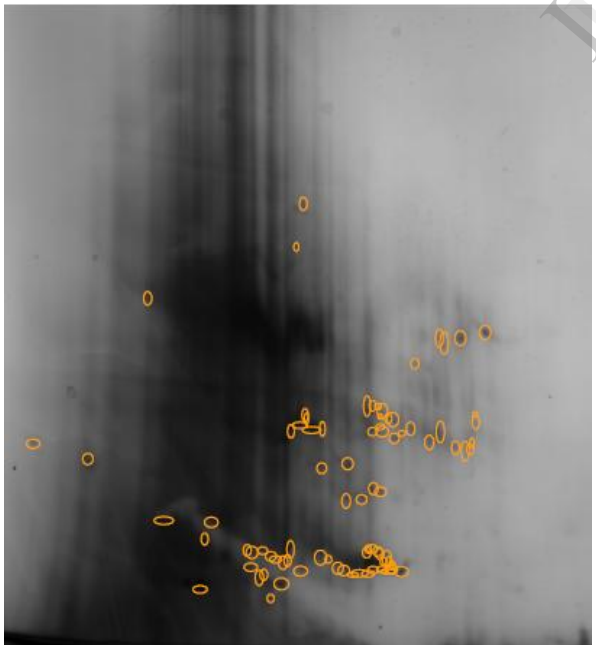
D) Database correlation analysis search parameters: Protein identification from MS and data: Program for data processing: Applied Biosystems GPS Explorer v3.6; search engine: Mascot (Matrix Science, Boston, MA); sample type: gel samples; digestion enzyme: trypsin; species: mouse; I.D focus: biological modifications; database: NCBInr or Swiss-Prot; search engine: type of search: MS and; Mascot (Matrix Science); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±0.3 - 1 Da; maximum missed cleavages: 1-2; variable modifications: oxidation (M), carbamidomethyl (C).

3. Results and Discussion

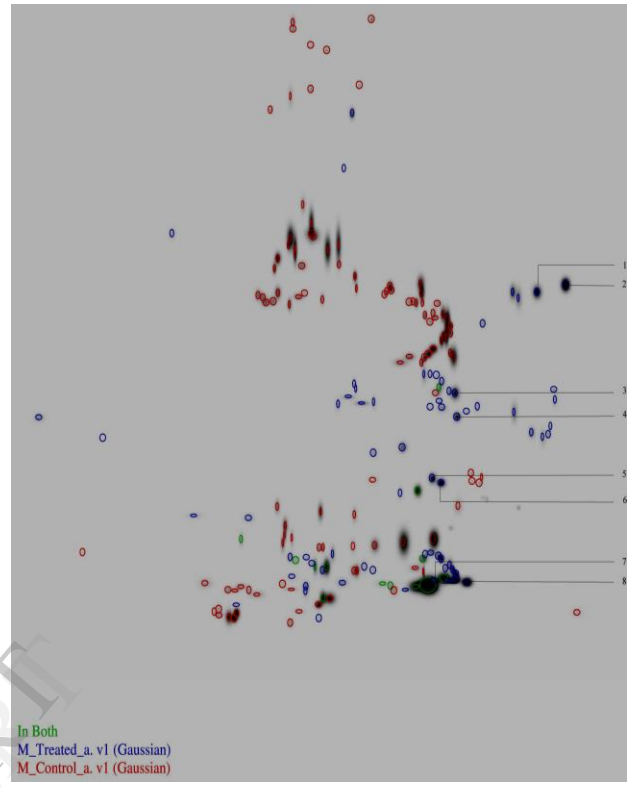
The results of 2 Dimensional Electrophoresis expose many proteins that are expressed differentially in the control and treated. The raw images of 2D gel electrophoresis of the control and treated samples, the master image is shown in figure 1. The mass spectrometry results of spots marked 1 and 2 are shown in figure 2.The Peptide Mass Fingerprinting of spots marked 1and 2 were identified to be protein subunits similar to 50S ribosomal protein L17, and Aspartate transcarbamoylase The Mascot search result for Peptide Mass Fingerprint of spot 1 was found to be 50S ribosomal protein L17, chloroplastic OS=Arabidopsis with Score : 58 for RK17_ARATH.Protein score is -10*Log(P), where P is the probability that the observed match is a random event.Protein scores greater than 58 are significant ($p<0.05$). The spot two has shown maximum similarity for Aspartate transcarbomylase of *Arabidopsis thaliana*. The expression of ribosomal subunit protein L17, a part of P protein complex, for coordinated and regulated synthesis of stress related proteins. Aspartate transcarbamoylase (ATCase; EC 2.1.3.2) catalyzes the committed step in the de novo synthesis of pyrimidine nucleotides(Hasegwa *et al*., 2004). The regulation of pyrimidine metabolism is tightly controlled in plants. Additionally, plants produce toxic secondary metabolites derived from

pyrimidines for use as defense compounds.(Chen et al., 2008).

(a)



(b)



(c)

Figure.1 Raw 2D images of (a) Control (b) treated and (c) Superimposed Master image showing proteins differentially expressed in control (red), in treated (blue) and expressed in both (green).

A comparative proteomics analysis was performed to screen and identify the proteins that respond to salinity stress.The proteomic approach is highly useful in identifying proteins that can act as candidates for development of transgenics with genes encoding these proteins that are helpful for plants in stress response or tolerance (Flowers, 2004; Sobhanian *et al*., 2011). These proteins may be involved in carbohydrate, nitrogen and energy metabolism and their rgulation, reactive oxygen species scavenging, mRNA and protein processing, and cytoskeleton stability. (Kosava *et al*., 2011)

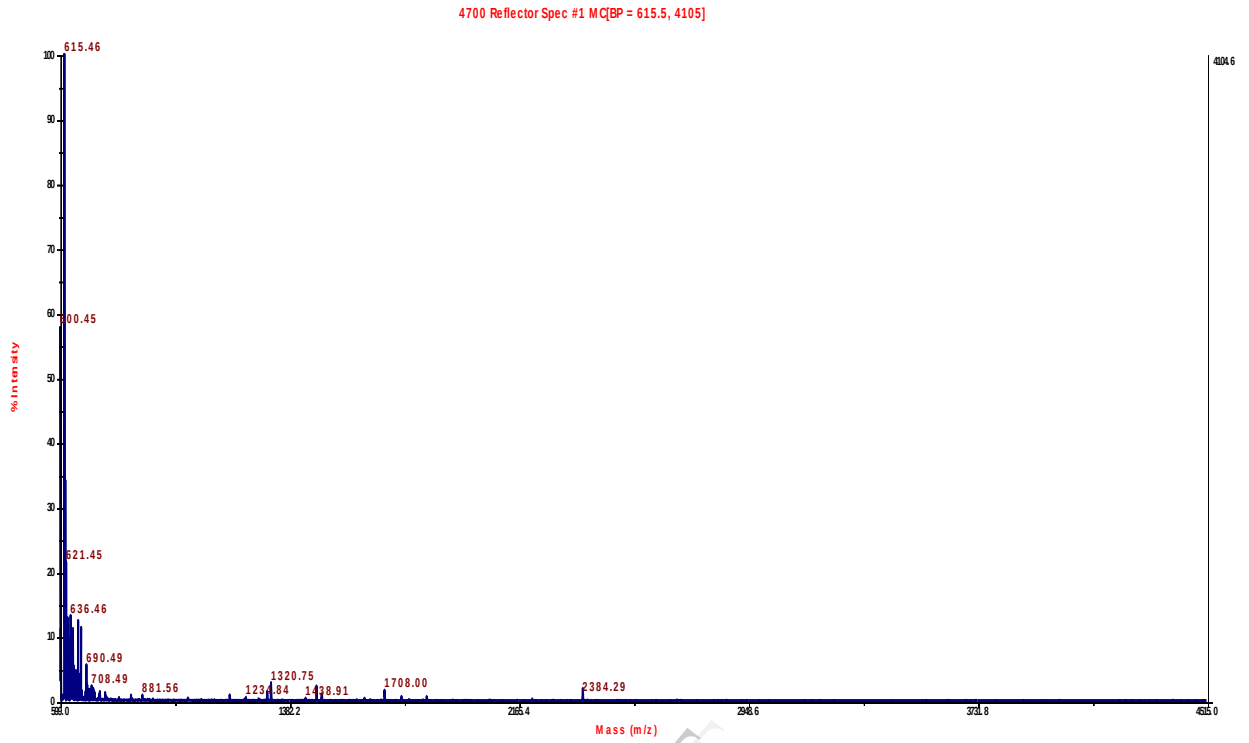


Fig 2. The PMF result of spot 1

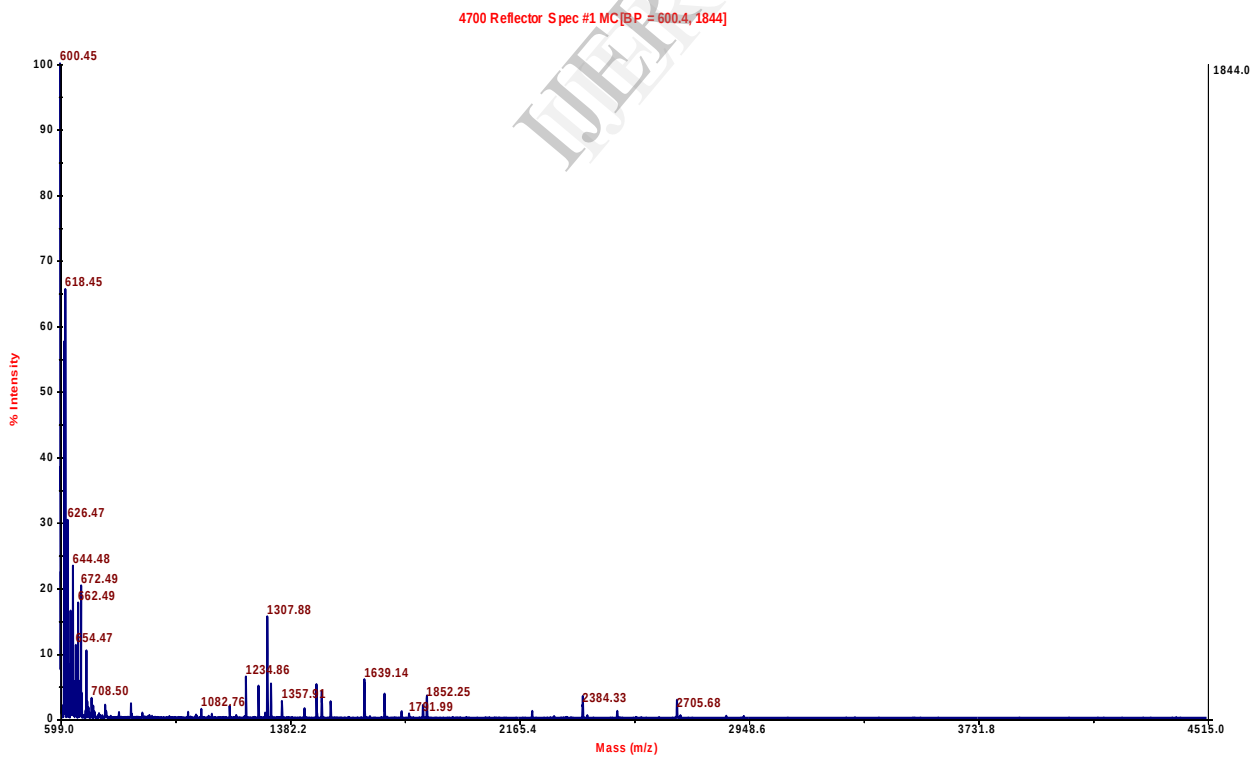


Fig 3. The PMF result of spot 2

At proteomic level, plant response to salinity has been examined in various plant species including both glycophytes and halophytes. Analysis of 2DE gels coupled with protein

identification by mass spectrometry has enabled to identify differentially expressed protein spots. However, a mere protein differential abundance does not give much information on protein function under salinity and therefore validation of comparative proteomics should be done by protein functional analysis. Therefore, other approaches (e.g., post-translational modifications (PTMs), protein-protein interactions, tissue and subcellular localization and phenotype influence on silencing or overexpressing of a gene encoding a protein of interest) have to be employed to unravel the role of the proteins in acquisition and development of salinity tolerance in plants.

References

1. Chattopadhyay, A.; Subba, P.; Pandey, A.; Bhushan, D.; Kumar, R.; Datta, A.; Chakraborty, S.; Chakraborty, N. Analysis of the grasspea proteome and identification of stress-responsive proteins upon exposure to high salinity, low temperature, and abscisic acid treatment. *Phytochemistry* **2011**, *72*, 1293–1307.
2. Chen CT, Slocum RD.Expression and functional analysis of aspartate transcarbamoylase and role of de novo pyrimidine synthesis in regulation of growth and development in Arabidopsis. *Plant Physiol Biochem*. 2008 Feb;46(2):150-9.
3. Flowers, T.J. Improving crop salt tolerance. *J. Exp. Bot.* **2004**, *55*, 307–319.
4. Hasegawa, P.M.; Bressan, R.A.; Zhu, J.K.; Bohnert, H.J. Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2000**, *51*, 463–499.
5. Jiang, Y.; Yang, B.; Harris, N.S.; Deyholos, M.K. Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. *J. Exp. Bot.* **2007**, *58*, 3591–3607.
6. Kosová, K.; Vítámvás, P.; Prášil, I.T.; Renaut, J. Plant proteome changes under abiotic stress—contribution of proteomics studies to understanding plant stress response. *J. Proteomics* **2011**,*74*, 1301–1322.
7. Munns, R.; Tester, M. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* **2008**, *59*, 651–681.
8. Sobhanian, H.; Aghaei, K.; Komatsu, S. Changes in the plant proteome resulting from salt stress:Toward the creation of salt-tolerant crops? *J. Proteomics* **2011**, *74*, 1323–133
9. Yan S.; Tang Z.; Su W.; Sun W. Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics* **2005**, *5*, 235–244.
10. Yan, J.X., Wait, R., Berkelman, T., Harry, R.A ., Westbrook, J.A., Wheeler, C.H., Dunn, M.J., (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization- mass spectrometry. *Electrophoresis* . 17, 3666-72
11. Zhang, H.; Han, B.; Wang, T.; Chen, S.; Li, H.; Zhang, Y.; Dai, S. Mechanisms of plant salt response: Insights from proteomics. *J. Proteome Res.* **2012**, *11*, 49–67.